Université de Montréal

Molecular characterization of extended-spectrum cephalosporin-resistance in *Escherichia coli* in pigs on-farm and from clinical cases throughout Quebec, Canada during 16 years

par
Seyedehameneh Jahanbakhsh

Département de pathologie et microbiologie
Faculté de médecine vétérinaire

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Résumé

Le développement de la multirésistance chez *Escherichia coli* est un problème important en médecine animale et humaine. En outre, l'émergence et la diffusion des déterminants de résistance aux céphalosporines à larges spectres de troisième génération (ESCs) parmi les isolats, incluant des céphalosporines essentielles en médecine humaine (ex. ceftriaxone et ceftiofur), est un problème majeur de santé publique. Cette thèse visait trois objectifs. D'abord étudier la dynamique de la résistance aux antimicrobiens (AMR) ainsi que la virulence et les profils génétiques de la AMR des *E. coli* isolées de porcs recevant une nourriture post-sevrage supplémentée avec de la chlortétracycline et de la pénicilline G, et, accessoirement, évaluer les effets d'additifs alimentaires sur cette dynamique en prenant pour exemple d'étude un minéral argileux, la clinoptilolite, étant donné son possible lien avec le gène *bla*$_{\text{CMY-2}}$ qui confère la résistance au ceftiofur. L'objectif suivant était d'investiguer les mécanismes menant à une augmentation de la prévalence du gène *bla*$_{\text{CMY-2}}$ chez les porcs qui reçoivent de la nourriture médicamentée et qui n'ont pas été exposés au ceftiofur Ici encore,nous avons examiné les effets d'un supplément alimentaire avec un minéral argileux sur ce phénomène. Enfin, notre dernier objectif était d'étudier, dans le temps, les génotypes des isolats cliniques d'*E. coli* résistant au ceftiofur, isolés de porcs malades au Québec à partir du moment où la résistance au ceftiofur a été rapportée, soit de 1997 jusqu'à 2012.

Dans l'étude initiale, la prévalence de la résistance à 10 agents antimicrobiens, incluant le ceftiofur, s'accroît avec le temps chez les *E.coli* isolées de porcelets sevrés. Une augmentation tardive de la fréquence du gène *bla*$_{\text{CMY-2}}$, encodant pour la résistance au ceftiofur, et la présence des gènes de virulence *iucD* et *tsh* a été observée chez les isolats. La nourriture supplémentée avec de la clinoptilolite a été associée à une augmentation rapide mais, par la suite, à une diminution de la fréquence des gènes *bla*$_{\text{CMY-2}}$ dans les isolats. En parallèle, une augmentation tardive dans la fréquence des gènes *bla*$_{\text{CMY-2}}$ et des gènes de virulence *iucD* et *tsh* a été observée dans les isolats des porcs contrôles, étant significativement plus élevé que dans les porcs ayant reçu l'additif au jour 28. La diversité, au sein des *E. coli* positives pour *bla*$_{\text{CMY-2}}$, a été observée au regard des profils AMR. Certaines lignées clonales d'*E.coli* sont devenues prédominantes avec le temps. La lignée clonale du phylotype A prédominait dans le groupe supplémenté, alors que les lignées

clonales du phylotype B1, qui possèdent souvent le gène de virulence *iucD* associé aux ExPEC, prédominaient dans le groupe contrôle. Les plasmides d'incompatibilité (Inc) des groupes, I1, A/C, et ColE, porteurs de *bla*$_{CMY-2,}$ ont été observés dans les transformants. Parmi les souches cliniques d'*E.coli* ESC-résistantes, isolées de porcs malades au Québec de 1997 à 2012, *bla*$_{CMY-2}$ était le gène codant pour une β-lactamase le plus fréquemment détecté; suivi par *bla*$_{TEM}$ et *bla*$_{CTX-M,}$. De plus, les analyses clonales montrent une grande diversité génétique. Par contre, des isolats d'*E. coli* avec des profils PFGE identiques ont été retrouvés dans de multiples fermes la même année mais aussi dans des années différentes. La résistance à la gentamicine, kanamycine, chloramphenicol, et la fréquence de *bla*$_{TEM}$ et de IncA/C diminuent significativement au cour de la période étudiée, alors que la fréquence de IncI1 et de la multirésistance à sept catégories d'agents antimicrobiens augmente significativement avec le temps. L'émergence d'isolats d'*E. coli* positifs pour *bla*$_{CTX-M}$, une β-lactamase à large spectre et produisant des ESBL, a été observée en 2011 et 2012 à partir de lignées clonales distinctes et chez de nombreuses fermes.

Ces résultats, mis ensemble, apportent des précisions sur la dissémination de la résistance au ceftiofur dans les *E. coli* isolées de porcs. Au sein des échantillons prélevés chez les porcs sevrés recevant l'alimentation médicamentée sur une ferme, et pour laquelle une augmentation de la résistance au ceftiofur a été observée, les données révèlent que les souches d'*E. coli* positives pour *bla*$_{CMY-2}$ et résistantes aux ESCs appartenaient à plusieurs lignées clonales différentes arborant divers profils AMR. Le gène *bla*$_{CMY-2}$ se répand à la fois horizontalement et clonalement chez ces *E. coli*. L'ajout de clinoptilotite à la nourriture et le temps après le sevrage influencent la clonalité et la prévalence du gène *bla*$_{CMY-2}$ dans les *E. coli*. Durant les 16 années d'étude, plusieurs lignées clonales différentes ont été observées parmi les souches d'*E. coli* résistantes au ceftiofur isolées de porc malades de fermes québécoises, bien qu'aucune lignée n'était persistante ou prédominante pendant l'étude. Les résultats suggèrent aussi que le gène *bla*$_{CMY-2}$ s'est répandu à la fois horizontalement et clonalement au sein des fermes. De plus, *bla*$_{CMY-2}$ est le gène majeur des β-lactamases chez ces isolats. À partir de 2011, nous rapportons l'émergence du gène *bla*$_{CTX-M}$ dans des lignées génétiques distinctes.

Mots clé: Porc; *E. coli*; gène de virulence; résistance antimicrobienne; plasmide, gène *bla*$_{CMY-2}$, gène *bla*$_{CTX-M}$, résistance aux ESC, argile minéral (clinoptilolite).

Abstract

Development of multidrug resistance in *Escherichia coli* is an important problem in animal and human medicine. Further, emergence and spread of determinants for resistance to third generation extended-spectrum cephalosporins (ESCs) such as the critically important cephalosporins in human medicine (e.g. ceftriaxone and ceftiofur) among isolates is a public health concern. Thus, the objectives of the present thesis were (1) to study the dynamic of antimicrobial resistance (AMR) phenotypes as well as virulence and AMR gene profiles in *E. coli* from pigs receiving a feed medicated with chlortetracycline and penicillin G following weaning and to study the effect of feed supplementation with a clay mineral, clinoptilolite, on this dynamic; (2) To investigate the mechanisms leading to an increase in the prevalence of *bla*$_{\text{CMY-2}}$ conferring resistance to ceftiofur in pigs receiving medicated feed but which had not received ceftiofur, and to examine the effect of feed supplementation with a clay mineral on this phenomenon; and (3) to investigate the temporal characterization of clinical isolates of ceftiofur-resistant *E.coli* from diseased pigs in Quebec, Canada from 1997, when ceftiofur resistance was first reported, to 2012.

In the initial study, prevalence of resistance to 10 antimicrobial agents, including ceftiofur, increased over time in *E.coli* isolates from weaned pigs. A late increase in the frequency of *bla*$_{\text{CMY-2}}$, the gene encoding resistance to ceftiofur, and the presence of virulence genes *iucD* and *tsh* were observed in the isolates. Feed supplementation with clinoptilolite was associated with an early increase but later decrease in the frequency of the *bla*$_{\text{CMY-2}}$ gene in isolates. Concurrently, a later increase in the frequency of the *bla*$_{\text{CMY-2}}$ and the virulence genes *iucD* and *tsh* was observed in the control pig isolates, being significantly greater than in the supplemented pigs at day 28. Diversity among the *bla*$_{\text{CMY-2}}$-positive *E. coli* isolates with respect to AMR patterns was observed. Certain clonal lineages of *E. coli* became predominant with time. The clonal lineage of phylotype A predominated in the supplemented group, whereas the clonal lineages of phylotype B1 which often possessed the ExPEC-associated virulence gene *iucD*, predominated in the control group. The *bla*$_{\text{CMY-2}}$-carrying plasmids of incompatibility (Inc) groups, I1, A/C, and ColE were observed in transformants. In ESC-resistant *E.coli* clinical isolates from

diseased pigs in Quebec, from 1997 to 2012, *bla*$_{CMY-2}$ was the most frequently detected β-lactamase gene, followed by *bla*$_{TEM}$ and *bla*$_{CTX-M}$ and clonal analysis showed high diversity. *E. coli* isolates with identical PFGE patterns were found in multiple farms in the same year and also in different years. Resistance to gentamicin, kanamycin, chloramphenicol, and the frequency of *bla*$_{TEM}$ and IncA/C significantly decreased over the study time, whereas the frequency of IncI1 and multidrug-resistance to seven antimicrobial categories significantly increased over time. Emergence of *bla*$_{CTX-M}$-positive, extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolates was observed in 2011 and 2012 from distinct clonal lineages and multiple farms.

Taken together, this work gives some insight into the spread of ceftiofur resistance in *E.coli* isolates from pigs. Results reveal that in weaned pigs receiving medicated feed on one farm in which an increase in ceftiofur resistance was observed, the *bla*$_{CMY-2}$-positive *E.coli* resistant to ESCs belonged to several different clonal lineages with diverse AMR patterns. The *bla*$_{CMY-2}$ gene spread both horizontally and clonally in *E. coli*. Feed supplementation with clinoptilolite and time period after weaning influenced the clonality and the prevalence of *bla*$_{CMY-2}$ gene in *E. coli*. In ceftiofur-resistant *E.coli* strains isolated from diseased pigs in farms throughout Quebec over a 16 year period, several different pathogenic clonal lineages were observed, although none was persistent or predominant over the study time. The results suggest that *bla*$_{CMY-2}$ gene spreads both horizontally and clonally on and between farms. Furthermore, *bla*$_{CMY-2}$ was the major β-lactamase gene in these isolates. From 2011, we report the emergence of *bla*$_{CTX-M}$ in distinct clonal lineages.

Keywords: Pig; *E. coli*; virulence gene; antimicrobial resistance; plasmid, *bla*$_{CMY-2}$ gene, *bla*$_{CTX-M}$ gene, ESC-resistance, clay mineral (clinoptilolite).

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List of abbreviations

AE : Attaching and effacing

afa/dra : Dr-antigen-binding adhesins

AFLP : Amplified fragment length polymorphism

AIDA : Adhesin involved in diffuse adherence

AMC : Amoxicillin/clavulanic acid

AMK : Amikacin

AMP : Ampicillin

AMR : Antimicrobial resistance

APEC : Avian pathogenic *Escherichia coli*

APZEC : Animal pathogenic and zoonotic *E. coli*

BFP : Bundle-forming pilus

BIGSdb : Bacterial isolate genome sequence database

CF : Colonization factor

CHL : Chloramphenicol

CIP : Ciprofloxacin

CIPARS : Canadian Integrated Program for Antimicrobial Resistance Surveillance

CLA : Clavulanic acid

CLSI : Clinical and Laboratory Standards Institute

CMT : Complex mutant TEM

CNF/*cnf* : Cytotoxic necrotizing factor

CRO : Ceftriaxone

CT : Cholera toxin

E. coli : *Escherichia coli*

E. cloacae : *Enterobacter cloacae*

EAST1 : Enteroaggregative *E. coli* heat-stable enterotoxin 1

ED : Edema disease

EDTA : ethylenediaminetetraacetic acid

EPEC : Enteropathogenic *Escherichia coli*

ESBLs : Extended-spectrum beta-lactamases

ESCs : Extended-spectrum cephalosporins

ETEC : Enterotoxigenic *Escherichia coli*

ExPEC : Extra-intestinal pathogenic *Escherichia coli*

FDA : Food and Drug Administration

FIS : Sulfisoxazole

FOX : Cefoxitin

GEN : Gentamicin

GRAS : Generally Regarded as Safe

HGMF : Hydrophobic grid membrane filter

HGT : Horizontal gene transfer

HUS : Hemolytic uremic syndrome

IMP : Imipenemase

Inc : Incompatibility

IRT : Inhibitor-resistant TEM

IS : Insertion sequences

iucD/*iutA* : Aerobactin receptor

KAN : Kanamycin

kpsMTII : Group 2 capsular polysaccharide units

LEE : Locus of enterocytes effacement

LPS : Lipopolysaccharide

LT : Heat-labile enterotoxin

MAPAQ : Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec

MDR : Multi-drug resistant

MGEs : Mobile genetic elements

MLST : Multilocus sequence typing

MLVA : Multiple-locus variable-number tandem-repeat analysis

MRSA : Methicillin-resistant *S. aureus*

NAL : Nalidixic acid

NARMS : National Antimicrobial Resistance Monitoring System

NDM : New Delhi metallo-beta-lactamase

PAIs : Pathogenicity islands

papA : P fimbriae structural subunit

papC : P fimbriae assembly

PCR : Polymerase chain reaction

PFGE : Pulsed-field gel electrophoresis

PRSA : Penicillin-resistant *S. aureus*

RAPD-PCR : Random amplified polymorphic DNA PCR

Rep-PCR : Repetitive element PCR

RFLP : Restriction fragment length polymorphism

SEPEC : Septicemic *E. coli*

SNP : Single nucleotide polymorphism

ST : Heat-stable enterotoxin

STEC : Shiga-toxin producing *Escherichia coli*

STR : Streptomycin

Stx : Shiga toxin

SXT : Trimethoprim-sulphamethoxazole

TE : Transposable elements

TET : Tetracycline

TIO : Ceftiofur

tsh : Temperature-sensitive hemagglutinin

TZB : Tazobactam

UPEC : Uropathogenic *Escherichia coli*

UTIs : Urinary tract infections

VIM : Verona integron-encoded metallo-beta-lactamase

wgMLST : Whole-genome MLST

WGS : Whole-genome sequencing

WHO : World Health Organization

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Introduction

The use of antimicrobials in food producing animals has been surveyed over the past two decades because of their impact on animal and human health associated with antimicrobial resistance (AMR). Development of AMR increases the risk of failure of antimicrobial treatment in animals and humans. In particular, emergence and prevalence of ESC-resistant *E. coli* in food producing animals is a public health concern, as cephalosporins (e.g. ceftriaxone, ceftiofur) are critically important β-lactam antimicrobials in human and animal medicine (WHO 2011). Ceftiofur, a third- generation cephalosporin, is used in pigs to treat respiratory disease, lameness and enteric disease (Deckert et al. 2010). As ceftiofur is in the same general class of antimicrobials as ceftriaxone, resistance to ceftiofur is a problem because shared resistance determinants may confer resistance to ceftriaxone. Ceftiofur has been administrated therapeutically in food producing animals since 1989 (Daniels et al. 2009) and ceftiofur resistance via the $bla_{\text{CMY-2}}$ gene was first reported in 1998 (Fey et al. 2000; Winokur et al. 2000). During the past decade, ESC-resistance has been reported among clinical isolates of *Enterobacteriaceae* from humans and animals in Canada (Mulvey et al. 2009; Mataseje et al. 2010). The Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec reported a significant increase in resistance to ceftiofur in *E. coli* isolated from pigs comparing two windows of time, 1993-2009 (4%) and 2010-2014 (19%), being 22% in 2014 (MAPAQ 2015).

Administration of antimicrobials as therapeutic agents, or as growth promoters, or for disease prevention during animal production can result in the development of AMR. These resistant bacteria may spread to humans directly or through food chain, resulting in potential public health risks (Johnston 2001; Wegener 2003). In addition, the resistant bacteria and/or resistance genes are shed into the environment where they may persist for long periods (Chantziaras et al. 2014). Thus, animal feeds may be supplemented with feed ingredients as an alternative for or a complement to, the administration of antimicrobials. For example, the clay mineral, clinoptilolite, has been used to improve performance and health but also as an alternative to the use of antimicrobials or together with antimicrobials

for prevention of post-weaning diarrhea in piglets (Papaioannou et al. 2004). However, the mechanisms of action of clinoptilolite are little understood. Some *in vitro* studies showed that DNA bound to clay minerals is more resistant to degradation by DNase I (Romanowski et al. 1991), and also, minerals induce bacterial mutation and boost genetic diversity of bacteria (Yoshida et al. 2004). Likewise, It has been shown in an *in vitro* study that clay minerals promote horizontal gene transfer of AMR genes in different bacterial species (Lotareva and Prozorov 2000; Rodriguez-Beltran et al. 2013). These data imply that clay minerals may modulate the frequency of AMR and virulence genes of bacteria in the animal intestinal. In weaned pigs, antimicrobials are used in feed mainly as a medication to prevent disease and thus reduce mortality and morbidity (Cromwell 2002). Penicillin and tetracycline are among the administrated in-feed antimicrobials in pigs and often in mixture (Akwar et al. 2008). Administration of in-feed antimicrobials in pigs has been related to raise resistance of fecal *E. coli* within and between antimicrobial classes (Kim et al. 2005; Akwar et al. 2008).

In *Enterobacteriaceae*, ESC-resistance has been related to production of AmpC-like β-lactamases (e.g. CMY-2) and ESBLs (e.g. CTX-M and OXA) encoded by genes often found on transferable plasmids. The CTX-M family are important ESBLs in human medicine and have become a threat to public health (Li et al. 2007). The increasing relation of resistance to other classes of antimicrobials such as aminoglycosides, sulphonamides, phenicols and tetracyclines in ESC-resistant *E. coli* has promoted multidrug-resistant (MDR) strains which severely limit therapeutic options (Pitout et al. 2007). CMY-2-encoding genes are commonly located on transferable elements (integrons, transposons, insertion sequences) carried by plasmids that facilitate the horizontal spread of resistance and most AmpC beta-lactamase producing strains may carry additional resistance genes. Hence, certain resistance genes can be conserved due to a link with the genes encoding resistance to other antimicrobials that are registered for use in animal production (Dunne et al. 2000; Allen and Poppe 2002; Funk et al. 2006; Singer and Hofacre 2006). Although the *E.coli* phylotypes are different between isolates from humans and animals (Johnson et al. 2003; Maynard et al. 2004), common replicon types of plasmids encoding β-lactamase genes were observed in *E. coli* isolates from humans and food producing animals (Carattoli

2009). Plasmids bearing both virulence and resistance genes may also spread in a pathogenic bacterial population due to antimicrobial selection pressure (Martinez and Baquero 2002). In animal production, a variety of risk factors related to farm management may be associated with the introduction and spread of AMR in bacteria, however, these are not yet fully elucidated. Further research is needed to understand factors that contribute to the circulation and persistence of antimicrobial resistance determinants among both commensal and pathogenic enteric bacteria on-farm and worldwide.

Hypothesis

Feed supplements influence the prevalence of virulence and antimicrobial resistance genes of *E. coli* in the intestine of pigs and could affect the fecal excretion of *E. coli* possessing these genes.

Objectives

The primary objective of this study was to study the temporal characterization of virulence genes and antimicrobial resistance in *E. coli* from pig fecal samples and the effect of a feed supplement.

In particular, the specific objectives of this research are:

1. To examine the dynamic of AMR phenotype, virulence and AMR gene profiles in *E. coli* isolates from pigs receiving a diet containing chlortetracycline and penicillin G at therapeutic doses following weaning on a commercial farm and to investigate the effect of simultaneous feeding of the clay mineral, clinoptilolite, on this dynamic.

2. To elucidate the mechanisms leading to an increase of the prevalence of $bla_{\text{CMY-2}}$ conferring resistance to ceftiofur in a nursery barn in pigs with no ceftiofur use but which received a feed medicated with chlortetracycline and penicillin G, and to

investigate the effect of feed supplementation with a clay mineral on this phenomenon.

3. To study the temporal characterization of clinical ceftiofur-resistant *E.coli* isolates from diseased pigs in Quebec-Canada from 1997 to 2012.

Chapter 1: Literature review

1 Escherichia coli

1.1 General aspects of *E.coli*

E. coli, a common inhabitant of the intestinal tract of humans and other warm-blooded animals is a gram negative, facultative anaerobic organism and a member of *Enterobacteriaceae* family (Kaper et al. 2004; Gyles and Fairbrother 2010). It was first described by Theodore Escherich in 1885 as a slim rod in the feces of an infant (Escherich 1988). *E. coli* typically colonizes the intestine shortly after birth and remains there as a part of the normal microflora of the gastrointestinal tract of animals (Gyles and Fairbrother 2010). *E. coli* may be part of both the resident and a transient population in the gastrointestinal tract. Resident strains may persist for months or years whereas transient strains may be present for only a few days or weeks (Caugant et al. 1981). In addition, *E. coli* is the most frequently used indicator bacterium for examining the spread of antimicrobial resistance (AMR) in different environments and host species, as it often displays multi-drug resistance (MDR) and is a common inhabitant of the gastrointestinal tract microbiota of humans and other animal species, and is also found in the environment (Guenther et al. 2011; Stedt et al. 2014).

E.coli can be classified into two main groups: a non-pathogenic commensal group and a pathogenic group, causing intestinal or extra-intestinal diseases. Most of *E. coli* strains live as commensals in a mutually beneficial association with the host. They partially occupy the intestine and can help to exclude other bacteria, including pathogens (Russo and Johnson 2000). However, these commensal *E. coli* strains can occasionally cause opportunistic infections in immune-compromised hosts or where the normal gastrointestinal barriers are breached (Russo and Johnson 2000). Only a small proportion of *E. coli* strains are pathogenic and responsible for a broad spectrum of diseases, being classified into pathotypes based on the type of virulence factors present and on the mechanisms by which they cause disease. In most diseases caused by *E. coli*, pathogenicity is associated with virulence genes located on plasmids, bacteriophages, or pathogenicity islands (PAIs) that can be mobilized into different strains to create novel combinations of virulence factors. For identification and characterization of pathogenic

E. coli, it is important to detect virulence factors that are unique to, or associated with, certain types of pathogenic *E. coli* (Levine 1987; Nataro and Kaper 1998; Milon et al. 1999; Kaper et al. 2004). The term virotype will be used to refer to variants within pathotypes, based on differences in the combination of virulence genes.

In animals, the most important pathotypes are Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Shiga toxin producing *E. coli* (STEC), and Extraintestinal pathogenic *E. coli* (ExPEC) (Gyles and Fairbrother 2010). *E.coli* pathotypes are important for separating pathogenic from non-pathogenic types and for epidemiological studies. The susceptibility of the host to these different categories of *E. coli* pathotypes is related to many factors such as age, immune status, diet, number of pathogenic bacteria encountered and genetic (Quinn et al. 2011). In addition, STEC may be highly pathogenic for humans whereas it is part of normal flora of cattle and other ruminants. Likewise, ExPEC and EPEC typically are considered as part of the normal intestinal microbiota of their host and may be considered as opportunistic pathogens. Altogether, the set of *E. coli* which are pathogenic in animals or causative of zoonotic diseases in humans may be referred to as animal pathogenic and zoonotic *E. coli* (APZEC) (Gyles and Fairbrother 2010).

Serotyping is a well-established assay that is based on differences in the O, K, and H antigens detected on the polysaccharide portion of lipopolysaccharide (LPS), capsular polysaccharide, and flagellar proteins, respectively (Scheutz et al. 2004). The other molecular based typing techniques that are used to characterize *E.coli* isolates include polymerase chain reaction (PCR)-based genotyping, pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA PCR (RAPD-PCR), amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST), restriction fragment length polymorphism (RFLP) method, repetitive element PCR (Rep-PCR), multiple-locus variable-number tandem-repeat analysis (MLVA) and whole genome sequencing (Foley et al. 2009).

1.2 *E. coli* pathotypes

1.2.1 Enterotoxigenic *E. coli* (ETEC)

ETEC is the most frequent cause of watery diarrhea in farm animals, and is a significant causative agent of diarrhea in children and travellers in developing countries. This pathotype is defined by producing enterotoxins and adhesins which are mostly regulated on large plasmids. Almost all ETEC bacteria are known to attach to specific receptors on the intestinal epithelium by fimbrial or non-fimbrial adhesion and promote colonization and produce enterotoxins without inducing pathological lesions. This action results in the hypersecretion of water and electrolytes and in reduced absorption. ETEC bacteria cause severe watery diarrhea during the first days of life and also in the first week following weaning in pigs. Enterotoxins of ETEC are heat stable (STa, STb, or enteroaggregative *E. coli* heat-stable enterotoxin 1 [EAST1]) or heat labile (LT) (Gyles and Fairbrother 2010). The LT is very similar physiologically, structurally, and antigenically to the cholera toxin (CT) which is expressed by *Vibrio cholerae* and also have a similar mode of action (Nagy and Fekete 2005; Svennerholm 2011). In pigs, the most common fimbrial adhesins of ETEC are F4 (K88), F5 (K99), F6 (987P), F41, and F18. A non-fimbrial adhesion (adhesin involved in diffuse adherence [AIDA-I]) has been recently found in certain ETEC in pigs. The enterotoxins STa and STb differ in structure and mechanism of action. Only toxins of the STa class have been associated with human disease whereas the STb toxin is involved with animal disease. Animal ETEC strains possess fimbrial adhesins such as F4 and F5, which are not found in human ETEC strains (Kaper et al. 2004).

A schematic representation of the steps involved in the pathogenesis of ETEC infection in pig is shown in Figure 1. ETEC is ingested by animals and enter the intestinal tract, and when present in sufficient numbers, colonize the small intestine following attachment by fimbrial adhesins to specific receptors on the small intestinal epithelium. The adherent bacteria produce enterotoxins which stimulate water and electrolytes production into the intestinal lumen. This causes watery diarrhea, which may lead to dehydration, listlessness, metabolic acidosis, and death (Figure 1) (Gyles

and Fairbrother 2010). Post weaning diarrhea (PWD) is a major cause of death in weaned pigs worldwide. Fimbrial adhesins F18 and F4 are the types that are commonly found on ETEC from PWD in pigs. The predominant O serogroup of ETEC associated with PWD in pigs worldwide is O149 (Fairbrother et al. 2005). ETEC: F5 causes diarrhea in neonatal pigs and F4-producing ETEC occasionally increase rapidly in the small intestine of young pigs and induce symptoms of shock and rapid death. The most important virotypes and O serogroups in ETEC in pigs are listed in Table 1.

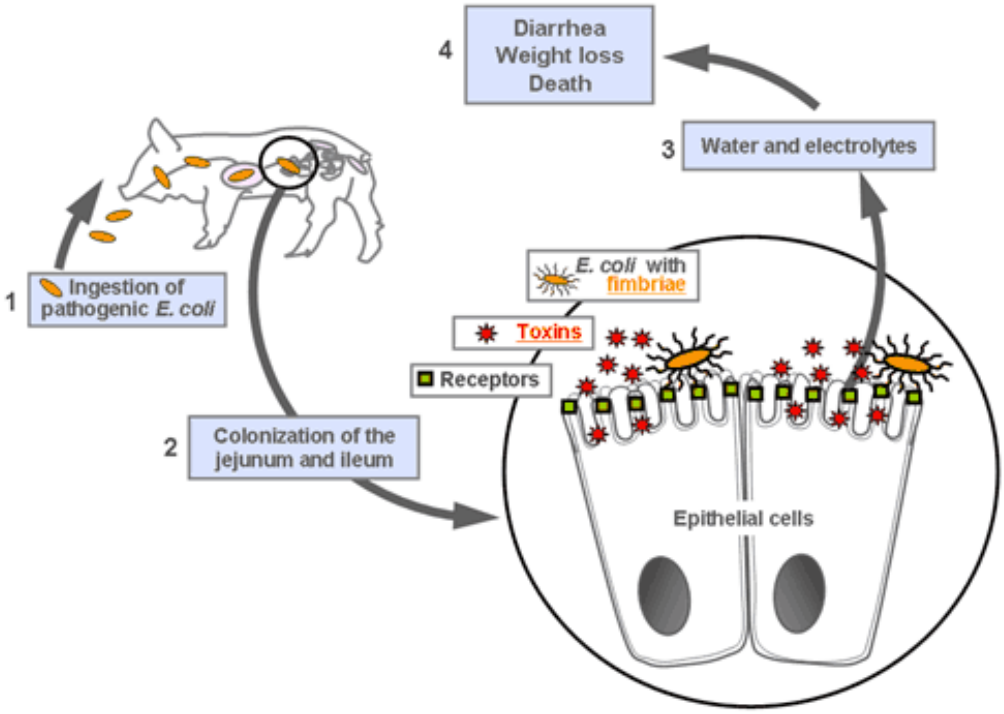

Figure 1. Schematic representation of the pathogenesis of ETEC infections in pig.

Source: http://www.ecl-lab.com/en/ecoli/pathogenesis.asp

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Table 1. Important pathotypes, virotypes, and O serogroups of *E. coli* causing disease in pigs.

Disease	Pathotype	Virotypes	O serogroups
Enteric			
Neonatal diarrhea	ETEC	STa:K99:F41, STa:F41, STa:987P, LT:STb:EasT1:K88ac, LT:STb:STa:EAST1:K88ac, STb:EAST1:AIDA	8,9,20,45,64,101,138, 141,147,149,157
Postweaning diarrhea	ETEC	LT:STb:EAST1:K88ac, LT:STb:STa:EAST1:K88ac, STa:STb, STa:STb:F18ac, STa:F18ac	8,138,139,141,147,149, 157
	EPEC	Eae, Tir, EspA, EspB, EspD, EspC (enterotoxin)	45,103,123
Edema disease	STEC	Stx2e:F18ab:(AIDA),_Hly+	138,139,141
Extraintestinal			
Colisepticemia	SEPEC	Aerobactin, F165-1(P fimbrial family), F165 - 2 (S fimbrial family), CNF1 or CNF2, CDT	6,8,9,11,15,17,18,20,45, 60,78,83,93,101,112, 115,116
Urogenital tract infection	UPEC	P, S, aerobactin, CNF1	1,4,6,18

Source: Adapted from (Gyles and Fairbrother 2010)

1.2.2 Enteropathogenic *E. coli* (EPEC)

EPEC is considered as one of causative agent of childhood diarrhea in developing countries and is implicated as an important cause of diarrhea in humans and animals. The EPEC cause attaching and effacing (AE) lesions on the intestinal mucosa, which are characterized by microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at sites of bacterial attachment. Ability to produce AE

lesions has also been found in strains of shiga toxin producing *E. coli* (Nataro and Kaper 1998; Kaper et al. 2004).

The genes for the production of AE lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island that harbor the genes encoding intimin, a type III secretion system, a number of secreted (Esp) proteins, and the translocated intimin receptor named Tir. Intimin is an outer membrane protein encoded by the *eae* (*E. coli* attaching and effacing) gene, responsible for the attachment of bacteria to enterocyte membranes Tir which is one of the EPEC translocated proteins. Tir is inserted into the host cell membrane, where it acts as a receptor to intimin (Trabulsi et al. 2002; Kaper et al. 2004).

