Resistance to extended-spectrum cephalosporins in Enterobacteriaceae from chickens, dogs, and pigs in Ontario

by

Pauline Lu Ching Zhang

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Pathobiology

Guelph, Ontario, Canada
© Pauline Lu Ching Zhang, September, 2017
ABSTRACT

RESISTANCE TO EXTENDED-SPECTRUM CEPHALOSPORINS IN ENTEROBACTERIACEAE FROM CHICKENS, DOGS, AND PIGS IN ONTARIO

Pauline Lu Ching Zhang
University of Guelph, 2017

Advisor: Dr. Patrick Boerlin

Little information is available on resistance to extended-spectrum cephalosporins (ESCs) in Enterobacteriaceae from animals in Canada. The study objectives were to determine the frequency of ESC resistance in clinical, cecal, and fecal isolates of Enterobacteriaceae from chickens, dogs, and pigs in Ontario using selective and non-selective methods, and characterize the ESC resistance genes. All isolates were tested for ESC susceptibility and resistant ones were screened for major ESC resistance gene families. Most ESC-resistant isolates were \textit{E. coli} and \textit{blaCMY} was the most frequent gene family followed by \textit{blaCTX-M} for all three animal species. There were high frequencies of intestinal carriage of ESC-resistant Enterobacteriaceae in all three animal species. Whole-genome sequencing of canine and swine CTX-M-positive isolates identified a diversity of sequence types, including human pandemic clones. The \textit{blaCTX-M} variants found in this study provide evidence supporting horizontal gene transfer of ESC-resistant bacteria and their genes between humans and these animals.
ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude towards Dr. Patrick Boerlin for being an amazing advisor throughout this journey. Thank you for taking me on as a graduate student in your lab, and for taking a chance with a Zoology student. I am extremely thankful for the many opportunities and experiences that helped me grow both academically and as an individual. It was wonderful to have had an advisor who genuinely cares for the success and well-being of his students. Thank you for always being exceptionally patient when teaching me about the field of antimicrobial resistance. I will always be grateful for your support, guidance, and enthusiasm. In addition, I would like to thank the members of my advisory committee Dr. Durda Slavic and Dr. Richard Reid-Smith for their invaluable input and advice over the past two years. Thank you both for taking the time to attend numerous meetings and share your knowledge and expertise with me.

I would like to express my heartfelt thanks to Gabhan Chalmers for teaching me the tips for success in the lab and answering my endless questions. The completion of my research would not have been possible without your patience and guidance. Thank you to Ashley Cormier for being an extraordinary companion in graduate studies in the Boerlin lab. Your company made late nights in the lab much more enjoyable. To both Gabhan and Ashley, thank you for all the laughs! I am also indebted to Vickie Xiao Shen for being an incredible research partner, contributing substantially to my canine research, and accompanying me to gather fecal samples across Southern Ontario. I would also like to thank Julie Cobean for travelling with us to get fecal samples and helping with the never-ending susceptibility tests. Thank you to Kristin Davis for your assistance and enthusiasm in preparing lysate plates for me. I would also like to thank Iman Gohari for constantly reminding me that “research is fun”! After this journey, I must say that I agree with you.

I would like to extend my gratitude to the Animal Health Laboratory Bacteriology team for their assistance with collecting and identifying isolates, with special thanks to Jennifer Zoethout for organizing the isolate collection and generating countless data reports for me. I would also like to thank Sarah Lippert and Kimani Williams for their help with the broth microdilution susceptibility testing. I am also grateful to Dr. Hani Dick and the technicians at IDEXX for their assistance with isolate collection. Thank you to everyone at the Canadian
Integrated Program for Antimicrobial Resistance for making the cecal sample collection possible: Dr. Anne E. Deckert, Dre. Danielle Daignault, Marie-Claude Deshaies, and Lien Mi Tien. I am grateful for the guidance provided by Dr. Nicol Janecko for obtaining fecal samples from off-leash dog parks. Lastly, I would also like to thank Dr. David Pearl for his help with statistical analyses.

I would like to acknowledge the funding agencies that made this project possible: NSERC, the Ontario Veterinary College Pet Trust Fund, and the Ontario Veterinary College Summer Career Opportunities and Research Experience Program.

Special thanks to my friends back at home and in Guelph for constantly encouraging me, cheering me on during difficult times, and reminding me that I need to take breaks once in a while. I would also like to thank my fellow graduate students at Pathobiology for their support and for making this department such a great place to work at. I would like to especially thank Amanda AuYeung for being an amazing friend and for all the support she has given me over the past two years. And last but not least, I would like to thank my family for their love, emotional support, understanding, and for always visiting me in Guelph when I needed food.
DECLARATION OF WORK PERFORMED

The work presented in this thesis was performed by Pauline Lu Ching Zhang, with the following exceptions:

1. The primary isolation of Enterobacteriaceae from clinical samples was performed by IDEXX Laboratories Inc. and the Animal Health Laboratory (AHL), University of Guelph.
2. The cecal samples of chickens and pigs were collected by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS).
3. Enrichment, isolation, and disk diffusion susceptibility testing of ESC-resistant Enterobacteriaceae from cecal and fecal samples was partly performed by Xiao Shen.
4. Broth microdilution susceptibility testing of generic *E. coli* isolates was partly performed by Gabhan Chalmers and the Animal Health Laboratory, University of Guelph.
5. Disk diffusion susceptibility testing was occasionally performed by Gabhan Chalmers and Julie Cobean.
6. Preparation of lysate plates for PCR screening of resistance genes was partly performed by Kristin Davis.
7. Illumina MiSeq whole-genome sequencing and Sanger sequencing was performed by the Advanced Analysis Centre Genomics Facility, University of Guelph.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

DECLARATION OF WORK PERFORMED ........................................................................... v

LIST OF TABLES .................................................................................................................. viii

LIST OF FIGURES ................................................................................................................ x

LIST OF ABBREVIATIONS ................................................................................................. xii

CHAPTER 1: LITERATURE REVIEW ................................................................................... 1
  1. ENTEROBACTERIACEAE ............................................................................................. 1
  2. ANTIMICROBIALS .................................................................................................... 6
  3. ANTIMICROBIAL RESISTANCE (AMR) ................................................................... 10
  4. THESIS PROPOSAL OVERVIEW ......................................................................... 20

CHAPTER 2: PREVALENCE AND MECHANISMS OF EXTENDED-SPECTRUM
CEPHALOSPORIN RESISTANCE IN CLINICAL AND COMMENSAL
ENTEROBACTERIACEAE ISOLATES FROM DOGS IN CANADA .................................. 26

ABSTRACT ......................................................................................................................... 27
  1. INTRODUCTION ...................................................................................................... 27
  2. MATERIALS AND METHODS ............................................................................... 29
  3. RESULTS ............................................................................................................... 32
  4. DISCUSSION .......................................................................................................... 35

ACKNOWLEDGEMENTS ..................................................................................................... 47

CHAPTER 3: PREVALENCE AND MECHANISMS OF EXTENDED-SPECTRUM
CEPHALOSPORIN RESISTANCE IN CLINICAL AND COMMENSAL
ENTEROBACTERIACEAE ISOLATES FROM CHICKENS AND PIGS IN ONTARIO ........ 48

ABSTRACT ......................................................................................................................... 49
  1. INTRODUCTION ...................................................................................................... 49
  2. MATERIALS AND METHODS ............................................................................... 51
  3. RESULTS ............................................................................................................... 53
  4. DISCUSSION .......................................................................................................... 56

ACKNOWLEDGEMENTS ..................................................................................................... 64

CHAPTER 4: DISCUSSION AND CONCLUSIONS ............................................................ 65
  1. SUMMARY OF ESC RESISTANCE ........................................................................ 65
  2. FUTURE DIRECTIONS ........................................................................................... 68
3. CONCLUSIONS.................................................................................................................. 69
REFERENCES .......................................................................................................................... 73
APPENDIX 1. SUPPLEMENTARY DATA FOR CHAPTER 2 .............................................. 92
APPENDIX 2. SUPPLEMENTARY DATA FOR CHAPTER 3 .............................................. 98
LIST OF TABLES

CHAPTER 2: Prevalence and Mechanisms of Extended-Spectrum Cephalosporin Resistance in Clinical and Commensal Enterobacteriaceae Isolates from Dogs in Canada

Table 2.1. ESC-resistant clinical and fecal isolates categorized by bacterial species and their corresponding genotypes.................................................................39

Table 2.2. Disk diffusion phenotypes of clinical and fecal *E. coli* isolates with reduced susceptibility to cefoxitin and ESCs, and their corresponding genotypes.........................40

Table 2.3. ESC resistance genes found in the CTX-M positive clinical and fecal isolates........41

Table 2.4. Results of logistic regression for gene frequencies and Fisher’s exact tests for blaCTX-M and other resistance genes.................................................................42

CHAPTER 3: Prevalence and Mechanisms of Extended-Spectrum Cephalosporin Resistance in Clinical and Commensal Enterobacteriaceae Isolates from Chickens and Pigs in Canada

Table 3.1. ESC-resistant clinical and cecal isolates from chickens and pigs and their corresponding genotypes.................................................................60

Table 3.2. Disk diffusion phenotypes of resistant clinical and fecal *E. coli* isolates from chickens and pigs through disk diffusion susceptibility testing and their corresponding genotypes........61

CHAPTER 4: Discussion and Conclusions

Table 4.1. Summary of the resistance genes found in ESC-resistant Enterobacteriaceae isolates from chickens, dogs, and pigs.................................................................70

APPENDIX 1: Supplementary Data for Chapter 2

Table 2.S1. Antimicrobial resistance genes found in the CTX-M positive ESC-resistant clinical isolates and fecal isolates from dogs.................................................................94
APPENDIX 2: Supplementary Data for Chapter 3

Table 3.S1. Antimicrobial resistance genes found in the CTX-M positive ESC-resistant clinical isolates and cecal *E. coli* isolates from swine.................................................................98

Table 3.S2. Colony counts of serial dilutions of broth cultures for *E. coli*, *K. pneumoniae*, and *E. cloacae* before and after two separate competition trials.........................................................101
LIST OF FIGURES

CHAPTER 1: Literature Review

Fig. 1.1. Schematic representation of the major targets of different antimicrobial classes in a bacterial cell showing the cell wall, cell membrane, DNA synthesis, protein synthesis, and metabolic pathways.................................................................7

Fig. 1.2. Schematic representation of the four major mechanisms of resistance for a bacterial cell.................................................................12

Fig. 1.3. A conceptual diagram outlining some of the different compartments with arrows representing a subset of potential transfer pathways of ESC resistance determinants and the red boxes highlight the focus of the project.................................................................21

CHAPTER 2: Prevalence and Mechanisms of Extended-Spectrum Cephalosporin Resistance in Clinical and Commensal Enterobacteriaceae Isolates from Dogs in Canada

Fig. 2.1. An overview of the different clinical and fecal isolate collections and the methodologies used in this study.................................................................43

Fig. 2.2. The frequency of reduced susceptibility to antimicrobials in generic E. coli isolates (n=217) from canine fecal samples with 95% confidence intervals.................................44

Fig. 2.3. Minimum spanning tree using wgMLST with 3223 genes showing the different STs of ESC-resistant clinical E. coli and fecal E. coli from enrichment...........................................45

Fig. 2.4. Minimum spanning tree using wgMLST with 3223 genes showing the distribution of CTX-M variants in clinical E. coli and fecal E. coli.........................................................46

CHAPTER 3: Prevalence and Mechanisms of Extended-Spectrum Cephalosporin Resistance in Clinical and Commensal Enterobacteriaceae Isolates from Chickens and Pigs in Canada

Fig. 3.1. Brief overview of the different clinical and cecal isolate collections and the methodologies used in this study.................................................................62
CHAPTER 4: Discussion and Conclusions

Fig. 4.1. Minimum spanning tree of all CTX-M-positive canine and swine E. coli isolates using wgMLST with 3073 genes .................................................................71

Fig. 4.2. Minimum spanning tree of all CTX-M-positive canine and swine isolates showing the different CTX-M variants using wgMLST with 3073 genes .................................72

APPENDIX 1: Supplementary Data for Chapter 2

Fig. 2.S1. The cities in Southern Ontario that were sampled for canine feces shown with Google Maps. Map data: © 2016 Google .................................................................92

Fig. 2.S2. The frequency of ESC-resistant Enterobacteriaceae fecal carriage after enrichment in cities of Southern Ontario with 95% confidence intervals ........................................93
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHL</td>
<td>Animal Health Laboratory</td>
</tr>
<tr>
<td>AIEC</td>
<td>Adherent and invasive <em>Escherichia coli</em></td>
</tr>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
</tr>
<tr>
<td>APEC</td>
<td>Avian pathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CIPARS</td>
<td>Canadian Integrated Program for Antimicrobial Resistance Surveillance</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohemorrhagic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended-spectrum β-lactamase</td>
</tr>
<tr>
<td>ESC</td>
<td>Extended-spectrum cephalosporin</td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser desorption ionization-time of flight</td>
</tr>
<tr>
<td>MAPAQ</td>
<td>Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
</tr>
<tr>
<td>OIE</td>
<td>World Organization for Animal Health (Office International des Epizooties)</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin-binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
</tr>
<tr>
<td>STEC</td>
<td>Shiga-toxin producing <em>Escherichia coli</em></td>
</tr>
</tbody>
</table>
UPEC  Uropathogenic *Escherichia coli*
USA  United States of America
UTI  Urinary tract infection
WGS  Whole-genome sequencing
WHO  World Health Organization
CHAPTER 1: LITERATURE REVIEW

1. ENTEROBACTERIACEAE

1.1 General overview

The Enterobacteriaceae are a very diverse family of bacteria that are ubiquitous in the environment and in humans, animals, plants, and insects (Brenner et al., 2005). Most species in this family are harmless as they are a part of the healthy gut microbiota of humans and animals and help prevent enteric pathogens from colonizing the digestive tract (Linton and Hinton, 1988). However, some species of Enterobacteriaceae can be pathogenic. Many are known as opportunistic pathogens (Linton and Hinton, 1988). The family includes important pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella enterica* subsp. *enterica*. Overall, Enterobacteriaceae have an enormous impact on the health of humans, animals and plants and they are of great economic importance. Because they are ubiquitous and generally easy to culture, Enterobacteriaceae have become the most studied family of bacteria.

Enterobacteriaceae are Gram-negative, straight rods and can be motile with peritrichous flagella (Brenner et al., 2005). Most of the members of this family are catalase-positive, oxidase-negative and reduce nitrate to nitrite. In addition, these bacteria are facultative anaerobes, meaning that they are capable of both respiratory and fermentative metabolism (Quinn et al., 2011). They are mesophiles and thus grow optimally at temperatures from 22-35°C and do not form endospores or microcysts (Brenner et al., 2005).

The Enterobacteriaceae are part of the phylum *Proteobacteria*, the class *Gammaproteobacteria*, and the order *Enterobacteriales*. Presently, the family consists of 53 different genera and 338 recognized species (LPSN; http://www.bacterio.net). The type genus of the family is *Escherichia* (Brenner et al., 2005). The members of Enterobacteriaceae have been re-organized and renamed multiple times. Whole-genome sequencing and 16S ribosomal RNA gene sequencing have played a major role in elucidating the relationships between genera and species (Iversen et al., 2004; Zhang and Qiu, 2015).

This literature review will mainly focus on *E. coli*, *S. enterica*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Serratia* spp., *Citrobacter* spp., and *Pantoea* spp. in chickens, dogs,
and pigs. *Escherichia coli* is the most well-known species of the genus *Escherichia* and it has many different serotypes. In North America, some of the most important *E. coli* pathotypes for chickens, dogs and pigs are avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), and enterotoxigenic *E. coli* (ETEC), respectively. The *Salmonella* genus is made up of only two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is relevant to agriculture and public health, whereas *S. bongori* is mainly restricted to reptiles. *Salmonella enterica* encompasses six subspecies and over 2500 serovars (Grimont and Weill, 2007). The only subspecies that causes clinical infections in chickens, dogs, and pigs is *S. enterica* subsp. *enterica*.

The genus *Klebsiella* comprises fifteen species, but only two typically cause illness in chickens and dogs: *Klebsiella oxytoca* and *K. pneumoniae*. The other genera *Citrobacter*, *Enterobacter*, *Proteus*, *Serratia*, and *Pantoea* are mainly opportunistic pathogens and only occasionally cause disease in chickens and pigs. From these genera, the more common species responsible for clinical illness in canines are *Enterobacter cloacae*, *Enterobacter aerogenes*, *Serratia liquefaciens*, *Serratia marcescens*, *Proteus mirabilis*, *Proteus vulgaris*, and *Pantoea agglomerans*. These bacterial species mainly cause urinary tract infections, septicemia, bacteremia, and post-surgical wound infections, but can also be part of mixed opportunistic infections.

**1.2 Enterobacteriaceae in chickens**

Most Enterobacteriaceae species do not cause disease in chickens with the exception of APEC and *S. enterica* subsp. *enterica*. Inflammation of the pericardium, air sacs, liver, subcutaneous tissue, umbilical stump, or oviduct may all be referred to as colibacillosis (Linton and Hinton, 1988; Gomis et al., 1997; Dho-Moulin and Fairbrother, 1999). Chickens can be infected at any age during the production process (Guabiraba and Schouler, 2015) and one way this can occur is through fecal dust entering the respiratory system (Chaudhari and Kariyawasam, 2014). Colibacillosis is typically characterized by systemic infection. In addition, some APEC serogroups are more frequently associated with colibacillosis than others, such as O1, O2, and O78 (Gao et al., 2013). APEC infections are often accompanied by infections with other microorganisms such as viruses and mycoplasma (Linton and Hinton, 1988). For example, co-infections of APEC and coronavirus can lead to swollen head syndrome, which is characterized
by swelling of the sinuses and/or neck muscle spasms (Morley and Thomson, 1984; Pattison et al., 1989). Overall, colibacillosis has negative effects on egg production and hatching rates. It also results in increased mortality and carcass condemnation at slaughter (Guabiraba and Schouler, 2015).

The other species of Enterobacteriaceae that primarily causes illness in chickens is S. enterica. Typically, the presence of S. enterica in chickens is subclinical (Dhanani et al., 2015). However, similar to E. coli, there are two host-adapted pathogenic serovars for chicken: Salmonella Gallinarum and Salmonella Pullorum (Linton and Hinton, 1988; Liu et al., 2015). These serovars are responsible for fowl typhoid and pullorum disease, respectively (Cheraghchi et al., 2014). Pullorum disease and fowl typhoid both result in systemic infections and high mortality (Yin et al., 2015; Zhu et al., 2015). These diseases are highly prevalent in developing countries, but they have been eradicated from Canada (Cheraghchi et al., 2014).

1.3 Enterobacteriaceae in dogs

Enterobacteriaceae typically have a symbiotic relationship with the host (Mackie et al., 1999). However, if they find their way into the urinary tract, they can lead to a urinary tract infection (UTI), which is a common medical issue in dogs. It is estimated that 14% of all dogs will have at least one UTI in their lifetime (Windahl et al., 2014). The species of Enterobacteriaceae that commonly cause UTIs are E. coli, K. pneumoniae, and P. mirabilis (Wong et al., 2015). However, E. coli is the most frequent urinary pathogen and can be isolated in up to 2/3 of UTI cases (Windahl et al., 2014). In an investigation on canine UTIs within an intensive care unit in Ontario, Canada, E. coli was isolated in 46.2% of cases (Ogeer-Gyles et al., 2006a). Some E. coli strains are classified as uropathogenic E. coli (UPEC), as they have acquired virulence factors which make them particularly successful at causing disease in the urinary tract (Mann et al., 2017).

In addition to UTIs, E. coli can cause diarrhea in dogs and these strains include enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), and adherent and invasive E. coli (AIEC) (Martinez-Medina et al., 2011; Quinn et al., 2011). These strains differ from others due to the expression of virulence factors such as adhesins and enterotoxins. However, their significance in dogs and their corresponding virulence factors have not been clearly defined
(Marks et al., 2011), as EPEC has been found in dogs with and without enteric disease in Brazil (Puño-Sarmiento et al., 2013). *Salmonella enterica* is also capable of causing diarrhea in dogs. Specifically, salmonellosis can manifest itself in the form of fever, diarrhea, lethargy, enteritis, and vomiting. Enteritis in dogs may lead to septicemia as well, especially if they are young. This is not always the case as many *S. enterica* infections in dogs can be subclinical. Worldwide, *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most frequent serotypes isolated from dogs (Carter and Quinn, 2000). However, there are geographical and time variations in this distribution. For instance, a study conducted in Ontario, Canada from 2005 to 2006, found *S. Typhimurium, Kentucky, Heidelberg, and Brandenburg* to be the most common serotypes (Leonard et al., 2011).

*Enterobacteriaceae* can contribute to secondary infections as well as cause other clinical diseases in dogs, such as septicemia, otitis, dermatitis, and surgical wound infections. Depending on the type of surgical procedure, infection rates of surgical sites have been documented ranging from 2-18% (Vasseur et al., 1988; Nicholson et al., 2002). These studies did not identify the causative bacterial species, but other studies have isolated *E. coli, Enterobacter* spp., *Klebsiella* spp., and *P. mirabilis* from postsurgical infections (Gibson et al., 2008; Meakin et al., 2013; Windahl et al., 2015). Another study identified *Citrobacter* spp. as the cause of fatal septicemia in two dogs (Galarneau et al., 2003).

1.4 *Enterobacteriaceae in pigs*

Similar to chickens, most Enterobacteriaceae do not cause clinical disease in pigs with the exception of enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC) and *S. enterica* subsp. *enterica* (Quinn et al., 2011). In Canada, STEC in pigs is of less importance compared to ETEC. The main virulence factors for porcine ETEC are colonization factors and enterotoxins. Colonization factors, which are protein antigens expressed as bacterial fimbriae, allow porcine ETEC to adhere to the walls of the intestine and the enterocytes (Xia et al., 2015). ETEC strains express a variety of colonization factors, such as K88 and 987P (or F4 and F6) (Nagy and Fekete, 1999; Gonzales-Siles and Sjöling, 2016). Once ETEC has successfully bound to the intestinal wall, enterotoxins are produced, such as the heat-labile toxins (LT) and the heat-stable toxins (Sta and Stb). These enterotoxins play a major role in disrupting osmoregulation and ionoregulation, ultimately resulting in watery diarrhea (Dubreuil, 2012; Gonzales-Siles and
Sjöling, 2016). ETEC is most frequently seen in neonatal piglets and post-weaning pigs (Nagy and Fekete, 1999). It had such an impact on neonatal piglets that in 1994, it accounted for 50% of piglet deaths worldwide (Adewole et al., 2016). Vaccinating sows has helped decrease the frequency of neonatal diarrhea, but not of post-weaning diarrhea and now ETEC has a greater impact on the swine industry (Fairbrother et al., 2005).

Unlike *E. coli*, *S. enterica* is not a commensal bacterial species in the swine gut. If *S. enterica* is present, it can cause a variety of clinical pictures, ranging from subclinical infection to severe diarrhea in pigs. The most prevalent *Salmonella* serovars can vary as in Alberta, the most common were Typhimurium, Derby and Infantis (Rajic et al., 2005) whereas in Saskatchewan, the most prevalent were Derby, Enteritidis, and California (Mainar-Jaime et al., 2008). Salmonellosis is one of the most common problems in post-weaning pigs (Haley et al., 2012). However, in comparison to ETEC, it accounts for fewer cases of neonatal diarrhea (Chan et al., 2013). This was seen in a retrospective study conducted in Ontario, Canada that investigated causes of neonatal diarrhea in piglets (Chan et al., 2013).

### 1.5 Enterobacteriaceae and public health

Diseases caused by Enterobacteriaceae species have a huge impact on public health (Paterson, 2006). For example, hospital-acquired infections are often caused by *E. coli*, *K. pneumoniae*, and *Proteus* spp. (Guentzel, 1996; Iliyasu et al., 2016; Xia et al., 2016). The consumption of water contaminated water with *E. coli* also cause disease, especially in developing countries (Julian, 2016). In some cases, animals, their products, and byproducts can be a source of Enterobacteriaceae causing human disease. This is mainly limited to *S. enterica* and STEC. It is important to note that there are human-specific strains of STEC different from those previously discussed for swine, and those that typically cause severe disease in humans are classified as enterohemorrhagic *E. coli* (EHEC) (Delannoy et al., 2013). Human infections typically occur through eating and handling raw and improperly cooked contaminated food intended for either human or animal consumption. For example, there have been outbreaks of *S. Heidelberg* in the USA and STEC in Canada that were linked to raw poultry meat and beef tartare, respectively (Gaulin et al., 2015; Routh et al., 2015). Similarly, companion animal owners are also at risk of *S. enterica* infection through exposure of contaminated pet food (Finley et al., 2008), as well as through increased canine fecal shedding of *S. enterica* if their dogs are on
raw diets, which are generally predominantly poultry-based (Lefebvre et al., 2008). Contamination of meat during slaughter can occur due to the intestinal carriage of pathogenic strains *E. coli* and *S. enterica* in food animals.

2. **ANTIMICROBIALS**

2.1 **Definition and modes of action**

Antimicrobials, herein meaning antibiotics and their synthetic and semi-synthetic derivatives, are agents capable of killing or inhibiting the growth of microorganisms, and are referred to as bactericidal or bacteriostatic, respectively (Giguère, 2013). In addition, some antimicrobials may be bacteriostatic at low concentrations and bactericidal at higher concentrations. Different classes of antimicrobials vary in terms of the range of microorganisms affected. Antimicrobials also differ in how they target bacteria as they may inhibit protein synthesis, disrupt DNA synthesis, cell wall synthesis, cell membrane synthesis and stability, or block metabolic pathways (Giguère, 2013) (Fig. 1.1). Some of the most important antimicrobial classes include β-lactams, sulfonamides, polymixins, tetracyclines, fluoroquinolones, macrolides/lincosamides, and aminoglycosides. β-lactams are particularly important in treating serious bacterial infections in both human and veterinary medicine (OIE, 2015; WHO, 2017).
Fig. 1.1. Schematic representation of the major targets of different antimicrobial classes in a bacterial cell showing the cell wall, cell membrane, DNA synthesis, protein synthesis, and metabolic pathways.

2.2 β-lactams and cell wall biosynthesis

In 1928 Alexander Fleming discovered the first antimicrobial penicillin, a β-lactam (Demain, 1991). All β-lactams are characterized by having a β-lactam ring and they work by targeting cell wall biosynthesis. A component of cell wall biosynthesis consists of cross-linking multiple glycan chains, N-acetylglucosamine and N-acetylmuramic acid, with peptide chains to form the polymer peptidoglycan or murein (Ghuysen, 1968). Peptidoglycan is present in the cell walls of both Gram-negative and Gram-positive bacteria, but the latter has a greater amount of it (Vollmer et al., 2008; Kong et al., 2010). There are multiple key enzymes involved in forming the peptidoglycan, including the DD-transpeptidases (also known as penicillin-binding proteins or PBPs) as they catalyze the cross-linking of the glycan chains (Hou and Poole, 1971). It is these enzymes that β-lactams irreversibly bind to, thereby inhibiting cell wall biosynthesis (Goffin and Ghuysen, 1998; Bassetti et al., 2013). The general theory is that loss of cell wall integrity results in the bacterial cell being incapable of coping with the osmotic pressure and rupturing (Park and Strominger, 1957). However, recent study results support the claim that β-lactams actually cause direct damage to cell wall synthesis machinery as well as an exhaustion of cellular resources (Cho et al., 2014).
2.2.1 Groups of β-lactams

Initially, the spectrum of activity for penicillin was largely limited to Gram-positive bacteria. As time went on, researchers experimented with creating semi-synthetic antimicrobials and it led to numerous derivatives including the aminopenicillins and carboxypenicillins (Demain and Elander, 1999). With the rise of resistant bacterial strains, other semi-synthetic β-lactams were produced with increasing spectrums of activity, potency, and stability against resistant strains (Demain and Elander, 1999). Currently, there are multiple groups of β-lactams, including the penicillins, cephalosporins, monobactams, and carbapenems. There are also β-lactamase inhibitors, compounds that are administered in conjunction with β-lactams to overcome resistant strains such as the combination amoxicillin and clavulanic acid (Drawz and Bonomo, 2010). Due to the low toxicity, broad-spectrum activity, high potency and high stability of β-lactams, they are presently one of the most widely used classes of antimicrobials (Frère and Page, 2014).

2.2.2 Cephalosporins

As the search continued for more antimicrobials, researchers Edward Abraham and Guy Newton isolated a natural compound from the species *Cephalosporium acremonium* that they later named cephalosporin C (Abraham and Newton, 1961). This agent differed from penicillin in that it had a 7-aminocephalosporinic acid nucleus instead of the penicillin's beta-lactam nucleus (Abraham and Newton, 1961). Furthermore, cephalosporins were more effective than the aminopenicillins at killing Gram-positive bacteria (Demain and Elander, 1999). Continued experiments on altering the molecular structure led to four separate generations of cephalosporins with generally broader spectrums of activity (Bassetti et al., 2013). Presently, the third and fourth generations of cephalosporins are the most widely used and together are called the extended-spectrum cephalosporins (ESCs). There are additional cephalosporins that have been referred to as fifth-generation cephalosporins. However, the term "fifth-generation" is not universally accepted because it falsely implies that the overall spectrum of activity has increased relative to the past generations; it is simply that they cover many more Gram-positive organisms (Bassetti et al., 2013). In Canada, ESCs are important for veterinary medicine. For example, the ESC ceftiofur is used in swine (Dutil et al., 2010) whereas ESCs cefpodoxime and cefovecin are
used to treat infections in dogs and cats (Stegemann et al., 2007; Sudhakara Reddy et al., 2014; Burke et al., 2017).

2.2.3 Cephamycins

The cephamycins were first discovered and isolated from multiple *Streptomyces* spp. by Stapley and colleagues in Spain (Stapley et al., 1972). In comparison to other β-lactams, cephamycins have an additional methoxyl group (Liras, 1999). Due to the increased spectrum of activity in comparison to other β-lactams at the time, cephamycins such as cefoxitin quickly became the most widely used antimicrobials in 1985 and continue to be so today (Sanders et al., 1985; Pratt, 2016). The cephamycins and cephalosporins are collectively referred to as the cephems.

2.2.4 Monobactams

First approved for clinical use in 1986, aztreonam is used to treat Gram-negative bacterial infections in humans (Ramsey and MacGowan, 2016). Monobactams only have one β-lactam ring whereas other β-lactam antimicrobials typically have two rings in their molecular structure (Bassetti et al., 2013). Presently, aztreonam is still the only monobactam used in practice for humans (Ramsey and MacGowan, 2016) and is infrequently used in veterinary medicine.

2.2.5 Carbapenems

In 1971, the compound carbapenem was isolated from *Streptomyces* sp. by Ramanathan Nagarajan and his colleagues (Nagarajan et al., 1971). In comparison to the other β-lactams, carbapenems have the widest spectrum of activity (Temkin et al., 2014). They are also quite stable and effective, even in the presence of high bacterial densities (Toussaint and Gallagher, 2015). Some of the more commonly used carbapenems are ertapenem, imipenem, and meropenem. In humans, imipenem is used to treat respiratory, urinary, and nosocomial infections (Barker et al., 2003). In companion animals, cases of bacteremia and sepsis can be treated with meropenem (Byun et al., 2016).
3. ANTIMICROBIAL RESISTANCE (AMR)

3.1 Definition and methods of acquiring AMR

As the use of β-lactams increased in the past couple of decades, antimicrobial resistance (AMR) against them has also risen. AMR has now become a severe problem in treating and preventing bacterial infections with β-lactams for both humans and animals (McEwen et al., 2008; Woolhouse and Ward, 2013). AMR is defined as the ability of microorganisms to survive treatment/interaction with an agent that would typically kill them or inhibit their growth (Boerlin and White, 2013). AMR can result from mutations in pre-existing chromosomal genes or from horizontal gene transfer (Newcombe and Hawirko, 1949). If mutations confer a selective advantage, such as AMR, they can be passed on to the next generation and clonally spread (Rolinson, 1971; Hummel et al., 1986; Dowson et al., 1989). Because these mutations are random, they have a smaller impact on AMR relative to horizontal gene transfer. Bacteria mainly acquire AMR through horizontal gene transfer, which is the process of exchanging genes between bacterial cells (von Wintersdorff et al., 2016). Horizontal gene transfer can occur through transformation, transduction, or conjugation. Transformation refers to uptake of DNA from the environment, transduction involves transfer of genes through a bacteriophage, and conjugation refers to transfer of genes from one bacterium to another through a process involving direct contact (Boerlin and White, 2013). Resistance genes are often found on mobile genetic elements, such as transposons and integrons. These genetic elements are often located on plasmids, which are extrachromosomal circular pieces of DNA capable of autonomous replication (Boerlin and White, 2013). Plasmids can quickly spread horizontally among bacterial populations, giving rise to AMR in bacteria that were previously susceptible. In addition, the use of antimicrobials contributes to selective pressure, which can lead to a greater overall prevalence of resistant bacteria.

It is important to note that even if a bacterial strain expresses resistance mechanisms, it does not necessarily mean that it will be clinically resistant. In certain cases, higher doses of the antimicrobial or physiological concentrations of the antimicrobial at the site of infection are sufficient to overcome low levels of resistance. Thus, there is a difference between a bacterial strain that has reduced susceptibility versus a resistant strain. One way of quantitatively measuring resistance is through assessing the minimum inhibitory concentration (MIC), the
lowest concentration of the antimicrobial agent that can inhibit bacterial growth. A bacterial strain is considered resistant when it passes a certain threshold, or MIC breakpoint, that is set by organizations such as the Clinical Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST), or other equivalents. In some cases, expression of multiple resistance mechanisms can be required for a bacterial strain to be resistant (Toussaint and Gallagher, 2015).
3.1.1 General mechanisms of AMR

Overall, chromosomal mutations and horizontal gene transfer in bacteria can lead to β-lactam resistance via four major mechanisms: target modification, enzymatic modification of the antimicrobial agent, reduced cell permeability, and active efflux pumps (Fig. 1.2).

Fig. 1.2. Schematic representation of the four major mechanisms of resistance for a bacterial cell where the green squares represent the antimicrobial, the yellow and black shape is the target, and the purple cylinders represent outer membrane porins. A) A situation without any mechanisms of resistance and the antimicrobial is capable of penetrating the cell freely and of binding to the target and exerting its effect. B) Downregulation of expression of porins results in reduced permeability of the cell membrane. C) Active efflux systems, represented by the green structures, pump the antimicrobial out of the cell. D) Antimicrobial modification by an enzyme, represented by the solid blue shape, renders it unable to bind to the target. E) Target modification results in the antimicrobial agent being unable to bind to the target; targets can also be modified by having proteins bind to it which may be referred to as target protection.
3.2 β-lactam resistance

The first report of penicillin resistance occurred in 1940, when Chain and Abraham discovered a penicillinase, an enzyme capable of hydrolyzing the β-lactam ring and nullifying its bactericidal activity (Abraham and Chain, 1988). They found this enzyme in *E. coli* five years before penicillin was approved for clinical use. Within a year of using penicillin, there were already cases of resistant *Staphylococcus aureus* isolates (Rammelkamp and Maxon, 1942). The frequency of resistant cases dramatically increased and by the 1980s, they had been documented in North America, Europe, Asia, and Africa (Klugman, 1990). This rise in penicillin resistance partly contributed to the push for finding new antimicrobials, as well as producing different generations of β-lactams. However, the number of new antimicrobials gradually declined while bacteria were seemingly finding more and more ways to combat existing antimicrobials (Kong et al., 2010; Lewis, 2013). Over time, resistance towards the extended-spectrum cephalosporins became widespread in many species, including those of Enterobacteriaceae (Paterson, 2006). β-lactams exert their bactericidal activity by reaching the peptidoglycan within the cell wall and binding to the PBPs to inhibit cell wall biosynthesis (Hou and Poole, 1971). Therefore, the two major mechanisms of resistance for β-lactams are through target modification and antimicrobial modification by an enzyme. To a much lesser extent, porin mutations can also lead to β-lactam resistance and is seen more frequently in Gram-negative bacteria such as *Pseudomonas aeruginosa* (Dé et al., 2001; Wolter and Lister, 2013). Mutations in efflux pumps such as the mar operon can also lead to β-lactam resistance in Enterobacteriaceae (George and Levy, 1983), but these mutations typically result in low levels of resistance (Alekshun and Levy, 1997). Target modification is seen more frequently in Gram-positive bacteria, such as methicillin-resistant *S. aureus* (Georgopapadakou, 1993). The primary mechanism of resistance in Enterobacteriaceae is through antimicrobial modification by an enzyme (β-lactamase; King et al., 2016).

3.2.1 β-lactamases and their classifications

β-lactamases are enzymes that hydrolyze the β-lactam ring, effectively rendering the antimicrobial useless (Abdallah et al., 2015). In Enterobacteriaceae, the β-lactamases are active in the periplasmic space (Bush, 1988). The majority of β-lactamases are encoded on transferrable genetic elements, which greatly contributed to their rapid spread. Presently, there are over 1400 different beta-lactamase enzymes recognized. Two methods are utilized for their classification.
The first is the Ambler classification, which is based on the amino acid sequences of the enzyme (Ambler, 1980). The second was created by Bush and Jacoby, which is based on the functional properties of the enzyme (Bush and Jacoby, 2010).

The Ambler classification categorizes the β-lactamases into the classes A, B, C, and D (Ambler, 1980). Classes A, C, and D are serine β-lactamases, and class B represents the metallo-β-lactamases, which refers to the specific active site used during hydrolysis. Class A includes the narrow-spectrum β-lactamases, extended-spectrum β-lactamases, and serine carbapenemases. The narrow-spectrum β-lactamases are classified as such because they are only capable of hydrolyzing penicillin. In contrast, the extended-spectrum β-lactamases are capable of hydrolyzing penicillins as well as the extended-spectrum cephalosporins. The class A enzymes are the most prevalent in Enterobacteriaceae. The class B enzymes and class C enzymes represent metallo-β-lactamases and cephalosporinases respectively, both of which can confer resistance to carbapenems. Class D represents some OXA-type enzymes which are of less importance in relation to Enterobacteriaceae because they are mainly produced by P. aeruginosa and Acinetobacter baumanii. Class D enzymes can confer resistance towards oxacillin, oxyimino β-lactams, and carbapenems.

For the Bush and Jacoby classification, the β-lactamases are separated into three functional groups, with their own subclasses as well. The only subclasses which hydrolyze cephalosporins in group 1 are 1 and 1e, (Bush and Jacoby, 2010). The functional group 2 encompasses all of the serine β-lactamases and it is also the largest functional group. It includes the subclasses 2a, 2b, 2be, 2br, 2ber, 2c, 2ce, 2d, 2de, 2df, 2e, and 2f. Collectively, group 2 can hydrolyze penicillins, carbenicillins, the early cephalosporins, extended-spectrum cephalosporins, and the carbapenems. The functional group 3 confers resistance to all of the β-lactams with the exception of the monobactams.