EPEC are grouped into typical and atypical strains and they differ in several characteristics. Typical EPEC strains possess a plasmid carrying EAF (EPEC adherence factor) whereas atypical EPEC do not contain the EAF plasmid (Trabulsi et al. 2002). This plasmid encodes bundle-forming pilus (BFP), which mediates inter-bacterial adherence and possibly adherence to epithelial cells (Kaper et al. 2004). Thus, typical EPEC strains are identified by the presence of both eae and *bfp* genes, whereas atypical EPEC strains have been defined as those which possess only *eae* gene (Henderson et al. 2009). Humans are a reservoir for typical EPEC, whereas for atypical EPEC, both animals and humans can be reservoirs suggesting that atypical EPEC may be a potential zoonotic cause of human diarrhea. Typical and atypical EPEC seem to be two groups of distinct organisms that have in common the LEE pathogenicity island (Trabulsi et al. 2002). Atypical EPEC strains have been detected among *E. coli* isolates from pork, indicating that pigs may also be potential reservoirs for the pathogen (Xia et al. 2010). The most important virotypes and O serogroups in EPEC in pigs are presented in Table 1.

1.2.3 Shiga toxin producing *E.coli* (STEC)

STEC refers to those *E. coli* strains that produce at least one member of a class of potent cytotoxins termed shiga toxin (Stx), because of the close relation to the Stx of

Shigella dysenteriae type 1 (Gyles 2007). This toxin was initially named verotoxin because of its distinct effect on Vero cells (Karmali 1989). The common feature of all STEC is the production of bacteriophage encoding Stx. STEC strains may cause watery diarrhea, hemorrhagic colitis, and/or hemolytic uremic syndrome (HUS) in human and edema disease (ED) in pigs (Fairbrother and Nadeau 2006).

STEC have been characterized by a variety of methods, including serotyping. O157:H7 strains, as well as an increasing frequency of certain non-O157 strains are zoonotic STEC (Fairbrother and Nadeau 2006). O157:H7 isolates are the most common STEC pathogens associated with outbreaks of foodborne diseases in North America, but isolates of other serotypes such as O26 and O111, can also cause disease and are more important than O157:H7 in other countries (Kaper et al. 2004). It is well demonstrated that cattle are a main reservoir of STEC O157:H7 in North America but in countries such as Australia, sheep are of greater significance (Gyles 2007). Attachment to intestinal epithelial cells is an early feature of STEC infection and two patterns of attachment and interaction have been observed, in relation to *eae*-positive and *eae*-negative STEC isolates (Kaper et al. 2004). The combined presence of the *eae* and stx_2 genes has been shown to be an important predictor of HUS (Ethelberg et al. 2004).

Shiga toxin (Stx) is the key virulence factor in STEC diseases. The ED in pigs is the only animal disease for which the role of Stx is clearly established and associated with STEC:F18. An overview of the relation of STEC with ED of pigs is shown in Figure. 2. The initial step is the ingestion of STEC bacteria by the animal and entry into the intestinal tract. Then bacteria with fimbrial adhesins F18 (eae-negative STEC) attach to specific receptors on the intestinal epithelial cells and colonize in the jejunum and ileum over three to six days. The adherent bacteria produce shiga toxin 2e (Stx2e) which is transported across the epithelial cells to the circulation. The Stx2e binds to receptors on the vascular endothelium in the central nervous system and other sites including the stomach and subcutaneous tissues of the forehead and eyelids, giving rise to edema, ataxia, and death (Figure 2). Pigs that lack the intestinal receptors for F18 fimbriae are resistant to ED. There are no receptors for F18 in newborn pigs, so ED usually occurs in older pigs after weaning (Gyles and Fairbrother 2010).

The two major *E. coli* Stx toxins are Stx1, which is identical to Stx of *Shigella dysenteriae*, and Stx2, which is 56% homologous to Stx1(Gyles and Fairbrother 2010). Stx1 and Stx2 share a similar function and have the same genetic operon structure, encoding an A (enzymatic toxin) and a B (cell receptor binding) subunit. Stx1 and Stx2 are further grouped into distinct genetic variants which differ in animal reservoirs, animal disease, and severity of disease in humans (Gyles 2007). The most important virotype and O serogroups in STEC in pigs are listed in Table 1.

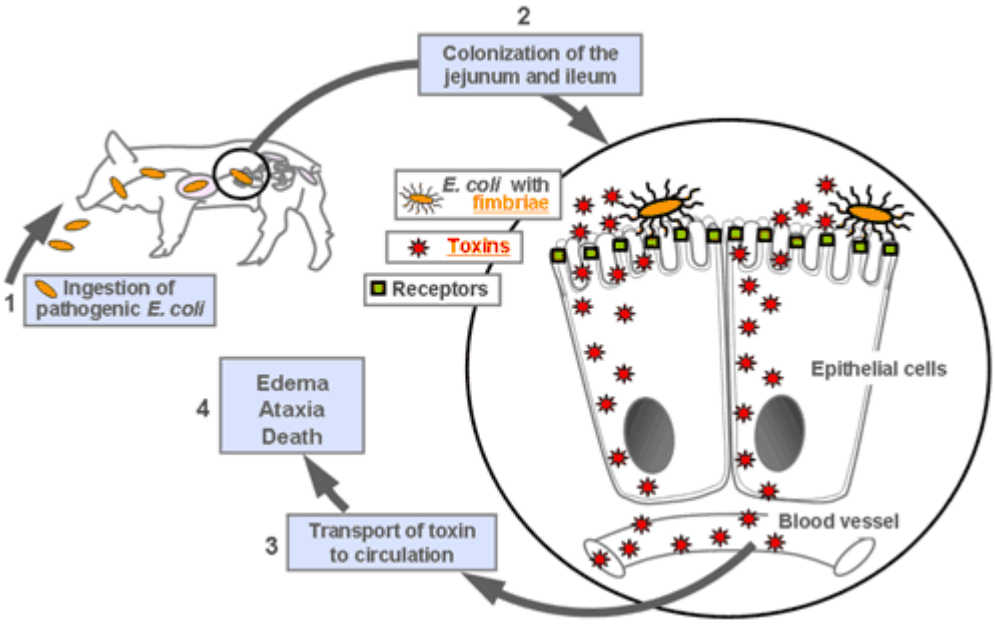

Figure 2. Schematic representation of the pathogenesis of STEC in edema disease in pig.

Source: http://www.ecl-lab.com/en/ecoli/pathogenesis.asp

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1.2.4 Extraintestinal pathogenic *E. coli* (ExPEC)

E. coli strains that induce diseases outside of the intestinal tract are termed extraintestinal pathogenic *E. coli* (Russo and Johnson 2000). These bacteria are considered to be opportunistic pathogens, as they are often found in the normal intestinal

microflora and colonize intestinal, respiratory or other mucosal surfaces, possibly due to fimbrial adhesins. When the animal is weakened, such as following a viral infection, ingestion of mycotoxins, or when a newborn has not received enough colostrum, bacteria pass more readily through the mucosa to the circulation (Gyles and Fairbrother 2010). Diarrheagenic *E. coli* do not generally cause extraintestinal diseases, and ExPEC strains do not normally induce diarrhea. ExPEC strains are phylogenetically and epidemiologically distinct from intestinal pathogenic strains (Johnson and Russo 2002; Russo and Johnson 2003). ExPEC cause a diversity of infections in all animal species and in humans, including septicaemia, urinary tract infections (UTIs), meningitis, genital tract and the mammary gland infection (Kaper et al. 2004; Smith et al. 2007; Gyles and Fairbrother 2010).

A schematic representation of the steps involved in the development of disease due to ExPEC in animals is shown in Figure 3. ExPEC strains are ingested by the animal host and colonize the intestinal, respiratory or other mucosal surfaces. In certain conditions, they pass through and between epithelial cells to gain access to the underlying tissue. These bacteria can resist the lethal effects of complement and phagocytes, possibly due to the presence of P fimbriae in pigs (Fairbrother and Ngeleka 1994). They can persist and multiply in the blood stream and are transported to distant organs. The toxins producing by the bacteria, such as cytotoxic necrotizing factor, may contribute to tissue damage (Fairbrother and Ngeleka 1994). Endotoxins released by dead bacteria may stimulate a cytokine response leading to shock and death of the animal. In localized infections, there may be bacterial interaction with extracellular matrices, leading to pneumonia, serositis, mastitis, metritis, urinary tract infection, meningitis, etc (Figure 3). Septicemic disease occurs in animals due to septicemic *E. coli* (SEPEC) that belong to a limited number of serotypes (Fairbrother and Ngeleka 1994). Bacteria persist and multiply in the blood and other extraintestinal sites mostly due to the presence of iron acquisition systems such as aerobactin and partly through their ability to adapt and grow in the iron-restricted extracellular environments of the host (Griffiths 1994). *E. coli* septicemia occurs in neonatal pigs and less frequently in suckling pigs (Fairbrother and Ngeleka 1994). It is characterized by an acute generalized infection, with signs of shock, often followed by death in 3-8 hours, with lethality of up

to 100%. (Gyles and Fairbrother 2010). The predominant O serotypes and virotypes associated with ExPEC in pigs are shown in Table 1.1.

ExPEC isolated from disease in humans and animals share several virulence factors and animals may be reservoirs of some of these pathogens that cause disease in humans (Gyles and Fairbrother 2010). In contrast to ETEC, EPEC, and STEC, ExPEC strains are not defined by the presence of a certain virulence factor or group of factors but usually have a large number of virulence factors which may vary greatly between strains. These factors contribute to bacterial colonization, invasion, iron acquisition, resistance to the bactericidal effects of complement and phagocytosis, and toxic activity (Gyles and Fairbrother 2010). A multiplex PCR protocol for identification of possible ExPEC in the various animal species has been developed in the Reference Laboratory for *E. coli* (EcL) based on detection of *tsh* (temperature sensitive hemagglutinin), *papC* (P fimbriae assembly), *iucD* (aerobactin receptor) and *cnf* (cytotoxic necrotizing factor) genes simultaneously (http://apzec.ca/en/Protocols). Another multiplex PCR has been described by Johnson et al. for identification of human ExPEC by screening of 5 virulence markers, including *papA* (P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), *sfa/foc* (S and F1C fimbriae subunits), *afa/dra* (Dr-antigen-binding adhesins), *kpsMTII* (group 2 capsular polysaccharide units), and *iutA* (aerobactin receptor). In this method, the criterion of "presence of two of the five markers," was used to differentiate ExPEC and non-ExPEC isolates (Johnson et al. 2003).

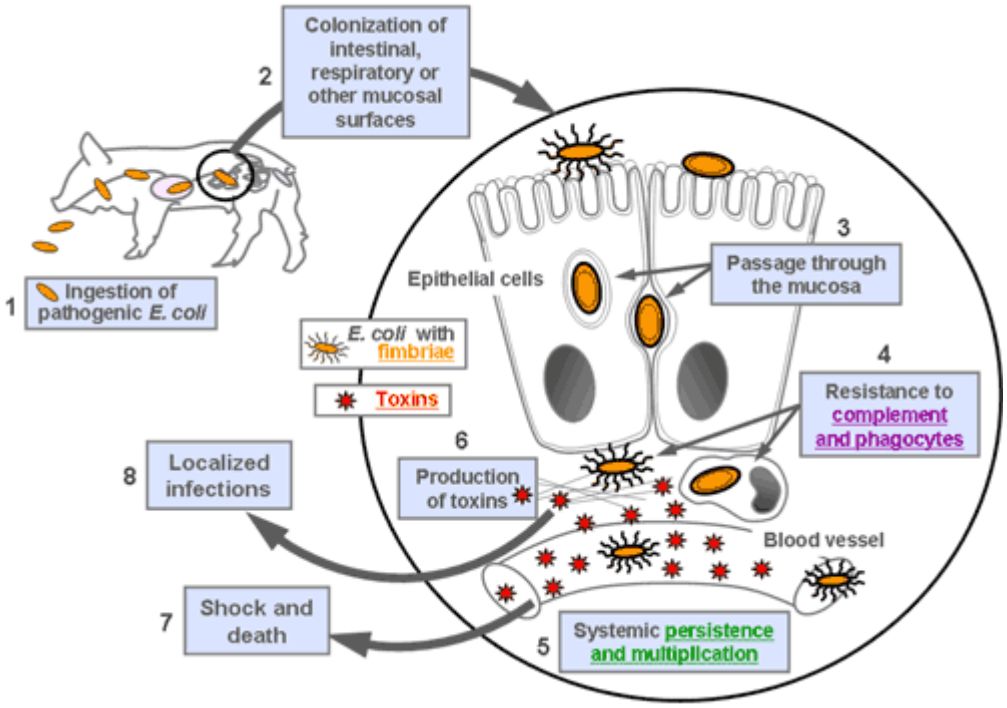

Figure 3. Schematic representation of the pathogenesis of ExPEC infections in animal.

Source: http://www.ecl-lab.com/en/ecoli/pathogenesis.asp

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1.3 Clonal diversity of *E. coli*

Clones are those isolates of a bacterial population that are identical or indistinguishable in genotype emphasizing they are the descendants of the same ancestor (Spratt 2004). However, since bacterial reproduction is not truly asexual, numerous mechanisms such as mutation, conjugation, transformation, and transduction are the sources of recombinational replacements that result in diversification of the ancestral genotype of a clone. Thus, according to a new definition, the term "Clonality" refers, more precisely, to a bacterial population that results from a restriction of genetic recombination to the extent that the prevalent pattern of clonal structure is not broken (Tibayrenc and Ayala 2012). Over the past decades, scientists demonstrated a strong association among particular pathogenic *E. coli* serotypes. Subsequent investigations led to a *E. coli* clone concept which emphasized that not all isolates of pathogenic species are equal and that in a bacterial population there are a small number of genotypes

(clones) that are greatly over-represented among those recovered from a particular phenotype or type of disease (Spratt 2004). Subsequently, the clonality of *E. coli* strains became an important aspect of epidemiological study. Dissemination of antimicrobial resistant *E. coli* clones was defined as one of the main mechanisms involved in multiple outbreaks around the globe. This included the dissemination of urinary tract infections (UTIs) in the United States due to trimethoprim-sulfamethoxazole resistant strains of *E. coli* clonal group A in 1990's (Burman et al. 2003) or the latest widespread infections in Canada, Europe, and Asia due to CTX-M-15 harbouring strains of *E. coli* sequence type 131 that exhibit extended-spectrum cephalosporin (ESC) resistance (Coque et al. 2008; Nicolas-Chanoine et al. 2008). More and more evidence suggests that *E. coli* clonal spread is a major contributor to emerging resistance. Thus, in addition to reducing selection pressure by limiting antimicrobial use, it was necessary to recognize and define *E. coli* clones to permit the interruption of transmission pathways.

To achieve this goal, the first step was to recognize that particular clones are much more strongly associated with a given pathogenicity and/or antimicrobial resistance than others. After exploration of the genetic structure of bacterial populations, certain methods were developed to distinguish the diversity of genotypes and to help in classifying *E. coli* clones.

1.3.1 Conventional techniques for *E. coli* typing

Serotyping is based on the immunogenicity of the bacterial surface structures. Initially, the combination of three major surface antigens (the O lipopolysaccharide (LPS), the flagellar H and the capsular K (O:K:H)) was considered for sub-typing of *E. coli* strains. As typing of the K antigen was difficult for most laboratories, serotyping based on the O and H antigens became the 'gold standard' (DebRoy et al. 2011). As O antigen has higher diversity of the protein in Gram-negative bacteria, it is a more common antigen to be targeted for *E. coli* serotyping (Sun et al. 2011). Gene-based methods (such as PCR and RFLP) have been widely used recently for serotype profiling. Nevertheless, the agglutination reaction-based serotyping for O group identification still

remains one of the most comprehensive and simple methods for testing O groups (DebRoy et al. 2011).

PCR genotyping takes advantage of the variability in genetic composition of microorganisms (due in part to horizontal gene transfer (HGT)) to subtype bacterial strains. In this method, multiple host-specific factors such as virulence and antimicrobial resistance genes are screened by PCR and the presence or absence of genetic factors is used to carry out **phylogenetic analysis and typing** (Foley et al. 2009). More advanced technologies such as multiplexed real-time PCR and Microarray have been developed for rapid and cost effective approaches of simultaneous detection of multiple genes (Bruant et al. 2006). Clermont et. al in 2000 described a triplex PCR strategy to assign *E. coli* isolates rapidly to one of the four major phylogenetic groups (A, B1, B2 and D) of *E. coli* strains based on PCR detection of 3 genes; *chuA* a heme transport factor in *E. coli* O157:H7, *yjaA* a gene from *E. coli* K-12 genome with unknown function and the DNA fragment TSPE4.C2 (Clermont et al. 2000). This method has been widely used in phylogenetic sub-typing of *E. coli* strains from human clinical samples (Alonso et al. 2015; Pietsch et al. 2015) as well as food-producing animal originated *E. coli* strains (Liao et al. 2015; Muller et al. 2016) and has been recently extended to become more discriminatory, identifying additional groups C, E, F (Clermont et al. 2013).

1.3.2 Advanced molecular fingerprinting of *E. coli* clones

Genotypic methods by molecular typing techniques have been designed as rapid methods for characterization of bacterial clones. Three main mechanisms of discrimination aid molecular epidemiologists and surveillance studies to identify clones: 1- restriction-based analysis of the bacterial DNA; 2- PCR amplification of particular genetic targets and 3- DNA sequence-based techniques to identify polymorphism at specific loci in the genome.

1.3.2.1 Restriction-based methods

Plasmid profiling is one of the earliest genotyping methods used for epidemiological studies of pathogens (Schaberg et al. 1981). This technique is based on the presumption that the bacteria from the same clonal lines typically carry the same plasmids. Following the plasmid isolation, the cell debris, proteins, and chromosomal DNA are removed. The plasmids are then separated by gel electrophoresis along with plasmids of known size, such as those from *E. coli 39R861,* to determine the sizes of the isolated plasmids. Finally, the number and size of plasmid bands are analyzed to define the plasmid profile for a particular isolate (Foley et al. 2009). The major drawback of this method is that the migration of plasmids during gel electrophoresis can be influenced by conformational changes in plasmids (linear versus supercoiled) (Olsen et al. 1993).

Restriction fragment length polymorphism (RFLP) analysis was developed based on the comparison of bacterial DNA fragmented by restriction endonuclease and separated by gel electrophoresis. However, due to great number of restriction sites on genomic DNA, the fragments have to be labeled with a probe for specific repetitive DNA fragments such as the ribosomal RNA genes (called **Ribotyping**) (Bouchet et al. 2008) or insertion sequences (such as IS1) (Fernandez et al. 2007) and the size and number of restriction fragments are used to compare bacterial strains.

Pulsed-field gel electrophoresis (PFGE) is similar to RFLP typing as it involves bacterial DNA digestion. However, utilizing rare restriction enzymes (like *XbaI*, *BlnI* or *SpeI* for *E. coli* isolates) will generate smaller fragments of a wide range of sizes after specialized electrophoresis (Foley and Walker 2005). PFGE has remarkable discriminatory power and reproducibility and it is a widely applicable method for comparative typing of most bacterial species (van Belkum et al. 2007). PFGE has been used successfully for identification of *E. coli* strains originating from food animals, especially when combined with other typing methods like serotyping (Fischer et al. 2014; Shin et al. 2014). PFGE was successfully used for molecular subtyping of ESBL-producing *E. coli* ST131 (Pitout et al. 2009b).

1.3.2.2 Amplification-based methods

Amplified fragment length polymorphisms (AFLP) is a combination of restriction digestion and PCR amplification. Basically, cutter enzymes such as EcoRI or MseI generate a large number of genomic DNA fragments followed by ligation of short adapter sequences which will be used as targets for PCR primers. Following PCR, the amplified fragments are electrophoretically separated and the separation profiles are used for inter-strain comparisons (Jonas et al. 2003). AFLP demonstrated a similar discriminatory index to PFGE in molecular fingerprinting of *E. coli* O157 (Tsai et al. 2005). AFLP was also used to provide evidence for selection of ciprofloxacin-resistant *E. coli* strains under antimicrobial pressure in clinical samples as well as for clonal dissemination of ciprofloxacin-resistant in patients (van Hees et al. 2011).

Repetitive element PCR (Rep-PCR) is another PCR-based typing method based on the repeated DNA sequence elements distributed throughout the genome. PCR primers specific to repeat elements are designed for amplification. The amplicons are then separated by gel electrophoresis to generate the pattern profiles which will be used to study the genetic relatedness of strains (Sukhumungoon et al. 2016). Rep-PCR (DiversiLab fingerprinting system) was used to identify *E. coli* clone ST131 producing β-lactamase CTX-M-15. In spite of the significant cost of procedure, the method was evaluated as a rapid standardized typing protocol for monitoring of the worldwide spread of *E. coli* clone ST131 (Pitout et al. 2009a).

Multiple locus variable number of tandem repeat analysis (MLVA) generates the profile of the bacterial genomic regions with repeated DNA motifs and utilizes the differences in the number of repeated copies at multiple loci among strains to carry out the genotyping analysis (Denoeud and Vergnaud 2004). The MLVA method demonstrated better discriminatory ability compared to PFGE for the study of *E. coli* O157:H7 (Noller et al. 2003).

1.3.2.3 Sequencing-based methods

Multilocus sequence typing (MLST) is a sequence-based molecular typing method that relies on specific nucleotide changes. The nucleotide sequences of housekeeping genes are the basis of this method as these genes are conserved among isolates within a species and are not subject to strong selective pressures. The relatedness between isolates is then determined by profiling of the polymorphisms within these target genes (Sullivan et al. 2005; Matsumura 2013). Web-based databases (developed for a number of bacterial species) facilitate the classification of tested sequences and assign the isolate to a particular sequence types (ST) and those with identical STs are defined as being clonal by MLST (Enright and Spratt 1999). Although MLST only examines approximately 0.1% of the core genome of bacterial (typically seven core loci), clonal assignments by MLST are confirmed by analysis of complete genome sequences (Feil 2004). Although it is an excellent method for tracing multidrug-resistant clones or STs across the globe (Sullivan et al. 2005), MLST often uses Sanger sequencing which makes it an expensive method for widespread use (Feil 2004). As in the case of other bacterial species, MLST provides a sketch of *E. coli* population by profiling its core genome (Wirth et al. 2006). MLST has been recognized as an accurate technique for characterization of bacterial isolates in cases of disease clusters, which has made it a suitable method for the prospective or retrospective study of outbreaks (Sullivan et al. 2005). Identification of a highly virulent extraintestinal *E. coli* clone, B2-O25b:H4-ST131-CTX-M-15 in 2008 is a good example to show the usefulness of MLST in case of pandemic outbreaks (Nicolas-Chanoine et al. 2008). However, Bednorz et al. (2013) described that MLST typing of the 181 *E.coli* clones defined by PFGE resulted in the assignment of 91 sequence types (STs), indicating MLST is less discriminatory than PFGE. Nevertheless, MLST data allows for comparison between laboratories (Bednorz et al. 2013). On the other hand, de Been et al. (2014) who used whole-genome sequencing (WGS) to study the relatedness of cephalosporin resistant *E. coli* from humans, chicken meat, poultry and pigs. WGS analysis revealed considerable heterogeneity between human and poultry-associated isolates which had previously been considered to be identical based on MLST, plasmid typing and AMR gene sequencing (de Been et al. 2014). By applying the whole-genome sequencing (WGS) technology we

are now able to recognize the genetic relationship among bacteria with a much higher resolution. The whole-genome MLST (wgMLST) that benefits the WGS technology is rapidly becoming a powerful discriminatory tool for the typing of isolates and will replace many of the above techniques. Nowadays, the publically accessible repositories like Bacterial Isolate Genome Sequence Database (BIGSdb) help researchers in determining the gene loci and allele number in bacterial species (Jolley and Maiden 2010).

Single nucleotide polymorphism (SNP) analysis is another technique that was developed to identify nucleotide mutations (called single nucleotide polymorphism or SNP) at specific loci in the bacterial genome to be used to differentiate isolates. Profiling of multiple SNPs (especially synonymous SNPs, those that do not change the identity of their encoded amino acid) in bacteria can be used to trace the relatedness of strains (Cebula et al. 2005). Several methodologies have been introduced to detect and study SNPs, including real-time PCR (Griffing et al. 2015) and DNA sequencing (Mortimer et al. 2004), although PCR is still the most popular approach used to screen bacteria through their SNPs profile (Mathers et al. 2015). Several studies have been designed to use SNPs for identification of *E. coli* clonality. In 2009, SNPs within the *mdh* and *gyrB* genes were used for detection of clonal groups among *E. coli* pathogens causing antimicrobial-resistant urinary tract infection (Johnson et al. 2009). Furthermore, Weissman and colleagues have mapped the sequences of *fum*C and *fim*H loci (called CH typing or clonotyping) to identify the *fimH30* ST131 lineage as clonotype CH40-30 (Weissman et al. 2012). Most recently, SNP genotyping technique was successfully used for genetic characterization and typing of a shiga toxin-producing *E. coli* O26:H11 strain in food producing animals (Ison et al. 2015).

1.3.3 Pathogenicity and AMR profiles of *E. coli* clones

The clonal dissemination of intestinal pathogenic *E. coli* (like O157:H7 strain) (Glode et al. 1977) as well as extraintestinal pathogenic *E. coli* (ExPEC) lineages including sequence types (ST) 95, ST73, ST393, ST69, and ST131 have been identified and traced geographically and all were associated with both community-onset and

healthcare-associated infections (Riley 2014). ST95 ExPEC strains are mostly associated with neonatal meningitis in humans and avian colibacillosis (Nandanwar et al. 2014). They belong to phylogenetic group B2 (Mora et al. 2013). In France, they were the most common clonal group among *E. coli* isolated from blood of patients with cirrhosis (Bert et al. 2010). Between 2007 and 2010, ST73 was the third most common ST in San Francisco hospitalized patients with Blood Stream Infection (BSI) symptoms (Adams-Sapper et al. 2013). ST73 strains belong to phylogroup B2 and serotype O6:H1 (Martinez-Medina et al. 2009) and are among the predominant CTX-M-15 ESBL-producing ExPEC in North Africa (Fam et al. 2011). ST393 was one of seven major ExPEC STs in the late 2000s in Canada (Peirano et al. 2012). It was the predominant ExPEC clonal group and one of the five most common ESBL-producing STs in Spain between 2005 to 2008 (Mora et al. 2011) and was also reported from Korea (Lee et al. 2010). Among all ExPEC *E. coli* clones, ST131 is currently the most frequently studied of the pandemic CTX-M–type ESBL–producing ExPEC *Escherichia coli* clonal lineage (Nicolas-Chanoine et al. 2008). It was initially identified by multi-locus sequence typing (MLST) during 2000–2006 and has been spread rapidly in several countries through food/water sources and/or via returning travellers from contaminated areas (Nicolas-Chanoine et al. 2008). So, sometimes EXPEC has been referred as a new class of food borne pathogens (Smith et al. 2007). The complete sequences of plasmids carrying CTX-M ESBLs within three different lineages of *E. coli* ST131 showed that IncFII plasmids harbouring $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1}}$ play a major role in the global spread of CTX-M-15 ESBLs in *E. coli* (Woodford et al. 2009). ST131 strains are also resistant to fluoroquinolones, owing to chromosomal gene mutations (*gyrA* and *parC*) (Johnson et al. 2013). Unfortunately, recent reports revealed that ST131 strains are gaining resistance to other classes of antimicrobials by expressing enzymes such as NDM-1 metallo-β-lactamase (Peirano et al. 2011). This clearly underlines the need for more research to better understand the epidemiology and biology of high-risk clonal *E. coli* lineages in order to find new strategies to prevent the evolution of drug resistance among pandemic strains.

2 Antimicrobials and resistance

Currently, a possible apocalyptic scenario would be the non-effective treatment of bacterial infections due to development of global resistance to antimicrobials. Development of resistance in zoonotic bacteria that mostly originate from food animals is now considered a major threat to public health, primarily through the increased risk of treatment failures in patients with infection complications.

Unfortunately, intensive use of antimicrobials in food animal production, especially as growth promoters and prophylactic purposes, exposes the bacterial species to substantial selective pressure that facilitates the spread of resistance. Notably, the selection occurs at the same rate among non-pathogenic bacteria which can transfer the resistance to pathogenic species. Due to the rapid and vast spread of antimicrobial-resistant bacteria from food animal reservoirs, the European Union decided in 2006 to ban the administration of antimicrobial agents as growth promoters in food animal production (EuropeanUnion 2006). In North American countries although the non-therapeutic usage of antimicrobials in animal production is not strictly banned, surveillance programs such as the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and National Antimicrobial Resistance Monitoring System (NARMS) in the United States have been developed to monitor trends in antimicrobial use and antimicrobial resistance in selected bacterial organisms from food animal sources nationwide.

E. coli has been the center of attention in most of these studies due to its zoonotic role that can lead to a wide variety of infections such as diarrhea, urinary tract infection, meningitis, peritonitis and septicemia in humans. Several studies reported the direct effect of administration of antimicrobials in pig farms and the risk of antimicrobial resistance development in *E. coli* to several classes of antimicrobials such as aminoglycosides (Bourque et al. 1980), tetracyclines (Wagner et al. 2008), quinolones (Taylor et al. 2009), sulphonamides (Rosengren et al. 2007) and β-lactams (Makita et al. 2015).

In *E. coli*, the importance of resistance to β-lactams (cephalosporins) has been highlighted in a report published by World Health Organization (WHO 2011). In this report, first- and second-generation cephalosporins are listed as highly important antimicrobial agents for the treatment of *E. coli* infection in humans whereas third- (cefovecin, cefpodoxime, and ceftiofur), and a fourth-generation (cefquinome) cephalosporins with wide range of application in veterinary medicine were regarded as critically important as they not only are alternatives to treat serious human disease but also their resistance genes are highly transmittable via non-human sources (WHO 2011).

Acknowledging this warning, the broad spectrum of activity and wide therapeutic applications of β-lactams makes them the most commonly used antimicrobials in the food animal industry, but also underlines the need for a special focus on the impact of these antimicrobials on livestock and public health.

2.1 β-lactam antibiotics and their mechanism of action

β-lactam antibiotics are the most common antimicrobial agents used against both human and animal infections caused by gram-positive and -negative bacteria. They have dominated the clinical market since their introduction in the 1940's and today consist of nearly 75% of the market. Their core structure consists of a nitrogen-containing β-lactam ring with the variable side chain R attached via the amide bridge to the ring carbon 6 atom. They act through inhibition of cell wall synthesis specifically by targeting a group of anchoring proteins in the cell membrane named penicillin-binding proteins (PBPs). Osmotic instability followed by autolysis occurs in bacteria after binding of the β-lactam ring to PBPs and alteration of the cell wall synthesis (Wivagg et al. 2014). Based on the spectrum of activity and binding affinity, several classes of β-lactams have been synthesized including penicillins, cephalosporins, monobactams and carbapenems. Some β -lactams, like penicillins and carbapenems, have a similar structure whereas penicillins and cephalosporins are quite different in architecture. In penicillins, the β-lactam ring is fused to thiazolidine, whereas in the cephalosporins the dihydrothiazine ring replaced the thiazolidine. The difference will be even more

complex by changing the lateral chains of the original β-lactam that results in the formation of different semi-synthetic penicillins or cephalosporins, either with broader antibacterial spectrum or with changes in their pharmacokinetic properties (Elander 2003).