3.3 Common ESC resistance determinants and their epidemiology

3.3.1 AmpC β-lactamases

After Abraham and Chain reported finding a penicillinase in E. coli, many scientists tried to determine the gene encoding it. Researchers worked with ampicillin-resistant strains of E. coli and found mutations in genes that were named ampA and ampB (Eriksson-Grennberg et al.,
1965; Eriksson-Grennberg, 1968). Unfortunately, it turned out that neither of these genes encoded the enzyme. It was not until Burman and his team discovered that mutations in a chromosomal gene led to reduced production of penicillinase; this gene was named \textit{ampC} (Burman et al., 1973). AmpC is now a class C beta-lactamase and is part of the functional group 1. Subsequent studies found that AmpC is encoded on the chromosomes of many Enterobacteriaceae species besides \textit{E. coli}, such as \textit{Citrobacter} spp., \textit{Enterobacter} spp., and \textit{S. marcescens} (Joris et al., 1986; Lindberg and Normark, 1986; Galleni et al., 1988; Preston et al., 2000; Naas et al., 2002). AmpC is typically expressed at low levels and does not result in clinically relevant levels of resistance. However, if there is overexpression of AmpC, it can result in resistance towards extended-spectrum cephalosporins and monobactams (Sanders and Sanders, 1986; Schmidtke and Hanson, 2006). The first case of plasmid-mediated AmpC was identified in 1990, when a new beta-lactamase called MIR-1 was found in clinical \textit{K. pneumoniae} isolates (Papanicolaou et al., 1990). The nucleotide sequence of MIR-1 was 90% identical to that of AmpC's and exhibited the properties of a class C $\beta$-lactamase (Papanicolaou et al., 1990). Since then, plasmid-mediated AmpC $\beta$-lactamases have been found in \textit{E. coli}, \textit{K. pneumoniae}, \textit{S. enterica}, \textit{P. mirabilis} (Jacoby, 2009), and other non-Enterobacteriaceae species. Presently, the AmpC $\beta$-lactamases group encompasses all class C $\beta$-lactamases that confer resistance to penicillins, extended-spectrum cephalosporins, and monobactams (Philippon et al., 2002). This includes the CMY, FOX, MOX, LAT, DHA, BIL-1, ACC-1, ACT and MIR families. Of these AmpC $\beta$-lactamases, CMYs have proliferated and spread the most (Denisuiik et al., 2013).

The first CMY $\beta$-lactamase CMY-1 was discovered in 1988 in a clinical \textit{K. pneumoniae} isolate in South Korea (Bauernfeind et al., 1989). It was named CMY because it confers resistance to cephamycins. Its presence also allowed for high levels of resistance to imipenem in a specific \textit{E. coli} isolate with decreased porin expression (Poirel et al., 2004a). The CMY gene family is currently composed of 153 unique CMY gene variants (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047). One of the most common CMY variants is CMY-2, first identified in a \textit{K. pneumoniae} isolate in Greece in 1990 (Bauernfeind et al., 1996). It is also one of the most common plasmid-mediated AmpC $\beta$-lactamase isolated from bacteria in humans. When studying the prevalence of AmpC $\beta$-lactamases in Canadian hospitals from 2007-2011, \textit{blaCMY-2} accounted for 64 of 65 \textit{E. coli} isolates with AmpC $\beta$-lactamase genes.
The gene *bla<sub>CMY-2</sub>* has also been documented in *E. coli* from a number of animal species worldwide, including chickens, dogs, and pigs (Li et al., 2007).

For Canada specifically, *bla<sub>CMY-2</sub>* has been found in *E. coli* isolated from swine feces and canine rectal swabs in Ontario (Kozak et al., 2009; Murphy et al., 2009). Another study identified *bla<sub>CMY-2</sub>* in *E. coli* isolated from canine UTIs in Ontario and Québec (Khashayar, 2009). As for the agriculture industry, a study found *bla<sub>CMY-2</sub>* in *Salmonella* spp. isolated from the organs, yolk sacs and intestines of turkeys as well as from one bovine fecal sample (Allen and Poppe, 2002). In addition, 134 *E. coli* and *S. enterica* isolates were collected from chickens, cattle, swine, and their retail meat from 1997-2007 and *bla<sub>CMY-2</sub>* was identified in all of them (Martin et al., 2012). In Ontario, *bla<sub>CMY-2</sub>* was also identified on a multi-drug resistant plasmid in *E. coli* isolated from a pig (Travis et al., 2006). Other than the studies previously mentioned, there have been few investigations in Canada on the prevalence of AmpC β-lactamases in dogs, chickens, and pigs, compared to some other countries. The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) regularly reports on the frequencies of ceftiofur/ceftriaxone resistance in *E. coli* and *S. enterica* from food animals, and has found evidence of *bla<sub>CMY-2</sub>* based on isolates collected for surveillance, particularly in chickens (Edirmanasinghe et al., 2017). However, CIPARS does not systematically investigate the genetic basis of resistance and only screens *E. coli* and *S. enterica* isolates on a routine basis and no other Enterobacteriaceae species.

### 3.3.2 Extended-spectrum β-lactamases (ESBLs)

The three main families of extended-spectrum β-lactamases (ESBLs) are TEM, SHV, and CTX-M. The use of the term ESBL has been confusing over the years. It was initially used to describe the TEM and SHV enzymes that hydrolyze ESCs and are inhibited by beta-lactamase inhibitors. Since its inception, another group of enzymes with a similar spectrum of hydrolytic activity was discovered, the CTX-M enzymes. In the literature, the term ESBL at times only refers to the TEM and SHV derivatives while in others, it includes the CTX-M enzymes. For the purposes of this review, ESBLs encompass all enzymes capable of hydrolyzing penicillins, monobactams, and ESCs, cannot hydrolyze cephamycins or carbapenems, and are inhibited by beta-lactamase inhibitors such as clavulanic acid (Livermore, 2013). In addition, it is important
to note that there are other types of ESBLs such as specific variants in the OXA, PER, VEB, BES-1 families, but compared to TEM, SHV, and CTX-M, they have spread significantly less.

ESBLs are part of class A and the functional groups 1e, 2be, and 2de. The first TEM was reported in Greece in *E. coli* isolated from a patient named Temoneira (thus the name TEM) (Datta and Kontomichalou, 1965). This TEM only exhibited resistance to penicillin and the first generation cephalosporins. This spectrum of hydrolytic activity was the same for the SHV (sulphydryl reagent variable) enzyme discovered in 1972 (Pitton, 1972). Soon after, mutant TEM and SHV enzymes capable of hydrolyzing ESCs were reported and had spread throughout Europe (Knothe et al., 1983). The first CTX-M was identified in *E. coli* from a newborn and named for its resistance to cefotaxime and its location (Munich, Germany; Bauernfeind et al., 1990). Since then, ESBLs have been reported in Enterobacteriaceae worldwide (Paterson, 2006).


In Canada, there have been many reports of ESBLs in clinical Enterobacteriaceae. One study screened *K. pneumoniae* over a 10 year period and found that the variants CTX-M-14 and CTX-M-15 were predominant in hospitals in Calgary, Alberta (Peirano et al., 2012a). The frequencies of TEMs and SHVs found in bacteria from humans are decreasing while CTX-M-14 and CTX-M-15 are on the rise. The increasing prevalence of CTX-M-14 and CTX-M-15 was seen in two other Canadian studies investigating ESBL-producing *E. coli* in various medical centers (Peirano et al., 2010, 2012b). Compared to humans, there have been fewer publications on ESBL-producing Enterobacteriaceae from animals in Canada. The literature seems to suggest that ESBL-producing Enterobacteriaceae are present at a lower prevalence in animals compared to humans. This is supported in part by phenotypic studies that have found low levels of ESC resistance in enterotoxigenic *E. coli* from pigs in Québec and Prince Edward Island (Maynard et al., 2003; Hariharan et al., 2004). In Canada, a study on chicken cecal samples and retail meat found that only 3.8% of *E. coli* and 1.4% of *S. enterica* carried *bla*SHV-2 or *bla*SHV-2a (Pouget et al., 2013). The frequency of these ESBL genes in *E. coli* and *S. enterica* from pigs was even lower (0.5% and 0%, respectively) (Pouget et al., 2013). In addition, *bla*SHV was identified in only 0.2% of isolates in a study that investigated ceftiofur-resistant *E. coli* isolated from chickens with colibacillosis (Chalmers et al., 2017). Chalmers and collaborators found that 10.6% of isolates
had blatEM, but these gene variants did not encode for ESBLs. CTX-M has only been recently described in Enterobacteriaceae from chickens and pigs in a few publications (Jahanbakhsh et al., 2016; Chalmers et al., 2017). On the other hand, TEM, CTX-M-14, and CTX-M-15 have been found in E. coli isolated from UTIs in dogs in Canada (Khashayar, 2009).

### 3.3.3 Carbapenemases

Due to the proliferation and high prevalence of ESBLs, carbapenems are used more frequently to treat severe infections (Temkin et al., 2014); thus there is great concern over the spread of carbapenemases. These enzymes can be separated into two main groups, the serine carbapenemases (Class A and D) and the metallo-β-lactamases (Class B) (Temkin et al., 2014). For the Bush and Jacoby classification, carbapenemases encompass the enzymes in functional groups 2f and 2df. In 1991 carbapenem resistance was discovered in Japan in a S. marcescens isolate (Ito et al., 1995). The gene that conferred the resistance was later named IMP-1. In 1996 a carbapenem-resistant strain of K. pneumoniae was isolated from a human in North Carolina; however, it was not discovered until a subsequent investigation in 2001 (Yigit et al., 2001). The carbapenemase of this isolate was later named KPC and prevalence studies led to finding more resistant cases in the USA, Greece, Italy, and now they are reported worldwide (Nordmann, 2014). KPC is the only class A serine carbapenemase to have spread significantly in humans.

The two main class B carbapenemases are the Verona integron-encoded (VIM) metallo-β-lactamases and the New-Delhi metallo-β-lactamases (NDM). VIM was first identified in 1997 in P. aeruginosa isolated from an Italian patient’s surgical wound in Greece (Lauretti et al., 1999) and gradually became widespread. VIM-producing K. pneumoniae initially accounted for less than 1% of isolates from patients in Greek intensive care units, but increased to 50% of isolates by 2006 (Vatopoulos, 2008). NDM was isolated from a Swedish patient in 2009 who had travelled to New Delhi, India (Yong et al., 2009). Subsequent studies demonstrated that NDM was widespread in the Indian subcontinent and now it has spread worldwide. Lastly, with the identification of OXA-48 in K. pneumoniae isolated from a patient in 2001 in Turkey, it became the first carbapenemase categorized under class D (Poirel et al., 2004b). These main groups of carbapenemases (IMP, KPC, NDM, VIM and OXA-48) are now distributed across the world and have contributed to increasing alarm about carbapenem resistance, including in Canada (Nordmann, 2014).
The first report of carbapenemases in Enterobacteriaceae isolated from animals was not until 2012, when Fischer and collaborators found VIM-producing *E. coli* on a German pig farm (Fischer et al., 2012). These researchers also found three VIM-producing *S. enterica* isolates from poultry and pig farms in Germany a year later (Fischer et al., 2013). In 2015, 11.32% of 116 *E. coli* isolates from retail chicken meat were positive for the gene encoding the NDM carbapenemase in Egypt (Abdallah et al., 2015). In the USA, a study carried out from 2009-2013 found that the OXA-48 gene had an overall prevalence of 19.1% in extraintestinal pathogenic *E. coli* isolated from dogs and cats (Liu et al., 2016). Spain has also reported VIM-producing *K. pneumoniae* isolated from dogs (González-Torralba et al., 2016). In addition, Algeria has also reported occurrences of OXA-48 and NDM-5 in companion animals (Yousfi et al., 2016). For Canada, there are still no reports of carbapenemases in Enterobacteriaceae from companion animals or from domestic agriculture (Simmons et al., 2016), although they have been detected in imported seafood (Rubin et al., 2014; Janecko et al., 2016). If the prevalence of carbapenemases is extremely low, surveillance carried out by programs such as CIPARS may be unable to detect them.

### 3.4 Current situation with resistance to extended-spectrum cephalosporins (ESCs)

Different approaches have been proposed to control increasing ESC resistance. One of them is the development of new antimicrobials. For instance, the Infectious Diseases Society of America put forth a program to develop 10 new antimicrobials by 2020 (Bassetti et al., 2013). Researchers are also looking for new beta-lactamase/inhibitor combinations. Recently, ceftolozane-tazobactam and ceftazidime-avibactam were approved for clinical use (Papp-Wallace and Bonomo, 2016). Another approach is to investigate potential reservoirs of resistance genes and their transmission pathways, and then implement procedures and policies and/or increase public awareness to prevent transmission. For example, there has been evidence supporting the transfer of resistant bacteria from dogs to humans, and direct transfer between livestock and farm workers (Dahms et al., 2015; Schultz et al., 2015). In addition, there have been studies documenting the transfer of resistance genes between commensal and pathogenic Enterobacteriaceae in ground meat, the intestinal tract of turkeys, and a simulation of the swine intestine (Blake et al., 2003; Poppe et al., 2005; Walsh et al., 2008). Overall, transmission pathways of ESC-resistant Enterobacteriaceae are extremely complex and difficult to elucidate.
(Ewers et al., 2012). There is limited information on the potential reservoirs and transfer pathways of ESC-resistant bacteria specifically, as well as their resistance genes. Therefore, there is a need to investigate the transfer of ESC resistance genes between commensals and pathogens in Enterobacteriaceae.

4. THESIS PROPOSAL OVERVIEW

4.1 Rationale

The frequency and diversity of ESC resistance determinants found in Enterobacteriaceae are steadily increasing (Nordmann, 2014). The World Health Organization has classified ESCs as critically important antimicrobials for human medicine (WHO, 2017). ESCs are also frequently used in animals (Gandolfi-Decristophoris et al., 2013), but there is limited information on the situation of ESC resistance for bacteria from animals in general and particularly in Canada. Furthermore, AMR studies have mainly focused on only E. coli and S. enterica. There is little information on whether other Enterobacteriaceae species such as Klebsiella spp., Citrobacter spp., Proteus spp, and Enterobacter spp. can serve as reservoirs for genetic determinants of ESC resistance. This is an important area to investigate because resistance genes can be transferred between commensal and pathogenic strains of Enterobacteriaceae in the intestinal tract (Blake et al., 2003). More information is needed to better understand the complex epidemiology of ESC resistance and the potential relations between animals and humans in this context. The three animal species chosen for this study were chickens, dogs, and pigs. Chickens and pigs were chosen in particular because they are two major livestock commodities in Ontario. Dogs were also chosen because of their importance as companion animals in Ontario and the close relationship they have with humans. In addition, all three animal species have the potential of transmitting resistant bacteria to humans.

4.2 Hypothesis

It was hypothesized that if there are barriers between the bacterial populations, and/or differences in the mobile genetic elements between bacterial and animal host species, it would result in compartmentalization of ESC resistance genes (Fig. 1.3). Secondly, it was hypothesized that due to widespread horizontal gene transfer, ESC resistance genes would be similar between commensals and pathogens from an animal host.
Fig. 1.3. A conceptual diagram outlining some of the different compartments with arrows representing a subset of potential transfer pathways of ESC resistance determinants and the red boxes highlight the focus of the project. The strains and species listed under ‘pathogenic bacteria’ can cause disease, but they can also be found in healthy animals.

4.3 Objectives

1. The first objective was to determine the prevalence of ESC resistance in Enterobacteriaceae isolated from chicken, canine, and swine clinical samples submitted to two major diagnostic laboratories in Ontario.

2. The second objective was to assess the frequency of chickens, dogs, and pigs carrying ESC-resistant Enterobacteriaceae in their intestine.
3. The third objective was to determine the prevalence of resistance to ESCs and other clinically relevant antimicrobials in generic fecal *E. coli* from dogs.

4. The fourth objective was to characterize and compare the distribution of major ESC resistance genes within each of the three animal species between Enterobacteriaceae species and between clinical and fecal isolates, as well as make overall comparisons between the animal host species.

5. The fifth and the last objective was to perform whole-genome sequencing on canine and swine CTX-M-positive isolates in order to investigate the genetic similarities between isolates, as well as identify the *blaCTX-M* variants, sequence types, and other AMR genes present. This information was necessary to determine if there were important clonal lineages present.

### 4.4 Methods

The goal of the study was to collect ESC-resistant *E. coli*, *S. enterica*, *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp., *Pantoea* spp., and *Serratia* spp. isolates (Fig. 1.4). The sample collection consisted of clinical isolates as well as commensal bacteria isolated from cecal and fecal matter. Sampling took place in Ontario from November 2015 to November 2016. Clinical isolates were obtained through the Animal Health Laboratory (AHL) of the University of Guelph and IDEXX Laboratories in Markham, Ontario. The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) routinely collects cecal matter from broiler chickens and pigs in abattoirs across Canada; for this study we restricted our collection to samples from pigs and chickens raised in Ontario. Thus, CIPARS provided aliquots of each cecal sample for isolation of ESC-resistant commensal Enterobacteriaceae. The target sample size of 200 fecal samples from presumably healthy dogs was reached through sampling in off-leash dog parks. Field sampling took place in the summer of 2016 and in ten different cities across Southern Ontario. For the purposes of this thesis, the term “clinical isolates” only represented Enterobacteriaceae isolated from clinical samples that were submitted to the two major diagnostic laboratories AHL and IDEXX. This included any Enterobacteriaceae that was isolated from fecal samples that were submitted to the diagnostic laboratories. The term “clinical isolates” does not necessarily mean all isolates referred in such a manner have the capability to
cause clinical disease; the term was used to describe the origins of the isolates. In addition, the terms “cecal isolates” and “fecal isolates” strictly represented those that were obtained through enrichment of cecal and fecal samples from CIPARS and dog parks, respectively. The terms were used in the context of these definitions for the entire thesis.

4.4.1 AHL Sample Collection

AHL receives an extremely high number of clinical chicken cases. Due to limitations in time and resources, systematic sampling was employed for chicken *E. coli* in which only isolates from the first clinically relevant chicken case of the day was collected. *E. coli* was collected from cases of colibacillosis or high mortality. Any *S. enterica* strains isolated from clinical samples were collected. For pigs, *S. enterica* and K88-positive *E. coli* from cases of neonatal diarrhea and post-weaning diarrhea were collected. Isolates of *S. enterica* were also collected from apparently healthy swine carriers. All Enterobacteriaceae species of interest were collected from canine cases of UTIs, otitis, dermatitis, wound infections, surgical site infections or septicemia/bacteremia.

4.4.2 IDEXX Sample Collection

As IDEXX only processes companion animal samples, the same criteria used for AHL canine samples were applied for IDEXX. However, only a subset of samples was examined because IDEXX receives a large number of samples. These included 1) the first 10 *E. coli* isolates, 2) the first 10 non-*E. coli* Enterobacteriaceae isolates of the month, and 3) the first 10 ESC-resistant Enterobacteriaceae of the month which was further split into 3 ESC-resistant *E. coli* isolates and 7 ESC-resistant non-*E. coli* Enterobacteriaceae isolates. The non-ESC-resistant Enterobacteriaceae isolates were screened for resistance via susceptibility testing to determine the prevalence of ESC-resistance in canine clinical isolates.

4.4.3 CIPARS Cecal Samples and Canine Fecal Samples

The CIPARS cecal samples were aliquoted and frozen inBrucella(1,3),(996,993) broth and glycerol. The canine fecal samples were processed within 24 hours of collecting them. ESC-resistant Enterobacteriaceae were isolated by enriching the cecal and fecal matter in a 1:10 dilution of Enterobacteriaceae Enrichment broth containing cefotaxime, and then plating it onto
chromogenic selective media containing ceftriaxone (Rapid Enterobacteriaceae *Escherichia coli* Coliform Agar). Oxidase and catalase tests were used for presumptive confirmation of Enterobacteriaceae species before final identification at the species level via MALDI-TOF mass spectrometry.

**4.4.4 ESC-resistant Isolate Characterization**

All clinical and commensal isolates were tested for susceptibility to cefoxitin, ceftriaxone, ceftazidime, and ertapenem (for the detection of carbapenem resistance) via the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines. The ESC-resistant Enterobacteriaceae were then screened using existing generic PCR protocols for the presence of major families of ESC resistance genes, *bla*<sub>CMY</sub>, *bla*<sub>CTX-M</sub>, and *bla*<sub>SHV</sub>. Any isolates showing reduced susceptibility to carbapenems were also screened for *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub>. Univariable statistical analyses such as logistic regression was used to compare the distribution of these genes among the different sources and bacterial species.