Derived from a fungi named *Penicillium*, penicillins were the most widely effective antibiotics particularly against gram-positive bacteria, with the least toxicity known (Kirby and Bulger 1964). Based upon their effectiveness and bactericidal action, penicillins are classified into four classes. The first clinically used penicillins were natural penicillins (penicillin V, penicillin G, benzathine, and procaine penicillin) that are mainly useful against gram-positive bacteria such as strains staphylococci and streptococci as well as a few gram-negative bacteria. The second class is the narrow-spectrum activity penicillinase-resistant penicillins (oxacillin, dicloxacillin, cloxacillin, methicillin, and nafcillin) that were mainly developed against penicillinase-producing staphylococci. Aminopenicillins (amoxicillin, bacampicillin, and ampicillin) are the next class of penicillins which are active agents against several gram-negative bacteria (like *E. coli*). These penicillins are resistant to acidic environment, so they could be administered orally. The fourth class, the extended spectrum Penicillins are sub-categorized to acylaminopenicillins (mezlocillin, piperacillin, and azlocillin) and alpha-carboxypenicillins (ticarcillin and carbenicillin). These penicillins have a similar spectrum of anti-bacterial activity to aminopenicillins although they can also be used against gram-negative species of the *Enterobacteriaceae* family (Moran et al. 2009; Brunton et al. 2011).

Cephalosporins are chronologically classified into five generations based on their activity. Alterations at R sites (side chains) form the variation among cephalosporin generations directly influences the spectrum of activity against different bacterial species, and longer half-life. Hence, each of the generations has added another level of advantage over the previous ones. The first generation of cephalosporins (eg. cefacetrile, cefadroxil, and cephalexin) were effective only against gram-positive bacteria. Each newer generation has significantly extended spectrum against gram-negative bacteria although in most cases the activity against gram-positive organisms was decreased. Later generations are often considered as extended-spectrum cephalosporins (ESCs) and

are reserved for use against penicillin resistant infections to prevent the spread of cephalosporin resistant bacteria. The third- (eg. ceftiofur, ceftiolene, and ceftriaxone), fourth- (eg. cefclidine, cefepime, and cefluprenam) and fifth-generation (ceftobiprole, ceftaroline, and ceftolozane) cephalosporins have true broad-spectrum activity (Moran et al. 2009; Brunton et al. 2011).

Due to widespread use of β-lactams, bacteria have evolved three mechanisms to combat the effects of these drugs (Babic et al. 2006). Production of β-lactamases that is encoded on chromosomes or by plasmids is known to be the most common and important mechanism in gram-negative bacteria like *E. coli* against β-lactam antibiotics (Livermore 1995). Other mechanisms of defence against β-lactam antibiotics includes the alteration of penicillin binding proteins (PBPs) that exhibit low affinity for β -lactam antibiotics; and inhibiting the expression of outer membrane proteins (Babic et al. 2006).

2.1.1 Classification of β-lactamases

Discovered in 1940, β-lactamase was first described as a penicillin-inactivating mechanism in bacteria that threatened the future use of β-lactam antibiotics and were originally named penicillinases (Abraham and Chain 1988). Basically, β-lactamases are enzymes that hydrolyze the amide bond of the β-lactam ring, deactivating the drug, although they are not covalently bound to the drug as PBPs are. Today β-lactamases are either directly or indirectly responsible for most of the multidrug resistance observed in gram-negative bacteria in isolates from both humans and animals. Since the discovery of β-lactamases, over 890 members of this class of enzyme have been identified in naturally occurring bacteria (Bush and Jacoby 2010). Initially, these enzymes have been classified (A through D) according to the amino acid sequences of the enzymes (Ambler 1980; Bush 1989). Three classes (A, C and D) catalyze the reaction using a serine residue, the B class of metallo-β-lactamases catalyzing the reaction using zinc (Ambler 1980; Bush 2010). Subsequently, a new functional grouping of β-lactamases has been introduced according to their ability to hydrolyze specific lactam classes and on the inactivation properties of the lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Bush et al. 1995). Hence, this classification scheme is much more

relevant to physicians and microbiologists in a diagnostic laboratory, because it considers β-lactamase inhibitors and β-lactam substrates that are clinically relevant. Table 1.2. depicts the most up-to-date version of this classification. Based on functional classification three major groups of β-lactamases have been identified.

2.1.1.1 Group 1 cephalosporinases

The first group consists of the cephalosporinases belonging to molecular class C that are encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms (Jacoby 2009). They mediate resistance to cefoxitin, cefotetan, ceftriaxone, cefotaxime, and most penicillins except carbapenems. These enzymes are inhibited by cloxacillin, oxacillin, and aztreonam (Bush et al. 1995). AmpC β-lactamase was the first bacterial enzyme reported to destroy penicillin in *E. coli* (Abraham and Chain 1988). AmpC lactamases are usually encoded by *bla* genes located on the bacterial chromosome, including CMY-2, P99, ACT-1, and DHA-1. However, plasmid-borne AmpC enzymes are also becoming more prevalent since 1989 although are less common than extended-spectrum β-lactamases (ESBLs) (Philippon et al. 2002) and they could be detected in those enterobacteria not expected to produce an AmpC β-lactamase. The *bla*$_{CMY}$ genes are the major group of this family with 43 currently known alleles (Jacoby 2009). Based on the origin, two classes of CMY are identified (Table 2). Six (CMY-1, -8, -9, -10, -11, and -19) are related to be chromosomally determined AmpC enzymes in *Aeromonas* spp., whereas the remainder (including CMY-2, the most common plasmid-mediated AmpC β-lactamase worldwide) are related to AmpC β-lactamases of *Citrobacter freundii* (Jacoby 2009). The plasmid-mediated AmpC β-lactamase not only are able to develop resistance to a broad spectrum of β-lactams but also the same plasmids could carry β-lactamases genes from group 2 of β-lactamases (Hanson et al. 1999; Chen et al. 2007). CMY-2 is an important cause of β-lactam resistance in livestock, with the broadest geographical spread. The first isolates in cattle and pigs were reported in 1998–2000 in United States (US) where CMY-2-producing *Salmonella* spp. were isolated and were even transferred from infected calves to the farmer's son (Fey et al. 2000; Winokur et al. 2000). Subsequently, CMY-2-producing *E. coli* isolates

were also found in fecal samples of healthy chickens in Spain (Brinas et al. 2003). Similar strains have also been isolated from cats, cattle, chickens, dogs, horses, pigs, and turkeys (Jacoby 2009). A study showed 60 percent of the tested *E. coli* isolates randomly collected from environment of community veterinary hospitals in southern Ontario, Canada were positive for the $bla_{\text{CMY-2}}$ gene from which ninety-five percent were resistant to ampicillin, amoxicillin-clavulanic acid and cefoxitin (Murphy et al. 2010).

2.1.1.2 Group 2 serine-lactamases

Functional group 2 serine β-lactamases, including molecular classes A and D, represent the largest group of β-lactamases, due primarily to the increasing identification of ESBLs during the past 20 years (Bush and Jacoby 2010). The spreading rate of genes encoding the enzymes of this group is very high because of their incorporation into plasmids, to gain great efficiency in transferring among different strains. The most abundant members of functional group 2 classify in molecular class A with more than 550 enzymes mostly reflecting the original TEM and SHV (at least 40 variants) (Bush and Fisher 2011). Hence, to avoid complexity the TEM- and SHV-derived β-lactamases were divided into two subclasses, 2a and 2b (Table 2). Subgroup 2a β-lactamases belong to Class A and mostly are chromosomally-encoded penicillinases with some exceptions. They do not cause significant clinical resistance for β-lactams other than penicillin (Bush and Jacoby 2010). Subgroup 2b β-lactamases are broad-spectrum β-lactamases that are capable of inactivating both penicillins and cephalosporins at the same rate. The best-known members of group 2b are β-lactamases TEM-1, TEM-2, and SHV-1. They are sub-divided into 2be,2ber and 2br. Subgroup 2be includes so-called ESBLs that are the results of mutations in the parent $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$ genes leading to a single amino acid change in the original β-lactamases or enzymes which enabled them to cleave efficiently both penicillins and cephalosporins of generations I-IV (oxyimino-cephalosporins). ESBLs include other β-lactamases of the CTX-M type which arose by plasmid transfer from pre-existing chromosomal ESBL genes from non-pathogenic commensal *Kluyvera spp.* CTX-M ESBLs can efficiently hydrolyze cefotaxime and are

inhibited by tazobactam more than by clavulanic acid (Walther-Rasmussen and Hoiby 2004). They represent important enzymes found in isolates from the food animals. Recently, the type of ESBL was significantly extended to other β-lactamases such as OXA type from class D β-lactamase mutants with increased enzymatic activity towards cefepime (Livermore 2008). There are also numbers of less common ESBLs unrelated to TEM, SHV, or CTX-M, including BEL-1, BES-1, SFO-1, TLA-1, TLA-2, and members of the PER and VEB enzyme families (Naas et al. 2008). The transmission of zoonotic ESBL-producing Gram-negative bacteria (eg. *E. coli*) between food-producing animals and humans is considered as a serious threat to public health in human medicine as well as increasingly in the veterinary context worldwide. Although TEM and SHV type β-lactamases were mainly detected in Europe in the 1990s, during the last decades ESBL-producing *Enterobacteriaceae*, particularly *E. coli*, have emerged globally and currently CTX-M β-lactamases are the most frequently detected ESBL in livestock in Europe (Ewers et al. 2012). A recent study in Germany revealed that among the isolates collected from cattle and pig farms, all isolated ESBL-producing bacteria from animal sources were *E. coli* and CTX-M was the most prevalent β -lactamase. Further investigation showed that isolates found in cattle feces shared an identical MLST sequence type and CTX-M allele to human isolates (Dahms et al. 2015).

2.1.1.3 Group 3 metallo-β-lactamases (MBLs)

MBLs constitute a unique group of β-lactamases. They are structurally different from other lactamases as they require zinc ion at their active site. They also differ functionally in the ability to hydrolyze carbapenems, although some serine-lactamases now have also acquired that ability. They are inhibited by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) and dipicolinic acid but are resistant to clavulanic acid and tazobactam inhibition (Laraki et al. 1999). Like serine-lactamases, the metallo enzymes have been functionally subdivided into two sub-groups 3a and 3b (Table 2) (Rasmussen and Bush 1997; Bush and Fisher 2011). Originally the *bla*$_{\text{MBL}}$ genes were located on the chromosome; however, due to evolutionary pressures in a variety of hosts, several dozen unique variants were generated and began to appear on

mobile genetic elements specially in important Gram-negative pathogens such as *Enterobacteriaceae* (Bush and Jacoby 2010). Several MBLs have been described, the most important types for epidemiological dissemination and clinical relevance being identified as imipenemase (IMP) and verona integron-encoded metallo- β-lactamase (VIM). Both are plasmid-encoded and belong to subgroup 3a, and include SPM and the most recently discovered New Delhi metallo- β-lactamase (NDM) (Cornaglia et al. 2011). NDM-1 was first detected in *E. coli* isolated in Sweden in 2008 from an Indian patient (Yong et al. 2009). The serious problem in infections with NDM-1-positive bacteria is that these bacteria have developed resistance to other antimicrobial classes such as aminoglycosides and fluoroquinolones, leaving few treatment options (Mochon et al. 2011). Recently, MBL genes (bla_{VIM} and $bla_{\text{NDM-1}}$) belong to subgroup 3a, have been found in different integrons from *Salmonella spp.* (Fischer et al. 2013), *E. coli* (Fischer et al. 2012), *Acinetobacter junii* and *Acinetobacter calcoaceticus* (Wang and Sun 2015) isolated from livestock farms. Subclass 3b of MBLs includes CphA from *Aeromonas spp.* (Hernandez Valladares et al. 1997) generally isolated from water/wastewater sources and Sfh-I from another environmental isolate, *Serratia fonticola* (Saavedra et al. 2003) (Table 2), both enzymes are mono-zinc that strongly prefer carbapenem substrates (Fonseca et al. 2011). Although, the identified bacterial species (in above studies) are not of human or animal origins, as part of environmental microbiota, they can constitute an important reservoir of genetic determinants of antibiotic resistance. In general, the presence of such carbapenemase-encoding genes, the importance of carbapenems for human treatment, and the location of genes on highly effective mobile genetic elements (MGEs) in the livestock environment underline the possibility of their transmission via food in the community and/or hospitals which is a potential threat for public health.

Table 2. Functional grouping of major β-lactamases aligned with molecular assignments.

Bush-Jacoby group	Molecular class	Defining characteristic(s)	Selected enzymes
1	C	Hydrolyzes cephalosporins and cephamycins, generally with higher k_{cat} values than penicillins; Not inhibited by CLA and TZB; High affinity for aztreonam	*Escherichia coli* and *Pseudomonas aeruginosa* AmpC, CMY-2, FOX-1, MIR-1, P99
1e	C	Hydrolysis of penicillins, cephamycins, expanded-spectrum cephalosporins, monobactamsb; Not inhibited by CLA and TZB	GC1, CMY-37
2a	A	Efficient hydrolysis of penicillins; Inhibited by CLA and TZB	PC1 and other staphylococcal penicillinases
2b	A	Efficient hydrolysis of penicillins and early cephalosporins (cephaloridine, cefazolin, cephalothin); Inhibited by CLA and TZB	SHV-1, TEM-1, TEM-2, TLE-1 (TEM-90)
2be	A	Hydrolysis of penicillins, expanded-spectrum cephalosporins, monobactams, Inhibited by CLA and TZB	ESBLsb: CTX-M-15, CTX-M-44 (Toho-1), PER-1, SFO-1, SHV-5, TEM-10, TEM-26, VEB-1
2br	A	Efficient hydrolysis of penicillins and early cephalosporins; Not well inhibited by CLA	IRTs: TEM-30, TEM-76, TEM-103, SHV-10, SHV-26
2ber	A	Hydrolysis of penicillins, expanded-spectrum cephalosporins, monobactams; Less efficiently inhibited by	CMTs: TEM-50, TEM-68, TEM-89
2c	A	Efficient hydrolysis of carbenicillin; Inhibited by CLA	PSE-1, CARB-3
2d	D	Efficient hydrolysis of cloxacillin or oxacillin; Not always inhibited by CLA	OXA-1, OXA-10
2de	D	Hydrolysis of penicillins and expanded spectrum cephalosporins; Not always inhibited by CLA	ESBLs: OXA-11, OXA-15
2df	D	Hydrolysis of carbapenems and cloxacillin or oxacillin; Not always inhibited by CLA	OXA-23, OXA-48
2e	A	Efficient hydrolysis of cephalosporins; Inhibited by CLA and TZB but not by aztreonam	CepA
2f	A	Hydrolysis of carbapenems, cephalosporins, penicillins, and cephamycins; Poorly inhibited by CLA, low inhibition by TZB	IMI-1, KPC-2, KPC-3, SME-1, GES-2
3a	B	Hydrolysis of all β-lactams except monobactams; Inhibited by EDTA and metal ion chelators, not inhibited by CLA and TZB	IMP-1, L1, NDM-1, VIM-1
3b	B	Preferential hydrolysis of carbapenems; Inhibited by EDTA and metal ion chelators, not inhibited by CLA and TZB	CphA, Sfh-1

Adapted from (Bush and Fisher 2011)

Abbreviations: CLA, clavulanic acid; CMT, complex mutant TEM; ESBL, extended spectrum β-lactamase; IRT, inhibitor-resistant TEM; TZB, tazobactam.

2.2 Acquisition and transfer of antimicrobial resistance genes

Acquiring resistance genes through the gene transfer process is one of the several mechanisms bacteria have developed to fight against the action of antimicrobials (Davies 1994). Generally, two ways has been identified for this process. Firstly, vertical gene transfer includes the intragenomic reorganization of genomic sequences during replication of bacteria and their transfer to daughter cells, Secondly, horizontal transfer refers to the acquisition of foreign DNA sequences from the same or different genera or bacterial species via MGEs (Davies 1994). Both type of transfer play an important role in bacterial evolution and adaptation through distribution of genes that increase virulence and resistance to antimicrobials (Rodriguez-Rojas et al. 2013).

2.2.1 Mobile genetic elements (MGEs)

MGEs are the mediators for horizontal gene transfer that carry fitness genes associated with survival in the host (Hayashi et al. 2001). Commonly, they have a huge impact on bacterial genomes with marked changes in genome size and/or pathogenicity. A good example would be the comparison between pathogenic *E. coli* O157:H7 with genome size of 5.5 Mb and 4.6 Mb non-pathogenic *E. coli* K-12, where the gain of MGEs is responsible for evolution of commensal *E. coli* to a pathogenic bacteria with the ability to survive in a range of environments inside and outside the host (Hayashi et al. 2001). Based on their sizes, structures, biological properties and ways of spreading, MGEs are categorized as plasmids, transposons, integrons, gene cassettes, and phages (Gyles and Boerlin 2014).

2.2.1.1 Plasmids

Plasmids are circular extra-chromosomal DNA molecules with self-replication ability and can be found in almost all bacteria. Ranging from 2 kb to more than 100 kb, they carry spectrum of genes that encode nonessential functions such as drug resistance, virulence factors, fitness or adaptation (Smillie et al. 2010). Plasmids containing

antimicrobial resistance genes have been reported to be transferred from agricultural bacteria to human pathogens (Schuurmans et al. 2014). A formal classification of plasmid is based on incompatibility (Inc) groups (Novick 1987). Incompatibility grouping method is based on the introducing, by conjugation or transformation, of a plasmid of an "unknown" Inc group into a strain harbouring a plasmid of a "known" Inc group. If the resident plasmid is destabilized in the progeny, the incoming plasmid is assigned to its same Inc group (Datta and Hedges 1971). Plasmids with the same replication control are "incompatible", whilst plasmids with different replication controls are "compatible". Therefore, two plasmids belonging to the same Inc group cannot be procreated in the same cell line (Datta and Hughes 1983; Couturier et al. 1988). These plasmids could carry different incompatibility (Inc) groups to improve bacterial adaptability. IncHI2, IncF, IncA/C and IncI1 harbouring plasmids are only a few examples of Inc groups that have recently been found in *E. coli* isolates from animal farms which transfer multiple resistance determinants such as penicillinases ($bla_{\text{TEM-1}}$, $bla_{\text{TEM-135}}$), ESBLs ($bla_{\text{SHV-12}}$ and $bla_{\text{CTX-M-1}}$) and plasmid-mediated AmpC β-lactamases ($bla_{\text{CMY-2}}$) to render bacteria resistant to different antimicrobial classes (Martin et al. 2012; Deng et al. 2015; Jones-Dias et al. 2015; Yang et al. 2015).

2.2.1.2 Transposons

Transposons or transposable elements (TE) are small DNA sequences that can translocate from one position to another on the genome and alter the cell's genome size (Schwarz and Chaslus-Dancla 2001). Similar to integrons, they must integrate into plasmids or chromosomal DNA but unlike plasmids they lack replication system. They can be found from a very small size (< 2.5 kb) with a simple organization called insertion sequence (IS) that can carry only one gene, to around 60 kb that often carry antimicrobial resistance genes. Insertion of transposons has no restriction which makes them very efficient at transferring antimicrobial resistance genes between bacteria species (Schwarz and Chaslus-Dancla 2001). Mobilisation of CTX-M genes via transposons onto plasmids has a key role in widespread dissemination and adaptation of CTX-M enzymes in pathogenic bacteria (Canton et al. 2012). Some families of

transposons such as Tn*3*, Tn*5053* and Tn*402*-like transposons are more involved in movement of antimicrobial resistance genes among species and carry antimicrobial resistance genes as part of class 1 integrons (El Salabi et al. 2013). The majority of Metallo-β-lactamase encoding genes are reported to be carried in the form of gene cassettes on class 1 integrons and/or Tn*402*-type transposons (Marchiaro et al. 2010).

2.2.1.3 Integrons and gene cassettes

Integrons are known as genetic elements with an attachment site that have the ability to capture and incorporate antimicrobials resistance genes known as gene cassettes in a site-specific recombination manner and as result to enhance the expression of the genes conferring resistance to antimicrobials (Mazel 2006). Although they are generally known as mobile elements in a plasmid-mediated form, some large integrons were detected recently on the chromosome (El Salabi et al. 2013). Expressing a unique capacity to cluster resistance genes into complex operons, integrons promote resistance gene dissemination by horizontal gene transfer (Carattoli 2001). According to the homology of the integrase, the enzymes that mediate the recombination process, five classes of mobile integrons have been identified to date (El Salabi et al. 2013). Class 1 represents the most common structure and plays an important role in disseminating of MBLs particularly the genes with high levels of resistance to carbapenems in *Enterobacteriaceae* such as $bla_{\text{NDM-1}}$, $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, bla_{IMP} and $bla_{\text{DIM-1}}$ (Yong et al. 2009; Zhao et al. 2009). Class 2 integrons are embedded on large transposon called Tn*7* and are likely to confer resistance to six antimicrobials. Class 3 integrons are even less frequently observed than class 2 integrons and finally Class 4 and 5 are mainly related to trimethoprim resistance in *V. cholerae*. (Mazel 2006). The prevalence of Class 1 and 2 integrons is high among gram-negative isolates in Europe and Middle East countries (Leverstein-van Hall et al. 2002; Al-Assil et al. 2013). Several recent studies reported the prevalence of class 1 and 2 integrons in multi-drug resistant *E.coli* isolates in humans (Lavakhamseh et al. 2015) as well as in food producing animals such as cattle (Bakhshi et al. 2014), pigs (Szmolka et al. 2015) and poultry (Cavicchio et al. 2015),

underlining the fact that plasmid-mediated integrons are one of the major elements of inter-species transfer of antimicrobial resistance.

2.2.2 Vertical gene transfer

The inheritance of genetic material via transmission of genes from ancestors to progeny during DNA replication is known as vertical gene transfer or vertical evolution (Kenneth Todar 2008). This is a very important event in spreading of new adaptive traits (e.g. antimicrobial resistance) resulting from genetic mutation or acquired to the bacterial population. The mutation is a very rare event in a single cell. For example, the rate for acquisition of resistance to streptomycin via gene mutation is 10^{-9} in *E. coli.* However, due to very fast growth rate of bacteria, under the selective environment of the antimicrobial, resistance is developed rapidly in a population (Kenneth Todar 2008).

2.2.3 Horizontal gene transfer

Lateral transfer of resistance genes from other bacteria or what so-called horizontal gene transfer is known as the major way of acquiring antimicrobial resistance (Binnewies et al. 2006). The dynamic structure of the bacterial genome is prone to constant modification by addition and deletion of genes in order to adapt to their environment (Dutta and Pan 2002). HGT is believed to be an evolutionary genetic adaptation mechanism against antimicrobials because, unlike other adaptive genetic changes like mutations, transferring of the mobile genetic elements can result in the acquisition of genes with entirely novel functions for the cell. For example, in case of plasmid-encoded β-lactamases, the recipient will acquire the ability to inactivate drugs (Culyba et al. 2015).

2.2.3.1 Transformation

Transformation has been described as the ability of bacteria to alter their genetics by uptake and introduction of foreign homologous genetic materials (DNA or RNA) from the environment which subsequently enables bacteria to acquire new adaptation traits such as resistance to antimicrobials (Johnston et al. 2014). Because not all bacteria have such competency, special chemical and physical conditions are required (eg. electroporation) to facilitate the process. It has been proposed that DNA internalization during transformation is a transient twostep process known as competence (Johnston et al. 2014), consisting of an initial transfer from the surface to the cytoplasmic membrane followed by crossing of DNA through highly conserved cytoplasmic membrane channels as was confirmed in *Helicobacter pylori* (Stingl et al. 2010; Kruger and Stingl 2011). It has been reported in *in vitro* study that administration of certain antimicrobials such as aminoglycosides and fluoroquinolones in sub-lethal concentrations can induce competence in *S. pneumoniae* (Prudhomme et al. 2006). Non-therapeutic dosage of antimicrobials can provide enough stress for such a reaction, especially in pathogens lacking the SOS response (an inducible DNA repair network). For instance, *Legionella pneumophila* is induced to competence for natural genetic transformation by fluoroquinolones and other DNA-damaging antimicrobials (Charpentier et al. 2011).

2.2.3.2 Transduction

Another mechanism of HGT is the transfer of genetic material by bacteriophages (Soucy et al. 2015). The process includes the integration of exogenous host genetic materials from chromosome DNA fragments to various mobile elements such as plasmids, islands, transposons and insertion elements, into a phage genome and transfer it to another organism recognized by the phage. Two methods of transduction have been demonstrated among bacteria: «Generalized» that happens during cell lysis with the incorporation of a random piece of the host DNA; and «Specialized», in which a prophage imprecisely excises itself from a host genome and incorporates some of the flanking host DNA. (Soucy et al. 2015). A good example of specialized transduction is λ in *E. coli*, when it can packaged into the bacteriophage and transferred to another

recipient cell (Huddleston 2014). Transduction is a major mechanism of horizontal gene transfer in nature. Bacterial genes of all types exist in up to 50% to 60% of bacteriophages (Dinsdale et al. 2008) showing their importance to serve as a reservoir for a spectrum of genes including antimicrobial resistance elements and to transfer them among bacteria. Recently, separate studies emphasised the role of bacteriophages in transfer of genes responsible for resistance to tetracycline (*tet*M) and gentamicin (*ant*2-I) between the same and different enterococcal species (Yasmin et al. 2010; Mazaheri Nezhad Fard et al. 2011). However, compared to natural transformation and conjugation, the role of this mechanism in transferring antimicrobial resistance genes has been neglected for decades, although growing evidence demonstrates that antimicrobial treatment can increase the number of bacterial antimicrobial resistance genes within the phage genome, probably through activation of the SOS response (Modi et al. 2013).

2.2.3.3 Conjugation

Conjugation involves the transfer of DNA fragments (plasmids or conjugative transposons) in varied sizes by physical contact between a donor and a recipient cell via a conjugation pilus or pore (Soucy et al. 2015). The conjugative pilus is described as part of the Type IV secretion machinery (Bacon et al. 2000). Generally, the autonomously replicating plasmids or circular molecules of DNA that replicate from the host chromosome are considered as conjugative machinery. Some plasmids are conjugative in nature, meaning they harbor genes for the transfer machinery and are self-transmissible. In contrast, the non-conjugative plasmids are not able to be mobilized in the absence of a helper self-transmissible plasmid (Huddleston 2014). The fact that, in addition to plasmids, other mobile genetic elements such as conjugative transposons, integrons or integrating conjugative elements can be transferred by conjugative machinery reveals the complicated role of conjugation in horizontal gene transfer. Interestingly, these elements also enable the non-conjugative plasmids to be transferrable to other cells (Salyers et al. 1995). The role of the conjugative machinery in dissemination of antimicrobial resistance genes has been studied extensively. The

transferability of conjugative plasmids of incompatibility group IncL/M type carrying ESBL genes such as *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{TEM-71}}$, *bla*$_{\text{SHV-1}}$ and *bla*$_{\text{PER-2}}$ was reported in *Serratia marcescens*, an important pathogen in nosocomial infections (Batah et al. 2015). The conjugal transfer of IncA/C plasmid harbouring antimicrobial resistance genes in *E. coli* has also been reported in pig farms where high-dose administration of chlortetracycline in the feed was carried out (Johnson et al. 2015).

2.3 Antimicrobial use in swine production

Compared to other food production species, antimicrobial use is even more substantial in the pig industry, due to intensive livestock farming conditions (Street and Gonyou 2008). The high density of animal in commercial herds promotes the risk of injuries and diseases. Such conditions increase the need for antimicrobials use as preventive, which are commonly administered as feed supplements (Callens et al. 2012). In addition, the ability of antimicrobials to suppress or inhibit the growth of certain microorganisms, made them a perfect choice to be used for improving the rate and efficiency of growth in pig farms (Cromwell 2002). The unfavorable impact of this practice is the dissemination of resistance among both pathogenic and commensal bacteria such as *E. coli* (van den Bogaard and Stobberingh 2000). In hog farms, animals receive in-feed antimicrobials in all stages of growth. The USDA has reported that 70 to 80% of starter and grower diets for pig are antimicrobial supplemented compared to 50 to 60% of finishers diets. However, records show that only 18% of all antimicrobials were used as growth promoters whereas 82% of cases were related to disease prevention and treatment (Cromwell 2002). It was also demonstrated that chlortetracycline was the most antimicrobial administered in all growth stages, followed by tylosin and oxytetracycline. Notably, the report underlined that 66.0% and 18.2% of total antimicrobials used in swine industry are classified as highly and critically important, respectively, according to (FDA/CVM) guidelines (Apley et al. 2012).

In Canada, the story seems to be similar. In 2006, Alberta Agriculture, Food and Rural Development (AAFRD) reported a very high usage of in-feed antimicrobials in

Alberta pig farms (Rajic et al. 2006). Based on the records, 90.9% of grower farms added antimicrobial agents to animal feed compared to 80% of finishers. Combinations of chlortetracycline, sulfamethazine, and penicillin were the most frequent antimicrobials administered in weaners and growers/finishers. However, the report data were more encouraging for public health compared to US, as the frequency of critically important antimicrobials in human medicine (quinolones and 3rd generation cephalosporins) were apparently low (Rajic et al. 2006). Subsequently in 2013, Ontario Swine Veterinary-based Surveillance (OSVS) reported that 80.4% of pig farms in Ontario, Canada used antimicrobials. Interestingly, the administration of antimicrobials in viral (e.g., porcine circovirus infection and influenza) or non-infectious (e.g., injury) cases were greater than actual bacterial infections (e.g. *Escherichia coli*) because of misdiagnosis or in some cases to avoid secondary bacterial infections although the treatment failure was fairly high (70%). The most commonly used antimicrobials reported in this study were almost the same as in the preceding report, i.e. penicillin (34.9%) and tetracyclines (10.7%). However, a new 3rd generation cephalosporin, ceftiofur (7.8%) was now becoming one of the most frequent used antimicrobials in pig farms. This report also emphasised that in more than 20% of records the combination of more than 2 antimicrobials were used for treatment and the highest ranking in this list were the combination of penicillin with either tetracycline or ceftiofur. The use of multiple antimicrobials was also shown to be associated with increased probability of antimicrobial treatment failure. (Glass-Kaastra et al. 2013). The most recent report published by The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) also showed that β-lactams were the fourth most common class of antimicrobial sold in the animal industry in 2013. Based on the same report, fluoroquinolones, a group of antimicrobials that have been classified as of critical importance by WHO and are extremely regulated in use due to antimicrobial resistance concerns, have been used 11% more frequently in animals compared to 2012 (CIPARS 2015). Considering the importance of growing AMR through antimicrobial use in animal production, the government of Canada has developed a collaborative *Federal Action Plan* in response to this threat. In order to improve the effectiveness of antimicrobial uses in farm animals, the framework has outlined two specific actions in

stewardship section: “1- To strengthen the promotion of the appropriate use of antimicrobials in human and veterinary medicine” and “2- To strengthen the regulatory framework on veterinary medicines and medicated feeds, including facilitating access to alternatives, and encourage the adoption of practices in order to reduce the use of antimicrobials” (PHAC 2015). Such frameworks would help governments to prevent the spread of infectious diseases, to reduce the need for treatment using antimicrobials, which in turn contributes to conserving the effectiveness of available treatment options.