**4.4.5 Whole-genome sequencing of CTX-M-positive isolates**

Whole-genome sequencing was performed on the canine and swine isolates that were PCR-positive for *bla*<sub>CTX-M</sub>. DNA was extracted from all the canine and swine isolates that were PCR-positive for *bla*<sub>CTX-M</sub> using the EpiCentre MasterPure DNA purification kits. MiSeq sequencing of these isolates was completed at the Advanced Analysis Centre of the Genomics Facility at the University of Guelph. Assembly, analysis, and assigning multi-locus sequence types (MLSTs) for the isolates were carried out with the BioNumerics v7.6 application. A brief overview of the sample collections and methodologies that were used in this study are presented in Fig. 1.4.
Fig. 1.4. Brief overview of the isolate collections and the sampling strategies employed in the project.
CHAPTER 2: PREVALENCE AND MECHANISMS OF EXTENDED-SPECTRUM CEPHALOSPORIN RESISTANCE IN CLINICAL AND COMMENSAL ENTEROBACTERIACEAE ISOLATES FROM DOGS IN CANADA

Authors: Pauline L. C. Zhang\textsuperscript{a}, Xiao Shen\textsuperscript{a}, Gabhan Chalmers\textsuperscript{d}, Richard J. Reid-Smith\textsuperscript{a,b,c}, Durda Slavic\textsuperscript{d}, Hani Dick\textsuperscript{e}, Patrick Boerlin\textsuperscript{a,*}.

\textsuperscript{a}Department of Pathobiology and \textsuperscript{b}Department of Population Medicine, Ontario Veterinary College, University of Guelph, 50 Stone Rd. E, Guelph, Ontario, N1G 2W1, Canada.
\textsuperscript{c}Centre for Food-borne, Environmental and Zoonotic Infectious Diseases, Public Health Agency of Canada, 160 Research Lane, Suite 103, Guelph, Ontario, N1G 5B2, Canada.
\textsuperscript{d}Animal Health Laboratory, Post Office 3612, Guelph, Ontario, N1H 6R8, Canada.
\textsuperscript{e}IDEXX Laboratories, 1345 Denison St., Markham, Ontario, L3R 5V2, Canada.

*Corresponding author.
E-mail address: pboerlin@uoguelph.ca

To be submitted for publication in the journal ‘Veterinary Microbiology’
ABSTRACT

There is relatively little information on the genetic basis of resistance to the critically important extended-spectrum cephalosporins (ESCs) in Enterobacteriaceae from dogs in Canada. This study assessed the frequency of ESC resistance in Enterobacteriaceae isolated from clinically ill and healthy animals, and the distribution of major ESC resistance genes in these bacteria. A total of 543 Enterobacteriaceae were isolated from 506 clinical samples from two diagnostic laboratories in Ontario. Eighty-eight ESC-resistant Enterobacteriaceae and 217 generic fecal Escherichia coli were isolated from 234 fecal samples from healthy dogs. These fecal isolates were tested for ESC resistance along with the clinical isolates. Isolates with reduced ESC susceptibility were screened for $\text{bla}_{\text{CMY}}$, $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{SHV}}$, and all CTX-M-positive isolates underwent whole-genome sequencing. The prevalence of ESC resistance in clinical Enterobacteriaceae was 10.4%. The average frequency of fecal carriage of ESC-resistant Enterobacteriaceae in healthy dogs was 26.5%, but they were present in low concentrations. The majority of ESC-resistant isolates were $E. \ coli$, suggesting that other commensal Enterobacteriaceae species may not serve as major reservoirs for ESC resistance genes. The results imply that there may be transfer of ESC resistance genes between clinical and fecal Enterobacteriaceae in dogs. The diversity of sequence types (including ST131 and ST648) and CTX-M variants (including CTX-M-14, 15, and 27) support the transfer of resistant bacteria between humans and dogs. CTX-M-1 was frequently found in canine fecal Enterobacteriaceae, while it is still rare in Enterobacteriaceae from clinical infections in humans in Canada, suggesting transfer of resistant bacteria to dogs from food animals or other sources.

1. INTRODUCTION

Enterobacteriaceae cause a variety of infections in dogs, with urinary tract infections (UTIs) being one of the most frequent (Windahl et al., 2014). Enterobacteriaceae such as Klebsiella pneumoniae and Proteus mirabilis are regularly isolated from canine UTIs, but the most common pathogen is Escherichia coli (Windahl et al., 2014). Successful treatment of such infections often requires antimicrobials, in particular $\beta$-lactams, but antimicrobial resistance (AMR) is increasing (Ball et al., 2008). $\beta$-lactam resistance is mostly caused by the production of $\beta$-lactamases, of which a variety can hydrolyze and inactivate extended-spectrum cephalosporins.
(ESCs). This is especially concerning because ESCs are considered critically important antimicrobials for human and veterinary medicine (OIE, 2015; WHO, 2017).

ESC resistance is mainly caused by the expression of AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs), and more recently carbapenemases (Courtice et al., 2016). ESC-resistant Enterobacteriaceae that produce β-lactamases such as CMY, TEM-52, CTX-M, and OXA-48 have been observed in dogs throughout North America (Khashayar, 2009; O’Keefe et al., 2010; Liu et al., 2016). Additionally, multi-drug resistance (MDR) has been increasingly reported in *E. coli* isolated from canine UTIs (Liu et al., 2016).

AMR in bacteria from dogs is also a potential public health risk because pets can serve as reservoirs of resistant bacteria to which humans are exposed (Guardabassi et al., 2004). The potential for transmission of *E. coli* between humans and companion animals is illustrated for instance by the isolation of an identical *E. coli* clone from a dog’s UTI and from its household members’ feces (Johnson et al., 2008). The transmission potential is also shown by the sequence type (ST) 131, which has successfully spread worldwide in humans and animals, and causes millions of resistant infections each year (Pitout and DeVinney, 2017). CTX-M-producing *E. coli* ST131 have been recovered from companion animals in North America, Europe, and Asia (Khashayar, 2009; Ewers et al., 2014; Kawamura et al., 2017). Most of the previous studies that investigated AMR in Enterobacteriaceae from dogs in Canada did not examine the associated resistance genes, and the information available is mainly limited to *E. coli* and *Salmonella* (Prescott et al., 2002; Authier et al., 2006; Ogeer-Gyles et al., 2006b; Ball et al., 2008; Murphy et al., 2009; Courtice et al., 2016). More complete knowledge in this area would help guide antimicrobial treatment, particularly since therapy is usually initiated before receiving susceptibility results and some treatment guidelines suggest that if resistance to a particular antimicrobial exceeds 10%, it should not be used for first-line treatment (Weese et al., 2011). In addition, UTIs are often caused by Enterobacteriaceae from the host’s own fecal flora, and there is evidence that AMR genes can be transferred from commensal to pathogenic Enterobacteriaceae (Blake et al., 2003). Therefore, knowing the frequency of AMR in canine fecal bacteria, without the bias present in passive diagnostic data, would be of interest as well.
The goal of this work was to study AMR among Enterobacteriaceae in dogs within the province of Ontario, with an emphasis on ESC resistance. It was focused on *E. coli*, *Salmonella enterica*, *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., and *Pantoea* spp. The study had four main objectives. The first was to assess the prevalence of ESC resistance in Enterobacteriaceae isolated from canine clinical samples submitted to diagnostic laboratories. The second was to determine the prevalence of resistance to ESCs and other clinically relevant antimicrobials in generic fecal *E. coli* from dogs. The third objective was to assess the frequency of fecal carriage of ESC-resistant Enterobacteriaceae in the local dog population. The last objective was to assess and compare the distribution of major ESC resistance genes and their variants between Enterobacteriaceae species as well as between clinical and fecal isolates.

2. MATERIALS AND METHODS

A total of 543 clinical isolates were collected from the Animal Health Laboratory (AHL), University of Guelph, Guelph, Ontario (n=211) and IDEXX Laboratories Inc., Markham, Ontario (n=332) from November 2015 to October 2016. For both laboratories, Enterobacteriaceae from the species of interest were collected from cases of UTIs, otitis, dermatitis, wound infections, surgical site infections, or septicemia. All the isolates available during the period of investigation were collected from AHL, while for IDEXX, a stratified systematic sampling strategy was used to collect the first ten *E. coli* isolates of each month, and the first ten non-*E. coli* Enterobacteriaceae isolates of each month, in order to determine the general prevalence of ESC resistance in *E. coli* and other Enterobacteriaceae (Fig. 2.1). Furthermore, since IDEXX systematically screens for ESC resistance, purposive sampling was used to collect the first ten ESC-resistant Enterobacteriaceae of the month in order to assess resistance determinants (Fig. 2.1). These ten isolates were further categorized into three ESC-resistant *E. coli* isolates and seven ESC-resistant non-*E. coli* Enterobacteriaceae isolates (Fig. 2.1). All clinical isolates were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany).
2.2 Fecal isolate collection

A total of 234 canine fecal samples were collected from off-leash dog parks in ten different cities in Southern Ontario. Locations were selected based on major urban areas within a driving distance from the laboratory of less than two hours (Appendix 1, Fig. 2.S1). A minimum of 20 samples were collected from two different dog parks at each location, with only one exception. The dog owners were provided with new unopened plastic bags and requested to provide each of their dog's feces to the research team. Samples were kept at 4°C and processed within 24 hours. To determine the frequency of resistance toward a range of antimicrobials in fecal *E. coli*, the samples were directly plated onto Rapid Enterobacteriaceae *Escherichia coli* Coliform Agar (REBECCA; bioMérieux, Marcy-l'Étoile, France) without antimicrobials and one presumptive *E. coli* colony was isolated per sample, when present (Fig. 2.1). The fecal samples were also enriched for ESC-resistant Enterobacteriaceae (Fig. 2.1) using 1.0 g of feces to inoculate 9.0 mL of EE Broth Mossel Enrichment (Becton Dickinson [BD], Cockeysville, MD, USA) supplemented with 2 µg/mL cefotaxime. After overnight 37°C incubation with agitation, 10 µL of the broth culture was streaked onto REBECCA supplemented with 1 µg/mL ceftriaxone (Sigma-Aldrich, St. Louis, MO, USA). Each different colony morphotype seen on this primary plate was subcultured and purified onto REBECCA supplemented with ceftriaxone. Oxidase tests (Sigma-Aldrich) and catalase tests were conducted on isolates to confirm presumptive Enterobacteriaceae identification. Further identification at the species level was done using MALDI-TOF MS (Bruker).

2.3 Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for ampicillin, ticarcillin, cefazolin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, cefoxitin, cefpodoxime, imipenem, trimethoprim/sulfamethoxazole, chloramphenicol, doxycycline, amikacin, and gentamicin were determined for the generic fecal *E. coli* isolates using the Sensititre COMPAN1F plate (TREK Diagnostics, Cleveland, OH, USA). Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015).

All clinical isolates, as well as fecal isolates from enrichment, were tested for ESC susceptibility by the disk diffusion method according to the CLSI guidelines. The antimicrobials
used were cefoxitin (FOX-30), cefotaxime (CTX-30), and ceftazidime (CAZ-30) (Becton Dickinson [BD]). Ertapenem (ETP-10) was also used to test for reduced susceptibility to carbapenems (Becton Dickinson [BD]). The reference strains E. coli ATCC 25922 and K. pneumoniae ATCC 700603 were used as quality controls. An overview of the different sample collections and methodologies used are shown in Fig. 2.1.

2.4 PCR screening of ESC resistance genes

All isolates with reduced susceptibility to cefoxitin and/or a third-generation cephalosporin (cefotaxime and ceftazidime) were screened for the presence of three main ESC resistance genes, *bla*<sub>CMY</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, using single and multiplex PCR amplification, as described previously (Kozak et al., 2009; Cottell et al., 2013). Lysates were obtained by boiling cell suspensions in PCR-grade water for 15 minutes and then centrifuged prior to PCR. In addition, all isolates with reduced susceptibility to ertapenem, as well as isolates with inhibition zones slightly above the intermediate breakpoint (18 mm - 22 mm), were screened for the presence of *bla*<sub>VIM</sub>, *bla*<sub>AMP</sub>, *bla*KPC, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub>, as described previously (Mataseje et al., 2012). Sanger sequencing was used to determine any *bla*<sub>SHV</sub> variants in non-Klebsiella spp. using the primers SHV-F; ATTACCGACCGGCATCTTTC and SHV-R; CCTTTATCGGCCTTCACTCA.

2.5 Genome sequencing and analysis

Due to CTX-M’s relatively recent emergence in North American animals, genomic DNA was extracted for MiSeq sequencing (Illumina, San Diego, CA, USA) from all of the ESC-resistant isolates that were PCR-positive for *bla*<sub>CTX-M</sub> (Fig. 2.1). DNA extractions were carried out using the EpiCentre MasterPure DNA Purification Kit (EpiCentre Biotechnologies, Madison, WI, USA), as per manufacturer’s instructions. Nextera XT kits (Illumina) were used to prepare libraries, and sequencing was carried out at the Advanced Analysis Centre of the Genomics Facility at the University of Guelph (Guelph, ON, Canada). Assembly was performed using the wgs application for BioNumerics v7.6 (Applied Maths, Austin, TX, USA) and the SPADES algorithm, as well as assembly-free and assembly-based allele calling. The multi-locus sequence types (MLST) for *E. coli* were assigned using the wgMLST application for BioNumerics and the BioNumerics *Escherichia coli/Shigella* Enterobase scheme. Sequence types were assigned for
the *K. pneumoniae* isolates using the PasteurMLST website (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public). Antimicrobial resistance genes were identified using the *E. coli* functional genotyping plugin for BioNumerics.

### 2.6 Statistical methods

Statistical analyses were carried out using STATA 14 (Stata Corporation, College Station, TX, USA). Mixed-effects logistic regression analyses were used to investigate potential clustering of canine fecal carriage of ESC-resistant Enterobacteriaceae by dog parks and by cities. In addition, logistic regression was used to determine if ESC resistance was associated with location. Logistic regression was also used to compare if the frequencies of *bla*<sub>CMY</sub> and *bla<sub>CTX-M</sub>* were significantly different between clinical and fecal *E. coli* isolates. For the isolates that underwent whole-genome sequencing (WGS), Fisher’s exact tests were used to determine if the five most frequent *bla<sub>CTX-M</sub>* genes were significantly associated with other AMR genes. A *p*-value of ≤0.05 was considered significant.

### 3. RESULTS

#### 3.1 Diversity, ESC susceptibility, and genotypes of clinical isolates

The overall prevalence of resistance to cefepime and/or ESCs was 10.4% (n=22) of 211 isolates from AHL. The majority of clinical ESC-resistant isolates from AHL were *E. coli* (n=15), followed by *Enterobacter cloacae* (n=3), *Enterobacter aerogenes* (n=2), *Citrobacter braakii* (n=1), and *Proteus mirabilis* (n=1; Table 2.1). One *E. coli* and two *E. cloacae* isolates had reduced susceptibility to ertapenem and inhibition zones close to the breakpoint of 19 mm (15-18 mm), but all three were negative for the carbapenemase genes tested.

The prevalence of resistance to cefepime and/or ESCs in IDEXX’s stratified systematic sampling isolates was 10.4% (n=24 of 231 isolates). The diversity of bacterial species was similar to those from AHL, with *E. coli* being the most frequent (n=16) followed by *P. mirabilis* (n=4), *E. cloacae* (n=2), and one isolate each of *Enterobacter asburiae* and *Enterobacter kobei* (Table 2.1). None of these isolates had reduced susceptibility to ertapenem. Overall, the pooled prevalence of cefepime and ESC resistance for clinical Enterobacteriaceae
isolates from AHL and IDEXX’s was 10.4% (n=46 of 442 isolates). Of these 442 isolates, 3.6% and 2.3% were confirmed by PCR to be positive for bla\text{CMY} and bla\text{CTX-M}, respectively. For \textit{E. coli} specifically, the prevalence of cefamycin and ESC resistance was 8.9% (n=22) and 10.5% (n=26 of 248 isolates), respectively.

For IDEXX’s purposive sampling of ESC-resistant isolates, a total of 101 isolates were collected. The diversity of bacterial species collected were similar to those of AHL and IDEXX’s systematic stratified sampling, with the exception of \textit{Klebsiella oxytoca} and \textit{K. pneumoniae}. The majority of ESC-resistant isolates were \textit{E. coli} (n=34), followed by \textit{E. cloacae} (n=21), \textit{P. mirabilis} (n=16), \textit{K. pneumoniae} (n=13), \textit{E. aerogenes} (n=5), \textit{E. asburiae} (n=3), \textit{E. kobei} (n=3), \textit{C. freundii} (n=2), \textit{E. ludwigii} (n=2), and \textit{K. oxytoca} (n=2; Table 2.1). The only SHV-positive \textit{E. cloacae} isolate carried a \textit{bla\text{SHV}}-12. There were six isolates (four \textit{E. cloacae}, one \textit{E. kobei}, and one \textit{E. asburiae}) with reduced susceptibility to ertapenem (14-16 mm), but all were negative for the carbapenemase genes tested.

The ESC-resistant clinical isolates categorized by bacterial species and their corresponding genotypes are shown in Table 2.1. In comparison to \textit{E. coli}, \textit{K. oxytoca}, \textit{K. pneumoniae}, and \textit{P. mirabilis}, there were species that had relatively high proportions of isolates that tested negative for all of the resistance genes tested: \textit{E. cloacae}, \textit{E. aerogenes}, \textit{E. asburiae}, \textit{E. kobei}, \textit{E. ludwigii}, \textit{C. braakii}, and \textit{C. freundii}. In addition, the majority of \textit{K. pneumoniae} isolates were \textit{bla\text{SHV}}-positive. The disk diffusion phenotypes of all the clinical \textit{E. coli} isolates with reduced susceptibility to cefoxitin and ESCs, and their corresponding genotypes are shown in Table 2.2. The gene \textit{bla\text{CMY}} was the most frequently detected in ESC-resistant \textit{E. coli}, followed by \textit{bla\text{CTX-M}}. All clinical ESC-resistant \textit{E. coli} were PCR-negative for \textit{bla\text{SHV}}, and all isolates that were only resistant to cefoxitin were negative for the resistance genes tested. Multiple isolates were PCR-positive for more than one gene: three \textit{E. coli} with \textit{bla\text{CMY}} and \textit{bla\text{CTX-M}}, ten \textit{K. pneumoniae} with \textit{bla\text{CMY}} and \textit{bla\text{SHV}}, and two \textit{K. pneumoniae} with \textit{bla\text{CTX-M}} and \textit{bla\text{SHV}}.

3.2 Diversity, antimicrobial susceptibility and genotypes of fecal isolates

The results of broth microdilution testing for clinically relevant antibiotics for 217 generic fecal \textit{E. coli} isolates recovered without antibiotic selection are shown in Fig. 2.2. AMR
prevalence was highest for ampicillin at 9.7% and lowest for amikacin and imipenem at 0%. The prevalence of resistance to cephemycins and/or ESCs was 3.2% (n=7 of 217 isolates).

ESC-resistant Enterobacteriaceae were detected in 62 out of 234 fecal samples after enrichment. Based on the presence of different colony morphotypes, 88 isolates were collected from these 62 samples. The weighted average frequency of canine fecal carriage of ESC-resistant Enterobacteriaceae was 26.5% ± 7.9% (Appendix 1, Fig. 2.S2). The species diversity of resistant isolates differed from the clinical ones and comprised E. coli (n=81), Pluralibacter gergoviae (n=2; formerly classified as Enterobacter gergoviae), E. cloacae (n=2), C. freundii (n=1), E. kobei (n=1), and P. mirabilis (n=1; Table 2.1). There were no isolates with reduced susceptibility to ertapenem. Also, the species C. freundii, E. cloacae, E. kobei, and P. gergoviae had a high proportion of isolates that were negative for all genes tested. The disk diffusion phenotypes of all the fecal E. coli isolates with reduced susceptibility to cefoxitin and ESCs, and their corresponding genotypes are shown in Table 2.2. The most frequent ESC resistance gene was blacMY, followed by blactXM (Table 2.2). All fecal E. coli with reduced susceptibility to cefoxitin and ESCs were PCR-negative for blashv.

3.3 Whole-genome sequencing of CTX-M-positive isolates

WGS was conducted for 28 CTX-M-positive isolates of clinical origin (24 E. coli, two K. pneumoniae, one E. cloacae, and one K. oxytoca). The most frequent ST in the E. coli isolates was ST131 (n=10), followed by two isolates each of ST38 and ST5612. There was one E. coli isolate each for ST44, ST69, ST117, ST224, ST354, ST457, ST648, ST1193, ST6478, and ST4891. The two K. pneumoniae isolates were ST711. K. oxytoca and E. cloacae were not assigned STs. The CTX-M variants identified in the clinical isolates were CTX-M-15 (n=12), CTX-M-27 (n=7), CTX-M-55 (n=5), CTX-M-14 (n=2), CTX-M-169 (n=1), and a new variant CTX-M-202 [n=1 (GenBank accession number MF195067)].

WGS was conducted for 19 ESC-resistant CTX-M-producing isolates of fecal origin (18 E. coli and one P. gergoviae). The most frequent ST in the fecal isolates was ST648 (n=5), followed by ST131 (n=2), and then one isolate each of ST10, ST38, ST58, ST68, ST69, ST372, ST393, ST1730, and ST4110. Two E. coli and the one P. gergoviae isolate were not assigned STs. The CTX-M variants in the fecal isolates were CTX-M-1 (n=6), CTX-M-15 (n=5), CTX-
M-14 (n=3), CTX-M-27 (n=2), CTX-M-55 (n=2), and CTX-M-124 (n=1). A minimum spanning tree based on 3223 genes was created using wgMLST for all CTX-M-positive isolates from the study on which the different STs (Fig. 2.3) and CTX-M variants (Fig. 2.4) are superposed. Overall, ST131 was the most frequent, followed by ST648, and both of these STs formed clear wgMLST clusters (Fig. 2.3). In contrast, the different CTX-M variants were not clearly associated with any specific wgMLST cluster (Fig. 2.4).

Overall, the most frequent CTX-M variants were \(\text{bla}_{\text{CTX-M-15}}\), \(\text{bla}_{\text{CTX-M-27}}\), \(\text{bla}_{\text{CTX-M-55}}\), \(\text{bla}_{\text{CTX-M-1}}\), and \(\text{bla}_{\text{CTX-M-14}}\) (Table 2.3). A variety of resistance genes for other antimicrobial agent classes were found in both the clinical and fecal isolates, including aminoglycosides, fluoroquinolones, \(\beta\)-lactams, chloramphenicol, fosfomycin, macrolides, quinolones, sulfonamides, trimethoprim, and tetracyclines (Appendix 1, Table 2.5). The most frequent resistance genes were \(\text{strA}\), \(\text{strB}\), \(\text{sul1}\), \(\text{sul2}\), \(\text{tet}(A)\), and \(\text{mph}(A)\). CTX-M-1 was only found in the resistant fecal isolates, and the resistance genes \(\text{oqxA}\) and \(\text{oqxB}\) were found in \(K.\ pneumoniae\) isolates.

The inclusion of parks and cities as random effects in our multi-level model were not statistically significant (Wald test=6.53, df=9, \(p\)-value=0.6856). The frequencies of \(\text{bla}_{\text{CMY}}\) and \(\text{bla}_{\text{CTX-M}}\) were not significantly different between ESC-resistant clinical and ESC-resistant fecal \(E.\ coli\) isolates (Table 2.4). Although it was not significant, statistical analyses suggested that the odds of ESC-resistant clinical \(E.\ coli\) carrying \(\text{bla}_{\text{CTX-M}}\) was two times that of ESC-resistant fecal \(E.\ coli\) (Table 2.4). The \(\text{bla}_{\text{CTX-M-15}}\) gene was significantly associated with \(\text{aac(3)-IIa}\), \(\text{aac(6')Ib-cr}\), \(\text{catB3}\), and \(\text{dfrA14}\) (Table 2.4). No significant associations were detected between \(\text{bla}_{\text{CTX-M-1}}\), \(\text{bla}_{\text{CTX-M-14}}\), \(\text{bla}_{\text{CTX-M-27}}\), \(\text{bla}_{\text{CTX-M-55}}\), and other AMR genes.

4. DISCUSSION

The prevalence of ESC resistance in clinical \(E.\ coli\) in Ontario in this study (10.4%) was higher than previous findings for Ontario and Québec (4-5% and 2%, respectively) in \(E.\ coli\) from canine UTIs (Khashayar, 2009). In addition, the prevalence of ESC resistance has exceeded the threshold of 10%, so ESCs should not be used for first-line treatment (Weese et al., 2011). For cefoxitin, this study had similar resistance frequencies (8.8%) to Khashayar’s report of 5% and 12% for Ontario and Québec, respectively. Other studies in Canada found that the
prevalence of ESC resistance was either much lower (1.8%) or absent in canine *E. coli* urinary isolates (Ogeer-Gyles et al., 2006a; Courtice et al., 2016). These differences in prevalence may reflect increases in AMR and geographical differences. Alternatively, they might be due to the inclusion of isolates from general clinical infections, as previous studies were only on UTI isolates. This is supported by results in the USA where the prevalence of resistance to cefoxitin and ESCs for clinical *E. coli* isolates from dogs ranged from 10.1-13.9% (Thungrat et al., 2015). Of clinical isolates in this study, 2.3% were CTX-M-producers, which is similar to previous studies in the USA where 1-3% among clinical *E. coli* from companion animals were ESBL-producers (O’Keefe et al., 2010; Liu et al., 2016). No carbapenemase-producing isolates were detected, suggesting that carbapenemases are still absent or rare in canine Enterobacteriaceae in Canada, despite their increasing presence in human pathogens (Logan and Weinstein, 2017).

Prevalence estimates for AMR based on passive surveillance of clinical samples, albeit valuable information, often suffer of diagnostic-associated bias and are likely overestimated. Thus, it is important to investigate AMR in generic fecal *E. coli* in dogs, which form the reservoir for disease-causing strains outside of a clinical setting. Our results showed that the prevalence of CTX-M-producers in generic fecal *E. coli* in presumably healthy dogs was 0.5%. This low frequency is similar to previous studies in Ontario and the UK, where ESBL-producing *E. coli* was either absent or infrequent (1.9%) in healthy companion animals visiting veterinarians (Murphy et al., 2009; Wedley et al., 2017).

Enrichment culture showed that approximately every fourth dog in urban areas in Southern Ontario sheds ESC-resistant Enterobacteriaceae in its feces. However, a comparison with the results without enrichment (3.2% ESC-resistant) suggests that, although widespread, ESC-resistant bacteria were only present in low concentrations. This is supported by previous studies that used non-selective media and found low frequencies of ESC resistance in *E. coli* in dogs recruited from dog parks (3.8% and 4.5%) (Leonard et al., 2012; Procter et al., 2014). The majority of ESC-resistant fecal isolates were *E. coli*, suggesting that other commensal Enterobacteriaceae may not serve as significant reservoirs of ESC resistance genes. Relatively high numbers of ESC-resistant isolates were negative for all of the resistance genes tested. However, many of these were from species other than *E. coli*, in particular *Enterobacter* spp. and *Citrobacter* spp. ESC resistance could have been caused by overexpression of chromosomal
AmpC β-lactamases which is frequently seen in *Enterobacter* spp. (Livermore, 1995) or by other mechanisms such as porin modifications which were not investigated in this study (Babouee Flury et al., 2016). To the best of our knowledge, this was the first report of *bla*<sub>SHV-12</sub> in *E. cloacae* from a dog in Canada, which is concerning as *bla*<sub>SHV-12</sub> is frequently located on MDR plasmids (Alonso et al., 2017). Most of the resistant *K. pneumoniae* isolates were positive for *bla*<sub>SHV</sub>, which was most likely the gene variant *bla*<sub>SHV-1</sub>, as it is frequently found on the chromosome of *K. pneumoniae* (Livermore, 1995). Similarly, the *oqxAB* genes found in *K. pneumoniae* were most likely chromosomally encoded (Guillard et al., 2016).

The minimum spanning tree of CTX-M-producing ESC-resistant *E. coli* isolates showed a large diversity of STs. The two most frequent STs were ST131 and ST648, which are both recognized human pandemic clones that have also emerged in companion animals (Ewers et al., 2014; Pitout and DeVinney, 2017). Interestingly, most isolates of the ST131 cluster (10/14) were clinical infections, while only one out of the six ST648 was of clinical origin. A variety of CTX-M subtypes similar to those described previously were found in both clusters (Ewers et al., 2014). The most frequent CTX-M variants in this study were also some of the most frequently found in past studies that investigated *E. coli* from companion animals in Japan and the USA (O’Keefe et al., 2010; Liu et al., 2016; Kawamura et al., 2017), as well as in *E. coli* from humans in general (Bevan et al., 2017). The main CTX-M variants were seen throughout the tree and were not restricted to a single cluster, thus illustrating once more the frequency of horizontal gene transfer of *bla<sub>CTX-M</sub>* genes (Bevan et al., 2017). The frequencies of *bla<sub>CMY</sub>* and *bla<sub>CTX-M</sub>* were not significantly different between ESC-resistant clinical and ESC-resistant fecal *E. coli*, suggesting transfer of resistance genes between these isolates. Alternatively, the pathogens and fecal flora may belong to the same population since the fecal flora could be the source of disease-causing isolates. In contrast to the other CTX-M variants, CTX-M-1 was only detected in fecal *E. coli* isolates, and not in clinical ones. Furthermore, CTX-M-1 has been extremely rare in human clinical isolates in Canada (M. Mulvey, personal communication) and is more frequently found in *E. coli* from poultry (Boerlin and Chalmers, unpublished data). Recently, CTX-M-1 was also identified in cecal *E. coli* from multiple pigs (Zhang and Boerlin, unpublished data). These findings suggest that CTX-M-1 may be entering bacteria from the gut flora of dogs in Canada through sources other than humans. The most likely origin of these may be poultry and/or pigs, either through the consumption of raw food or other transmission pathways.
Overall, resistance to antimicrobial agents other than β-lactams in CTX-M-positive isolates was highest for aminoglycosides, sulfonamides, tetracyclines, and macrolides. Unsurprisingly, the corresponding genes [strA, strB, sul1, sul2, tet(A)] are also the most frequently observed AMR genes in Enterobacteriaceae from animals in general, and confer resistance to antimicrobials that have been used for decades. The significance of the widespread presence of the macrolide resistance gene mph(A) in bacteria considered naturally resistant to macrolides, is more difficult to assess and may warrant further investigation. The statistically significant associations between blaCTX-M-15 and the other resistance genes suggest that they are found on the same plasmid. This is very likely for blaCTX-M-15 and aac(6')Ib-cr, indicating a worrisome co-location that has already been reported in a variety of organisms and geographical locations (Carattoli, 2009).

Our study provided novel data on the prevalence and genetic basis of ESC resistance in clinical and fecal canine Enterobacteriaceae. Based on the results, ESCs should not be used as first-line treatment in dogs. We observed a high frequency of fecal carriage of ESC-resistant Enterobacteriaceae in dogs in Southern Ontario, but they seemed to be present in low concentrations only, as ESC resistance was infrequent in generic fecal E. coli isolated using non-selective methods. To our knowledge, this was also the first report of SHV-12-positive E. cloacae in a Canadian animal. Furthermore, although CTX-M β-lactamases appear to be widespread, albeit not common, in both commensal and pathogens in dogs, carbapenemase-producing Enterobacteriaceae are still absent or at least extremely rare in dogs in Ontario. The specific STs and CTX-M variants of canine clinical and fecal isolates provided evidence for the transfer of resistant bacteria and their ESC resistance genes between humans and dogs. However, as suggested by the situation with CTX-M-1, other sources than humans may also be implicated in the emergence of ESBLs in bacteria from dogs in Ontario.
## Tables

Table 2.1. ESC-resistant clinical and fecal isolates categorized by bacterial species and their corresponding genotypes.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Origin</th>
<th>Total no. of resistant isolates</th>
<th>No. of isolates (%)</th>
<th>bla&lt;sub&gt;CMY&lt;/sub&gt;</th>
<th>bla&lt;sub&gt;CTX-M&lt;/sub&gt;</th>
<th>bla&lt;sub&gt;SHV&lt;/sub&gt;</th>
<th>No gene detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrobacter braakii</td>
<td>Clinical</td>
<td>1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>Clinical</td>
<td>2</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (100)</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Clinical</td>
<td>65</td>
<td></td>
<td>34 (52.3)</td>
<td>24 (36.9)</td>
<td>0</td>
<td>10 (15.4)</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>88</td>
<td></td>
<td>55 (62.5)</td>
<td>19 (21.6)</td>
<td>0</td>
<td>14 (15.9)</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>Clinical</td>
<td>7</td>
<td></td>
<td>1 (14.3)</td>
<td>0</td>
<td>0</td>
<td>6 (85.7)</td>
</tr>
<tr>
<td>Enterobacter asburiae</td>
<td>Clinical</td>
<td>4</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>Clinical</td>
<td>26</td>
<td></td>
<td>0</td>
<td>1 (3.8)</td>
<td>1 (3.8)</td>
<td>24 (92.3)</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>2</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Enterobacter kobei</td>
<td>Clinical</td>
<td>4</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4 (100)</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Enterobacter ludwigi</td>
<td>Clinical</td>
<td>2</td>
<td></td>
<td>1 (50.0)</td>
<td>0</td>
<td>0</td>
<td>1 (50.0)</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>Clinical</td>
<td>2</td>
<td></td>
<td>0</td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
<td>0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Clinical</td>
<td>13</td>
<td></td>
<td>11 (84.6)</td>
<td>2 (15.4)</td>
<td>12 (92.3)</td>
<td>0</td>
</tr>
<tr>
<td>Pluralibacter gergoviae</td>
<td>Fecal</td>
<td>2</td>
<td></td>
<td>0</td>
<td>1 (50.0)</td>
<td>0</td>
<td>1 (50.0)</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>Clinical</td>
<td>21</td>
<td></td>
<td>19 (90.5)</td>
<td>0</td>
<td>0</td>
<td>2 (9.5)</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>1</td>
<td></td>
<td>1 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.2. Disk diffusion phenotypes of clinical and fecal *E. coli* isolates with reduced susceptibility to cefoxitin and ESCs, and their corresponding genotypes.

<table>
<thead>
<tr>
<th>Phenotypes of reduced susceptibility</th>
<th>Origin</th>
<th>Total no. of resistant isolates</th>
<th>No. of isolates (%)</th>
<th>bla&lt;sub&gt;CMY&lt;/sub&gt;</th>
<th>bla&lt;sub&gt;CTX-M&lt;/sub&gt;</th>
<th>bla&lt;sub&gt;SHV&lt;/sub&gt;</th>
<th>No gene detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefoxitin and ESCs</td>
<td>Clinical</td>
<td>40</td>
<td>34 (85.0)</td>
<td>4 (10.0)</td>
<td>0</td>
<td>5 (12.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>73</td>
<td>55 (75.3)</td>
<td>4 (5.5)</td>
<td>0</td>
<td>14 (19.2)</td>
<td></td>
</tr>
<tr>
<td>Cefoxitin only</td>
<td>Clinical</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ESCs only</td>
<td>Clinical</td>
<td>20</td>
<td>0</td>
<td>20 (100)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>15</td>
<td>0</td>
<td>15 (100)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.3. ESC resistance genes found in the CTX-M positive clinical and fecal isolates.

<table>
<thead>
<tr>
<th>Antimicrobial resistance gene</th>
<th>Bacterial Species</th>
<th>Total no. of isolates (%; n=47)</th>
<th>No. of isolates (%) from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Clinical (n=28)</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;ACT-5&lt;/sub&gt;</td>
<td>E. cloacae</td>
<td>1 (2.1)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CMY-2&lt;/sub&gt;</td>
<td>E. coli</td>
<td>3 (6.4)</td>
<td>3 (10.7)</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CTX-M-1&lt;/sub&gt;</td>
<td>E. coli</td>
<td>6 (12.8)</td>
<td>0</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CTX-M-14&lt;/sub&gt;</td>
<td>E. coli</td>
<td>5 (10.6)</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CTX-M-15&lt;/sub&gt;</td>
<td>Total</td>
<td>17 (36.2)</td>
<td>12 (42.9)</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>K. oxytoca</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>P. gergoviae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CTX-M-27&lt;/sub&gt;</td>
<td>E. coli</td>
<td>9 (19.1)</td>
<td>7 (25.0)</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CTX-M-55&lt;/sub&gt;</td>
<td>E. coli</td>
<td>7 (14.9)</td>
<td>5 (17.9)</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CTX-M-115&lt;/sub&gt;</td>
<td>E. coli</td>
<td>1 (2.1)</td>
<td>0</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;CTX-M-169&lt;/sub&gt;</td>
<td>E. coli</td>
<td>1 (2.1)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td><em>bla</em>&lt;sub&gt;OXY-2-7&lt;/sub&gt;</td>
<td>K. oxytoca</td>
<td>1 (2.1)</td>
<td>1 (3.6)</td>
</tr>
</tbody>
</table>
Table 2.4. Results of logistic regression for gene frequencies and Fisher’s exact tests for \textit{bla}_{CTX-M}\ and other resistance genes.

<table>
<thead>
<tr>
<th>Test</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>\textit{p}-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of \textit{bla}_{CMY} between ESC-resistant and ESC-resistant fecal \textit{E. coli}</td>
<td>0.58</td>
<td>0.3-1.1</td>
<td>0.1083</td>
</tr>
<tr>
<td>Frequency of \textit{bla}_{CTX-M} between ESC-resistant and ESC-resistant fecal \textit{E. coli}</td>
<td>2.05</td>
<td>0.1-4.2</td>
<td>0.0515</td>
</tr>
<tr>
<td>\textit{bla}_{CTX-M-15} and \textit{aac(3)}-\text{IIa}</td>
<td>8.0</td>
<td>1.4-54.2</td>
<td>0.0094</td>
</tr>
<tr>
<td>\textit{bla}_{CTX-M-15} and \textit{aac(6')Ib-cr}</td>
<td>25.8</td>
<td>2.6-1181.5</td>
<td>0.0006</td>
</tr>
<tr>
<td>\textit{bla}_{CTX-M-15} and \textit{catB3}</td>
<td>25.8</td>
<td>2.6-1181.5</td>
<td>0.0006</td>
</tr>
<tr>
<td>\textit{bla}_{CTX-M-15} and \textit{dfrA14}</td>
<td>8.0</td>
<td>1.4-54.2</td>
<td>0.0094</td>
</tr>
</tbody>
</table>
Fig. 2.1. An overview of the different clinical and fecal isolate collections and the methodologies used in this study.¹

¹ The MIC susceptibility testing used a variety of antimicrobial classes while the disk diffusion susceptibility testing only used cefoxitin, cefotaxime, ceftazidime, and ertapenem.
Fig. 2.2. The frequency of reduced susceptibility to antimicrobials in generic *E. coli* isolates (n=217) from canine fecal samples with 95% confidence intervals.
Fig. 2.3. Minimum spanning tree using wgMLST with 3223 genes showing the different STs of ESC-resistant clinical *E. coli* and fecal *E. coli* from enrichment. The grey circles indicate the clinical isolates whereas white circles indicate fecal isolates. Two isolates were not assigned STs.²

² The two identical ST648 isolates originated from the same fecal sample, whereas the two identical ST131 isolates were from clinical samples of different animals.
Fig. 2.4. Minimum spanning tree using wgMLST with 3223 genes showing the distribution of CTX-M variants in clinical *E. coli* and fecal *E. coli*. The grey circles indicate the clinical isolates whereas white circles indicate fecal isolates.

---

3 The two identical isolates carrying *blaCTX-M-1* originated from the same fecal sample, whereas the two identical *blaCTX-M-15* isolates were from clinical samples of different animals.
Conflict of interest

None to declare.