2.3.1 Association between antimicrobial use and antimicrobial resistance in pig farms

Food animals are often exposed to antimicrobials either for treatment/prevention of infectious disease or in low concentration doses to promote growth. The use of antimicrobials as growth promotor and preventive (mostly as feed supplements) remains a concern in swine industry, as this practice provides the conditions which result in selective pressure for antimicrobial-resistant bacteria. The major controversy concerns the usage of antimicrobials that are identical to or closely resemble human drugs. The acquired resistance subsequently transfers via resistance genes and bacteria among other animals, animal products (e.g. meat), and the environment. There is also evidence that the antimicrobial resistance genes in commensal bacteria (e.g generic *E. coli*) may be transferred to zoonotic enteropathogens such as *E. coli* O157:H7 (Salyers et al. 2004). Among different bacteria acquiring antimicrobial resistance, the ESBL-producing zoonotic bacteria (e.g. *E. coli*) are of major concern as they can transfer resistance to some of the highly and critically important antimicrobials such as commonly-used extended-spectrum (third-generation) cephalosporins (i.e., ceftazidime, cefotaxime and ceftriaxone) as well as to β-lactam-lactamase inhibitor combinations (i.e., piperacillin tazobacatam, etc.) (McEwen and Fedorka-Cray 2002; CIPARS 2015).

The association between antimicrobial use in farm animals and development of antimicrobial resistance seems to be a complex species-dependent process as some on-farm and experimental studies failed to show such relationship in specific bacteria

(Dargatz et al. 2000; McEwen and Fedorka-Cray 2002). Nonetheless, antimicrobial use in animals apparently contributes to the spread of resistance among populations of bacteria by the selection process. A recent study on prevalence of antimicrobial resistance among *E. coli* isolates from food animals and consumption of in-feed antimicrobials among European farms showed a significant correlation between the two parameters for certain antimicrobial classes such as tetracyclines, fluoroquinolones and third-generation cephalosporins with Spearman's rank correlations of 0.92, 0.85 and 0.703 respectively (Chantziaras et al. 2014). Earlier, Kim et al. had also shown that resistance to antimicrobials (apramycin, carbadox, and chlortetracycline) increases among zoonotic enteropathogens (*E. coli*) isolates from growing piglets when these antimicrobials were used as feed additives. However, upon removal of apramycin, resistance in *E. coli* declined suggesting that resistance to antimicrobials in *E. coli* is a drug-dependent event (Kim et al. 2005). Another study on prevalence and patterns of antimicrobial resistance of fecal *E. coli* in farms carrying out administration of antimicrobials as growth promotor and preventive, revealed similar results. In this study 72% of farms used antimicrobials as in-feed additives. Interestingly, 68% of *E. coli* isolates collected from these farms were resistant to at least one antimicrobial, more than 3 time greater than what was observed in isolates categorized as being associated with "No in-feed medication". Further analysis showed that prevalence of MDR in farms using in-feed antimicrobials reaches to 80%, much greater than 52% in farms not using such antimicrobials (Akwar et al. 2008).

The Canadian provincial and national reports reveal similar statistics but in greater scale. Based on the last CIPARS report in 2013, 65% of *E. coli* isolated from pig farms were resistant to at least one antimicrobial, higher than the prevalence of 25% in cattle/beef. At the same time, the prevalence of resistance to ceftriaxone in *E. coli* isolates was less than 4% in pig farms and neither carbapenem- nor fluoroquinolone-resistant isolates were found. Although the report confirms the presence of multi-class resistant *E. coli* on farms, no clear statistics are provided (CIPARS 2015). The provincial report published by the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ), confirms that in the same period (2013), clinical *E. coli* isolates from pig farms were highly resistant to tetracycline, ampicillin,

trimethoprim, neomycin, and ceftiofur, with a prevalence of 90%, 72%, 50%, 29%, and 19%, respectively, demonstrating an increase of 5-10% in resistance to almost all antimicrobials as compared to 2012. The report did not provide information about antimicrobial administration, although the increase observed in prevalence of resistance in one year (2012-2013) could be partly due to an increase in antimicrobial usage in pig farms, either for therapeutic purposes or as a growth promoter (MAPAQ 2014).

2.3.2 Public health concerns

The WHO has referred to antimicrobial resistance as one of the greatest threats to public health at present (WHO 2014). Antimicrobial resistance can be passed to humans from resistant bacteria (pathogenic and/or commensal) through mobile genetic elements, such as plasmids or transposons, by either food or direct contact with animals or release of animal waste into the environment (Silbergeld et al. 2008). In the swine industry, use of antimicrobials classified as highly important or critically important for human medicine by WHO, such as tetracyclines (chlortetracycline), potentially provide selective pressure for the maintenance or emergence of resistant bacteria in humans. The incidence of antimicrobial-resistant infection outbreaks in European countries (Maguire et al. 1993; Molbak et al. 1999) involving strains originating from swine herds, convinced European Union (in 2006) to ban the use of antimicrobials as growth promoters in all food-producing animals (European Union 2005). In North American countries, the rules are less strict as both Canada and the United States implemented plans for a voluntary reduction in antimicrobial use for the purpose of growth promotion in food producing animals (United States Food and Drug Administration 2013; Health Canada 2014). However, to be aligned with WHO global action plan against AMR threat, the government of Canada stepped forward and developed a *Federal Action Plan* in order to limit and control the impact of AMR on public health through deliverable actions in three areas: surveillance, stewardship, and innovation (PHAC 2015). The action plan is specifically focuses on improvement of the veterinary usage of antimicrobials by modernization of the regulatory frameworks for medicated feeds, by supporting the industry to validate the commercially available alternatives to in-feed

antibiotics and by straightening the control over the importation of veterinary active pharmaceutical ingredients (PHAC 2015). Although these action plans are at the starting point, optimistically after implementation, some farms might start raising antimicrobial-free animals which would be a major step to avoid an apocalyptic outbreak of multi-drug resistant infections.

2.4 Antimicrobial resistance in *E. coli*

E. coli is the cause of various bacterial infections and at the same time is the most prevalent commensal inhabitant of the gastrointestinal tracts in both humans and animals (Guenther et al. 2011; Stedt et al. 2014). These two characteristics together with that the ability of *E. coli* to easily adapt to its environment with genetic flexibility, make this species a primary candidate for the acquisition of a great number of antimicrobial resistance mechanisms. Hence, it is not surprising that *E. coli* is frequently used as an indicator bacterium for addressing the circulation of antimicrobial resistance in different environments and host species which often demonstrate MDR (Guenther et al. 2011; Stedt et al. 2014). In addition, due to similarities in physiology and sharing identical genetic determinants of resistance with similar mechanisms of genetic dissemination, *E. coli* has been shown to be capable of exchanging resistance genes with other members of *Enterobacteriaceae* (Mann et al. 2011). Currently, *E. coli* is known as an important reservoir of resistance genes to "critically important antimicrobials" in human medicine, including β-lactams (cephalosporins), fluoroquinolones, aminoglycosides and sulfonamides (trimethoprim–sulfamethoxazole - SXT) (Pitout 2012). In 2010, over 20% of *E. coli* causing urinary infection in the United States were resistant to SXT, hence this drug will no longer be acceptable for treatment of urinary tract infections (Sanchez et al. 2011). As with SXT, incidence of fluoroquinolone (ciprofloxacin) resistance seems to be higher in urinary *E. coli* isolates from hospitals than the community settings (Fasugba et al. 2015).

The transmission of antimicrobial-resistant bacteria to humans through livestock products has become a concern to public health since it has been shown that they reduce

the efficacy of antimicrobial drugs in humans (Swann et al. 1969). The emergence and dissemination of resistant *E.coli* in pig farms, as one of the most commonly human food sources, has specially received attention from relevant international organizations including the World Health Organization (WHO) and World Organisation for Animal Health (OIE) (Dehaumont 2004; Orand 2012). The resistance patterns and their frequencies are considerably varied in pig originated *E. coli* isolates which is believed to be associated with antimicrobial uses in farms (European Food Safety Authority 2014). A recent report on faecal *E.coli* isolates from Irish pig farms revealed a strong correlation between high frequencies of resistance to tetracycline, trimethoprim/sulphamethoxazole and streptomycin and consumption of in-feed antibiotics. Besides, the use of trimethoprim/sulphonamide was found to be associated with the occurrence of resistance to other antimicrobials (Gibbons et al. 2016). In North America, the association between extensive use of both narrow-spectrum and broad-spectrum antimicrobials used as in-feed medication, and resistance in fecal *E. coli* in pigs was mentioned in several reports (Dunlop et al. 1998; Kim et al. 2005; Bunner et al. 2007). Also, the use of antimicrobials in weaner and finisher pigs was shown to contribute to the selection of resistance among fecal *E.coli* not only "within" but also "between" classes of antimicrobials (Akwar et al. 2008). Considering these studies and the increasing number of other reports warning about the situation of veterinary antimicrobial usage and prevalence of antimicrobial-resistant *E.coli* isolates from food-producing animals, necessitate more decent research to better understand the contributing factors such as bacterial properties (virulence factors and other genetic determinants) that affect the occurrence of antimicrobial resistance.

2.4.1 Extended spectrum cephalosporin (ESC) -resistance in *E. coli*

Among antimicrobial classes, resistance to β-lactams in *E. coli*, especially the third generation extended spectrum cephalosporins (ESCs), is of a major concern to public health not only because this group of drugs is used to treat serious community-onset or hospital-acquired infections caused by *E. coli* but also the ESC-resistant isolates are often resistant to other families of antimicrobials (Livermore 2008). Resistance to

ESCs is commonly emerging in *E. coli* via production of plasmid-mediated ESBLs (e.g. CTX-M types) and/or AmpC β-lactamases (e.g. CMY types).

The CTX-M family of ESBLs have emerged as the most prevalent ESBLs enzymes in *E. coli* causing bacteremia and intra-abdominal infections in human (Canton et al. 2012). In Europe, CTX-M producing *E. coli* are also the most frequently detected ESBL in livestock and has been recognized as the causative agent in animal infections such as bovine mastitis (Timofte et al. 2014). The most common CTX-M types reported in food-producing animals (particularly in pigs) are CTX-M-14 and CTX-M-15 (Horton et al. 2011; Tamang et al. 2013). Insertion sequences such as IS*10*, IS*26*, and IS*903* have been observed flanking the ORF region of *bla*$_{\text{CTX-M}}$ genes (Arduino et al. 2002). The *bla*$_{\text{CTX-M}}$ genes are carried on plasmids belonging to different incompatibility groups, including IncK, IncHI2, IncHI1, IncN, IncFIB, IncF or IncI1 (Girlich et al. 2007; Liao et al. 2015). In pigs, *bla*$_{\text{CTX-M-1}}$ carried on IncN plasmids was found to be identical to that isolated from farm workers which confirmed the within-farm transmission between human and porcine commensal *E. coli* (Moodley and Guardabassi 2009).

Plasmid-mediated AmpC β-lactamases are the second determinant, although less commonly encountered, with a broader spectrum of resistance compare to ESBLs. They confer resistance to 3rd generation cephalosporins (e.g. ceftiofur, ceftriaxone) in *E. coli* isolates from food animal farms (Lutz et al. 2011). In many gram negative bacteria, AmpC β-lactamase genes are chromosomal, although in *E. coli* these are poorly expressed and are mainly acquired by plasmids (Jacoby 2009). CMY-2 β-lactamases are the most common AmpC-type β-lactamases that are widely distributed via plasmids and have recently been recognized among animal farms in North American (Daniels et al. 2007), Asian (Ewers et al. 2012) and European countries (Batchelor et al. 2005) with a frequency ranging from 2% to 31% . The majority of the *bla*$_{\text{CMY-2}}$ plasmids identified in *E. coli* and *Salmonella* spp. in the United States belong to IncA/C and I1 (Carattoli 2009) and less frequently to other incompatibility groups such as IncF, K and ColE (Mata et al. 2012). IncA/C plasmids often carry the qnrA1 gene conferring resistance to fluoroquinolones and have been characterized as the most multi-resistant plasmids in bovine originated *E. coli* (Martin et al. 2012). IncI1 plasmids were also reported to be

the predominant plasmids carrying *bla*$_{CMY-2}$ in human *E. coli* isolates (Sidjabat et al. 2014).

The high prevalence of ESBLs and AmpC-producing *E. coli* isolates in the food animal industry demonstrates the risk of their diffusion in the human food chain and underlines the need to develop appropriate strategies to limit the spread of multidrug-resistant bacteria from food-producing animals to the community.

2.4.2 Antimicrobial resistance in *E. coli* and its association with virulence genes

Mobile genetic elements (plasmids, transposons, and integrons) are the core for epidemiological studies of antimicrobial resistance traits. However, AMR genes are not the only sequences carrying by mobile genetic elements. It is probable that virulence genetic determinants on the same genetic platform as antimicrobial resistance genes co-mobilize under antimicrobial selective pressure so the virulent strains will become stable by acquiring resistance properties (Da Silva and Mendonca 2012).

Among *E. coli* pathotypes that may transfer from animals and contaminated human foods, *E. coli O157:H7* (EHEC pathotype) is one of the most threatening serotypes to human health. Shiga toxin (*stx1, stx2*)-producing *E. coli O157:H7* from feedlot beef cattle revealed resistance to ciprofloxacin indicating that the use of in-feed fluoroquinolones may trigger the development of resistance in *E. coli* O157 in food-producing animals (Galland et al. 2001; Manna et al. 2006). In another study, 50% of *E. coli* O157 isolated from bovine, caprine and ovine milk possessed virulence genes (*stx1*, *stx2*, *eae* and *ehxA*) and all of them were found to be resistant to ampicillin and streptomycin (Solomakos et al. 2009). Presence of some virulence genes alone or in combinations seems to be correlated with antimicrobial resistance. In a study on *E. coli* isolated from meat, the prevalence of resistance to multiple antimicrobials was much higher in strains were carrying both *astA* and *iucD* genes compared to strains harboring only the *iucD*. The strains with the *pap* gene were more resistant to several antimicrobials than those lacking the *pap* (Badri et al. 2009).

Ngeleka and colleagues have reported that virulent EPEC and ETEC *E. coli* isolated from piglets (both diarrheic and non-diarrheic) expressed high levels of resistance to gentamicin, neomycin, and sulphametoxazol-trimethoprim (Ngeleka et al. 2003). This observation agrees with more recent similar studies reporting the correlation between resistance to ceftiofur and presence of *faeG* and *aidA* in *E. coli* isolated from diseased pigs (Wang et al. 2010) as well as the association between multi-drug resistance to certain antimicrobials (cefoxitin, gentamicin, kanamycin, streptomycin, tetracycline, and ceftiofur) and APEC-associated virulence genes (*cvaC*, *iss*, *iutA*, and *traT*) in avian-source *E. coli* (Johnson et al. 2012).

Genetically, a strong association between the resistance gene for tetracycline (*tetA*) and ETEC virulence genes (*estA, paa*) and between the apramycin resistance gene (*aac(3)IV*) and *faeG* were observed in pig-originated *E. coli* strains. It was also noteworthy that the difference observed in prevalence of antimicrobial resistance among isolates from ETEC and non-ETEC sick animals was assumed as a proof for existence of linkages between resistance and virulence genes (Boerlin et al. 2005). The linkage hypothesis was used again in another similar study on *E. coli* isolated from healthy pigs where a strong positive association between *fedA* and two gentamicin resistance genes *aadA1* and *aadA2* was believed to reveal their co-localization on the same mobile genetic elements (e.g., plasmids, transposons) (Lay et al. 2012).

The co-mobilization of AMR and virulence genes is still a controversial phenomenon, although accumulating evidence indicates that in certain strains there is a correlation between virulence and antimicrobial resistance. Hence, elucidating the genetic mechanisms regulating such hypothetical linkage will be necessary in order to help us to understand the interplay of resistance and virulence at the genetic level with the aim to improve our management of infectious diseases

3 Alternatives to antimicrobials as growth promoters

Antimicrobials have played a major role in the growth and development of the swine production for more than 50 years. Their efficiency in increasing growth rate,

improving feed utilization and reducing mortality from clinical disease is well documented (Cromwell 2002). The emergence of multidrug resistant bacteria is increasing, which is a particular concern on livestock farms. Many countries have banned or are banning the use of antimicrobials in swine diets as a growth promoter (van der Fels-Klerx et al. 2011). As a result, intensive studies have been focused on the development of alternatives to antimicrobials to maintain swine health and performance. There are a number of commonly used non-therapeutic alternatives to antimicrobial growth promoters in swine production.

Probiotics are widely used live microbial supplements which improve the host's health by modifying the intestinal microflora (Fuller 1989). *Bacillus*, yeasts, and lactic acid-producing bacteria are the three categories of organisms that are commonly referred to as probiotics (Heo et al. 2013). The anti-diarrhea potential of probiotics has been stressed in the animal industry and specifically in weaning piglets due to the emergence of antimicrobial-resistant microbes (Lalles et al. 2007). Probiotics provide beneficial effects via different mechanisms. They compete with pathogenic microorganisms for binding sites in the intestinal mucosa. *Lactobacillus* spp. has been shown to reduce the adhesion of pathogenic *E. coli* ETEC K88 to piglet ileal mucus by approximately 50% (Blomberg et al. 1993). Probiotics can also increase nutrient availability and inhibition of pathogen growth by production of organic acids (Vondruskova et al. 2010).

Prebiotics are selectively ingredients which cannot be digested by host but are specifically fermented by the intestinal microbiota and can change both the composition and (or) activity of the gastrointestinal microbiota (Gibson and Roberfroid 1995). To be qualified as a prebiotic, the ingredient should be resistant to hydrolysis and gastric acidity, be fermentable by the intestinal microbiota and could selectively promote growth and activity of well-being related intestinal bacteria. Thus, only two groups of fructo-oligosaccharides (FOS) and transgalacto-oligosaccharides (TOS) are categorized as prebiotics (Roberfroid 2007).Prebiotics can especially support the growth and/or activities of probiotic microorganisms in the gastrointestinal tract, such as *Bifidobacteria*, *Lactobacilli* and *Eubacteria* (Rayes et al. 2009). The evaluation of dietary TOS addition on swine nutrient digestibility revealed an increase in beneficial

species in the ileal and fecal bacteria population and an improvement in ileal short-chain fatty acid (SCFA) production, an indicator of intestinal fermentation (Smiricky-Tjardes et al. 2003).

Bacteriocins are another alternatives to traditional antibiotics. They are a subgroup of antimicrobial peptides produced by certain bacteria and play as a specific immunity mechanism against other bacterial species (Cotter et al. 2013). Certain properties of bacteriocins make them a very viable alternative to antimicrobials such as their in vitro potency against clinically important pathogens such as *Escherichia coli*, *Streptococcus pneumoniae* and *Clostridium difficile* (Piper et al. 2009) as well as their low toxicity for the treated host either human or animal (Haste et al. 2012). To control foodborne pathogenic bacteria (e.g *E.coli*) in livestock, a variety of bacteriocin have been tested (Diez-Gonzalez 2007). However, among all bacteriocins, colicins hold a great deal of promise. Colicins are a class of bacteriocins produced by and effective against *E. coli* and closely related bacteria. It was shown that the daily addition of colicin E7-producing *E. coli* to cattle feed could reduce the fecal shedding of *E.coli* serotype O157:H7 (Schamberger et al. 2004). It has been also indicated that colicins are effective against *E. coli* ETEC strains responsible for postweaning diarrhea and edema disease in swine (Stahl et al. 2004) and dietary inclusion of colicin E1 would help to prevent postweaning diarrhea caused by F18-positive *E. coli* (Cutler et al. 2007).

Organic acids such as citric, fumaric, lactic and formic acids, are also considered as dietary supplements in the swine industry to improve growth performance and health (Tsiloyiannis et al. 2001). Several benefits were observed for dietary supplementation of organic acids including, bactericidal effects due to reduction in pH and the ability to penetrate bacterial wall and destroy some specific microorganisms (Pettigrew 2006), improving enzymatic digestion and nutrient absorption, and increasing the performance of weaned and growing piglets (Vondruskova et al. 2010). The impact of organic acids on reduction of the intestine *E. coli* ETEC K88 population and reducing the severity of pig post-weaning diarrhea have been discussed previously (Tsiloyiannis et al. 2001). Another meta-analysis study also confirmed the positive effect of organic acids and their salts on improvement of growth and feed efficiency in piglets (Partanen and Mroz 1999).

Zinc is an abundant trace element in the body and is involved in a variety of general cellular functions, including replication, transcription and maintaining the epithelial barrier integrity (Vondruskova et al. 2010). Dietary supplementation of zinc in the form of **zinc oxide** was shown to be beneficial not only for the stability of the intestinal microbiota (Hahn and Baker 1993) but also by expressing antimicrobial activities to inhibit the growth of some pathogenic bacteria (Sawai 2003) in weaned piglets. In high dose, feeding zinc to weaned piglets has been also demonstrated to reduce pig post-weaning diarrhea incidence (Stensland et al. 2015). It was shown that zinc oxide protects the intestinal epithelia through two major pathways; it maintains the integrity of the cell membrane, reducing damage via translocation of redox-active metal ions (Srivastava et al. 1995) and by involvement in protein expression associated with glutathione metabolism and oxidative stress (Wang et al. 2008). On the other hand, the zinc oxide antimicrobial mechanism of action is believed to be partly due to its ability to bind to bacterial fimbriae and interfere with bacterial adhesion, specifically of pathogenic species such as *E. coli* ETEC (Jin and Zhao 2000). Recently, it was also shown that feed supplementation with zinc increases the proportion of multidrug resistant *E. coli* and clonal diversity of *E .coli* population in piglets (Bednorz et al. 2013).

Copper (like Zinc) is another essential trace elements that perform several biological functions. It is increasingly used, as copper sulfate ($CuSO_4$), as an alternative to in-feed antibiotics in weaned piglets for prevention of diarrheal disease (Yazdankhah et al. 2014; Agga et al. 2015). Although its mechanism of action is not clear, it has been shown that in presence of copper sulfate, gut microbiota are altered to reduce fermentation loss of nutrients and to suppress gut pathogens (Hojberg et al. 2005). However, over feed supplementation with copper activates several mechanisms of metal tolerance in bacteria that might promote the spread of antimicrobial resistance via co-selection not only at the farm level, but also in the environment (Seiler and Berendonk 2012).

Other feed alternatives such as enzymes, herbs, plants extracts and neutraceuticals like clay minerals, egg yolk antibodies, essential oils, eucalyptus oil-medium chain fatty acids and rare earth elements are also used as feed additives in pork

industry with the aim to maintain swine health and performance (McEwen and Fedorka-Cray 2002; Verstegen and Williams 2002; Thacker 2013).

3.1 Clay minerals

One of the alternatives to antimicrobials growth promoters that has been suggested as a feed additive for use in swine production is the clay (Trckova et al. 2009; Song et al. 2012; Subramaniam and Kim 2015). Clays are crystalline, hydrated aluminosilicate molecules composed of alkali and alkaline earth cations along with small amounts of various other elements. Clay minerals are formed by a net of stratified tetrahedral and octahedral layers and channels capable of trapping a wide variety of molecules. As a result of this structure, clay minerals are considered as a simple and effective tool for the prevention of the negative effects of toxic compounds. They have molecules of silicon, aluminium and oxygen. The layers can be inter-connected by hydrogen bonds or a group of cations, and this space is called an interlayer. A common feature of clay minerals is a high adsorption capacity determined by their stratified structure. The natural extracted clays (bentonites, zeolite, kaolin) are a mixture of various clays differing in chemical composition. The best-known are montmorillonite (MMT), smectite, illite, kaolinite, biotite and clinoptilolite (Vondruskova et al. 2010; Subramaniam and Kim 2015).

3.1.1 Clay minerals as animal food supplements

Administration of clays as a feed additive has been shown to improve weight gain and feed conversion in pigs (Papaioannou et al. 2004; Trckova et al. 2009; Duan et al. 2013). One of the most likely explanations for the improvement in performance is the fact that supplementation of the diet with clay minerals has been shown to increase nutrient digestibility (Shurson et al. 1984; Subramaniam and Kim 2015). The supplementation of a diet with 1% to 3% of clay minerals is generally recommended (Vondruskova et al. 2010). Several studies have described the decontamination

properties of clays with respect to mycotoxins (Lindemann et al. 1993; Phillips 1999; Marroquin-Cardona et al. 2009), heavy metals (Xu et al. 2004; Yu et al. 2008), and other toxins (Knezevic and Tadic 1994).

One of the known clay minerals, clinoptilolite is a natural zeolite which is mined from sedimentary deposits. Zeolites have diverse applications based on their unique adsorption, cation-exchange, dehydration–rehydration, and catalytic properties (Mumpton 1999; Papaioannou et al. 2005). Clinoptilolite is authorized by the European Union and registered as a technological additive in feed stuffs for all animal species at max 20,000 mg/kg complete animal feed (E- 568) (EFSA 2013). In Canada according to Veterinary Drugs Directorate (VDD) and Canadian Food Inspection Agency (CFIA) recommendation, using zeolites as animal feed additive are permitted however they should not exceed 2% of the complete feed (HC 2012). Also, it is considered as a GRAS (Generally Regarded as Safe), as listed by the Food and Drug Administration (FDA).

Recently, use of clinoptilolite in animal nutrition has been increased mainly to improve performance and health (Katsoulos et al. 2006) and also is considered as an alternative to antimicrobials in prevention of diarrhea in weaned piglets (Papaioannou et al. 2004). It has been reported that the performance of pigs fed 2 % clinoptilolite in comparison with a control group after weaning until market weight showed a 5.3% improvement in weight gain for pigs fed clinoptilolite, the response being greater in younger pigs than older pigs. Also, it has been shown that weight gain increased by 14.3 % and feed conversion by 2.9 % as a result of feeding 2 % clinoptilolite to growing pigs (Subramaniam and Kim 2015). There is evidence that oral administration of clinoptilolite can decrease the rate of passage of food through the intestine and improve average body weight gain and feed conversion and nutrient utilization in pigs (Papaioannou et al. 2004; Papaioannou et al. 2005; Leung et al. 2007). Clinoptilolite has been widely used in swine diets to protect against mycotoxins (Schell et al. 1993).

3.1.2 Antimicrobial activity of clay minerals

It has been reported that clays alter the microbial composition of the gastrointestinal tract resulting in a more favorable gut microflora (Subramaniam and Kim 2015). The ability of clay minerals to reduce the number of pathogenic bacteria in the intestinal tract has been demonstrated *in vivo* in pigs (Trckova et al. 2009) and *in vitro* (Hu et al. 2002; Haydel et al. 2008). Haydel et al. (2002), in an *in vitro* study, reported that clay mineral (CsAg02) induced a bactericidal activity against pathogenic *E. coli*, extended-spectrum β-lactamase (ESBL)-producing *E. coli*, *S. enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Mycobacterium marinum* and a combined bacteriostatic/bactericidal effect against *Staphylococcus aureus*, penicillin-resistant *S. aureus* (PRSA), methicillin-resistant *S. aureus* (MRSA), and *Mycobacterium smegmatis* (Haydel et al. 2008).

Additionally, it has been shown that administration of clinoptilolite as a feed supplement resulted in a reduction in total flora colony counts in the proximal and distal intestine in chickens (Olver 1983; Mallek et al. 2012). Likewise, it has been reported that feed supplementation with clinoptilolite reduced total viable counts of *E. coli* in the cecal contents of broiler chicks (Wu et al. 2013). The ability of clay minerals to allow adsorption of pathogens and enterotoxins to the surface layer or into the interlayer sheets to degrade them in the intestinal tract has been shown *in vivo* study (Trckova et al. 2009). However, little is known of the mechanism of action of clinoptilolite.

3.1.3 Effect of clay minerals on horizontal gene transfer

Yoshida et al. (2004) demonstrated that clay minerals induce bacterial mutation and promote genetic variability of bacteria. Where bacteria were cultured in Luria-Bertani medium with and without caly minerals. Chromosomal DNA was extracted from both group and underwent Random amplified polymorphic DNA PCR and band pattern were compared. By comparison of amplified DNA they reported that clay minerals induce mutation in bacteria and may play roles in microbial evolution (Yoshida et al. 2004) and that chromosomal and plasmid DNA bound to clay minerals are more

resistant to degradation by DNase I than free DNA (Romanowski et al. 1991; Khanna and Stotzky 1992; Paget et al. 1992; Romanowski et al. 1992). Several *in vitro* studies have shown that clay minerals promote direct horizontal transfer of AMR genes in different bacterial species. For instance, Rodríguez-Beltrán et al. (2013), who described that clay minerals (e.g. sepiolite) increase direct horizontal gene transfer between bacterial species, due to plasmid transfer in certain conditions such as presence of septiolite (final concentration of 100 µg/ml) and increased friction forces, the latter of which may have been provided by peristalsis in the intestine of the animal receiving continuously clay mineral as a food additive. This phenomenon may be exacerbated by the use of antimicrobials as growth promoters. Moreover, it has been shown that clay minerals montmorillonite and kaolinite increased the frequency of transformation in competent *Bacillus subtilis* cells (Richaume et al. 1989; Romanowski et al. 1993; Lotareva and Prozorov 2000; Rodriguez-Beltran et al. 2013). Influence of clay minerals on horizontal transfer of AMR genes is concentration dependence and previous *in vitro* studies demonstrated that higher concentrations of clay minerals resulted in a decrease in the number of transformed cells, in other words, a discount in HGT (Richaume et al. 1989; Lotareva and Prozorov 2000).

Chapter 2: Rationales and Objectives

Rationale

Emergence of resistance to clinically important antimicrobials such as cephalosporins (ceftiofur, ceftriaxone, etc) through widespread use of antimicrobials in animal production is a major public health concern. *E. coli* pathotypes (e.g. ETEC, STEC, EPEC and ExPEC) cause diseases in pigs and often carry antimicrobial resistance genes. In pigs, antimicrobials are used in feed primarily to treat disease and thus reduce mortality and morbidity but also for promoting growth and for disease prevention. Furthermore, administration of in-feed antimicrobials in pigs has been associated with increased resistance of fecal *E. coli* within and between classes of antimicrobials.

Ceftiofur is a critically important antimicrobial, being in the same general class as ceftriaxone which is important in human medicine for treating serious infections especially for use in children. It has been hypothesised that the widespread use of ceftiofur on animal farms has resulted in food-borne ceftriaxone-resistant enteric bacteria. To maintain the efficacy of this clinically important antimicrobial, it is important to know the frequency of ESC-resistant bacteria in livestock and to understand the mechanisms involved in their selection and persistance. As ceftiofur is extensively used in swine production, ceftiofur-resistant *E.coli* isolates from pigs are an appropriate subject for further investigation of the evolution with time of ceftiofur-resistant *E. coli* isolates from when ceftiofur resistance was first reported.

In addition, certain supplements are routinely used in animal feed to improve growth. For instance, the clay mineral clinoptilolite has been used in an attempt to improve performance and health but also as an alternative to the use of antimicrobials or together with antimicrobials for prevention of diarrhea in weaned piglets. However, little is known about the mechanism of action of clinoptilolite. Some *in vitro* studies have shown that clay minerals induce bacterial mutation and promote genetic variability of bacteria, and clays protect DNA degradation by DNAase I. Moreover, several *in vitro* studies reported that clay minerals promote direct horizontal transfer of AMR genes in different bacterial species. These data suggest that clay minerals may modulate the prevalence of AMR and virulence genes of *E.coli* in the animal gut. In addition, clay minerals may effect on certain *E. coli* clones and plasmids.

Hypothesis

Feed supplements influence the prevalence of virulence and antimicrobial resistance genes of *E. coli* in the intestine of pigs and could affect the fecal excretion of *E. coli* possessing these genes.

Objectives

The primary objective of this study was to study the temporal characterization of virulence genes and antimicrobial resistance in *E. coli* from pig fecal samples and the effect of a feed supplement.

In particular, the specific objectives of this research are:

1- To examine the dynamic of AMR phenotype, virulence and AMR gene profiles in *E. coli* isolates from pigs receiving a diet containing chlortetracycline and penicillin G at therapeutic doses following weaning on a commercial farm and to investigate the effect of simultaneous feeding of the clay mineral, clinoptilolite, on this dynamic.

2- To elucidate the mechanisms leading to an increase of the prevalence of $bla_{\text{CMY-2}}$ conferring resistance to ceftiofur in a nursery barn in pigs with no ceftiofur use but which received a feed medicated with chlortetracycline and penicillin G, and to investigate the effect of feed supplementation with a clay mineral on this phenomenon.

3- To study the temporal characterization of clinical ceftiofur-resistant *E.coli* isolates from diseased pigs in Quebec-Canada from 1997 to 2012.

Chapter 3: Articles

Details on the role of the candidate in the conception of the article: I am the first author of the article. I actively participated in the study design and; I substantially carried out research, analyzed data and wrote the paper.

Article 1. Impact of medicated feed along with clay mineral supplementation on *Escherichia coli* resistance to antimicrobial agents in pigs after weaning in field conditions

Seyedehameneh Jahanbakhsh[a], Kiswendsida Paul Kabore[a], Philippe Fravalo[b], Ann Letellier[b], John Morris Fairbrother[a]

OIE Reference Laboratory for *Escherichia coli* (EcL), Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, QC, Canada[a].

Research Chair in Meat Safety, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, QC, Canada[b].

Address for correspondence: John M. Fairbrother, Département de pathologie et microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, 3200 rue Sicotte, Saint-Hyacinthe QC, Canada, J2S 2M2.

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Abstract

The aim of this study was to examine changes in antimicrobial resistance (AMR) phenotype and virulence and AMR gene profiles in *E. coli* from pigs receiving in-feed antimicrobial medication following weaning and the effect of feed supplementation with a clay mineral, clinoptilolite, on this dynamic. Eighty *E .coli* strains isolated from fecal samples of pigs receiving a diet containing chlortetracycline and penicillin, with or without 2% clinoptilolite, were examined for antimicrobial resistance to 15 antimicrobial agents. Overall, an increased resistance to 10 antimicrobials was observed with time. Supplementation with clinoptilolite was associated with an early increase but later decrease in $bla_{\text{CMY-2}}$, in isolates, as shown by DNA probe. Concurrently, a later increase in the frequency of $bla_{\text{CMY-2}}$ and the virulence genes *iucD* and *tsh* was observed in the control pig isolates, being significantly greater than in the supplemented pigs at day 28**.** Our results suggest that, in the long term, supplementation with clinoptilolite could decrease the prevalence of *E. coli* carrying certain antimicrobial resistance and virulence genes.

Keywords: Pig; *E. coli*; virulence gene; antimicrobial resistance; clay mineral (clinoptilolite).

Introduction

Antimicrobial use in animal production has been monitored over the past two decades because of potential adverse effects on animal and human health related to antimicrobial resistance. Administration of antimicrobials in animal production began early after their initial discovery, primarily for treatment of diseases, but also for promoting growth and for disease prevention. The latter is particularly important because animals are commonly housed at high densities that can facilitate the spread of disease. Nevertheless, there are disadvantages associated with antimicrobial use in animal production. For instance, administration of antimicrobials may select, or co-select for the presence of AMR genes in the commensal or pathogenic bacterial populations in animals and lead to a breakdown in the treatment of associated diseases or transfer of resistance pathogenic bacteria to humans by contaminated meat. AMR genes may also be transferred to human pathogens and lead to problems in the treatment of disease in human patients (Johnston 2001, Wegener 2003). In light of these disadvantages, animal feeds maybe supplemented with other feed additives as alternatives for or a complement to, the use of antimicrobials. For example, the clay mineral clinoptilolite has been used in an attempt to improve performance and health but also as an alternative to the use of antimicrobials or together with antimicrobials for prevention of diarrhoea in weaned piglets (Papaioannou, Kyriakis et al. 2004). However, little is known of the mechanism of action of clinoptilolite. It has been demonstrated that clay minerals induce bacterial mutation and promote genetic variability of bacteria (Yoshida, Naka et al. 2004), chromosomal and plasmid DNA bound to clay minerals being more resistant to degradation by DNase I than free DNA (Romanowski, Lorenz et al. 1991). Some *in vitro* studies have shown that clay minerals promote direct horizontal transfer of AMR genes in different bacterial species (Lotareva and Prozorov 2000, Rodriguez-Beltran, Rodriguez-Rojas et al. 2013). These data suggest that clay minerals may modulate the prevalence of AMR and virulence genes of bacteria in the animal gut.

In weaning pigs, antimicrobials are used in feed primarily as a medication to prevent disease and thus reduce mortality and morbidity (Cromwell 2002). Among the most frequently used in-feed antimicrobials are penicillin and tetracycline, often in

combination (Akwar, Poppe et al. 2008). Use of in-feed antimicrobials in pigs has been associated with increased resistance of fecal *E. coli* within and between classes of antimicrobials (Kim, Gray et al. 2005, Akwar, Poppe et al. 2008). The main objective of the present study was to examine the dynamic of AMR phenotype and virulence profiles in *E.coli* and AMR gene profiles in these isolates from pigs receiving a diet containing chlortetracycline and penicillin in therapeutic doses following weaning on a commercial farm and to investigate the effect of simultaneous feeding of the clay mineral, clinoptilolite, on this dynamic.

Materials and methods

Antimicrobial medication regime

Pigs from a commercial crossbred genetic line were weaned at 21 days of age and transferred to pens in the nursery barn. Pigs received a standard commercial diet, three different rations (1, 2 and 3) being given continuously in feeders during the 28 days of the trial. The feed contained the following antimicrobials: chlortetracycline (Aureomycin® 220 G) in therapeutic doses as prescribed for protection of respiratory problems and penicillin G (Pen-P 110) at a metaphylactic dose as prescribed to prevent *Streptococcus suis* infections. Antimicrobials were used in feed as follows: chlortetracycline (1100 g/ton) from day 0 to day 7; chlortetracycline (660 g/ton), penicillin G (198.6 g/ton) and IVOMEC from day 8 to day 14; and chlortetracycline (660 g/ton) and penicillin G (198.6 g/ton) from day 15 to day 28. On days 3 and 5 after weaning, all pigs received a circovirus (Circumvent® PCV) and mycoplasma (Myco Silencer® Once) vaccine, respectively.

Trial design, collection and preparation of samples

This study was conducted on 168 pigs. At day 0 (first day of placement in the nursery), the pigs were randomly divided into 2 groups: control (C) and supplemented

(S). Feeding and housing conditions were identical for the two groups, except for the addition of 2% clinoptilolite to the feed of the supplemented group. The control group received a standard commercial feed (basal diet) and the supplemented group received the basal commercial diet with 2% clinoptilolite. Both groups were housed in the same room, each group allocated into 3 different pens, 28 pigs being placed in each pen, so that the average pen weights were similar.

Twelve pigs were tagged in each group to permit individual follow-up. Samples were collected at days 0, 2, 7, 14, and 28 after weaning, either directly from the rectum of the tagged pigs using a cotton swab or by pooling faeces from five sites on the floor of the pens as described by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (CIPARS 2008). On each sampling day, 8 samples were collected per group and were shipped on ice to the laboratory for further analysis. The method used for sample preparation was a modification of the CIPARS protocol (CIPARS 2008). Briefly, floor fecal samples were diluted to 1/10 (weight-for-weight) in buffered peptone water and 200 µl of diluted fecal samples and rectal swabs were inoculated into 5 ml Luria Bertani broth (LB – Difco, USA) and incubated overnight at 37°C. DNA templates were prepared from the processed samples by boiled cell lysis for examination by PCR, as described previously by Maluta et al. (Maluta, Fairbrother et al. 2014).

Processed fecal samples (a total of 80 samples consisting of 60 floor samples and 20 rectal samples) were serially diluted in peptone water to 10^{-5}, from which a last dilution was made in peptone water containing 1% tween 80 to obtain a 10^{-7} diluted subsample (approximately 10^{2} CFU/ml) (Sharpe and Peterkin 1988). A volume of 2 ml of this final dilution was filtered through a hydrophobic grid membrane filter (HGMF) using a Spreadfilter (Filtaflex, Almonte, Ontario, Canada). Filters were placed onto MacConkey agar plates and incubated overnight at 37°C to obtain HGMF master filters bearing predominantly *E. coli* lactose-positive colonies.

Bacterial isolation and identification

A total of 80 presumptive *E.coli* isolates were randomly selected from HGMF master filters to represent the different sampling days (except for day 2) and the two groups (n=40 for each of the groups supplemented or not with clinoptilolite). Isolates were identified as *E. coli* by biochemical tests (Simmons Citrate Agar, mobility and indole) and by PCR for the presence of *uidA E.coli* housekeeping gene, which encodes Beta-glucuronidase (Table 1 in the supplemental material).

Antimicrobial susceptibility testing

The selected *E. coli* strains (n=80) were examined for susceptibility to the same 15 antimicrobial agents examined in the CIPARS surveillance program in Canada (CIPARS 2008) using the disk-diffusion (Kirby-Bauer) assay. Bacterial strains grown overnight on blood agar were mixed in tubes containing 10 ml of sterile water to reach a turbidity of 0.5 McFarland Standard. The contents of the tubes were placed onto Mueller-Hinton agar plates using a sterile swab. The plates were incubated at 37°C for 24 h, and the diameters of the zones of complete inhibition were measured. The strains were recorded as susceptible, intermediate, or resistant according to the zone diameter interpretative standards recommended by Clinical and Laboratory Standards Institute (CLSI) in 2010 (CLSI 2010) for most of the antimicrobials and in 2008 for ceftiofur (CLSI 2008). The following antimicrobial disks were used; amikacin (30µg), kanamycin (30µg), gentamicin (10µg), streptomycin (10µg), ceftriaxone (30µg), ceftiofur (30µg), cefoxitin (30µg), ciprofloxacin (5µg), nalidixic acid (30µg), trimethoprim-sulfamethoxazole (23.75µg), sulfisoxazole (250µg), ampicillin (10µg), amoxicillin/Clavulanic acid (30µg), chloramphenicol (30µg), and tetracycline (30µg).

PCR for determination of virulence and AMR genes

Boiled cell lysates from the samples and the 80 selected *E. coli* strains were tested by multiplex PCR to determine the presence of the virulence genes which define the *E. coli* pathotypes commonly found in animals: Enterotoxigenic *E. coli* (*eltB, estA, estB*), Enteropathogenic *E. coli* (*eae*), Shiga toxin-producing *E. coli* (*stxA, stx2A*) and

Extraintestinal pathogenic *E. coli* (*cnf, papC, iucD, and tsh*). PCR procedures for detection of these genes were performed according to the protocol of the Reference Laboratory for *Escherichia coli* (EcL- Faculté de Médecine Vétérinaire de L'Université de Montréal) available in the animal pathogenic zoonotic *Escherichia coli* website (http://apzec.ca/en/Protocols). The presence of the AMR genes bla_{TEM}, $bla_{\text{CMY-2}}$, *tetA*, and *aadA1* was determined by PCR using primers and conditions as shown in Table 1 in the supplemental material.

Phylogenetic analysis

Phylogenetic grouping was carried out for the 80 selected *E. coli* strains using a multiplex PCR-based assay as described by Clermont et al. (Clermont, Bonacorsi et al. 2000). Based on the presence or absence of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2), strains were classified into four main *E.coli* phylogenetic groups (A, B1, B2, or D).

Detection of AMR and virulence genes in an extended collection of *E. coli* isolates

A HGMF Interpreter (Filtaflex, Almonte, Ontario, Canada) was used to count presumptive *E. coli* colonies on HGMF master filters, which were then replicated using a HGMF Replicator (Filtaflex, Almonte, Ontario, Canada), placed onto MacConkey agar plates, and incubated overnight at 37°C to obtain HGMF replicates. The positive and negative control strains used in the hybridization process were also filtered and replicated as described above. To generate DNA hybridization probes to detect each of the virulence or antimicrobial resistance genes, templates were prepared from overnight LB cultures of the appropriate control strains. Digoxigenin (DIG) alkaline phosphatase labelled probes were generated by using the specific primers (Table 1 in the supplemental material and (http://apzec.ca/en/Protocols) and a PCR DIG Probe Synthesis Kit (Roche Diagnostics) following instructions. The bacterial colonies on the HGMF replicates were pre-treated as described by Todd et al. (Todd, Szabo et al. 1999). Briefly, the HGMFs were incubated in pre-treatment solution (45 ml of 10 mM Na2PO4

[pH 6.0] [1.42 g/liter], 9 ml of 1 M NaHCO3, 135 ml of Lugalvan G35 detergent), then in 3 ml of lysis solution (150 mM NaOH in 70% ethanol) at 37°C for 30 min. HGMFs were then heated in a microwave oven for 30s, and gently shaken for 60 min at 37°C in 0.01% proteinase K solution [Sigma Chemical Co., St. Louis, Mo.] in 2X SSC [1X SSC is 0.15 M sodium chloride plus 0.15 M sodium citrate] supplemented with 0.1% sodium dodecyl sulfate [SDS]).

The DNA was cross-linked to the HGMFs by exposure to UV light. HGMFs containing cross-linked DNA were hybridized with DIG-labeled probes for the detection of the appropriate virulence or antimicrobial resistance genes. The procedure was carried out at 68°C using 20 ng /ml of denatured specific probe in hybridization solution (100 mg of herring sperm DNA [Sigma] per ml in 6X SSC supplemented with 0.5% SDS).

DIG-labeled DNA probes were detected, after hybridization to target nucleic acids, by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate, anti-DIG-AP) according to the instructions for the DIG Nucleic Acid Detection Kit (Roche Diagnostics). The presence of specific genes was revealed colorimetrically with the alkaline phosphatase substrate, 5-bromo-chloro-3-indolyl phosphate (BCIP), and nitroblue tetrazolium salt (NBT). A total of 7232 presumptive *E.coli* isolates were examined for the presence of selected genes.

Statistical analysis

Statistical analysis was carried out using SAS v.9.3 and JMPv.7 (SAS Institute, Cary, NC, USA). The Chi-square test (95% confidence intervals) was used to compare the effect of feed ingredient and time on the prevalence of AMR (genotypic and phenotypic) and virulence genes. Fisher's exact test was also used to examine the differences among groups constrained across days. Odds ratios (OR) (95% confidence intervals) based on HGMF results calculated with SAS were used to evaluate the associations between genes. For each comparison, a P value of <0.05 was considered to denote significant differences.

Results

Evolution of antimicrobial resistance in fecally shed *E. coli* from healthy pigs after weaning

Overall, the most frequently observed resistance in the randomly selected *E.coli* strains (n=40 for each of the groups supplemented or not with clinoptilolite) was to tetracycline (92.5%), ampicillin (83.7%), sulfisoxazole (80%), trimethoprim-sulfamethoxazole (77.5%), streptomycin (77.5%), amoxicillin/clavulanic acid (65%), chloramphenicol (40%), kanamycin (22.5%), ceftriaxone (20%), ceftiofur (20%) and cefoxitin (20%), gentamicin (18.75%) (data not shown). Furthermore, thirty-three AMR patterns were distinguished among *E.coli* strains and only 3 strains (3.75%) were susceptible to all antimicrobials tested, whereas 70 (87.5%) were classified as multi-drug resistant (MDR) as described by Magiorakos et al., a strain being MDR if it is non-susceptible to at least 1 antimicrobial agent in 3 or more antimicrobial defined categories (Magiorakos, Srinivasan et al. 2012) (Table 1).

At weaning, resistance to streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, ampicillin, amoxicillin/clavulanic acid, chloramphenicol, and tetracycline was observed in up to 60% of the strains (Fig. 1). The resistance prevalence increased significantly from day 0 to day 7 for most of these antimicrobials, remaining high until the end of the study or decreasing slightly in the case of streptomycin, sulfisoxazole, and trimethoprim-sulfamethoxazole. On the other hand, resistance remained low for kanamycin, gentamicin, ciprofloxacin and nalidixic acid (Fig. 1). All strains were susceptible to amikacin. No significant difference was observed between control and supplemented groups for any of the antimicrobials (supplementary Fig. 1). In contrast, no strains resistant to ceftiofur, ceftriaxone, or cefoxitin were observed at weaning, and the prevalence of resistance to these antimicrobials increased from 7 days after weaning, the increase being significant at day 28. Interestingly, the resistance to these cephalosporins was only observed in the supplemented pigs at days 7 and 14, being significantly different to the control group ($P<0.05$) (Fig. 2). On the other hand, in the control group, resistance to these cephalosporins was only observed at day 28, the

prevalence being similar to that observed in the supplemented group. One AMR pattern was predominant in extended-spectrum cephalosporin (ESC)-resistant *E.coli* strains in both groups, with co-resistance to streptomycin, cefoxitin, trimethoprim-sulfamethoxazole, sulfisoxazole, amoxicillin/clavulanic acid, ampicillin, chloramphenicol, and tetracycline. Nevertheless, five AMR patterns were observed in ESC-resistant *E. coli* strains, all being classified as MDR, all being non-susceptible to at least 1 agent in 5 or more antimicrobial categories (Table 1).

Presence of *bla*$_{\text{CMY-2}}$ gene in fecal samples

Since AMR gene *bla*$_{\text{CMY-2}}$ is a candidate for conferring resistance to clinically important cephalosporins such as ceftriaxone, ceftiofur and cefoxitin, DNA templates from the processed fecal samples from the weaned pigs were examined by PCR for the presence of this gene. The proportion of samples positive for the *bla*$_{\text{CMY-2}}$ gene in the supplemented group rapidly increased from day 2, reaching 100% at days 7 and 14, and decreasing at day 28 (Fig. 3). In contrast, no samples positive for *bla*$_{\text{CMY-2}}$ were observed in the control group until day 14, a gradual increase in the proportion of positive samples being observed at day 28. The proportion of positive samples was significantly higher in the supplemented group than in the control group at days 2, 7, and 14 (P<0.05); furthermore, as an increase in frequency of resistance to amoxicillin/clavulanic acid, ampicillin, tetracycline and streptomycin was observed with time in the randomly selected *E. coli* strains, fecal samples were examined by PCR for the presence of the AMR genes: *bla*$_{\text{TEM}}$, *aadA* and *tetA*. Results revealed an overall high prevalence of these genes in samples that was not affected by either the supplementation or time (data not shown).

Distribution of *E. coli* virulence genes in fecal samples

DNA templates from the processed fecal samples from post weaning pigs were tested by multiplex PCR for the presence of virulence genes defining the *E. coli* pathotypes commonly found in animals (http://apzec.ca/en/Protocols). Overall, the

proportion of samples positive for the ExPEC virulence genes *iucD*, *tsh* and *papC* was high, greater than 90% for all groups, no significant differences being observed with respect to the supplementation or the time after weaning. The proportion of samples positive for *eae* increased with time after weaning, from 37% to 75%, no significant differences being observed with respect to the supplementation. The proportion of samples positive for *estB* varied considerably between groups whereas *cnf* was rarely detected. In contrast, *estA , eltB*, *StxA,* and *Stx2A* were not detected in any sample (data not shown).

Prevalence of *bla*$_{\text{CMY-2}}$ and ExPEC genes *iucD*, *tsh, papC,* and *cnf* in *E. coli*

To further investigate the effect of time after weaning and supplementation with clinoptilolite on the presence of *bla*$_{\text{CMY-2}}$ and the ExPEC virulence genes in fecally shed *E. coli*, we initially examined the 80 randomly selected *E.coli* strains previously examined for antimicrobial susceptibility. As observed for resistance to ceftiofur, ceftriaxone, and cefoxitin, frequency of the *bla*$_{\text{CMY-2}}$ gene was significantly higher in the supplemented group than the control group at days 7 and 14 after weaning (P<0.05) (Table 2), correlation between the cephalosporin resistance and presence of *bla*$_{\text{CMY-2}}$ being 100%. In addition, the frequencies of ExPEC virulence genes *iucD* and *tsh* were higher in the control group than the supplemented group at day 28.

In order to confirm these trends and to further investigate the relation between the presence of *bla*$_{\text{CMY-2}}$, *iucD,* and *tsh* in isolates, we examined a more extensive collection of 7232 presumptive *E.coli* isolates obtained from the fecal samples by HGMF, using DNA probe colony hybridization. In the supplemented group, the proportion of isolates with the *bla*$_{\text{CMY-2}}$ gene increased at day 7, being significantly higher (P<0.05) than in the control group, and thereafter decreased. On the other hand, in the control group, the proportion of isolates with the *bla*$_{\text{CMY-2}}$ gene increased later, at day 14, being significantly greater (P<0.05) than in the supplemented group at both days 14 and 28 after weaning. In addition, an increase in the proportion of isolates positive for *iucD* or *tsh* was also observed on days 14 and 28 for the control group but not the supplemented group, being significantly greater (P<0.05) than in the supplemented

group at day 28 after weaning. Most of the isolates possessed only one of the genes *iucD*, *tsh*, and *bla*$_{CMY-2}$, although about one third of the *bla*$_{CMY-2}$-positive isolates from the control group at days 14 and 28 also possessed *iucD* or *tsh,* or both of these genes (Table 3). Odds ratio using the data of HGMF DNA probe hybridization confirmed the positive association between the *bla*$_{CMY-2}$ and *iucD* genes [OR (95%CI) =3.75 (2.67-5.27)], *bla*$_{CMY-2}$ and *tsh* genes [OR (95%CI) =3.95 (2.63-5.93)] and *iucD* and *tsh* genes [OR (95%CI) =5.05 (3.22-7.91)].

Distribution of *bla*$_{CMY-2}$-positive *E. coli* among phylogenetic groups

In order to gain some insight into whether the effect of clinoptilolite on *bla*$_{CMY-2}$-positive isolates may be clonal, we determined the phylogenetic group of each of the *bla*$_{CMY-2}$-positive strains among the 80 randomly selected *E. coli* strains previously examined for antimicrobial susceptibility. In the supplemented group, all *bla*$_{CMY-2}$-positive *E. coli* strains belonged to phylotype A at days 7 (n= 4) and 14 (n=3) after weaning and 3 out of 4 *bla*$_{CMY-2}$-positive *E. coli* strains belonged to phylotype A at day 28 . On the other hand, in the control group, *bla*$_{CMY-2}$-positive *E. coli* strains, found only at day 28 (n=5), belonged to phylotypes B1 (n= 3), D (n=1) and A (n=1) which indicates they belong to different clones than the *bla*$_{CMY-2}$-positive *E. coli* strains of the supplemented group (Fig. 4).

Discussion

Emergence of multidrug resistance in bacteria is one of the most important problems in animal and human medicine. In the present study an increased in frequency of resistance to streptomycin, ceftriaxone, ceftiofur, cefoxitin, trimethoprim-sulfamethoxazole, sulfisoxazole, ampicillin, amoxicillin/clavulanic acid, chloramphenicol, and tetracycline in *E. coli* strains from pigs with time could be a result of continuous administration of chlortetracycline and penicillin G in the feed. These results are in good agreement with the data of Akwar et al. (2008) and Kim et al. (2005), who showed that use of antimicrobials in feed in pigs, may exert a selection pressure

resulting in increased resistance of fecal *E. coli* within and between classes of antimicrobials (Kim, Gray et al. 2005, Akwar, Poppe et al. 2008). Indeed, Looft et al. (2012) found that use of chlortetracycline, penicillin, and sulfamethazine (known as ASP250) may increase the prevalence of resistance genes not only for the used antimicrobial but also for antimicrobials of other classes that were not administered, likely due to co-selection. For instance, they found an increased frequency of the following genes associated with feeding of ASP250: the *aph(3'')-Ib*,*aph(6')-Ic* gene conferring resistance to streptomycin, the *ImrA* gene related to lincomycin resistance; the *emrD, mdfA, mdtH, bcr* multi-drug resistance efflux pump genes conferring resistance to chloramphenicol, tetracycline, deoxycholate, fosfomycin, florfenicol, and sulfathiazole; and the *acrA*multi-drug resistance efflux pump gene responsible for resistance to many antimicrobial classes including phenicols, aminoglycosides, macrolides, and β-lactams (Looft, Johnson et al. 2012). Funk et al. (2006), in a phenotypic resistance study, also found that inclusion of chlortetracycline in the pig diet has an effect on resistance to multiple antimicrobials, including ampicillin, ceftriaxone and tetracycline, in the gram negative fecal flora of swine (Funk, Lejeune et al. 2006). Interestingly, in our study, whereas prevalence of resistance to antimicrobials in the same classes as the medicated feed, such as tetracycline and ampicillin, increased and remained high throughout the study, during which time pigs continued to be medicated, prevalence of resistance to antimicrobials of other classes, such as streptomycin, trimethoprim-sulfamethoxazole and sulfisoxazole, initially increased and then decreased with time. An exception was chloramphenicol, for which prevalence of resistance increased and remained high until the end of the study.

Identification of risk factors for development of AMR in food animals is complex, as it has been established that antimicrobial administration and selection or co-selection pressure may not be the only factors increasing AMR with time in farm conditions. Hence, other mechanisms such as vertical transmission of resistant *E. coli*, horizontal gene transfer between bacteria, adaptation, and maintenance of some sets of resistant strains could also have been responsible for the development of AMR with time on the studied farm (Blahna, Zalewski et al. 2006, Nilsson, Borjesson et al. 2014, Mazurek, Bok et al. 2015). On the other hand, in agreement with a previous study on a

cattle farm, in the present study the pigs in the control and supplemented groups were housed separately from each other, with no contact between pigs in the different groups. Nevertheless, animal to animal (some nose to nose and body contact) resistant strain transmission within each group could be a possible factor to increase AMR with time (Sharma, Munns et al. 2008). In addition, it has been reported that *E. coli* originating from fecal material, and contaminated food or water may be considered a possible source in cattle and that the feedlot environment is a critical source of new strains (Hoyle, Yates et al. 2005). Hence, it is possible that the farm conditions could play an important role in resistance dissemination over time in the present study.

It is interesting to note in our study that resistance to all antimicrobials except the cephalosporins and ciprofloxacin was present in pigs at weaning, before they received the medicated feed. The latter antimicrobials are not used in pigs in Canada, with the exception of injectable ceftiofur (CIPARS 2008). Although the number of tested strains for antimicrobial susceptibility was relatively low, the finding of resistance to ceftiofur, which was first observed at day 7 and continued to increase in prevalence through to day 28, was unexpected, as ceftiofur was not used in this batch of pigs. We confirmed a positive association between ceftiofur-resistance and the presence of *bla*$_{\text{CMY-2}}$ gene in *E.coli* isolated from healthy pigs. This is in accordance with previous investigations on the prevalence of *bla*$_{\text{CMY-2}}$-positive isolates in different animal species (Diarra, Silversides et al. 2007, Mataseje, Baudry et al. 2010). It is possible that the use of ceftiofur in previous batches of pigs, along with inadequate pen sanitation, allowed the *bla*$_{\text{CMY-2}}$ genes to persist in the environment. Indeed, we recovered *bla*$_{\text{CMY-2}}$-positive isolates from the pens before introduction of the pigs of this batch (results not shown). In addition, the resistance to ceftiofur in the piglets could also be associated with the microflora of the sows and the fact that resistance to ceftiofur was not observed before day 7 might be due to the low level of this phenotype in piglets initially and effect of selection pressure over time. The usage of chlortetracycline may have resulted in subsequent expansion of the *bla*$_{\text{CMY-2}}$ gene. For instance, in a recent 26 day metagenomic study in cattle, administration of chlortetracycline led to exacerbation of the number of *bla*$_{\text{CMY-2}}$ gene copies/gram of feces following ceftiofur therapy (Kanwar, Scott et al. 2014). Moreover, in our study, resistance to chloramphenicol increased up to

50%, even though this antimicrobial has not been used in pig production in Canada since the 1980s and florfenicol was not used in this batch. This is in agreement with Harada et al (2006), who showed that co-selection with other approved antimicrobials in pig production is a possibility for persistence of chloramphenicol resistant *E. coli* (Harada, Asai et al. 2006). Diarra et al. (2007) showed that, in the absence of antimicrobial selection pressure, certain resistance genes can be conserved due to a link with the genes encoding resistance to other antimicrobials that are registered for use in animal production (Diarra, Silversides et al. 2007). In our study, almost all ESC-resistant *E.coli* strains were co-resistant to streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, amoxicillin/clavulanic acid, ampicillin, chloramphenicol, and tetracycline. The association on the same plasmid of the $bla_{\text{CMY-2}}$ gene and genes conferring resistance to other antimicrobials, such as aminoglycosides, sulphonamides, phenicols and tetracyclines has been described by Doublet (Doublet, Carattoli et al. 2004), and co-transmission of resistance to non-β-lactam antimicrobials is often associated with CMY-2 (Blanc, Cortes et al. 2008).