ACKNOWLEDGEMENTS

The authors are grateful to the technicians at the Animal Health Laboratory and IDEXX Laboratories for their help with collecting isolates. In addition, we would like to thank Julie Cobean for technical assistance, Dr. Nicol Janecko for sharing protocols on fecal sample collection, and Dr. David Pearl for his help with statistical analyses. This work was supported by the NSERC [grant number 2015-03962]; the Ontario Veterinary College Pet Trust Fund; and the Ontario Veterinary College Summer Career Opportunities and Research Experience Program.
CHAPTER 3: PREVALENCE AND MECHANISMS OF EXTENDED-SPECTRUM CEPHALOSPORIN RESISTANCE IN CLINICAL AND COMMENSAL ENTEROBACTERIACEAE ISOLATES FROM CHICKENS AND PIGS IN ONTARIO

Authors: Pauline L.C. Zhang\textsuperscript{a}, Richard J. Reid-Smith\textsuperscript{a, b, c}, Durda Slavic\textsuperscript{d}, Anne E. Deckert\textsuperscript{b, c}, Patrick Boerlin\textsuperscript{a,*}

\textsuperscript{a}Department of Pathobiology and \textsuperscript{b}Department of Population Medicine, Ontario Veterinary College, University of Guelph, 50 Stone Rd. E, Guelph, Ontario, N1G 2W1, Canada.
\textsuperscript{c}Centre for Food-borne, Environmental and Zoonotic Infectious Diseases, Public Health Agency of Canada, 160 Research Lane, Suite 103, Guelph, Ontario, N1G 5B2, Canada.
\textsuperscript{d}Animal Health Laboratory, Post Office 3612, Guelph, Ontario, N1H 6R8, Canada.

*Corresponding author.
E-mail address: pboerlin@uoguelph.ca

To be submitted for publication in the journal ‘Veterinary Microbiology’
ABSTRACT

The current situation of resistance to extended-spectrum cephalosporins (ESCs) in Enterobacteriacea in chickens and pigs in Canada needs to be investigated because of the potential of transmission to humans. The study objectives were to determine the frequency of ESC resistance and ESC resistance genes in clinical and fecal Enterobacteriacea in chickens and pigs. A total of 369 and 170 clinical Enterobacteriacea isolates were collected from chickens and pigs, respectively. Additionally, 538 and 59 ESC-resistant Enterobacteriacea isolates were obtained from 242 chicken and 50 swine cecal samples, respectively. Polymerase chain reaction was used to screen for $\text{bla}_{\text{CMY}}$, $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{SHV}}$ in isolates with reduced ESC susceptibility, and whole-genome sequencing was performed on all CTX-M-positive swine isolates. The prevalence of ESC resistance in clinical Enterobacteriacea was 17.3% and 8.8% for chickens and pigs, respectively. Intestinal ESC-resistant Enterobacteriacea was almost ubiquitous for both animal species, but there were only low concentrations of resistant bacteria. The results suggest that resistance genes may transfer between pathogenic and commensal Enterobacteriacea in chickens and pigs, and that other than $E. \text{coli}$, commensal Enterobacteriacea do not act as major sources of ESC resistance genes. The diverse sequence types included, among others, ST10, ST453, and ST744, supporting the global transfer of resistant bacteria. The identification of CTX-M-14, 15, and 27 in swine isolates provide strong evidence for the transfer of resistant bacteria and their genes between humans and pigs. Although CTX-M-1 is infrequent in human Enterobacteriacea, it was found in cecal swine $E. \text{coli}$; more research is needed to determine underlying transmission pathways.

1. INTRODUCTION

$Escherichia \text{coli}$ and $Salmonella \text{enterica}$ frequently cause infections in chickens and pigs, leading to significant losses annually in both industries. Infections with pathogenic strains of $E. \text{coli}$ can result in colibacillosis in chickens, as well as neonatal and post-weaning diarrhea in pigs (Dho-Moulin and Fairbrother, 1999; Nagy and Fekete, 2005). Infections in chickens and pigs may be treated and prevented with antimicrobial use, but there have been increases in antimicrobial resistance (AMR; Public Health Agency of Canada, 2016a). For $\beta$-lactams specifically, the rise in the frequency of $\beta$-lactamases have led to problems in treating animals...
and humans (Ewers et al., 2012). The increased AMR is especially evident for extended-spectrum cephalosporins (ESCs), a group of β-lactams that are critically important for human and veterinary medicine (OIE, 2015; WHO, 2017).

In 2013, *S. enterica* was the most frequently reported cause of gastrointestinal illness in Canada (Government of Canada, 2014). These infections are typically self-limiting, but severe salmonellosis, which can include sepsis and other extra-intestinal infections, requires treatment with ESCs or other antimicrobials, especially for children, pregnant women, and the immunocompromised (Otto et al., 2014). Therefore, Enterobacteriaceae that are resistant to ESCs in food animals are a major public health concern. In an attempt to preserve the clinical efficacy of ESCs for humans, the Canadian poultry industry has banned the preventative use of ESCs since 2014. This was in part due to analysis of data from the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) that strongly correlated ceftiofur use in hatcheries with the frequency of isolating ceftiofur-resistant *Salmonella* Heidelberg in chicken retail meat, as well as the frequency of ESC-resistant *S. Heidelberg* human infections (Dutil et al., 2010). In contrast, no similar correlations have been made between ESC-resistant human illness and ESC-resistant swine Enterobacteriaceae. ESCs such as ceftiofur are still used to treat and prevent infections in swine (Deckert et al., 2010). In addition, CIPARS has low recovery of *S. enterica* from swine and even though there is high recovery of *E. coli*, comparisons cannot be made as there are no systematic collections of human *E. coli* isolates.

A variety of β-lactamases cause ESC resistance, such as AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs), and carbapenemases (Seiffert et al., 2013). Worldwide, ESBL-producing Enterobacteriaceae have been isolated from food animals and meat products (Ewers et al., 2012; Seiffert et al., 2013). In Canada, the β-lactamases CMY, CTX-M-1, TEM, SHV-2, and SHV-2a have been reported in *E. coli* and *S. enterica* from chickens, pigs, and retail meat (Aslam et al., 2012; Martin et al., 2012; Sheikh et al., 2012; Pouget et al., 2013; Chalmers et al., 2017); however, few studies have investigated the specific ESC resistance genes present in clinical Enterobacteriaceae from chickens and pigs in Canada and in Ontario in particular. Also, little is known about ESC resistance in the commensal bacteria of these animals other than in *E. coli*, and the genetic basis of that resistance. This is of importance, as it has been demonstrated that
commensal Enterobacteriaceae can transfer AMR genes to pathogenic strains (Blake et al., 2003).

This study investigated ESC resistance in Enterobacteriaceae from chickens and pigs in Ontario, which are two major livestock commodities in this province. Specifically, the genera and species of interest were *Escherichia coli*, *Salmonella enterica*, *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp., *Serratia* spp., and *Pantoea* spp. The first objective was to determine the prevalence of ESC resistance in *E. coli* and *S. enterica* isolated from chicken and swine clinical samples submitted to a major diagnostic laboratory in Ontario. Secondly, this work aimed to evaluate the frequency of healthy chickens and pigs carrying ESC-resistant Enterobacteriaceae in their gut in Ontario. The third objective was to analyse and compare ESC resistance genes between Enterobacteriaceae species, as well as between clinical and cecal isolates from chickens and pigs. The last objective was to use whole-genome sequencing to compare *blaCTX-M* variants in ESC-resistant isolates from pigs, as well as determine if there were dominant clonal lineages in this population.

2. MATERIALS AND METHODS

2.1 Clinical isolate collection

From November 2015 to October 2016, 336 chicken and 170 swine Enterobacteriaceae isolates were obtained from clinical samples through the Animal Health Laboratory (AHL), University of Guelph, Guelph, Ontario. For chickens, *E. coli* and *S. enterica* were collected from cases of colibacillosis and salmonellosis. If *S. enterica* was present in any cases of colibacillosis, these isolates were also collected. For pigs, K88-positive *E. coli* and *S. enterica* were collected from cases of neonatal and post-weaning diarrhea (Fig. 3.1). *S. enterica* isolated from pigs that had no clinical signs were also collected, in order to include healthy carriers of *S. enterica*. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik GmbH, Bremen, Germany) was used to identify all clinical isolates to the species level.
2.2 Cecal isolate collection

From November 2015 to October 2016, cecal samples were taken at slaughter from a total of 242 chickens and 50 pigs for the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). All the animals were from Ontario. Cecal samples were supplemented with *Brucella* Broth and glycerol to a concentration of 15% and kept at −80°C until time of processing. For each sample, 1.8 mL of thawed cecal content (90 mg of cecal content) was used to inoculate 16.2 mL of EE Broth Mossel Enrichment (Becton Dickinson [BD], Cockeysville, MD, USA) supplemented with 2 µg/mL cefotaxime, for enrichment of ESC-resistant Enterobacteriaceae (Fig. 3.1). After incubating the broth cultures overnight with agitation at 37°C, 10 µL was plated out onto Rapid Enterobacteriaceae *Escherichia coli* Coliform Agar (REBECCA; bioMérieux, Marcy-l'Étoile, France) with 1 µg/mL ceftriaxone. Every colony morphotype from the primary plate was then subcultured and purified onto REBECCA with ceftriaxone. Oxidase tests (Sigma-Aldrich, St. Louis, MO, USA) and catalase tests were used for presumptive confirmation of Enterobacteriaceae isolates and MALDI-TOF MS (Bruker) was used to identify them at the species level.

2.3 Antimicrobial susceptibility testing

Disk diffusion susceptibility testing was carried out for all clinical and cecal isolates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015). Cefoxitin (FOX-30), cefotaxime (CTX-30), ceftazidime (CAZ-30) (Becton Dickinson [BD]) were used to screen for ESC resistance. Additionally, the isolates were tested for carbapenem susceptibility with ertapenem (ETP-10; Becton Dickinson [BD]). The quality control strains were *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603. The different isolate collections and the techniques are summarized in Fig. 3.1.

2.4 PCR screening of ESC resistance genes

PCR for the major ESC resistance gene families *bla*<sub>CMY</sub>, *bla<sub>CTX-M</sub>*<sub>-</sub>, and *bla*<sub>SHV</sub> was carried out on all isolates that exhibited reduced susceptibility to cefotaxime, ceftazidime, and/or cefoxitin (Kozak et al., 2009; Cottell et al., 2013). In addition, PCR for *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, *blaNDM*, and *blaOXA-48* (Mataseje et al., 2012) was carried out on isolates that displayed reduced
susceptibility to ertapenem, as well as those with results slightly above the breakpoint (18 mm - 22 mm).

2.5 Genome sequencing and analysis

In Canada, reports of isolating CTX-M-producing Enterobacteriaceae from animals began relatively recently compared to other countries and are still sparse. Therefore, all the clinical and cecal CTX-M-producing swine isolates were further investigated by genome sequencing. Following the manufacturer’s protocols, DNA was extracted with the EpiCentre MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), and underwent MiSeq sequencing (Illumina, San Diego, CA, USA) at the Advanced Analysis Centre of the Genomics Facility at the University of Guelph (Guelph, ON, Canada). Libraries were prepared with Nextera XT kits (Illumina). Assembly, assembly-free and assembly-based allele calling was accomplished using BioNumerics v7.6 (Applied Maths, Ghent, Belgium), the wgs application, and the SPAdes algorithm. Furthermore, the Bionumerics v7.6 (Applied Maths) wgMLST application in conjunction with the BioNumerics Escherichia coli/Shigella Enterobase scheme, assigned multi-locus sequence types (MLST) for all the E. coli isolates. The E. coli functional genotyping plugin was used to determine the antimicrobial genes in the isolates.

2.6 Statistical methods

STATA 14 (Stata Corporation, College Station, Texas, USA) was used to carry out logistic regression in determining whether there were significant differences in the frequencies of $bla_{CMY}$ and $bla_{CTX-M}$ between resistant clinical and resistant cecal E. coli isolates for both chickens and pigs. Fisher’s exact tests were also carried out to investigate if there were any significant associations between the top five most frequent $bla_{CTX-M}$ genes and other AMR genes. A $p$-value of $\leq 0.05$ was considered significant.

3. RESULTS

3.1 ESC susceptibility, phenotypes, and genotypes of clinical isolates

Sixty-four out of 369 (17.3%) clinical isolates from chicken were resistant to cefoxitin and/or ESCs (95% CI, 13.8-21.5). The majority of them was E. coli (n=63/366), and only one
was *S. enterica* (n=1/3; Table 3.1). Overall, 57 (15.4%) and 3 (0.8%) of these isolates were positive for *bla*<sub>CMY</sub> and *bla*<sub>CTX-M</sub>, respectively. The only ESC-resistant *S. enterica* isolate was positive for *bla*<sub>CMY</sub> and it was the serotype *Salmonella* Heidelberg.

The prevalence of resistance to cephamycins and/or ESCs in clinical isolates from swine was 8.8% (15/170; 95% CI, 5.4-14.0). The majority of these isolates from pigs was also *E. coli* (n=11/80; Table 3.1). There were only four resistant *S. enterica* isolates (n=4/90) and only two isolates were serotyped: *Salmonella* Derby and *Salmonella* Infantis. Out of 170 swine Enterobacteriaceae, ten (5.9%) and two (1.2%) isolates were positive for *bla*<sub>CMY</sub> and *bla*<sub>CTX-M</sub>, respectively.

The gene *bla*<sub>SHV</sub> was not detected in any of the chicken nor swine isolates. The susceptibility testing results of all the clinical resistant isolates as well as their corresponding genotypes are shown in Table 3.2. A low proportion of ESC-resistant isolates had no ESC resistance genes detected, but for the most part, practically all of the resistant isolates were accounted for by *bla*<sub>CMY</sub> and *bla*<sub>CTX-M</sub>. One *E. coli* isolate from a chicken and one from a pig had reduced susceptibility to ertapenem and zones of inhibition diameters close to the breakpoint of 19 mm (15-18 mm). However, both isolates were negative for the carbapenemase genes tested.

### 3.2 Diversity, antimicrobial susceptibility, and genotypes of cecal isolates

After enrichment, ESC-resistant Enterobacteriaceae were detected in 98.3% (238/242) chicken cecal samples and 74.0% (35/50) swine cecal samples (95% CI, 95.8-99.4 and 95% CI, 56.2-80.1, respectively). Based on the different colony morphotypes, 538 and 79 ESC-resistant isolates were collected from the chicken and swine cecal samples, respectively. The ESC-resistant isolates from chickens mainly comprised *E. coli* (n=536), followed by one isolate each of *Escherichia fergusonii* and *P. mirabilis* (Table 3.1). Similarly, the ESC-resistant isolates from swine comprised *E. coli* (n=77) and *P. mirabilis* (n=2). Overall, 494 (91.8%) and 42 (7.8%) of the 538 ESC-resistant chicken isolates were positive for *bla*<sub>CMY</sub> and *bla*<sub>CTX-M</sub>, respectively. Out of 79 swine Enterobacteriaceae isolates, 57 (72.2%) and 22 (27.8%) of the ESC-resistant isolates were positive for *bla*<sub>CMY</sub> and *bla*<sub>CTX-M</sub>, respectively. Furthermore, all cecal ESC-resistant Enterobacteriaceae isolates from both chickens and pigs were negative for *bla*<sub>SHV</sub>. The results from disk diffusion susceptibility testing for isolates from chickens and pigs, as well as
associated genotypes are shown in Table 3.2. There were two cecal isolates, one chicken and one swine, with reduced susceptibility to ertapenem, but both isolates were negative for the carbapenemase genes tested.

3.3 Whole-genome sequencing of swine CTX-M-producing ESC-resistant isolates

Whole-genome sequencing (WGS) was conducted for twenty-three ESC-resistant CTX-M-producing isolates (22 cecal and 1 clinical). Overall, the two most frequent sequence types (ST) in the clinical isolates were ST453 (n=3) and ST744 (n=3), followed by ST10 (n=3), ST398 (n=2) and ST641 (n=2). There was one isolate each for ST23, ST88, ST101, ST189, ST367, ST648, and ST4891. Three E. coli isolates were not assigned STs. The CTX-M variants consisted of CTX-M-1 (n=9), CTX-M-14 (n=8), CTX-M-27 (n=3), CTX-M-15 (n=2), and CTX-M-115 (n=1). Minimum spanning trees were created using wgMLST comprising 3325 genes, displaying the different STs and CTX-M variants (Fig. 3.2). There was no obvious clustering of STs and CTX-M variants in the tree.

Resistance genes other than blaCTX-M were found in both the clinical and cecal isolates for a variety of antimicrobials, including aminoglycosides, chloramphenicols, macrolides, quinolones, sulfonamides, trimethoprim, and tetracyclines (Appendix 2, Table 3.S1). The most frequent resistance genes were aadA5, dfrA17, sul2, tet(A), and tet(B).

3.4 Statistical analyses of resistance genes distribution

No significant differences were found in the frequencies of blacMY and blaCTX-M between resistant clinical E. coli and resistant cecal E. coli for chickens (OR=0.7; 95% CI, 0.3-1.7; p-value=0.4516 and OR=0.6; 95% CI, 0.2-2.0; p-value=0.3535, respectively). Similarly, blacMY and blaCTX-M frequencies were not statistically different for resistant clinical and resistant cecal E. coli from swine (OR=1.5; 95% CI, 0.4-5.7; p-value=0.5402 and OR=4.0; 95% CI, 0.5-33.1; p-value=0.1317, respectively). There were also no significant differences in the frequencies of blacMY and blaCTX-M in resistant clinical E. coli isolates between chickens and pigs (OR=4.6; 95% CI, 1.1-19.7; p-value=0.0501 and OR=0.5; 95% CI, 0.05-5.3; p-value=0.5851, respectively). However, even though the difference was not significant, our data suggest that blacMY may be more frequent in resistant chicken clinical E. coli compared to resistant swine...
clinical E. coli. The odds of finding blaCMY in resistant chicken cecal E. coli were greater than finding it in resistant swine cecal E. coli (OR=4.2; 95% CI, 2.3-7.6; p-value < 0.001).

Furthermore, there were greater odds of finding blaCTX-M in resistant swine cecal E. coli isolates than those from chickens (OR=4.7; 95% CI, 2.6-8.5; p-value <0.001). The blaCTX-M-14 gene was significantly associated with aadA5 (OR=12; 95% CI, 1.153-159.550, p-value=0.0228), dfrA17 (OR=12; 95% CI, 1.153-159.550, p-value=0.0228), sul2 (OR=0.1; 95% CI, 0.007-1.011; p-value=0.0257). There were no significant associations between blaCTX-M-1, blaCTX-M-15, blaCTX-M-27, blaCTX-M-115, and other AMR genes.

4. DISCUSSION

To the best of our knowledge, there were no previous studies that investigated the prevalence of ESC resistance in clinical E. coli in chickens in Ontario. Compared to this study, there were much higher levels of resistance in Québec, as approximately 30% of E. coli isolated from chicken colibacillosis cases were resistant to ceftiofur (Gouvernement du Québec, 2015). For pigs, the prevalence of resistance (8.8%) is in agreement with a study conducted by Boerlin and collaborators between 2001 and 2003, which found ESC resistance levels of 11-13% in E. coli isolated from diarrheic pigs in Ontario (Boerlin et al., 2005). In 2014, it was reported that the prevalence of ceftiofur resistance in clinical E. coli isolates from swine in Québec had reached 22% (MAPAQ, 2015). It appears that the prevalence of ESC resistance may have remained high or even increased in clinical E. coli from pigs in Canada, as other previous studies either did not detect any or found only low levels (2-5%) of ESC resistance in swine pathogenic E. coli (Amezcua et al., 2002; Maynard et al., 2003; Hariharan et al., 2004; Jahanbakhsh et al., 2016).

The frequencies of chickens and pigs carrying ESC-resistant Enterobacteriaceae in their intestine were very high. However, routine CIPARS surveillance, which uses non-selective media, found that the national ESC resistance prevalence in 2014 was only 16% and 2% of cecal E. coli from chickens and pigs, respectively (Government of Canada, 2016). Therefore, even though the majority of chickens and pigs in Ontario carry ESC-resistant E. coli, these bacteria seem to be present in relatively low concentrations and most carriers are only detectable with selective media. There has been a clear decline in the prevalence of ESC resistance since the preventative use of ceftiofur in the poultry industry was banned in April 2014, as determined by
standard non-selective means (Public Health Agency of Canada, 2016b). Despite this, the frequency of animals carrying ESC-resistant *E. coli*, apparently in subpopulations detectable by selective media, is still extremely high in chickens. Thus, if the use of ceftiofur were to be implemented again, the prevalence of ESC resistance would likely rebound, as was already observed by Dutil and collaborators, when the Québec poultry industry started using ceftiofur again after an earlier voluntary withdrawal (Dutil et al., 2010). The main substitute for ceftiofur after its ban has been lincospectin, a combination drug of lincomycin and spectinomycin (Agunos et al., 2017). Because of the potential for co-selection, the effects of other antimicrobials should also be closely monitored, as spectinomycin use in Québec likely co-selected for gentamicin resistance (Chalmers et al., 2017).

Extremely few ESC-resistant isolates of Enterobacteriaceae species other than *E. coli* were recovered in this study, despite the use of media aimed at isolating Enterobacteriaceae in general (competition assays with representative isolates were performed with these media to ensure that other Enterobacteriaceae were not overgrown by *E. coli*; Appendix 2, Table 3.S2) which suggests that these other bacterial species do not represent a major reservoir of ESC resistance genes in the intestine of either chickens or pigs. Similar to our results, a previous study on clinical *E. coli* isolates from chickens in Québec found that CMY was the main cause of ceftiofur resistance, and to a much lesser extent CTX-M-1 (Chalmers et al., 2017). As *blaCTX-M* only recently emerged in clinical *E. coli* from pigs (Jahanbakhsh et al., 2016), our finding of a higher frequency of *blaCMY* (63.6%) than *blaCTX-M* (9%) in clinical isolates from swine was expected.

In agreement with the very low prevalence of *blasHV* previously found in *E. coli* and *Salmonella* from swine and chicken in Canada (Pouget et al., 2013) and with the low MICs for ESCs associated with their most frequent variants (SHV-2 and SHV-2a), no SHV-producing Enterobacteriaceae were isolated from either chickens or pigs. The results also showed that carbapenemase-producing Enterobacteriaceae have not yet emerged in chickens and pigs in Ontario, at least at detectable levels.

No significant differences were seen in the frequencies of *blaCMY* and *blaCTX-M* between clinical and cecal *E. coli* in either chickens or pigs. Thus, these results support the hypothesis of
transfer of ESC resistance genes occurring between commensal and pathogenic *E. coli* within a host species. Interestingly, *bla*CMY was more frequent among ESC-resistant cecal *E. coli* from chickens than swine and a similar but not significant trend was seen in clinical isolates. The reverse was true for *bla*CTX-M, which was more frequently observed in ESC-resistant cecal isolates from swine. These findings suggest that there are different dynamics and epidemiology of *bla*CMY and *bla*CTX-M in these two animal species.

The most frequent STs present in the swine *bla*CTX-M-positive *E. coli* (ST10, ST744, and ST453) have been found worldwide, causing infections in humans and various animals (Goldstone et al., 2014; Abraham et al., 2015; Lee and Yeh, 2017). Furthermore, many of the specific ST and CTX-M combinations found in this study have been identified in *E. coli* from pigs, pig farmers, and pig slaughterhouse workers in Germany, such as ST10/CTX-M-1, ST453/CTX-M-1, ST744/CTX-M-14 (Dohmen et al., 2015, 2017; Fischer et al., 2017). The combinations of ST10/CTX-M-15 and ST4891/CTX-M-15 in *E. coli* found in our study have also been reported in cases of piglet diarrhea in Taiwan (Lee and Yeh, 2017). The only other documented report of ST641/CTX-M-1 occurred in fecal *E. coli* from an equine patient in the Netherlands (Apostolakos et al., 2017). Interestingly, no isolates in this study were ST131 and only one was ST648, even though these human pandemic clones have been found in companion animals (Ewers et al., 2014; Pitout and DeVinney, 2017; Zhang et al., submitted).

There was no obvious clustering of CTX-M variants in our minimum spanning tree and the most frequent variants such as CTX-M-1, -14 and -27 were found in completely unrelated isolates. Thus, extensive horizontal gene transfer of CTX-M between porcine *E. coli* strains and/or repeated introduction of unrelated CTX-M-positive strains from other origins are likely occurring in swine in Ontario. The results also support the transfer of AMR genes or *E. coli* strains between humans and pigs, as CTX-M-14, -15, and -27 are frequently identified in human *E. coli* isolates (Ewers et al., 2012; Denisuik et al., 2013). In Canada, CTX-M-1 has only recently been detected in *E. coli* from dogs (Zhang et al., submitted) and food animals (Chalmers et al., 2017), but is still rare in human clinical isolates (M. Mulvey, personal communication). However, it is the most prevalent CTX-M variant in Europe (Ewers et al., 2012). This suggests that CTX-M-1-producing *E. coli* strains have moved globally and are possibly spreading in bacteria from animals in Ontario and Canada faster than in clinical human strains. Overall, the
most frequent resistance genes in CTX-M-positive isolates were \textit{aadA5}, \textit{dfrA17}, \textit{sul2}, \textit{tet(A)}, and \textit{tet(B)} which encode resistance to antimicrobials that have been used for decades: aminoglycosides, trimethoprim, sulfonamides, and tetracyclines. Furthermore, the genes \textit{aadA5}, \textit{dfrA17}, and \textit{sul2} were strongly associated with \textit{bla}_{\text{CTX-M-14}}, which suggests that they are likely located on the same plasmid.

Our study presented novel insight on the status of ESC resistance in clinical and cecal Enterobacteriaceae in chickens and pigs in Ontario, as previous studies on ESC resistance in these animals in Canada did usually not determine the specific genes or variants. Overall, ESC-resistant \textit{E. coli} strains are extremely widespread in chickens and pigs in Ontario, but are mostly detectable by selective methods only. There may be different dynamics for \textit{bla}_{\text{CMY}} and \textit{bla}_{\text{CTX-M}} in chicken and swine \textit{E. coli}, which would explain the different frequencies of these genes between the two animal species. In addition, the CTX-M variants identified support the transfer of ESC-resistant bacteria between humans and pigs. However, as illustrated by CTX-M-1, the pathways and directionality of this transfer may vary. Furthermore, our finding of isolates from STs described on other continents or worldwide support the global transfer of clonal lineages and may have implications on public health. To the best of our knowledge, this was the first report of CTX-M-1 in swine \textit{E. coli} in Canada. More investigation is needed to understand the emergence of this gene variant in animals in Canada.
Table 3.1. ESC-resistant clinical and cecal isolates from chickens and pigs and their corresponding genotypes.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Bacterial species</th>
<th>Origin</th>
<th>Total no. of resistant isolates</th>
<th>No. of isolates (%)</th>
<th>bla\textsubscript{CMY}</th>
<th>bla\textsubscript{CTX-M}</th>
<th>bla\textsubscript{SHV}</th>
<th>No genes found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td><em>Escherichia coli</em></td>
<td>Clinical</td>
<td>63</td>
<td>56 (88.9)</td>
<td>3 (4.8)</td>
<td>0</td>
<td>4 (6.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>536</td>
<td>492 (91.8)</td>
<td>42 (7.8)</td>
<td>0</td>
<td>3 (0.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Escherichia fergusonii</em></td>
<td>Cecal</td>
<td>1</td>
<td>1 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Salmonella enterica</em></td>
<td>Clinical</td>
<td>1</td>
<td>1 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Proteus mirabilis</em></td>
<td>Cecal</td>
<td>1</td>
<td>1 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td><em>Escherichia coli</em></td>
<td>Clinical</td>
<td>11</td>
<td>7 (63.6)</td>
<td>1 (9.1)</td>
<td>0</td>
<td>3 (27.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>77</td>
<td>56 (72.7)</td>
<td>22 (28.6)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Salmonella enterica</em></td>
<td>Clinical</td>
<td>4</td>
<td>3 (75.0)</td>
<td>1 (25.0)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Proteus mirabilis</em></td>
<td>Cecal</td>
<td>2</td>
<td>1 (50.0)</td>
<td>0</td>
<td>0</td>
<td>1 (50.0)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Disk diffusion phenotypes of resistant clinical and fecal *E. coli* isolates from chickens and pigs through disk diffusion susceptibility testing and their corresponding genotypes.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Phenotypes of reduced susceptibility</th>
<th>Origin</th>
<th>Total no. of resistant isolates</th>
<th>No. of isolates (%)</th>
<th>bla&lt;sub&gt;CMY&lt;/sub&gt;</th>
<th>bla&lt;sub&gt;CTX-M&lt;/sub&gt;</th>
<th>bla&lt;sub&gt;SHV&lt;/sub&gt;</th>
<th>No gene detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>Cefoxitin and ESCs</td>
<td>Clinical</td>
<td>55</td>
<td>54 (98.2)</td>
<td>0</td>
<td>0</td>
<td>1 (1.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>495</td>
<td>491 (99.2)</td>
<td>3 (0.6)</td>
<td>0</td>
<td>2 (0.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cefoxitin only</td>
<td>Clinical</td>
<td>2</td>
<td>2 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>1</td>
<td>1 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ESCs only</td>
<td>Clinical</td>
<td>6</td>
<td>0</td>
<td>3 (50.0)</td>
<td>0</td>
<td>3 (50.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>40</td>
<td>0</td>
<td>39 (97.5)</td>
<td>0</td>
<td>1 (2.5)</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>Cefoxitin and ESCs</td>
<td>Clinical</td>
<td>8</td>
<td>7 (87.5)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>56</td>
<td>56 (100)</td>
<td>1 (1.8)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cefoxitin only</td>
<td>Clinical</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ESCs only</td>
<td>Clinical</td>
<td>1</td>
<td>0</td>
<td>1 (100)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cecal</td>
<td>21</td>
<td>0</td>
<td>21 (100)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.1. Brief overview of the different clinical and cecal isolate collections and the methodologies used in this study.
Fig. 3.2. Minimum spanning tree using wgMLST with 3325 genes showing the different STs and the CTX-M variants of ESC-resistant clinical *E. coli* and cecal *E. coli* from enrichment for swine isolates only. Three isolates were not assigned STs.  

The two identical ST744 isolates were obtained from cecal samples of two different animals that were sampled in the same time period.
ACKNOWLEDGEMENTS

The authors are grateful to the technicians at the Animal Health Laboratory and CIPARS for their help with collecting samples and isolates. In addition, we would like to thank Gabhan Chalmers, Kristin Davis, and Julie Cobean for technical assistance. This work was supported by the NSERC [grant number 2015-03962].
CHAPTER 4: DISCUSSION AND CONCLUSIONS

1. SUMMARY OF ESC RESISTANCE

The prevalence of resistance to cephemycins and/or ESCs in clinical Enterobacteriaceae isolates from chickens, dogs, and pigs are shown in Table 4.1. The prevalence of ESC resistance was greatest for chickens, which may reflect the high levels of ceftiofur use in the poultry industry in the past and the resulting selective pressures (Agunos et al., 2017). It is important to note that only the isolates considered ESC-resistant from disk diffusion susceptibility testing were screened for resistance genes. Thus, there may have been some clinical isolates carrying \textit{bla}_{CMY} and \textit{bla}_{CTX-M} with low MICs which could have been missed due to being classified as ‘susceptible’. However, using the study results to extrapolate absolute frequencies of these ESC resistance genes suggests that \textit{bla}_{CMY} is more frequent in \textit{E. coli} from chickens in comparison to dogs and pigs: the overall prevalence of \textit{bla}_{CMY} in clinical \textit{E. coli} was 15.2\% in chickens compared to 3.6-4.1\% in dogs and pigs. In contrast, the absolute frequencies of \textit{bla}_{CTX-M} may not be as different between the animal species as its overall prevalence in clinical \textit{E. coli} was fairly similar (0.6-2.3\%) for all three animal species. The specific manner in how ceftiofur was used in chickens in terms of treatment regimen and dosage, may have selected for \textit{bla}_{CMY} more than \textit{bla}_{CTX-M}. Additionally, the selective pressures may have been much higher in chickens compared to the other animal species when only \textit{bla}_{CMY} was present. However, now that the preventative use of ceftiofur has been banned in chickens, the selective pressures have decreased and it has allowed \textit{bla}_{CTX-M} to emerge in chickens. It could be speculated that \textit{bla}_{CMY} is more frequent in chickens due to \textit{bla}_{CTX-M} potentially emerging in chickens much later than pigs. However, this is not likely the case because comprehensive screening of ESC-resistant isolates from CIPARS for 2010 and 2011 only found CTX-M-positive isolates from poultry, and not from swine (Boerlin and Chalmers, unpublished data). The results may also indicate that \textit{E. coli} carrying \textit{bla}_{CMY} have a greater advantage in colonizing chickens compared to \textit{E. coli} with \textit{bla}_{CTX-M}.

Interestingly, there were no \textit{bla}_{SHV} genes in any of the \textit{E. coli} isolates for all three animal species, suggesting that \textit{bla}_{SHV} is extremely rare. Alternatively, it may be due to the fact that Enterobacteriaceae carrying \textit{bla}_{SHV} have only slightly elevated MICs for ESCs, as is the case with SHV-2 and SHV-2a, so these strains are unable to grow on the selective media used in this
study. Furthermore, using different ESCs in the selective media (cefotaxime in the enrichment broth and ceftriaxone in the agar plates) may have led to potential biases in the proportions of \( \text{bla}_{\text{CMY}} \) and \( \text{bla}_{\text{CTX-M}} \) in the resistant commensal Enterobacteriaceae isolates. This is because CMY-producing Enterobacteriaceae have different MICs for ESCs compared to those that produce CTX-M (Briñas et al., 2003). Similarly, the selective media may have also affected the relative proportions of CTX-M variants, since different CTX-M variants confer differing levels of ESC resistance (Bonnet, 2004; Shin et al., 2017).

The intestinal carriage of ESC-resistant Enterobacteriaceae was very high for chickens, dogs, and pigs (Table 4.1). Even though these resistant bacteria appear to be present in low concentrations and mainly detectable only by selective media, they are still widespread. This indicates that these resistant bacteria may also be widespread in other animals, and that future studies should investigate this area using both selective media and non-selective media. It also raises concerns for public health as there is evidence supporting transfer of resistant bacteria between humans and each individual animal species (Johnson and Clabots, 2006; Dahms et al., 2015). Humans and their dogs often have close contact, allowing for transmission of resistant bacteria. In addition, retail meat can be contaminated with intestinal or fecal material during slaughter of chickens and pigs. Consumption of improperly cooked and contaminated retail meat would then transmit resistant bacteria and their genes to humans in the same way as foodborne pathogens are transmitted to humans (Larkin et al., 2004). Improperly cooked meat could also transmit resistant bacteria to dogs, particularly if they are fed raw diets (Finley et al., 2008). Other transmission pathways include, among many others, contamination of produce with manure and cross-contamination of meat while cooking.

A large diversity of STs and CTX-M variants was identified in CTX-M-positive canine and swine isolates. A combined minimum spanning tree for both species demonstrates that the \textit{E. coli} populations in dogs and pigs are mainly distinct from one another (Fig. 4.1). This is seen by the different STs found in dogs compared to pigs, as the only STs found in both animal species were ST10, ST648, and ST4891. The major STs in canine isolates were ST131 and ST648, whereas the major ones in swine isolates were ST453, ST744, ST10, ST641. Interestingly, the major STs found in both animal species are all recognized as human pandemic clones, with the
exception of ST641. This suggests that there may be different dynamics underlying the emergence of human pandemic clones in these animals.

There was no major clustering of CTX-M variants in specific lineages (Fig. 4.2). Although there were different STs present in dogs compared to pigs, the most frequent CTX-M variants were the same in both animal species (CTX-M-1, -14, -15, and -27), with the exception of CTX-M-55 in canine isolates. This provides evidence, yet again, of the horizontal gene transfer of CTX-M and how it has effectively spread within bacterial populations in different animal species. Furthermore, CTX-M-14, -15, -27 and -55 are typically found in human isolates. Contrary to the other major CTX-M variants, CTX-M-1 has been found here in E. coli from both dogs and swine, but is very infrequent in clinical isolates from humans in Canada (M. Mulvey, personal communication). In addition, CTX-M-1 has also been identified in E. coli from Alberta beef cattle (Boerlin and Cormier, unpublished data) and in chicken in Québec (Chalmers et al., 2017) and Ontario (Boerlin, personal communication). It is difficult to understand how CTX-M-1 is disseminating apparently widely in bacteria from Canadian animals, but not yet in humans. It may be possible that CTX-M-1 was introduced to Canadian food animals through import of livestock from Europe, as CTX-M-1 is the most frequently isolated variant in that region (Ewers et al., 2012). Subsequently, CTX-M-1 may have gradually entered the intestinal flora of dogs through the food chain. This may be supported by the fact that CTX-M-1 has only been found in cecal and fecal isolates. Except for chicken, in which it was first detected in APEC and in Canada, CTX-M-1 may not have made its way yet into specific pathogens from other species in Canada, including humans. There is the possibility that CTX-M-1 may actually already be present in humans, but mainly in commensal E. coli strains that typically do not belong to the main disease-associated clonal lineages. This would be in agreement with the rarity of CTX-M-1 in human clinical isolates, and its main presence in cecal/fecal isolates. Unfortunately, it is difficult to investigate this as there are no systematic collections of fecal E. coli isolates from humans in Canada. It is also interesting that CTX-M-1 was not identified in any ST131 isolates, an ST which is more virulent than others, and yet it was found in other clones that are widespread in humans worldwide such as ST10, ST648, and ST744.
2. FUTURE DIRECTIONS

This study provided results on ESC resistance gene distribution within animal host species as well as between them. Overall, the relative distribution of \( \text{bla}_{\text{CMY}} \) and \( \text{bla}_{\text{CTX-M}} \) did not appear to be significantly different between resistant cecal/fecal and resistant clinical \( E.\ coli \) strains for dogs, chickens, and pigs. Performing conjugation experiments of the plasmids carrying \( \text{bla}_{\text{CMY}} \) and \( \text{bla}_{\text{CTX-M}} \) would be the next step in determining if transfer of resistance genes between commensals and pathogens is likely to occur, as well as if the frequency of resistance gene transfer is different between commensals and pathogens. The results also suggested that there were differences in the distributions of \( \text{bla}_{\text{CMY}} \) and \( \text{bla}_{\text{CTX-M}} \) in intestinal flora between chickens and pigs. Specifically, \( \text{bla}_{\text{CMY}} \) was more likely to be found in resistant cecal \( E.\ coli \) from chickens, whereas \( \text{bla}_{\text{CTX-M}} \) was more likely to be found in resistant cecal \( E.\ coli \) from pigs. In contrast, no similar significant differences were detected in resistant clinical \( E.\ coli \) isolates from these animals. The low numbers of resistant clinical isolates from pigs may have reduced the power of the statistical analyses to identify significant differences. Future studies on ESC resistance genes in clinical \( E.\ coli \) from chickens and pigs should investigate this, as more information is required to clarify the situation.

Due to time and funding constraints, whole-genome sequencing was not performed for the CTX-M-positive isolates from chickens. Ideally, this should be completed in the future in order to have a comprehensive overview of the STs, CTX-M variants, and other AMR genes present in all three animal species. A variety of resistance genes was found in the CTX-M-positive isolates for both dogs and pigs. Statistical analyses suggest that \( \text{bla}_{\text{CTX-M-15}} \) and \( \text{aac(3)}-\text{IIa}, \text{aac(6')}\text{-Ib-cr}, \text{catB3}, \) and \( \text{dfrA14} \) are likely to be located on the same plasmid. This was already seen when CTX-M-15 started to emerge, with plasmid pC15-1a carrying the resistance genes \( \text{bla}_{\text{CTX-M-15}}, \text{aac(3)}-\text{IIa}, \text{aac(6')}\text{-Ib-cr}, \) and \( \text{catB3} \). The plasmid pC15-1a was isolated from an \( E.\ coli \) strain that caused a large outbreak in long-term care facilities in Toronto, Ontario and was also found in multiple strains from hospitals in Vancouver, British Columbia (Boyd et al., 2004). Future experiments could carry out plasmid preparations with the strains from our current study and create transformants. Subsequent PCR screening and antimicrobial susceptibility testing could confirm if the genes are located on the same plasmid. This information would be valuable in knowing if there is the potential for co-selection of ESC resistance when using other
antimicrobials. Additionally, these plasmids could be characterized in terms of incompatibility groups, as well as subtyping by plasmid MLST (Carattoli, 2009). Other methods of classifying plasmids include replicon typing and sequencing the relaxase genes (Francia et al., 2004; Carattoli et al., 2005). If feasible, sequencing the plasmids could help elucidate phylogenetic relationships with other plasmids. Comparing this information with past studies would provide more insight on transmission pathways of resistance, especially if CTX-M-1 starts to emerge in human *E. coli* isolates (Carattoli, 2009; Cantón et al., 2012; Damborg et al., 2015).