The present investigation is, to our knowledge, the first to provide insights regarding the effect of a clay mineral, clinoptilolite, as a feed ingredient, on the level of fecal excretion of ESC-resistant *E. coli in vivo* in pigs in farm conditions. We have demonstrated that feeding of clinoptilolite was associated with an early temporary increase in the proportion of fecally excreted *E. coli* carrying the gene responsible for ESC-resistance, $bla_{\text{CMY-2}}$. On the other hand, a delayed increase in the proportion of fecally excreted *E. coli* carrying $bla_{\text{CMY-2}}$, or one of the virulence genes *iucD* and *tsh*, was observed in pigs which did not receive clinoptilolite in the feed, the proportion of these isolates being significantly higher than in the group having received clinoptilolite. The initial temporary increase in frequency of isolates carrying $bla_{\text{CMY-2}}$ in the supplemented group is compatible with data from Rodriguez-Beltran et al. (2013) who reported that clay minerals (e.g. sepiolite) promote direct horizontal gene transfer (HGT) between bacterial species in *in vitro* conditions, which may be exacerbated by the use of antimicrobials as growth promoters. In addition, these authors showed that the promotion of HGT was due to plasmid transfer in certain conditions such as presence of septiolite (final concentration of 100 µg/ml) and increased friction forces, the latter of

which may have been provided by peristalsis in the intestine of the pigs receiving continuously clay mineral as a food additive (Rodriguez-Beltran, Rodriguez-Rojas et al. 2013). On the other hand, the subsequent significant decrease in the frequency of isolates carrying *bla*$_{\text{CMY-2,}}$ gene, and the lack of increase in the frequency of isolates carrying the plasmid-encoded virulence genes *iucD* and *tsh* in the supplemented group could be related to accumulation of clinoptilolite in the gastrointestinal tract of pigs over time after weaning which may lead to a decline in the HGT. For instance, previous *in vitro* studies demonstrated that higher concentrations of clay minerals result in a decrease in the number of transformed cells, in other words, a reduction in HGT (Richaume, Angle et al. 1989, Lotareva and Prozorov 2000). Alternatively, the later decrease in frequency of isolates carrying *bla*$_{\text{CMY-2}}$ observed in the supplemented group could be due to a direct or indirect antimicrobial effect of clinoptilolite or an interaction between clinoptilolite and chlortetracycline or penicillin G. The ability of clay minerals to reduce the number of pathogenic bacteria has been reported *in vivo* and *in vitro* (Haydel, Remenih et al. 2008, Vondruskova, Slamova et al. 2010). Likewise, Wu et al. (2013) found that the total number of *Lactobacillus acidophilu*s bacteria was significantly increased from days 22 to 42 in broiler chickens fed with clinoptilolite and also they showed that small intestinal and cecal PH values in the treatment group were significantly lower than those in the control group (Wu, Wang et al. 2013). Since probiotics containing *L. acidophilus* may inhibit growth of pathogenic bacteria by production of organic acids and antibiotic-like compounds (Lordelo, Marinho et al. 2007, Vondruskova, Slamova et al. 2010), an increase in *L. acidophilus* numbers due to the presence of clinoptilolite in the current study may have led to the later decrease in the proportion of *bla*$_{\text{CMY-2}}$, *iucD,* and *tsh*-positive isolates. Although the potential interaction of clay minerals and antimicrobials is controversial, nevertheless, a potential interaction between clinoptilolite and some antimicrobials has been demonstrated. For instance, in *in vitro* conditions, chlortetracycline, tylosin, and metronidazole can be absorbed strongly to clinoptilolite or other clay minerals. The interference is probably related to polarity of the antimicrobial molecules, since no interaction was confirmed with sulfamethoxazole and β-lactams, which are highly polar compounds (Papaioannou, Katsoulos et al. 2005, Allaire, Del Castillo et al. 2006).

The finding that the phylotype of *bla*$_{CMY-2}$-positive strains differed in the supplemented and control groups led us to speculate that the clinoptilolite supplementation may result in predominance of certain clones, that is, those of phylotype A. On the other hand, we observed a positive association between *bla*$_{CMY-2}$ and *iucD, bla*$_{CMY-2}$ and *tsh*, and *iucD* and *tsh*. The association between the *iucD* and *tsh* genes has been described previously, both genes being present on the pAPEC-1 plasmid of *E. coli* O78:K80:H9 and on pAPEC-02-ColV plasmid (Mellata, Touchman et al. 2009). Similarly, *iucD* and *bla*$_{CMY-2}$were found on the same plasmid, pCVM29188_10, isolated from *S. Kentucky* and transferable to *S. Kentucky, S. enteric* serovar Newport, and *E. coli* (Fricke, McDermott et al. 2009). Taken together, our results indicate that clinoptilolite may also have a selection effect on specific plasmids.

In conclusion, our results reveal an increased level of resistance, lasting at least 4 weeks, to several antimicrobials in *E. coli* from pigs, both within and between classes with respect to antimicrobials used for medicated feeding of these pigs following weaning. Our results also show that feed supplementation with clinoptilolite decreases the proportion of fecally shed *E. coli* possessing certain virulence and antimicrobial resistance genes, at least in the first month after weaning. Since in farm conditions, various factors may contribute, further studies involving different farms and a longer time after weaning will be required to confirm our findings over the long term. Future experiments should also more thoroughly investigate the relative role of bacterial clones and plasmids in the circulation of *bla*$_{CMY-2}$ gene in pigs and their environment over time after weaning, and the effect of feed supplementation with clinoptilolite on this circulation.

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Tables and figures

Table 1. Antimicrobial resistance patterns for 80 *E. coli* strains based on the number of antimicrobials to which each strain was resistant.

No. of antimicrobials	AMR pattern[a]	No. of strains with AMR pattern (%)
0	Susceptible	3 (3.75)
1	TET	3 (3.75)
	NAL	1(1.25)
	STR	1 (1.25)
2	STR, TET	1 (1.25)
3	SXT, FIS, TET	1 (1.25)
	AMP, CHL, TET	1 (1.25)
	AMP, AMC, TET	1 (1.25)
4	SXT, FIS, CHL, TET	1 (1.25)
	SXT, FIS, AMP, AMC	1 (1.25)
	SXT, FIS, AMP, TET	2 (2.5)
	AMP, AMC, CHL, TET	2 (2.5)
	STR, SXT, FIS, TET	1 (1.25)
5	KAN, STR, SXT, FIS, TET	1 (1.25)
	STR, SXT, FIS,AMP, TET	3 (3.75)
	KAN , FIS,AMP, AMC, TET	1 (1.25)
	STR, AMP, AMC, CHL, TET	2 (2.5)
	STR, FIS, AMP, AMC, TET	1 (1.25)
6	STR, SXT, FIS, AMP, AMC, TET	7 (8.75)
	STR, SXT, FIS,AMP, CHL, TET	3 (3.75)
	GEN, STR, SXT, FIS,AMP, TET	2 (2.5)
	CRO,TIO, FOX,AMP, AMC, TET[b]	1 (1.25)
7	KAN, STR, SXT, FIS,AMP, AMC, TET	2 (2.5)
	GEN, KAN, STR, SXT, FIS; AMP,TET	3 (3.75)
	STR, SXT, FIS, AMP, AMC, CHL, TET	9 (11.25)
	KAN, STR, SXT, FIS,AMP, CHL, TET	1 (1.25)
	GEN, STR, SXT, FIS, AMP, AMC, TET	1 (1.25)
8	GEN, KAN, STR, SXT, FIS, AMP, AMC, TET	7 (8.75)
9	KAN, STR,CIP, NAL, SXT,FIS,AMP, CHL, TET	1 (1.25)
	GEN, KAN, STR,NAL, SXT,FIS,AMP, AMC, TET	1 (1.25)
	STR, CRO, TIO, FOX, SXT, FIS, AMP, AMC, TET [b]	1 (1.25)
10	STR, CRO, TIO, FOX, SXT, FIS, AMP, AMC, CHL, TET [b]	12 (15)
11	STR, CRO,TIO, FOX,NAL, SXT, FIS,AMP, AMC, CHL, TET[b]	1 (1.25)
	GEN, KAN, STR, CRO,TIO, FOX, SXT, FIS,AMP, AMC, TET[b]	1 (1.25)
Total	33	80

[a]Abbreviations for antimicrobials are as follows: amikacin (AMK), kanamycin (KAN), gentamicin (GEN), streptomycin (STR), ceftriaxone (CRO), ceftiofur (TIO), cefoxitin (FOX), ciprofloxacin (CIP), nalidixicacid (NAL), trimethoprim-sulfamethoxazole (SXT), sulfisoxazole (FIS), ampicillin (AMP), amoxicillin/clavulanicacid (AMC),

chloramphenicol (CHL), tetracycline (TET). [b] Five AMR patterns in ESC-resistant *E. coli* strains.

Table 2. Recovery and distribution of *E. coli* positive strains for $bla_{\text{CMY-2}}$ and ExPEC virulence genes in fecal samples from weaned pigs as detected by PCR.

Day of sampling[a]	Group[b]	No. of tested strains[c]	No. of positive strains for AMR and virulence genes (%)				
			***bla*$_{\text{CMY-2}}$[d]**	***iucD*[e]**	***tsh*[e]**	***papC*[e]**	***cnf*[e]**
Day 0	C	10	0	1(10)	1(10)	1(10)	1(10)
	S	10	0	1(10)	0	0	1(10)
Day 7	C	10	0	3(30)	0	1(10)	0
	S	10	4(40)*	1(10)	0	0	1(10)
Day 14	C	10	0	0	0	0	0
	S	10	3(30)*	0	0	0	0
Day 28	C	10	5(50)	3(30)	3(30)	1(10)	1(10)
	S	10	4(40)	1(10)	1(10)	0	0
Total		80	16(20)	10(12.5)	5(6.25)	3(3.75)	4(5)

[a] Sampling days after starting supplement with clinoptilolite

[b] Animals receiving feed supplemented (S) or not (C) with clinoptilolite

[c] Number of strains tested by PCR

[d] Antimicrobial resistance gene (conferring resistance to β-lactam antibiotics)

[e] ExPEC, extraintestinal pathogenic *E. coli*, opportunistic agent that may cause extraintestinal infections.

*Value is significantly different to that of the supplemented or control group on the same day using the Fisher's exact test ($P<0.05$).

Table 3. Prevalence of *iucD*, *tsh* and *bla*$_{CMY-2}$ genes in fecal isolates from weaned pigs receiving or not clinoptilolite in the feed, as detected by the HGMF method

Day of sampling[a]	Group[b]	No. of tested isolates [c]	No. of isolates positive for antimicrobial and virulence gene combinations (%)						
			***bla*$_{CMY-2}$**[d]	***iucD*[e]**	***tsh*[e]**	***bla*$_{CMY-2}$*:iucD***	***bla*$_{CMY-2}$*:tsh***	***bla*$_{CMY-2}$*:iucD:tsh***	***iucD:tsh***
Day0	C	477	0	4(0.8)	2(0.4)	0	0	0	0
	S	403	0	14(3.5)	6(1.5)	0	0	0	1(0.2)
Day2	C	817	24(2.9)	0	4(0.5)	0	1(0.1)	0	0
	S	819	14(1.7)	4(0.5)	11(1.3)	0	0	0	0
Day7	C	774	18(2.3)	26(3.4)	3(0.4)	2(0.3)	0	0	0
	S	790	122(15.4)*	16(2)	10(1.3)	1(0.1)	1(0.1)	0	0
Day14	C	737	50(6.8)*	47(6.4)	31(4.2)	8(1.1)	11(1.5)	3(0.4)	15(2)
	S	787	33(4.2)	35(4.4)	29(3.7)	5(0.6)	0	2(0.3)	0
Day28	C	627	38(6.1)*	36(5.7)*	19(3)*	12(1.9)	7(1.1)	3(0.5)	0
	S	1001	33(3.3)	16(1.6)	2(0.2)	8(0.8)	1(0.09)	0	0
Total		7232	343	198	117	36	21	8	16

[a] Sampling days after starting supplement with clinoptilolite

[b] Animals receiving feed supplemented (S) or not (C) with clinoptilolite

[c] Number of tested isolates by HGMF DNA probe hybridization.

[d] Antimicrobial resistance gene (conferring resistance to β-lactam antibiotics)

[e] ExPEC, Extraintestinal pathogenic *E. coli*, opportunistic agent that may cause extraintestinal infections

* Value is significantly different to that of the supplemented or control group on the same day using the Chi-Square test ($P<0.05$).

Supplementary Table 1. List of primers used in the PCR and HGMF DNA probe hybridization, PCR conditions, and control strains.

Product or antimicrobial	Gene	Primer[a]	Amplicon size (bp)	Annealing temperature	Control strain	Reference
β- glucuronidase	*uidA*	for5'GCGTCTGTTGACTGGCAGGTGGTGG rev5'GTTGCCCGCTTCGAAACCAATGCCT	530	65°C	ECL3482	(Walk et al., 2009)
Tetracycline	*tetA*	for 5'GTGAAACCCAACATACCCC rev5'GAAGGCAAGCAGGATGTAG	888	55°C	ECL3482	(Maynard et al., 2003)
Ampicillin	bla_{TEM}	for 5'GAGTATTCAACATTTTCGT rev 5'ACCAATGCTTAATCAGTGA	857	50°C	ECL3482	(Maynard et al., 2003)
Ceftiofur	bla_{CMY-2}	for 5'GACAGCCTCTTTCTCCACA rev 5'TGGAACGAAGGCTACGTA	1000	50°C	ECL3482	(Zhao et al., 2001)
Streptomycin Spectinomycin	*aadA1*	for 5'CATCATGAGGGAAGCGGTG rev5'GACTACCTTGGTGATCTCG	786	50°C	ECL3482	(Gow et al., 2008)

[a] The other primers used in the PCR and HGMF technique are available at http://apzec.ca/en/Protocols

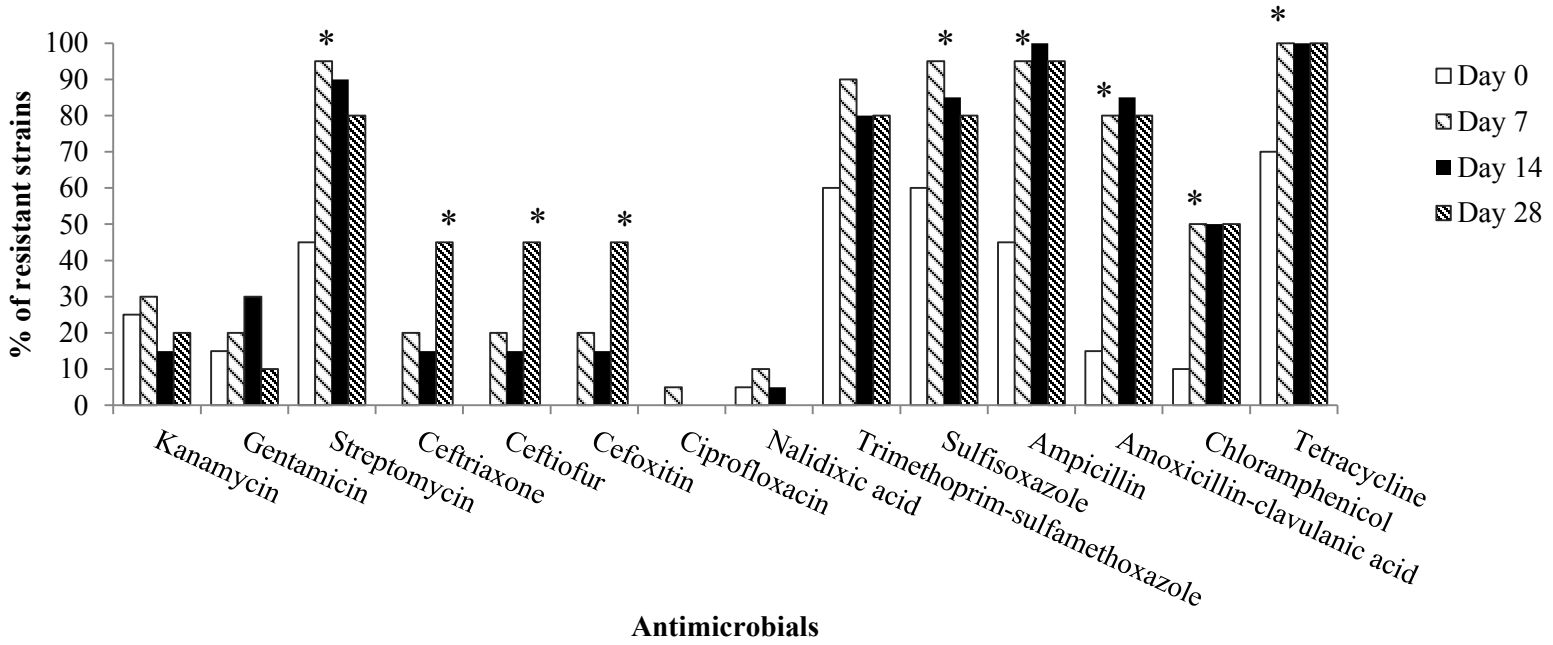


Figure 1. Effect of time on resistance profiles of 80 *E. coli* strains from weaned pigs. The percentage of resistance to most antimicrobials increased over time.

Asterisks indicate antimicrobials for which there is a significant association between days and frequency of resistance using the Chi-Square test ($P<0.05$).

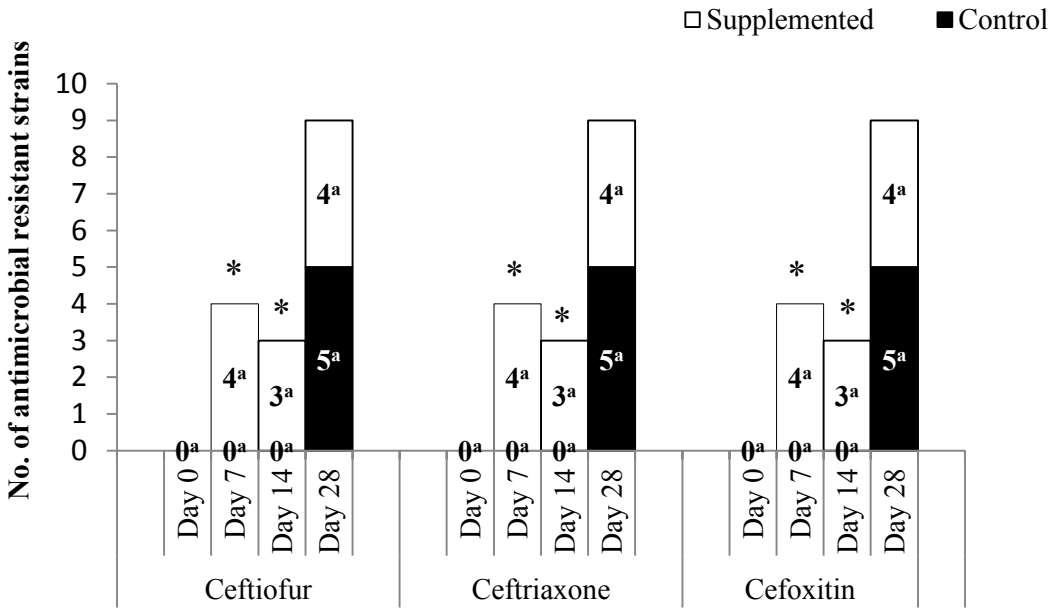


Figure 2. Effect of dietary supplementation with clinoptilolite on the frequency of ceftiofur, ceftriaxone and cefoxitin in 80 *E. coli* strains isolated from weaned pigs over time.

Asterisks indicate antimicrobials for which there is a significant association between group and frequency of resistance on each day of sampling using the Fisher's exact test ($P<0.05$).

[a] Number of ceftiofur, ceftriaxone and cefoxitin co-resistant strains

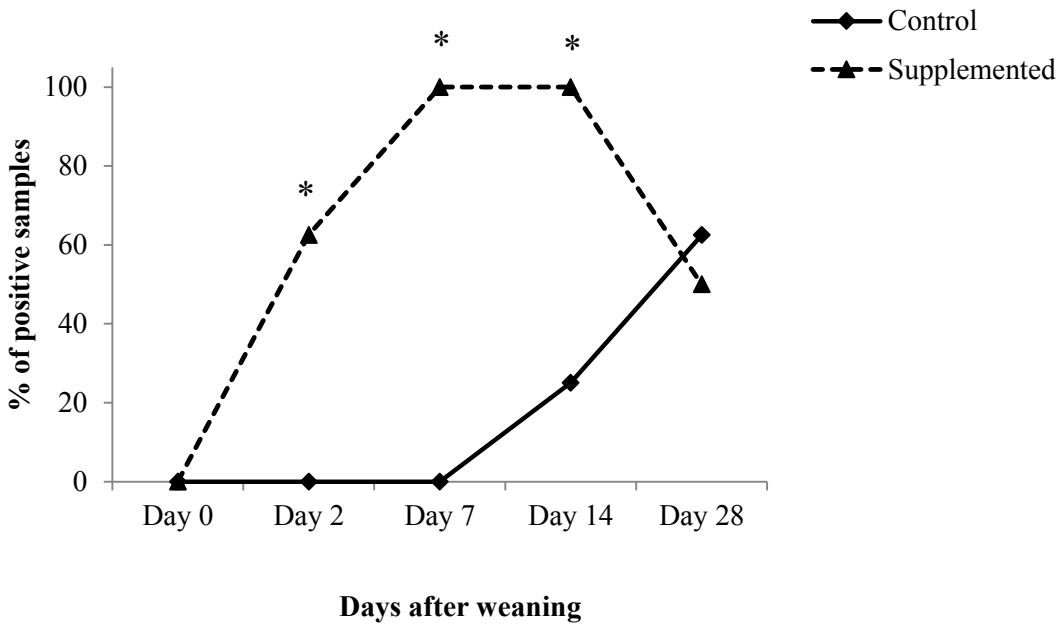


Figure 3. Trends in the presence of *bla*$_{\text{CMY-2}}$ gene in 80 fecal samples from weaned pigs over time, as detected by PCR.

Asterisks indicate days on which the frequency of the *bla*$_{\text{CMY-2}}$ gene was significantly higher in the supplemented group than in the control group using the Chi-Square test ($P<0.05$).

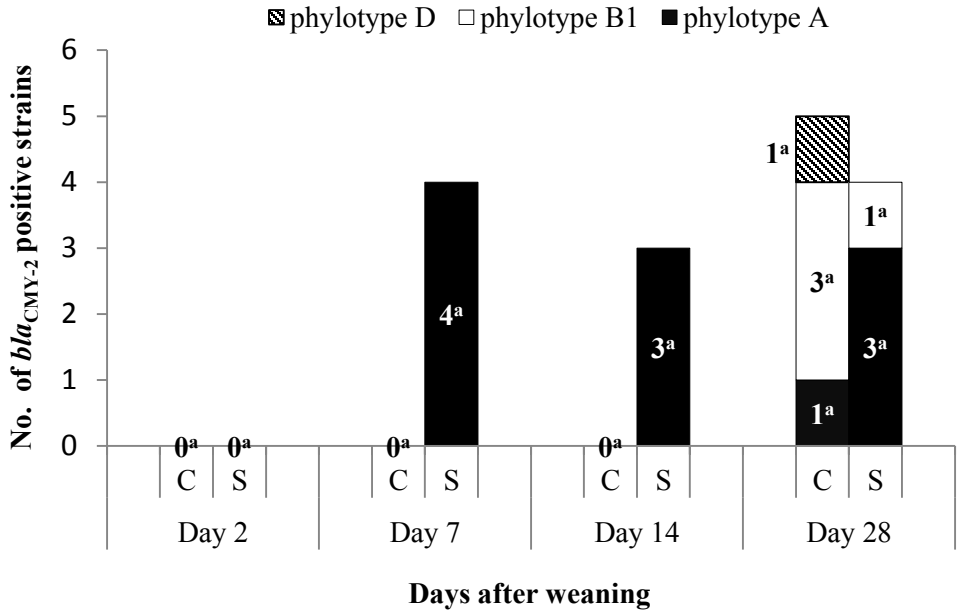
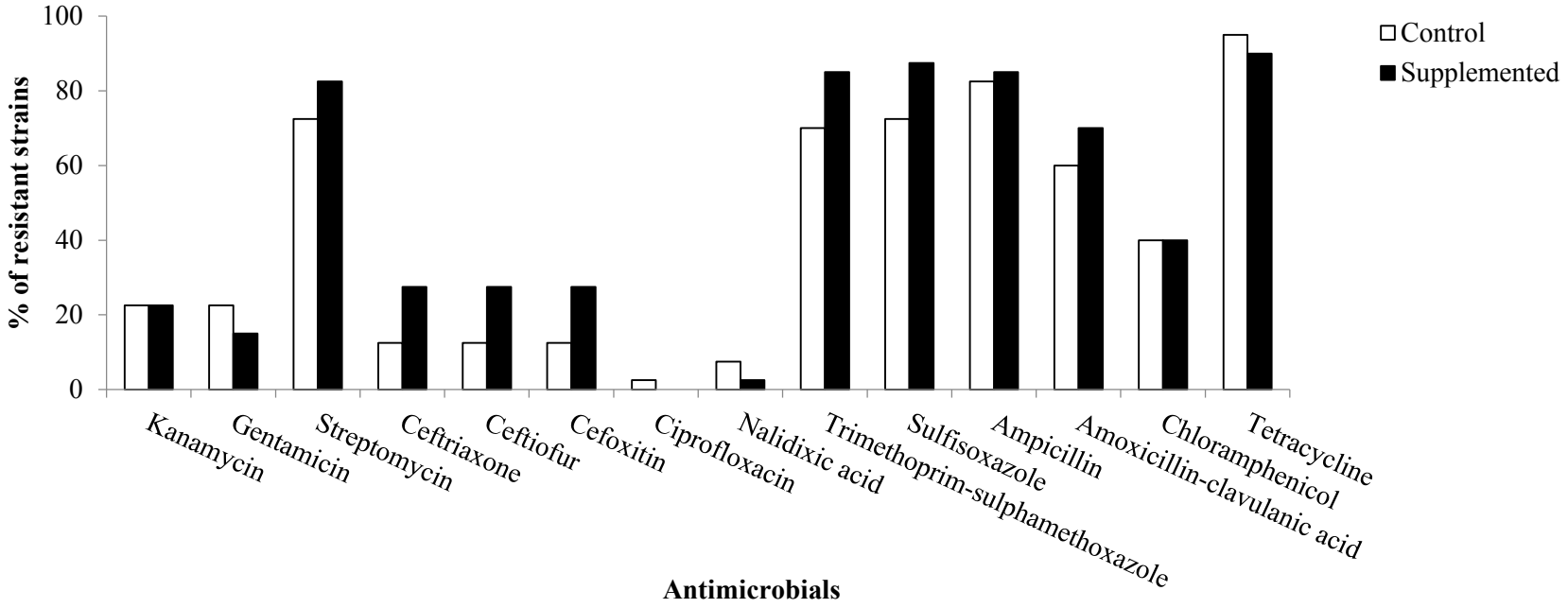


Figure 4. Association between presence of bla_{CMY-2} and phylogenetic grouping in 80 *E. coli* strains isolated from weaned pigs overtime.

Animals receiving (S) or not (C) clinoptilolite

[a] Number of bla_{CMY-2}-positive strains



Supplementary Figure. 1. Effect of dietary supplementation of clinoptilolite on the frequency of AMR in 80 *E. coli* strains from weaned pigs.

No significant difference was observed between the control and supplemented groups for any of the antimicrobials. Resistance to ceftriaxone, ceftiofur and cefoxitin was two times greater in the supplemented group than in the control group; however the difference was not statistically significant (Chi-square test, P=0.09).

Details on the role of the candidate in the conception of the article: I am the first author of the article. I actively participated in the study design and; I substantially carried out research, analyzed data and wrote the paper.

Article 2. Circulating of CMY-2 β-Lactamase gene in weaned pigs and their environment in a commercial farm and the effect of feed supplementation with a clay mineral

Seyedehameneh Jahanbakhsh[a], Ann Letellier[b], John Morris Fairbrother[a]

OIE Reference Laboratory for *Escherichia coli* (EcL), Centre de Recherche en Infectiologie Porcine et Aviaire (CRIPA), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada[a].

NSERC Industrial Research Chair in Meat Safety, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, QC, Canada[b].

Address for correspondence: John M. Fairbrother, Département de pathologie et microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, 3200 rue Sicotte, Saint-Hyacinthe QC, Canada, J2S 2M2.

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Abstract

Aims: To investigate the mechanisms leading to an increase in the prevalence of *bla*$_{CMY-2}$ conferring resistance to ceftiofur in pigs receiving a feed medicated with chlortetracycline and penicillin and to examine the effect of supplementation with a clay mineral on this phenomenon.

Methods and Results: In 138 *bla*$_{CMY-2}$-positive *E. coli* isolates from feces of pigs receiving feed supplemented or not with 2% clinoptilolite, from day 2 to day 28 after weaning, isolates from the two groups differed significantly with respect to their phylogenetic group: phylotype A predominated in the supplemented group whereas phylotypes B1 and D predominated in the control group, as determined by PCR. In 36 representative isolates, pulsed-field gel electrophoresis and antimicrobial susceptibility testing revealed that the *bla*$_{CMY-2}$-positive *E.coli* isolates were polyclonal with diverse antimicrobial resistance patterns and *bla*$_{CMY-2}$-carrying plasmids of incompatibility (Inc) groups, I1, A/C and ColE were observed in transformants as detected by PCR. *Enterobacter cloacae* possessing *bla*$_{CMY-2}$-carrying IncA/C plasmids were found in the pens before introduction of this batch of pigs. The *bla*$_{CMY-2}$ -positive *E. coli* isolates were more clonally diverse in the control group than the supplemented group.

Conclusions: The *bla*$_{CMY-2}$ gene appears to have spread both horizontally and clonally in this batch of pigs and may have spread from previous batches of pigs via plasmids carried by *E. cloacae* and expanded in animals of the present batch in the presence of the selection pressure due to administration of chlortetracycline and penicillin in the feed. Feed supplementation may have an effect on clonal diversity of *bla*$_{CMY-2}$ -positive isolates.

Significance and Impact of Study: Implementation of improved hygiene measures, decreased administration of certain antimicrobials on farm, and feed supplementation with certain ingredients may limit antimicrobial resistance spread between and within batches of animals.

Keywords: Pigs, *E.coli*, *bla*$_{CMY-2}$-positive plasmid, antimicrobial resistance, clay mineral (clinoptilolite)

Introduction

The emergence of *Escherichia coli* resistant to extended-spectrum cephalosporins (ESCs) is of great concern because third generation cephalosporins such as ceftriaxone and ceftiofur are clinically important β-lactam antimicrobials in human and animal medicine. Plasmid-mediated AmpC beta-lactamases, including CMY-2, confer resistance to cephalosporins, the latter being encoded by the $bla_{\text{CMY-2}}$ gene (Dunne et al. 2000; Martin et al. 2012). Antimicrobial use during animal production can result in the emergence of antimicrobial resistance (AMR) in bacteria which are shed into the environment where they may persist for long periods (Chantziaras et al. 2014). CMY-2-encoding genes are located commonly on transferable elements (integrons, transposons, insertion sequences) carried by plasmids that facilitate the horizontal spread of resistance and most AmpC beta-lactamase producing strains may carry resistance genes for other antimicrobials. Hence, certain resistance genes can be conserved due to a link with the genes encoding resistance to antimicrobials that are registered for use in animal production (Dunne et al. 2000; Allen and Poppe 2002; Funk et al. 2006; Singer and Hofacre 2006). Although the *E.coli* phylotypes are often different between isolates from humans and animals (Johnson et al. 2008), common replicon types of plasmids encoding beta-lactamase genes were observed in *E. coli* isolates from humans and food producing animals (Carattoli 2009). Moreover, IncA/C plasmids with fingerprint similarities of greater than 90% have been found to circulate in *E. coli* from human and animal sources (Mulvey et al. 2009). Furthermore, transmission of CMY-2 AmpC beta-lactamase plasmids between *E. coli* and *Salmonella* among humans and animals has been reported (Winokur et al. 2001). Plasmids bearing both virulence and resistance genes may also be spread in a pathogenic bacteria population and the virulence traits will be selected due to antimicrobial selective pressure (Martinez and Baquero 2002). Further research is needed to understand mechanisms contributing to the circulation and persistence of antimicrobial resistance determinants among both commensal and pathogenic enteric bacteria on-farm and worldwide.

On one commercial farm, we observed the appearance of a high level of resistance to ESCs (e.g. ceftiofur, ceftriaxone) in *E. coli* in a batch of weaned pigs which were fed a diet containing chlortetracycline and penicillin G in therapeutic doses for protection of

respiratory problems and to prevent *Streptococcus suis* infections but which had not been treated with other antimicrobials, including ceftiofur. Supplementation of the feed of pigs in this batch with the clay mineral clinoptilolite decreased the proportion of *E. coli* possessing *bla*$_{CMY-2}$, the gene responsible for ESC-resistance, excreted in the feces, at least in the first month after weaning (Jahanbakhsh et al. 2015). We hypothesized that both bacterial clones and plasmids were involved in the spread of *bla*$_{CMY-2}$ gene in pigs and feed supplementation with clinoptilolite may have an effect on certain of these *E. coli* clones and plasmids. Thus, the main objective of the present study was to characterize the *bla*$_{CMY-2}$ isolates from this batch of pigs in order to elucidate the mechanisms leading to an increase in the prevalence of *bla*$_{CMY-2}$ conferring resistance to ceftiofur in pigs which had not been treated with this antimicrobial and to investigate the effect of feed supplementation with a clay mineral on this phenomenon.

Materials and methods

E. coli isolation and identification

In our previous on farm experiment, pigs from one batch in the nursery had been randomly divided into 2 groups following weaning: control (C) and supplemented (S). The control group received a standard commercial feed containing chlortetracycline and penicillin G and the supplemented group had received the same feed but also containing 2% clinoptilolite. None of the pigs had been treated with other antimicrobials, including ceftiofur, during the experiment (Jahanbakhsh et al. 2015). Four hundred and eight *bla*$_{CMY-2}$-positive isolates, either alone (n=343) or in combination with *iucD* and *tsh* ExPEC-associated virulence genes (n=65) had been detected in 7232 *E. coli* isolates examined by hydrophobic grid membrane filter (HGMF) method. For the present study, 138 of the 408 *bla*$_{CMY-2}$-positive isolates from the previous study were selected to represent sampling days and groups. Hence, 138 *bla*$_{CMY-2}$-positive *E. coli* isolates (2 or 3 isolates per sample) were selected from the control (n=66) and supplemented (n=72) groups at days 2, 7, 14, and 28 after weaning. No *bla*$_{CMY-2}$-positive *E. coli* isolate had been detected at day 0. In addition,

101 *bla*$_{CMY-2}$-negative *E. coli* isolates were recovered from the control (n=53) and supplemented (n=48) groups representing all sampling days (0, 2, 7, 14 and 28). Isolates were identified as *E. coli* by biochemical tests (Simmons Citrate Agar, mobility and indole) and by PCR for the presence of *uidA E.coli* housekeeping gene, which encodes beta-glucuronidase (Walk et al. 2009). However, *E.coli* phylotype D isolates were negative for *uidA* gene, and were thus confirmed by API 20E kit system (BioMerieux, Canada). In addition, *uidA*-negative isolates were further identified as *E. coli* by sequencing of the housekeeping gene *adk*, from the *E. coli* MLST scheme as described by Maheux et al. (Maheux et al. 2014).

PCR for determination of *bla*$_{CMY-2}$ and ExPEC-associated virulence genes

A loop from the confluent growth or individual colonies on MacConkey agar plates were inoculated into 5 mL Luria Bertani (LB – Difco, USA) broth and incubated overnight at 37°C. DNA templates were prepared from the samples by boiled cell lysis for examination by PCR, as described previously by Maluta et al. (Maluta et al. 2014).

The presence of the *bla*$_{CMY-2}$ gene was confirmed in boiled cell lysates from the 138 representative *bla*$_{CMY-2}$-positive *E. coli* isolates by PCR as described by Zhao et al. (Zhao et al. 2001). Likewise, all these isolates were tested by multiplex PCR to determine the presence of ExPEC-associated virulence genes (*cnf, papC, iucD, and tsh*). PCR procedures for detection of these genes were performed according to the protocol of the Reference Laboratory for *Escherichia coli* (EcL- Faculté de Médecine Vétérinaire de L'Université de Montréal) available in the animal pathogenic zoonotic *Escherichia coli* website (http://apzec.ca/en/Protocols)

Phylogenetic grouping

Phylogenetic grouping was performed for the *bla*$_{CMY-2}$-positive *E. coli* isolates (n=138) using a multiplex PCR-based assay as described by Clermont et al. (Clermont et al. 2000). Based on the presence or absence of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment (TSPE4.C2), isolates were classified into four main *E.coli* phylogenetic groups (A, B1, B2, or D).

Antimicrobial susceptibility testing

A subset of 25% (n=36) of 138 *bla*$_{CMY-2}$-positive isolates representing criteria described above along with consideration of phylotypes and ExPEC-associated virulence genes were selected, and tested for antimicrobial susceptibility. These isolates, from control (n=17) and supplemented (n=19) groups from each sampling day except day 0, were examined for susceptibility to the same 15 antimicrobial agents examined in the CIPARS surveillance program in Canada (CIPARS 2008) using the disk-diffusion (Kirby-Bauer) assay as described previously (Jahanbakhsh et al. 2015). The isolates were recorded as susceptible, intermediate, or resistant according to the zone diameter interpretative standards recommended by Clinical and Laboratory Standards Institute (CLSI) in 2010 (CLSI 2010) for most of the antimicrobials and in 2008 for ceftiofur (CLSI 2008). The following antimicrobial disks were used; amikacin (AMK, 30µg), kanamycin (KAN, 30µg), gentamicin (GEN, 10µg), streptomycin (STR, 10µg), ceftriaxone (CRO, 30µg), ceftiofur (TIO, 30µg), cefoxitin (FOX, 30µg), ciprofloxacin (CIP, 5µg), nalidixic acid (NAL, 30µg), trimethoprim-sulfamethoxazole (SXT, 23.75µg), sulfisoxazole (FIS, 250µg), ampicillin (AMP, 10µg), amoxicillin/clavulanic acid (AMC, 30µg), chloramphenicol (CHL, 30µg), and tetracycline (TET, 30µg).

O Serotyping

The O serotyping was determined for *bla*$_{CMY-2}$-positive *E. coli* isolates (n=36) by standard agglutination methods (Edwards and Ewing 1972) for 86 O serogroups associated with swine disease. O antisera were produced at the EcL (Faculté de médecine vétérinaire, Université de Montré al, Saint-Hyacinthe, Quebec, Canada) according to standard methods (Edwards and Ewing 1972).

PFGE analysis

The *bla*$_{CMY-2}$-positive *E. coli* isolates (n=36) were analyzed by PFGE using XbaI restriction enzyme as described by PulseNet (Ribot et al. 2006). PFGE Profiles were analyzed using the BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were generated by the unweighted pair-group method using arithmetic averages, based on the Dice similarity coefficient (optimization 1%; tolerance 1.2%). The

definition of isolates and clones was based on that previously described by Bednorz et al. Accordingly each *E. coli* colony as recovered after initial screening on MacConkey agar plates was considered to be an individual isolate. A clone was defined as an *E. coli* group of isolates with a similar PFGE pattern differing by a maximum of one band. Hence, two clones differed by more than one band (Bednorz et al. 2013).

Plasmid characterization

Plasmids were extracted from wild type *bla*$_{CMY-2}$-positive *E. coli* isolates (n=36) using the Qiagen Plasmid Mini in accordance with manufacturer's instructions (Qiagen, Inc., Mississauga, ON). Transformations were performed by electroporation in MicroPulser with *E. coli* ElectroMAX DH10B™ as the recipient in accordance with manufacturer's instructions (Invitrogen, Carlsbad, CA). As previously described by Martin et al., (2012) transformants were selected on Mueller Hinton agar (BD, Franklin Lakes, NJ) supplemented with 8 µg/ml ceftiofur (Pfizer Animal health, Canada) (Martin et al. 2012). We analyzed up to five transformants per transformation experiment, whenever available. Transformants were examined by PCR to confirm the presence of *bla*$_{CMY-2}$ gene and by antimicrobial susceptibility testing (described above). Plasmid incompatibility grouping was carried out using PCR replicon typing as previously described for 21 Incompatibility (Inc) groups (Carattoli et al. 2005; Garcia-Fernandez et al. 2009). Detection of class 1 and 2-integrons in plasmids was performed by PCR as previously described (Ruiz del Castillo et al. 2013).

Conjugation mating experiments

In order to ascertain whether *bla*$_{CMY-2}$-positive isolates were present in the pen environment, pooled environmental swabs were taken from the walls and floors of pens before the introduction of the weaned pigs. Inoculation of environmental swabs on ceftiofur-supplemented MacConkey agar yielded only *Enterobacter cloacae* (*E. cloacae*) isolates (n=15). *E. cloacae* isolates were identified by API 20E kit system (BioMerieux, Canada). As purification of the *bla*$_{CMY-2}$-positive plasmid from *E. cloacae* isolates was not possible by transformation (electroporation) using electrocompetent *E coli* DH10B, conjugation was done using competent *E. coli* DH5α (Invitrogen).

E. cloacae was mated to competent *E. coli* DH5α by the broth-mating method (Usui et al. 2012). Briefly, overnight cultures of donor (20 µl) and recipient (20 µl) bacteria were mixed with 160 µl of fresh LB broth and incubated at 37ºC overnight. Transconjugants were selected on Mueller Hinton agar supplemented with 50 µg/ml nalidixic acid (Sigma-Aldrich, St. Louis, MO) and 8 µg/ml ceftiofur (Pfizer Animal health, Canada).

Statistical analysis

Statistical analysis was carried out using JMPv.10 (SAS Institute, Cary, NC, USA). The Chi-square test (95% confidence intervals) was used to compare the effect of feed ingredient and time on the prevalence of phylotypes and AMR (phenotypic). Fisher's exact test was also used to examine the differences among groups constrained across days. Odds ratios (OR) (95% confidence intervals) were used to evaluate the associations between phylotypes and ExPEC- associated virulence genes. Statistical significance for each comparison was deemed at P value of <0.05.

Results

Phylogenetic grouping and association of ExPEC-associated virulence genes and phylotypes

The *bla*$_{\text{CMY-2}}$-positive *E. coli* isolates belonged to phylotypes A, B1, and D (Table 1, Fig. 1) whereas the *bla*$_{\text{CMY-2}}$-negative *E. coli* isolates belonged only to phylotypes A and B1 (Fig. 1). In the supplemented group, all *bla*$_{\text{CMY-2}}$-positive *E. coli* isolates belonged to phylotype A and D at days 2, 7, 14 and phylotype B1 was only found at day 28 (Fig. 2). On the other hand, in the control group, *bla*$_{\text{CMY-2}}$-positive *E. coli* isolates belonged to phylotypes A, B1 and D at all sampling days. Interestingly, in the *bla*$_{\text{CMY-2}}$-positive *E. coli*, the frequency of phylotype A was significantly higher in the supplemented group than in the control group at all sampling days, whereas phylotypes B1 and D were significantly more frequently found in the control group than in the supplemented group (Fig. 2). On the

other hand, among the *bla*$_{CMY-2}$-negative *E. coli* isolates, the frequency of phylotypes did not differ in the control and supplemented groups (Fig. 1).

The *iucD* gene was the most commonly detected ExPEC-associated virulence gene and was significantly more frequent in the control group than in the supplemented group (data not shown). Likewise, some association between prevalence of virulence genes and phylotype in *bla*$_{CMY-2}$-positive *E. coli* isolates was observed, almost all isolates positive for ExPEC-associated virulence genes belonging to phylotype B1 (Table 1). A positive association was found between phylotype B1 and ExPEC-associated virulence genes in *bla*$_{CMY-2}$-positive *E. coli* isolates, whereas the presence of ExPEC-associated virulence genes showed negative association with phylotypes A and D (Table 1). The most common virulence gene combinations in B1 isolates were *iucD* alone (55%) and *tsh* alone (22%).

PFGE and O serogroup of *bla*$_{CMY-2}$-positive *E.coli*

Relatedness of each DNA fingerprint was clustered using BioNumerics software and isolates were divided into 7 clusters (I to VII) at ≥90% similarity, two of these clusters being singletons (II and VII). All phylotype A isolates belonged to serogroup O101 and to PFGE cluster I, consisting of 2 distinct clones. Phylotype D isolates were O83 and demonstrated a unique clone. Indistinguishable fingerprints were present in isolates from different sampling days indicating the persistence of clones during the course of study. On the other hand, Phylotype B1 isolates were non-typable, except for one O64 isolate, and exhibited more heterogeneity than those of phylotypes A and D, being grouped into four distinct clusters (IV, V, VI and VII) with four distinct clones.

In general, phylotype A isolates tended to cluster in the supplemented group, whereas phylotype B1 isolates tended to cluster in the control group which indicates the *bla*$_{CMY-2}$-positive *E. coli* isolates belong to different clones in the control and supplemented groups and are more clonally related in the supplemented group (Fig. 3).

Antimicrobial susceptibility profiles of *bla*$_{CMY-2}$-positive *E. coli* isolates

Overall, all isolates were resistance to ceftiofur, ceftriaxone, cefoxitin, ampicillin, amoxicillin/clavulanic acid, tetracycline, and followed by sulfisoxazole (94.4%), trimethoprim-sulfamethoxazole (94.4%), streptomycin (91.6%), chloramphenicol (44.4%),

kanamycin (37.1%), gentamicin (37.1%), and nalidixic acid (2.8%). All isolates were susceptible to amikacin and ciprofloxacin. Furthermore, seven AMR patterns were distinguished among *bla*$_{CMY-2}$ -positive *E. coli* isolates, all being classified as multi-drug resistant (MDR) as described by Magiorakos et al., an isolate being MDR if it is non-susceptible to at least one antimicrobial agent in three or more defined antimicrobial categories (Magiorakos et al. 2012). The predominant AMR patterns in ESC-resistant *E.coli* isolates were STR, CRO, TIO, FOX, SXT, FIS, AMP, AMC, CHL, TET (Pattern 3; n=14) which was observed in phylotype A and B1 isolates and GEN, KAN, STR, CRO, TIO, FOX, SXT, FIS, AMP, AMC, TET (Pattern 1; n=12) found in phylotype D isolates, indicating that AMR patterns differed in relation to phylotypes (Fig. 3). The two predominant AMR patterns were found in both control and supplemented groups from day 2 to day 28 after weaning. No significant difference was observed between control and supplemented groups for any of the antimicrobials (supplementary Fig. 1).

Characterization of plasmids harboring *bla*$_{CMY-2}$

PCR-based replicon typing of the transformants revealed that IncI1 was the most prevalent replicon in *bla*$_{CMY-2}$-positive plasmids from transformants, being present in 86% (31/36) of isolates (Fig. 3), and followed by two IncA/C, two ColE and one non-typeable *bla*$_{CMY-2}$-positive plasmids. Multiple replicon types of transferable *bla*$_{CMY-2}$-positive plasmids were not observed in any transformants. Interestingly, IncA/C *bla*$_{CMY-2}$-carrying plasmids were only observed in phylotype B1 isolates in the control group at day 28 in cluster IV. Moreover, ColE *bla*$_{CMY-2}$-positive plasmid was observed only in phylotype A at days 2 and 7 in clusters II and III in both groups.

Antimicrobial susceptibility testing showed that transfer of resistance to antimicrobials other than beta-lactams was observed in the transformants with IncA/C plasmids, with co-resistance to streptomycin, sulfisoxazole, chloramphenicol and tetracycline. However, it should be mentioned that as DH10B is resistant to streptomycin, this drug could not be evaluated in the transformation experiments. All of the transformants with IncI1 and ColE plasmids were resistant to all of the beta-lactam antimicrobials but susceptible to all the non-beta-lactam antimicrobials tested (Fig. 3). Class 1 and 2-integrons were not detected in any *bla*$_{CMY-2}$-positive plasmids. In addition,

all transformed plasmids were negative for the presence of the ExPEC-associated virulence genes (data not shown). As IncA/C *bla*$_{CMY-2}$-positive plasmids were only found in transformants from isolates of the control group at day 28, all of the *bla*$_{CMY-2}$-positive *E. coli* wild type isolates were examined for the presence of IncA/C and IncI1 plasmids. All isolates were positive for the presence of IncI1 plasmid. On the other hand, IncA/C plasmids were detected only in those *bla*$_{CMY-2}$-positive *E. coli* wild type isolates resistant to chloramphenicol, both in the control and supplemented groups from day 2 to day 28. Frequency of the presence of IncA/C plasmids was two times greater in the supplemented group than in the control group, although the difference was not statistically significant (Chi-square test, P=0.08). Also, the frequency of IncA/C plasmid in the *bla*$_{CMY-2}$-positive *E. coli* wild type isolates increased, although not significantly, over the course of the study, even though IncA/C *bla*$_{CMY-2}$-positive plasmids were not detected in any transformants before day 28 (Fig. 3). In contrast, IncI1 and IncA/C plasmids were not found in any of 20 *bla*$_{CMY-2}$-negative *E. coli* wild type isolates examined (data not shown).

Spread of *bla*$_{CMY-2}$ between batches of weaned pigs

E. cloacae isolated from environmental swabs were *bla*$_{CMY-2}$-positive, resistant to ceftiofur and MDR, showing a unique AMR pattern similar to that observed in *bla*$_{CMY-2}$-positive *E. coli* isolates, the only difference being resistance to kanamycin in the *E. cloacae* isolates (wild type) (Table 2). Transconjugants carrying IncA/C plasmid from *E. cloacae* showed a unique AMR pattern similar to that observed in IncA/C plasmid from *E. coli* isolates. However, it should be noted that *E. coli* DH5α is resistant to nalidixic acid so this drug could not be evaluated in the conjugation experiments. Nevertheless, the two patterns differed in resistance to kanamycin and trimethoprim-sulfamethoxazole as they are underlined in AMR pattern from *E. cloacae* and class 1-integron was detected in IncA/C plasmids from *E. cloacae* (Table 2).

Spread of *bla*$_{CMY-2}$-positive *E. coli* within a batch of pigs following weaning

As shown in a schematic representation of the circulation of *bla*$_{CMY-2}$-positive *E. coli* isolates with respect to time after weaning and supplementation of feed with

clinoptilolite (Fig. 4), *bla*$_{CMY-2}$-positive isolates of phylotype A: cluster III and phylotype D: cluster I were first observed at day 2 after weaning in both the control and supplemented groups, which had been housed in separate pens. Isolates of these two clusters remained in both groups until day 28. Phylotype D showed no changes in clonal type over time, whereas phylotype A revealed a new clone at day 7 in cluster II, although the predominant clone of phylotype A was in cluster III over time. However, the proportion of *bla*$_{CMY-2}$-positive *E. coli* isolates was greatest at day 7 in the supplemented group, consisting of phylotypes A and D, whereas the proportion of *bla*$_{CMY-2}$-positive *E. coli* isolates was greatest at day 28 in the control group, consisting of phylotypes A, B1 and D. For isolates of phylotype B1, there was relationship between sampling day and clonal type, as at day 7 a new clone was detected in the control group in cluster VI. Likewise, at day 28, new clones of phylotype B1 were found and grouped in cluster IV in the control group and in a singleton, cluster VII, in the supplemented group. Thus, overall, the *bla*$_{CMY-2}$-positive *E. coli* isolates were more clonally related in the supplemented group than the control group.

Discussion

The purpose of this study was to elucidate the mechanisms leading to an increase in the prevalence of *bla*$_{CMY-2}$ conferring resistance to ceftiofur in pigs which had not been treated with this antimicrobial and to investigate the effect of supplementation with a clay mineral on this phenomenon. The first step was to study the clonal relatedness of *bla*$_{CMY-2}$-positive *E. coli* isolates resistant to ESCs with respect to PFGE type, phylotype and O serotype. Clonal analysis by PFGE showed diversity among the *bla*$_{CMY-2}$-positive *E. coli* isolates and revealed 7 distinct clones among the 36 *bla*$_{CMY-2}$-positive *E. coli* isolates tested. This is in good agreement with previous studies which showed CMY-2-producing *E. coli* from livestock to be highly polyclonal (Daniels et al. 2007; Hiki et al. 2013; Guo et al. 2014). On the other hand, a recent study in a pig farrowing farm based on MLST and PFGE analysis suggested the clonal spread of *bla*$_{CMY-2}$-positive *E. coli* isolates. Although they found different clones on the farm, one clone predominated, being found in (7/13)

tested isolates (Deng et al. 2015). Further, Schmidt et al (2013) reported that clonal spread of *bla*$_{CMY-2}$ -positive *E. coli* strains was observed more than horizontal gene transfer of IncA/C plasmids between *E. coli* strains in cattle feedlot. Nonetheless they found diverse clones but some clones predominated (Schmidt et al. 2013).

Moreover, PFGE patterns with 100% similarity between at least 2 isolates were observed. This suggests that *bla*$_{CMY-2}$ spreads both horizontally and clonally in this study, and some clones are more common than others. Although the number of tested isolates for clonal analysis by PFGE was relatively low, taken together, the phylotyping and PFGE results show that the *bla*$_{CMY-2}$ -positive *E. coli* isolates were more clonally diverse in the control group than the supplemented group. On the other hand, Bednorz et al. (2013) showed higher diversity of multi-resistant *E.coli* in zinc supplemented pigs compared to the control group (Bednorz et al. 2013). Nevertheless, our results suggest that feed supplementation may have an effect on clonal diversity of *bla*$_{CMY-2}$ -positive isolates, an aspect which warrants further investigation. Further, *bla*$_{CMY-2}$-positive *E. coli* genotypes were diverse and influenced by sampling time within both groups in our study, possibly as a result of continuous administration of chlortetracycline and penicillin G in the feed. This is in accordance with a previous study which demonstrated a clear effect of treatment and time on genetic diversity of resistant *E.coli* in fecal samples from cattle (Alexander et al. 2009). In the present study, the *bla*$_{CMY-2}$-positive *E. coli* isolates were classified into three phylotypes A, B1 and D, as also observed by Guo et al. (2014) in food producing animals (Guo et al. 2014). It has been previously reported that phylotype D *E.coli* is associated with disease in humans such as urinary tract infections (Johnson et al. 2005; Jakobsen et al. 2010). However, although phylotype D/cluster I and phylotype A/cluster III were present in similar proportions in the control and supplemented groups at day 2, from day 7 phylotype A was predominant in isolates from the supplemented group and the majority of *bla*$_{CMY-2}$-positive *E. coli* isolates in the control group belonged to phylotypes B1 and D. Our findings revealed that certain clones became predominant. The phylotype A clone predominated in the supplemented group, whereas the phylotype B1 clones which often possess the ExPEC-associated virulence gene *iucD*, predominated with time, especially in the control group. This finding could support the conjecture of Mathers et al. (2015) that some ExPEC virulence genes such as *sat*, *iutA*, *malX*, *usp*, *iha*, *hra*, and *ompT* may play a

part in the fitness and adaptation of ST131 *E. coli* isolates (Mathers et al. 2015). Furthermore, it has been reported most commensal *E. coli* strains belong to phylogenetic group A (Clermont et al. 2000), thus they can be favourable. In the current study, the phylotype A isolates, which were mostly negative for tested virulence-associated genes, predominated in the supplemented group. We speculate this might be related to the positive effect of clay mineral on the establishment of favourable bacteria in the intestine, as described by Chalvatzi et al, who showed that a more favourable microbiota was found in clay mineral supplemented groups in comparison with control groups in a 18 week-study on laying pullets (Chalvatzi et al. 2016).

In the present study, *bla*$_{\text{CMY-2}}$ was located on three types of plasmids in transformants: IncI1, IncA/C and IncColE. Several studies have shown that IncI1 and IncA/C are the most common *bla*$_{\text{CMY-2}}$-positive plasmids in humans and animals worldwide (Carattoli 2009; Mataseje et al. 2010; Martin et al. 2012; Hiki et al. 2013). As observed in a previous study in broilers (Agerso et al., 2014), we found a high frequency of IncI1 carrying *bla*$_{\text{CMY-2}}$ plasmids in transformants on all sampling days in the present study. Similarly, as previously observed, we found that IncI1 plasmids carrying *bla*$_{\text{CMY-2}}$ confered resistance only to beta-lactam antimicrobials, including ceftiofur, ceftriaxone, cefoxitin, ampicillin, amoxicillin/clavulanic acid (Folster et al. 2011; Martin et al. 2012; Hiki et al. 2013). The high frequency of IncI1 *bla*$_{\text{CMY-2}}$-positive plasmids and resistance to ESCs in the absence of ceftiofur use could be a result of continuous administration of penicillin G in the feed. Similarly, Cameron-Veas et al. (2015) reported that the administration of amoxicillin resulted in an increase in the frequency of cephalosporin resistant-*E. coli* in pigs (Cameron-Veas et al. 2015) and Agerso et al. (2014) showed that use of aminopenicillins influenced the persistence and spread of ESC-producing *E. coli* in broiler production (Agerso et al. 2014). Interestingly, all isolates in clusters I and III showed the same plasmid profile, whereas for the B1 clusters, V and VI, and even in the same identical clone (in cluster VI), only some of the isolates were IncA/C positive, suggesting a transmission of the plasmid within the clone. In the case of clusters I and III, the plasmids may be strongly associated with the clones. Similarly, Yamamoto et al. (2014) demonstrated a correlation between plasmid profiles, PFGE profile and farms in multi-antibiotic resistant *E.coli* isolated from beef cattle (Yamamoto et al. 2014). IncA/C

plasmids, which are known to be multi-resistance plasmids (Mulvey et al. 2009; Martin et al. 2012; Hiki et al. 2013), were detected in the *bla*$_{CMY-2}$-positive *E. coli* wild type isolates from both the control and supplemented groups from day 2. However, as IncA/C *bla*$_{CMY-2}$-positive plasmids were not observed in any transformants before day 28, we cannot say if these IncA/C plasmids were positive for *bla*$_{CMY-2}$. In our study, the frequency of *E. coli* carrying IncA/C plasmid increased to 54%, possibly a result of continuous high-dose administration of chlortetracycline in the feed. Similarly, Johnson et al. (2015) showed that high-dose administration of chlortetracycline (350 g/ton) significantly increased the prevalence of *E.coli* carrying IncA/C plasmid in pig feces (Johnson et al. 2015). All *bla*$_{CMY-2}$-positive *E. coli* were resistant to tetracycline. Hence, such *bla*$_{CMY-2}$ -positive *E. coli* isolates which also carry gene(s) that confer resistance to tetracycline would probably survive and persist in the presence of the selection pressure due to administration of chlortetracycline. It has been shown that *E. coli* can carry multiple plasmids (Johnson et al. 2008; Abraham et al. 2014; Yamamoto et al. 2014)(unpublished data). Therefore, in the present study, despite the high frequency of the *bla*$_{CMY-2}$ encoding IncI1 plasmid which only conferred resistance to beta-lactam antimicrobials, coexistence of multiple resistance plasmids harboring tetracycline-resistance genes with the IncI1 plasmid could explain the circulation of the *bla*$_{CMY-2}$ gene and ESC-resistance.

Our results have also demonstrated that a possible source of the *bla*$_{CMY-2}$ gene for a new batch of weaned pigs could be the presence of this gene in the pen environment before animal arrival. This might be due to use of ceftiofur in previous production batches, combined with inadequate pen sanitation, allowing the *bla*$_{CMY-2}$ gene to persist in the environment. We recovered *bla*$_{CMY-2}$-positive *E. cloacae* isolates, carrying *bla*$_{CMY-2}$ encoding IncA/C plasmids, from the pens before introduction of the pigs of this batch. In addition, we confirmed the transferability of IncA/C plasmid harbouring *bla*$_{CMY-2}$ from *E. cloacae* to *E. coli* DH5α *in vitro* by conjugation, being a natural horizontal gene transfer between bacteria which thus could have occurred on this pig farm. Also, transfer of AmpC beta-lactamase plasmids and AMR genes between *E. coli* and *Salmonella* among humans and animals has been documented (Winokur et al. 2001; Blake et al. 2003). Alternatively, as transmission between sows and piglets has been shown, sow therapy with ceftiofur may lead to expansion of resistant *E. coli* isolates in piglets. The inability to detect resistance to

ceftiofur at day 0 in this batch could be due to the low level of this phenotype in piglets initially and effect of selection pressure of antimicrobials over time (Thompson et al. 2008; Schierack et al. 2009; Volkova et al. 2012). It should be noted that in the current study the most frequently observed *bla*$_{CMY-2}$ encoding plasmid was IncI1, whereas only IncA/C *bla*$_{CMY-2}$ encoding plasmids were detected in *E. cloacae* isolates from the pens prior to introduction of the pigs. It is possible that the plasmid-borne *bla*$_{CMY-2}$ gene was mobilized from IncA/C to IncI1. Yassine et al. (2015) showed the critical role of IS1294b (IS91 family) in the mobilization of the *bla*$_{CMY-2}$ gene from IncA/C to IncI1 in *Enterobacteriaceae* and its contribution to the evolution of diverse incompatibility group plasmids (Yassine et al. 2015).

Seven AMR patterns were found among *bla*$_{CMY-2}$-positive *E. coli* isolates, all being multidrug resistant. Particular AMR patterns were observed in certain clones, phylotype D showing a unique AMR pattern, just as some associations between phylotypes and antimicrobial resistance have been demonstrated previously (Mosquito et al. 2015; Massot et al. 2016). Similar AMR patterns were obtained in a previous study with a similar panel of antimicrobial agents evaluated in human and animal sources (Mulvey et al. 2009). PFGE profiles correlated well with phylotypes, O serotypes and AMR pattern. A similar correlation between antibiotic resistance, O serogroup and PFGE profile has been seen in ceftiofur-resistant *E. coli* isolates from dairy calves (Donaldson et al. 2006). Several studies have suggested that virulent clonal groups belong to phylogenetic group B2 and, to a lesser extent, to group D, which are more related to human clinical isolates, while commensal strains and strains derived from veterinary species belong to groups A and B1 (Clermont et al. 2000; Boerlin et al. 2005). In present study, Phylotype A predominated in *bla*$_{CMY-2}$-positive *E. coli* isolates, followed by phlotype B1 and D, whereas in comparison, ExPEC-associated virulence genes studied here were mostly harboured in phylotype B1 and most phylotypes A and D isolates were negative for ExPEC-associated virulence genes. In contrast, Tan et al. (2012) showed that isolates of phylogenetic groups B2 and D contained more ExPEC virulence genes in *E.coli* isolated from pigs (Tan et al. 2012). On the other hand, the study of *E. coli* isolates from diseased pigs showed that the presence of virulence genes did not differ among the four phylogenetic groups (Wang et al. 2010). The association between virulence genes and phylotypes has remained unclear in animals.