3. CONCLUSIONS

Resistance to extended-spectrum cephalosporins is a global threat to human and veterinary medicine. In Canada, ESBL- and cephemacinase-producing Enterobacteriaceae are widespread in companion and food animals. Therefore, ESC use must be limited as much as possible to help preserve their clinical efficacy. Furthermore, future studies on ESC resistance and AMR in general should systematically identify the specific resistance genes and variants responsible for phenotypic resistance. This is becoming more feasible as the price of whole-genome sequencing continues to decrease. Knowing the specific AMR genes and variants would allow for a greater understanding of potential transmission pathways, as well as insight into preventing transmission of resistant bacteria.

Overall, this study has provided insight into the current situation of ESC resistance in Enterobacteriaceae from chickens, dogs, and pigs. In all three animal species, there was high intestinal carriage of ESC-resistant Enterobacteriaceae, and *bla*CMY and *bla*CTX-M were identified in both cecal and clinical isolates. In addition, there may be different dynamics for *bla*CMY and *bla*CTX-M in *E. coli* between the three animal species. Fortunately, carbapenemase-producing Enterobacteriaceae were not detected in this study. The specific STs and CTX-M variants identified strongly support the transfer of resistant bacteria and of resistance genes between bacteria from humans and dogs, as well as between humans and pigs. More research is needed to determine the exact transmission pathways that are present.
Table 4.1. Summary of the resistance genes found in ESC-resistant Enterobacteriaceae isolates from chickens, dogs, and pigs.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Origin</th>
<th>Prevalence of ESC resistance$^5$</th>
<th>Total no. of resistant E. coli isolates</th>
<th>No. of E. coli isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enterobacteriaceae</td>
<td>E. coli only</td>
<td></td>
<td>bla&lt;sub&gt;CMY&lt;/sub&gt;</td>
</tr>
<tr>
<td>Chicken</td>
<td>Clinical</td>
<td>17.3%</td>
<td>17.3%</td>
<td>63/366</td>
</tr>
<tr>
<td></td>
<td>Cecal</td>
<td>98.3%</td>
<td>98.3%</td>
<td>536</td>
</tr>
<tr>
<td>Dog</td>
<td>Clinical</td>
<td>10.4%</td>
<td>10.6%</td>
<td>31/248</td>
</tr>
<tr>
<td></td>
<td>Fecal</td>
<td>26.5%</td>
<td>24.4%</td>
<td>88</td>
</tr>
<tr>
<td>Pig</td>
<td>Clinical</td>
<td>8.8%</td>
<td>6.5%</td>
<td>11/80</td>
</tr>
<tr>
<td></td>
<td>Cecal</td>
<td>74.0%</td>
<td>74.0%</td>
<td>77</td>
</tr>
</tbody>
</table>

$^5$ The values shown for the clinical isolates is the prevalence of ESC resistance for all clinical Enterobacteriaceae isolates, excluding IDEXX’s purposive sampling, and the prevalence of ESC resistance for all clinical E. coli isolates only. The values shown for the cecal/fecal isolates is the percentage of cecal/fecal samples positive for ESC-resistant Enterobacteriaceae and ESC-resistant E. coli only.
Fig. 4.1. Minimum spanning tree of all CTX-M-positive canine and swine *E. coli* isolates using wgMLST with 3073 genes. Clusters of major STs are highlighted in blue. Five isolates were not assigned STs.\(^6\)

\(^6\) The two identical ST744 isolates were from cecal samples of two different pigs sampled in the same time period. The two identical ST648 isolates were from the same canine fecal sample, and the two identical ST131 isolates were from clinical samples of two different dogs.
Fig. 4.2. Minimum spanning tree of all CTX-M-positive canine and swine isolates showing the different CTX-M variants using wgMLST with 3073 genes.

* indicate isolates from pigs and their absence indicates canine isolates.\(^7\)

\(^7\) The identical CTX-M-14 isolates were from cecal samples of two different pigs sampled in the same time period. The identical CTX-M-1 isolates were from the same canine fecal sample, and the identical CTX-M-15 isolates were from clinical samples of two different dogs.
REFERENCES


APPENDIX 1. SUPPLEMENTARY DATA FOR CHAPTER 2

Fig. 2.S1. The cities in Southern Ontario that were sampled for canine feces shown with Google Maps. Map data: © 2016 Google.
Fig. 2.S2. The frequency of ESC-resistant Enterobacteriaceae fecal carriage after enrichment in cities of Southern Ontario with 95% confidence intervals.
Table 2.S1. Antimicrobial resistance genes found in the CTX-M positive ESC-resistant clinical isolates and fecal isolates from dogs.

<table>
<thead>
<tr>
<th>Antimicrobial(s) and antimicrobial resistance gene</th>
<th>Bacterial Species</th>
<th>Total no. of isolates (%; n=47)</th>
<th>No. of isolates (%) from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Clinical (n=28)</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-IIa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P. gergoviae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>aac(3)-IId</td>
<td>E. coli</td>
<td>7 (14.9)</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>aac(3)-VIa</td>
<td>E. coli</td>
<td>1 (2.1)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>aadA1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>6</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P. gergoviae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>aadA2</td>
<td>E. coli</td>
<td>3 (6.4)</td>
<td>1 (3.6)</td>
</tr>
<tr>
<td>aadA5</td>
<td>E. coli</td>
<td>14 (29.8)</td>
<td>7 (25.0)</td>
</tr>
<tr>
<td>aph(3')-la</td>
<td>E. coli</td>
<td>7 (14.9)</td>
<td>3 (10.7)</td>
</tr>
<tr>
<td>strA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>22</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P. gergoviae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>strB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>22</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P. gergoviae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Aminoglycoside and fluoroquinolone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(6')-Ib-cr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E. cloacae</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K. oxytoca</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

94
<table>
<thead>
<tr>
<th>β-lactam</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. gergoviae</strong></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

| **E. cloacae** | 1 (2.1) | 1 (3.6) | 0 |
| **E. coli** | 3 (6.4) | 3 (10.7) | 0 |
| **E. coli** | 6 (12.8) | 0 | 6 (31.6) |
| **E. coli** | 5 (10.6) | 2 (7.1) | 3 (15.8) |
| **Total** | 17 (36.2) | 12 (42.9) | 5 (26.3) |

| **K. pneumoniae** | 2 | 2 | 0 |
| **E. cloacae** | 1 | 1 | 0 |
| **K. oxytoca** | 1 | 1 | 0 |
| **P. gergoviae** | 1 | 0 | 1 |

| **blactx-M-27** | **E. coli** | 9 (19.1) | 7 (25.0) | 2 (10.5) |
| **blactx-M-55** | **E. coli** | 7 (14.9) | 5 (17.9) | 2 (10.5) |
| **blactx-M-115** | **E. coli** | 1 (2.1) | 0 | 1 (5.3) |
| **blactx-M-169** | **E. coli** | 1 (2.1) | 1 (3.6) | 0 |
| **blactx-M-202** | **E. coli** | 1 (2.1) | 1 (3.6) | 0 |
| **blaoxa-1** | **Total** | 9 (19.1) | 7 (25.0) | 2 (10.5) |
| **E. coli** | 4 | 3 | 1 |
| **K. pneumoniae** | 2 | 2 | 0 |
| **E. cloacae** | 1 | 1 | 0 |
| **K. oxytoca** | 1 | 1 | 0 |
| **P. gergoviae** | 1 | 0 | 1 |

| **blaoxy-2-7** | **K. oxytoca** | 1 (2.1) | 1 (3.6) | 0 |
| **bashv-83** | **K. pneumoniae** | 2 (4.3) | 2 (7.1) | 0 |
| **blastem-34** | **E. coli** | 1 (2.1) | 0 | 1 (5.3) |
| **blatem-1-b** | **Total** | 17 (36.2) | 13 (46.4) | 4 (21.1) |
| **E. coli** | 12 | 9 | 3 |
| **K. pneumoniae** | 2 | 2 | 0 |
| **E. cloacae** | 1 | 1 | 0 |
| **K. oxytoca** | 1 | 1 | 0 |
| **P. gergoviae** | 1 | 0 | 1 |

<table>
<thead>
<tr>
<th>Phenicols</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

| **catA1** | **Total** | 6 (12.8) | 2 (7.1) | 4 (21.1) |
| **E. coli** | 3 | 0 | 3 |
| **E. cloacae** | 1 | 1 | 0 |
| **K. oxytoca** | 1 | 1 | 0 |
| **P. gergoviae** | 1 | 0 | 1 |

| **catA2** | **Total** | 2 (4.3) | 2 (7.1) | 0 |
| **E. coli** | 2 | 2 | 0 |

<p>| <strong>catB3</strong> | <strong>Total</strong> | 9 (19.1) | 7 (25.0) | 2 (10.5) |
| <strong>E. coli</strong> | 4 | 3 | 1 |</p>
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>floR</th>
<th>Fluoroquinolone</th>
<th>oqxA</th>
<th>K. pneumoniae</th>
<th>2 (4.3)</th>
<th>2 (7.1)</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td>6 (12.8)</td>
<td>6 (21.4)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K. oxytoca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. gergoviae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>floR</td>
<td>Fluoroquinolone</td>
<td>oqxB</td>
<td>K. pneumoniae</td>
<td>2 (4.3)</td>
<td>2 (7.1)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td>6 (12.8)</td>
<td>6 (21.4)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K. oxytoca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. gergoviae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qnrB1</td>
<td>Total</td>
<td>Fluoroquinolone</td>
<td></td>
<td></td>
<td>5 (10.6)</td>
<td>4 (14.3)</td>
<td>1 (5.3)</td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K. oxytoca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. gergoviae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qnrS1</td>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td>5 (10.6)</td>
<td>3 (10.7)</td>
<td>2 (10.5)</td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K. oxytoca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. gergoviae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>fosA</td>
<td></td>
<td></td>
<td></td>
<td>3 (6.4)</td>
<td>3 (10.7)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td>19 (40.4)</td>
<td>9 (32.1)</td>
<td>10 (52.6)</td>
</tr>
<tr>
<td>Macrolide</td>
<td>erm(B)</td>
<td></td>
<td></td>
<td></td>
<td>3 (6.4)</td>
<td>1 (3.6)</td>
<td>2 (10.5)</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>mph(A)</td>
<td></td>
<td></td>
<td></td>
<td>19 (40.4)</td>
<td>9 (32.1)</td>
<td>10 (52.6)</td>
</tr>
<tr>
<td></td>
<td>mph(B)</td>
<td></td>
<td></td>
<td></td>
<td>1 (2.1)</td>
<td>1 (3.6)</td>
<td>0</td>
</tr>
<tr>
<td>Sulfonamide</td>
<td>sul1</td>
<td></td>
<td></td>
<td></td>
<td>19 (40.4)</td>
<td>11 (39.3)</td>
<td>8 (42.1)</td>
</tr>
<tr>
<td></td>
<td>sul2</td>
<td></td>
<td></td>
<td></td>
<td>29 (61.7)</td>
<td>19 (67.9)</td>
<td>10 (52.6)</td>
</tr>
<tr>
<td></td>
<td>sul3</td>
<td></td>
<td></td>
<td></td>
<td>2 (4.3)</td>
<td>2 (7.1)</td>
<td>0</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>dfrA1</td>
<td></td>
<td></td>
<td></td>
<td>1 (2.1)</td>
<td>1 (3.6)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>dfrA8</td>
<td></td>
<td></td>
<td></td>
<td>1 (2.1)</td>
<td>1 (3.6)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>dfrA12</td>
<td></td>
<td></td>
<td></td>
<td>3 (6.4)</td>
<td>1 (3.6)</td>
<td>2 (10.5)</td>
</tr>
<tr>
<td></td>
<td>dfrA14</td>
<td></td>
<td></td>
<td></td>
<td>11 (23.4)</td>
<td>8 (28.6)</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td></td>
<td>dfrA17</td>
<td></td>
<td></td>
<td></td>
<td>16 (34.0)</td>
<td>9 (32.1)</td>
<td>7 (36.8)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Total</td>
<td>E. coli</td>
<td>K. pneumoniae</td>
<td>E. cloacae</td>
<td>K. oxytoca</td>
<td>P. gergoviae</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------</td>
<td>---------</td>
<td>---------------</td>
<td>-----------</td>
<td>------------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>tet(A)</td>
<td>27 (57.4)</td>
<td>19 (67.9)</td>
<td>8 (42.1)</td>
<td>E. coli</td>
<td>22</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>K. pneumoniae</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E. cloacae</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>K. oxytoca</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P. gergoviae</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(B)</td>
<td>E. coli</td>
<td>8 (17.0)</td>
<td>3 (10.7)</td>
<td>5 (26.3)</td>
<td>E. coli</td>
<td>1 (2.1)</td>
<td>0</td>
</tr>
<tr>
<td>tet(M)</td>
<td>E. coli</td>
<td>1 (2.1)</td>
<td>0</td>
<td>1 (5.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# APPENDIX 2. SUPPLEMENTARY DATA FOR CHAPTER 3

Table 3.S1. Antimicrobial resistance genes found in the CTX-M positive ESC-resistant clinical isolates and cecal *E. coli* isolates from swine.

<table>
<thead>
<tr>
<th>Antimicrobial(s) and antimicrobial resistance gene</th>
<th>Total no. of isolates (%; n=23)</th>
<th>No. of isolates (%) from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Clinical (n=1)</td>
</tr>
<tr>
<td><strong>Aminoglycoside</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aadA1</em></td>
<td>2 (8.7)</td>
<td>1 (100)</td>
</tr>
<tr>
<td><em>aadA2</em></td>
<td>4 (13.0)</td>
<td>1 (100)</td>
</tr>
<tr>
<td><em>aadA5</em></td>
<td>8 (34.8)</td>
<td>0</td>
</tr>
<tr>
<td><em>aadA12</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>aph(3')-la</em></td>
<td>2 (8.7)</td>
<td>0</td>
</tr>
<tr>
<td><em>strA</em></td>
<td>5 (21.7)</td>
<td>0</td>
</tr>
<tr>
<td><em>strB</em></td>
<td>5 (21.7)</td>
<td>0</td>
</tr>
<tr>
<td><strong>β-lactam</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bla</em>CARB-2</td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaCMY-2</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaCTX-M-1</em></td>
<td>9 (39.1)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaCTX-M-14</em></td>
<td>8 (34.8)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaCTX-M-15</em></td>
<td>2 (8.7)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaCTX-M-27</em></td>
<td>3 (13.0)</td>
<td>1 (100)</td>
</tr>
<tr>
<td><em>blaCTX-M-115</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaTEM-1-A</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaTEM-1-B</em></td>
<td>4 (17.4)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaTEM-1-C</em></td>
<td>2 (8.7)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaTEM-34</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>blaTEM-57</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Chloramphenicol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>catA1</em></td>
<td>4 (17.4)</td>
<td>0</td>
</tr>
<tr>
<td><em>cmlA1</em></td>
<td>2 (8.7)</td>
<td>1 (100)</td>
</tr>
<tr>
<td><em>floR</em></td>
<td>2 (8.7)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Macrolide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>mph(A)</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Quinolone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>qnrS1</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Sulfonamide</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>sul1</em></td>
<td>6 (26.1)</td>
<td>0</td>
</tr>
<tr>
<td><em>sul2</em></td>
<td>16 (69.6)</td>
<td>0</td>
</tr>
<tr>
<td><em>sul3</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td><strong>Trimethoprim</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dfrA5</em></td>
<td>1 (4.3)</td>
<td>1 (100)</td>
</tr>
<tr>
<td><em>dfrA12</em></td>
<td>2 (8.7)</td>
<td>1 (100)</td>
</tr>
<tr>
<td><em>dfrA14</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>dfrA16</em></td>
<td>1 (4.3)</td>
<td>0</td>
</tr>
<tr>
<td><em>dfrA17</em></td>
<td>9 (39.1)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Tetracycline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tet(A)</em></td>
<td>14 (60.9)</td>
<td>0</td>
</tr>
<tr>
<td><em>tet(B)</em></td>
<td>8 (34.8)</td>
<td>1 (100)</td>
</tr>
<tr>
<td><em>tet(M)</em></td>
<td>1 (4.3)</td>
<td>1 (100)</td>
</tr>
</tbody>
</table>
Competition Trial Protocol:

Broth cultures of *E. coli*, *K. pneumoniae*, and *E. cloacae* were made by inoculating 20 mL of Luria-Bertania Broth (Becton Dickinson [BD], Cockeysville, MD, USA) with a 10 µL loop of bacteria. Two replicates were made for each bacterial species. All of the broth cultures were incubated with shaking at 37°C until an optical density of 0.50 at 600 nm was measured using a spectrophotometer (Novaspec ® Plus Spectrophotometer). Subsequently, 50 µL of each LB broth culture was pipetted into three separate tubes of 20 mL of EE Broth Mossel Enrichment (Becton Dickinson [BD]) in the following combinations: *E. coli* with *K. pneumoniae*, *E. coli* with *E. cloacae*, and *K. pneumoniae* with *E. cloacae*. The inoculated EE broth was incubated overnight with agitation at 37°C. Serial dilutions of the inoculated EE broth were made before and after incubation to determine the concentrations of the three bacterial species. The colonies were counted and the results found that *E. coli* did not outcompete *E. cloacae* or *K. pneumoniae* (Table S2). Also, *E. cloacae* and *K. pneumoniae* were both detectable after their competition trial. The protocol was repeated a second time with a different set of strains and the results were the same as the first trial; there was no overgrowth of any bacterial species.
Table 3.S2. Colony counts of serial dilutions of broth cultures for *E. coli*, *K. pneumoniae*, and *E. cloacae* before and after two separate competition trials.

<table>
<thead>
<tr>
<th>Bacterial Species Combination</th>
<th>First Competition Trial</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st Replicate</td>
<td>2nd Replicate</td>
<td>1st Replicate</td>
<td>2nd Replicate</td>
<td></td>
</tr>
<tr>
<td>No. of colonies before trial</td>
<td>No. of colonies after trial</td>
<td>No. of colonies before trial</td>
<td>No. of colonies after trial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>166</td>
<td>123</td>
<td>141</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>194</td>
<td>130</td>
<td>137</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>76</td>
<td>55</td>
<td>167</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>E. cloacae</td>
<td>136</td>
<td>270</td>
<td>241</td>
<td>322</td>
<td></td>
</tr>
<tr>
<td>E. cloacae</td>
<td>289</td>
<td>390</td>
<td>250</td>
<td>478</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>194</td>
<td>27</td>
<td>186</td>
<td>59</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacterial Species Combination</th>
<th>Second Competition Trial</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st Replicate</td>
<td>2nd Replicate</td>
<td>1st Replicate</td>
<td>2nd Replicate</td>
<td></td>
</tr>
<tr>
<td>No. of colonies before trial</td>
<td>No. of colonies after trial</td>
<td>No. of colonies before trial</td>
<td>No. of colonies after trial</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>102</td>
<td>177</td>
<td>100</td>
<td>227</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>67</td>
<td>117</td>
<td>74</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>98</td>
<td>258</td>
<td>140</td>
<td>263</td>
<td></td>
</tr>
<tr>
<td>E. cloacae</td>
<td>110</td>
<td>135</td>
<td>138</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>E. cloacae</td>
<td>120</td>
<td>198</td>
<td>175</td>
<td>218</td>
<td></td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>53</td>
<td>116</td>
<td>104</td>
<td>162</td>
<td></td>
</tr>
</tbody>
</table>