In conclusion, our results reveal that the sub-population of *bla*$_{CMY-2}$-positive *E.coli* resistant to ESCs which expanded in a batch of weaned pigs not treated with this antimicrobial belonged to several different clones with diverse AMR patterns and possessed one or more *bla*$_{CMY-2}$-carrying plasmids of the Inc groups I1, A/C, and ColE. Our results suggest that the *bla*$_{CMY-2}$ gene spread both horizontally and clonally in these pigs. The *bla*$_{CMY-2}$ gene may have spread from previous batches of pigs via IncA/C plasmids carried by *E. cloacae* and capable of resisting disinfection measures used in the pen environment and expanded in animals of the present batch by means of multiple resistance IncI1, IncA/C and IncColE plasmids carried by *E. coli,* in the presence of the selection pressure due to administration of chlortetracycline and penicillin G in the feed. Phylotyping and PFGE results showed that the *bla*$_{CMY-2}$ -positive *E. coli* isolates were more clonally diverse in the control group than the group supplemented with the clay mineral clinoptilolite, suggesting that feed supplementation may have an effect on clonal diversity of *bla*$_{CMY-2}$ -positive isolates, an aspect which warrants further investigation. Taken together, our results suggest that implementation of improved hygiene measures, decreased administration of certain antimicrobials on farm, and feed supplementation with certain ingredients may limit antimicrobial resistance spread between and within batches of animals.

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Tables and figures

Table 1. Association of ExPEC-associated virulence genes and phylogenetic groups in *bla*$_{CMY-2}$ positive isolates.

Category	No. of *bla*$_{CMY-2}$[a] positive isolates	No. of isolates for phylogenetic grouping (%)		
		A	B1	D
With ExPEC virulence gene[b]	38	3(7.9)	33(86.8)	2(5.2)
Without ExPEC virulence gene	100	54(54)	9(9)	37(37)
P value		<0.0001	<0.0001	0.0002
OR[c] (95% CI)		0.07 (0.02-0.25)	66.73 (20.8-231.6)	0.09 (0.02-0.41)
Total	138	57	42	39

[a] Antimicrobial resistance gene (conferring resistance to β-lactam antibiotics)

[b] Extraintestinal pathogenic *E.coli* genes (*iucD*, *tsh*, *papC*)

[c] Odds ratio (OR) above 1 represents positive associations, and below 1 represents negative associations. A total of 138 *bla*$_{CMY-2}$-positive *E. coli* isolates were examined.

Table 2. A summary of antimicrobial resistance pattern in *E. cloacae* isolated from pens before introduction of pigs and *E. coli* isolated from pigs and in IncA/C *bla*$_{CMY-2}$-positive plasmids from both bacterial species.

Bacterial species	AMR pattern[a] (wild type)	AMR pattern for IncA/C plasmids (transformants or conjugants)	Class 1-integron
E.coli	STR,CRO,TIO,FOX, SXT,FIS,AMP,AMC,CHL,TET	STR[b],CRO,TIO,FOX,FIS,AMP,AMC,CHL,TET	-
E. cloacae	KAN[d],STR,CRO,TIO,FOX,SXT,FIS,AMP,AMC,CHL,TET	KAN[e],STR,CRO,TIO,FOX,NAL[c],SXT,FIS,AMP,AMC,CHL,TET	+

[a] Abbreviations for antimicrobials are as follows: kanamycin (KAN), streptomycin (STR), ceftriaxone (CRO), ceftiofur (TIO), cefoxitin (FOX), nalidixic acid (NAL), trimethoprim-sulfamethoxazole (SXT), sulfisoxazole (FIS), ampicillin (AMP), amoxicillin/clavulanicacid (AMC), chloramphenicol (CHL), tetracycline (TET).

[b] Electrocompetent *E coli* DH10B is resistant to streptomycin so this drug could not be evaluated in the transformation experiments.

[c] *E. coli* DH5α is resistant to nalidixic acid so this drug could not be evaluated in the conjugation experiments.

[d] AMR patterns from *E. coli* and *E. cloacae* differ in resistance to KAN (wild type).

[e] IncA/C plasmids AMR patterns from *E. coli* and *E. cloacae* differ in resistance to KAN and SXT (transformants and conjugants).

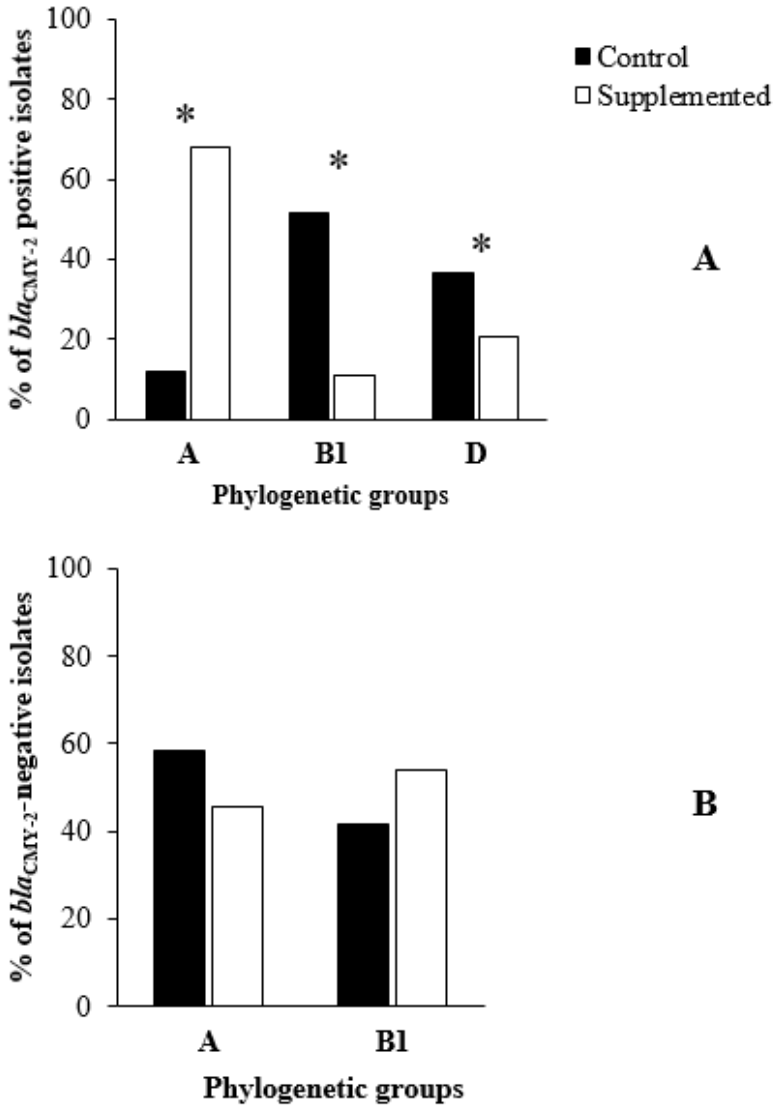


Figure 1. The frequency of *bla*$_{CMY-2}$-positive (n=138) (A) and *bla*$_{CMY-2}$-negative (n=101) (B) *E. coli* isolates among phylogenetic groups.

In A, asterisks indicate that, regardless of time, the frequency of the phylotype A was significantly higher in the supplemented group than in the control group and the frequency of the phylotypes B1 and D were significantly higher in the control group than in the supplemented group using the Fisher's exact test ($P<0.05$). In B, there was no difference in frequency of *bla*$_{CMY-2}$-negative strains among phylogenetic groups. In addition, phylotype D was not detected in *bla*$_{CMY-2}$-negative isolates.

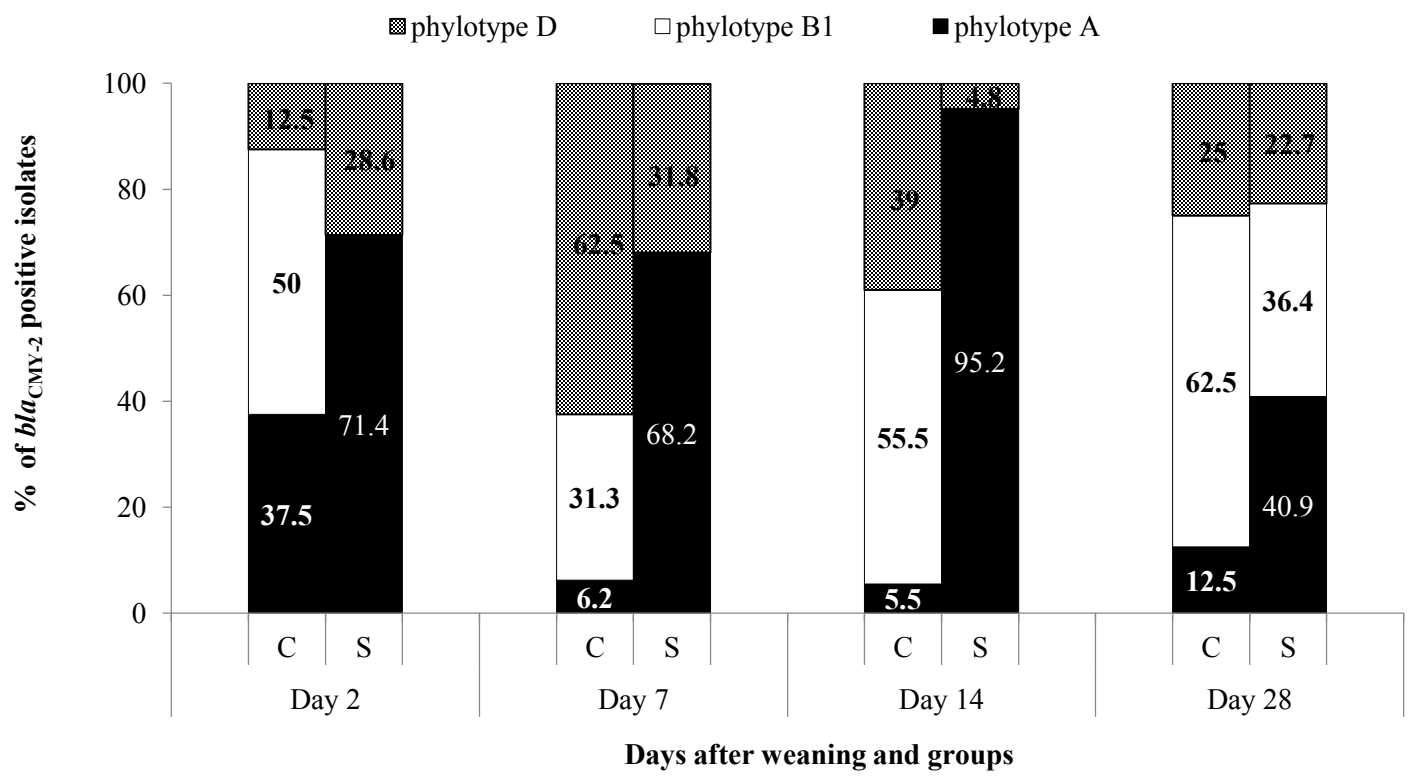


Figure 2. Distribution of 138 *bla*$_{CMY-2}$ -positive *E. coli* isolates among phylogenetic groups in relation to time after weaning and supplementation with clinoptilolite.

Phylotype A predominated in the supplemented group. Phylotypes B1and D predominated in the control group. The value is significantly different to that of the supplemented or control group on the same day using the Fisher's exact test ($P<0.05$), with except for phylotype D where significant differences were only observed on day 14.

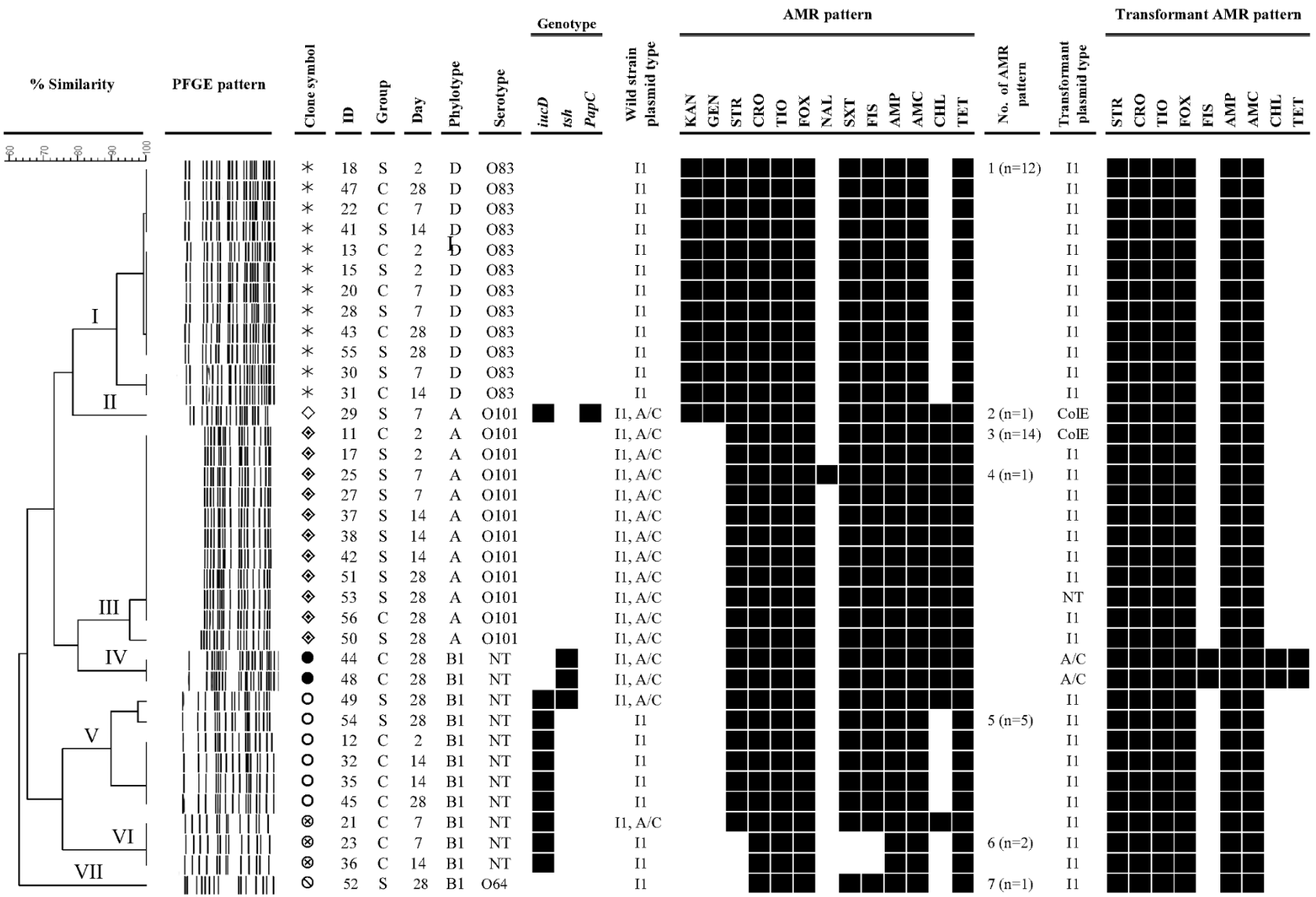

% Similarity
PFGE pattern
Clone symbol
ID
Group
Day
Phylotype
Serotype
Genotype
iucD
tsh
PapC
Wild strain plasmid type
AMR pattern
KAN
GEN
STR
CRO
TIO
FOX
NAL
SXT
FIS
AMP
AMC
CHL
TET
No. of AMR pattern
Transformant plasmid type
Transformant AMR pattern
STR
CRO
TIO
FOX
FIS
AMP
AMC
CHL
TET
I
II
III
IV
V
VI
VII

Figure 3. Cluster analysis of 36 *bla*$_{CMY-2}$ positive *E. coli* isolates based on PFGE patterns obtained by digestion of bacterial genomic DNA with XbaI.

The dendrogram was generated using the Dice coefficient and unweighted-pair group method. Using a similarity index of ≥90%, *E. coli* isolates are separated into 7 PFGE clusters (I to VII). Abbreviations for antimicrobials are as follows: kanamycin (KAN), gentamicin (GEN), streptomycin (STR), ceftriaxone (CRO), ceftiofur (TIO), cefoxitin (FOX), nalidixicacid (NAL), trimethoprim-sulfamethoxazole (SXT), sulfisoxazole (FIS), ampicillin (AMP), amoxicillin/clavulanicacid (AMC), chloramphenicol (CHL), tetracycline (TET). Black fields with "+" indicate the presence of ExPEC-associated virulence genes (*iucD*, *tsh* and *papC*) or the resistance to described antimicrobial agents. NT-non typable. C-control group and S-supplemented group.

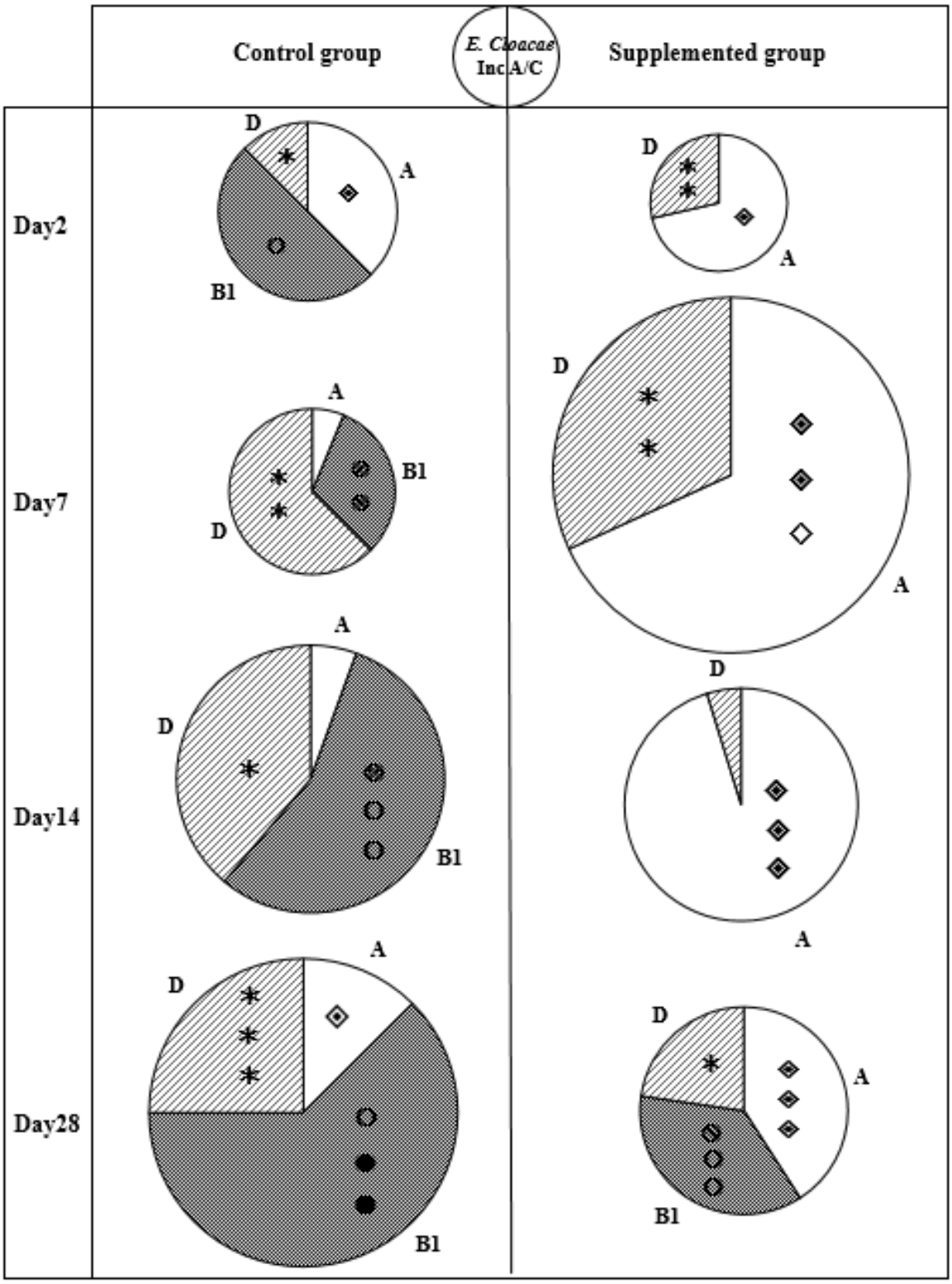
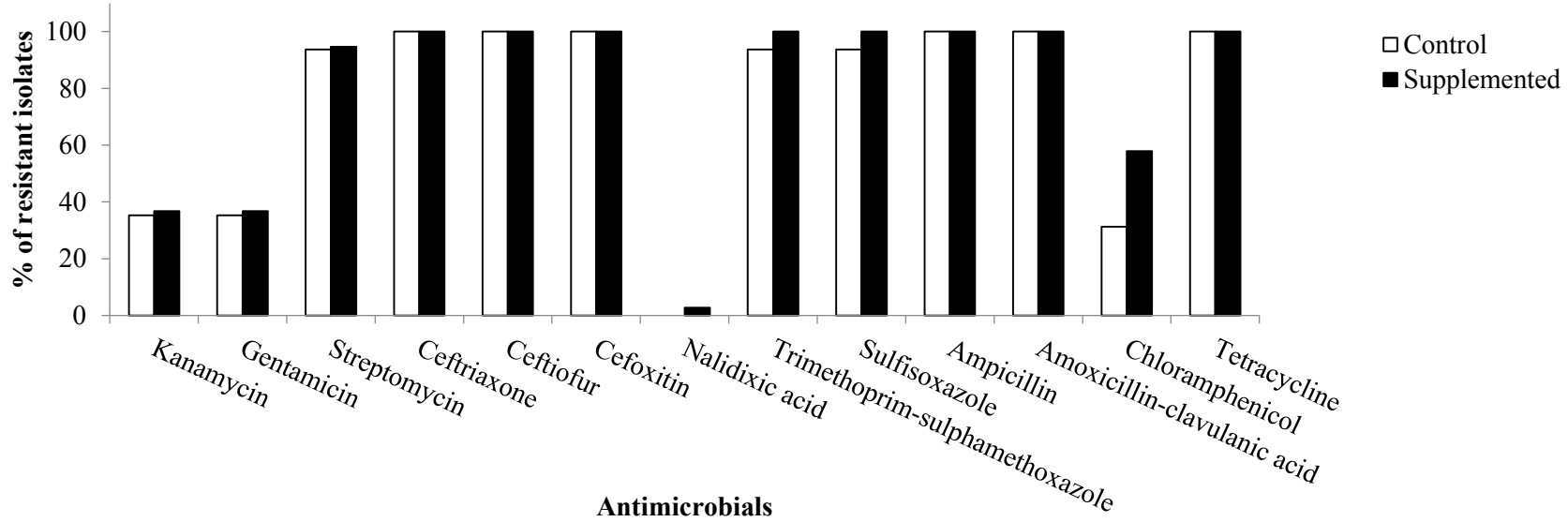

Control group
E. Cloacae Inc A/C
Supplemented group
Day2
Day7
Day14
Day28
D
A
B1

Figure 4. Schematic representation of circulation of *bla*$_{CMY-2}$-positive *E. coli* in the control and supplemented groups, with respect to the frequency of *bla*$_{CMY-2}$-positive isolates and phylogenetic grouping in relation to PFGE cluster/clone over time after weaning.

Size of each pie is based on the frequency of *bla*$_{CMY-2}$-positive isolates as detected by hydrophobic grid membrane filter (HGMF) method in an extended collection of *E. coli* isolates from our initial study (Jahanbakhsh et al. 2015) and proportion of phylotypes in each pie is in regardto phylogenetic grouping of 138 *bla*$_{CMY-2}$-positive *E. coli* isolates in each group and sampling day. Also, *E. cloacae* with IncA/C *bla*$_{CMY-2}$-positive plasmid was detected in both groups from pens before introduction of pigs. Symbols indicate as follows: ✳ clones from cluster I with IncI1 plasmid, ◇ clones from cluster II with IncColE plasmid, ◈ clones from cluster III with IncI1 or IncColE plasmids, ● clones from cluster IV with IncA/C plasmid, ⭘ clones from cluster V with IncI1 plasmid, ⊗ clones from cluster VI with IncI1 plasmid and ⦸ clones from cluster VII with IncI1 plasmid.



Supplementary Figure 1. Effect of dietary of supplementation clinoptilolite on the frequency of AMR in 36 *E. coli* isolates from weaned pigs.

No significant difference was observed between the control and supplemented groups for any of the antimicrobials. Resistance to chloramphenicol was two times more in supplemented group than in the control group however the difference was not statistically significant (Chi-square test, P=0.08)

Details on the role of the candidate in the conception of the article: I am the first author of the article. I actively participated in the study design and; I substantially carried out research, analyzed data and wrote the paper.

Article 3. Dynamics of extended-spectrum cephalosporin-resistance in pathogenic *Escherichia coli* isolated from diseased pigs in Quebec-Canada

Seyedehameneh Jahanbakhsh[a], Matthew G. Smith[c], Hamid-Reza Kohan-Ghadr[a], Ann Letellier[b], Darren J. Trott[c], John Morris Fairbrother[a]

OIE Reference Laboratory for *Escherichia coli* (EcL), Centre de Recherche en Infectiologie Porcine et Aviaire (CRIPA), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada[a].

NSERC Industrial Research Chair in Meat Safety, Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, QC, Canada[b].

School of Animal and Veterinary Sciences, the University of Adelaide, Roseworthy Campus Mudla Wirra Rd, Roseworthy, Australia 5371[c].

Address for correspondence: John M. Fairbrother, Département de pathologie et microbiologie, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, 3200 rue Sicotte, Saint-Hyacinthe QC, Canada, J2S 2M2

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Abstract

The aim of this study was to investigate the evolution with time of ceftiofur-resistant *E. coli* clinical isolates from pigs in Québec, Canada, between 1997 and 2012 with respect to *E. coli* pathotypes, clones, and antimicrobial resistance. Eighty-five ceftiofur-resistant *E. coli* isolates were obtained from the OIE (World Organisation for Animal Health) Reference Laboratory for *Escherichia coli* (EcL). The most prevalent pathovirotypes were enterotoxigenic *E. coli* (ETEC):F4 (40%), extraintestinal pathogenic *E. coli* (ExPEC) (16.5%) and shiga toxin-producing *E. coli* (STEC):F18 (8.2%). Susceptibility testing for 15 antimicrobial agents revealed a high prevalence of resistance to 13 antimicrobials, all isolates being multidrug-resistant. *bla*$_{\text{CMY-2}}$ (96.5%) was the most frequently detected β-lactamase gene, followed by *bla*$_{\text{TEM}}$ (49.4%) and *bla*$_{\text{CTX-M}}$ (3.5%). Pulsed-field gel electrophoresis (PFGE) applied to 45 *E. coli* isolates revealed that resistance to ceftiofur is spread both horizontally and clonally. In addition, the emergence of extended-spectrum β-lactamase (ESBL)-producing *E. coli* isolates carrying-*bla*$_{\text{CTX-M}}$ was observed in 2011 and 2012 in distinct clones. The most predominant plasmid incompatibility (Inc) groups were IncFIB, IncI1, IncA/C and IncFIC. Resistance to gentamicin, kanamycin, chloramphenicol, and the frequency of *bla*$_{\text{TEM}}$ and IncA/C significantly decreased over the study time, whereas the frequency of IncI1 and multidrug-resistance to seven antimicrobial categories significantly increased over time. Our findings reveal that extended-spectrum cephalosporins (ESCs)-resistant porcine *E. coli* isolates in Québec belong to several different clones with diverse antimicrobial resistance patterns and plasmids. Furthermore, *bla*$_{\text{CMY-2}}$ was the major β-lactamase gene in these isolates. From 2011, we report the emergence of *bla*$_{\text{CTX-M}}$ in distinct clones.

Keywords: Pig, *E.coli*, plasmid, *bla*$_{\text{CMY-2}}$ gene, *bla*$_{\text{CTX-M}}$ gene, ESC-resistance

Introduction

The emergence and prevalence of extended-spectrum cephalosporins (ESCs)-resistant *Escherichia coli* in food-producing animals is a global public health concern. Cephalosporins are used in both animals and humans to treat various bacterial infections. Ceftiofur, a third-generation cephalosporin, is used in pigs to treat respiratory disease, lameness and enteric disease, the regulation of use varying from country to country (Deckert et al. 2010). Third-generation cephalosporins are considered to be a critically important class of antimicrobial as they are of very high importance to human medicine (WHO 2011). For example, the third-generation cephalosporin ceftriaxone is one of the drugs of choice to treat invasive pediatric salmonellosis (Fey et al. 2000). Resistance to ceftiofur in food-producing animals is a potential public health issue because ceftiofur resistance determinants also confer cross-resistance to ceftriaxone and other third-generation cephalosporins. Ceftiofur has been used therapeutically in food-producing animals since 1989 and ceftiofur resistance mediated via the $bla_{\text{CMY-2}}$ gene was first reported in 1998 (Daniels et al. 2009). Since 1997, prevalence of ceftiofur-resistance in *E. coli* isolates from clinically ill pigs has increased in Quebec, reaching about 22% in 2014 (MAPAQ 2015). In *Enterobacteriaceae*, ESC-resistance has been associated with production of AmpC β-lactamases (e.g. CMY-2) and ESBLs (e.g. CTX-M and some OXA) encoded by genes on transferable plasmids. The CTX-M and CMY families of β-lactamase producing gram negative bacteria are a major public health threat due to the limited therapeutic options to treat infections with these bacteria as a result of co-association of resistance to that of other classes of antimicrobials, including aminoglycosides, sulphonamides, phenicols and tetracyclines, giving rise to multidrug-resistant (MDR) strains (Mathers et al. 2015).

During the past decade, ESC-resistance has been reported among clinical isolates of *Enterobacteriaceae* from humans and animals in Canada (Mulvey et al. 2009; Mataseje et al. 2010). The objective of this study was to investigate the evolution with time of ceftiofur-resistant *E.coli* isolates from diseased pigs on different farms in Quebec, Canada from 1997 to 2012 with respect to *E. coli* pathotypes, clones, plasmid types, and antimicrobial resistance.

Materials and methods

Source of isolates

A total of 85 ceftiofur-resistant *E. coli* isolated from clinically diseased pigs from 1997 to 2012 were obtained from the strain collection of the OIE (World Organisation for Animal Health) Reference Laboratory for *Escherichia coli* (EcL) (EcL- Faculté de Médecine Vétérinaire de L'Université de Montréal). Isolates from possible cases of *E. coli* infection from the routine bacteriology diagnostic laboratories were submitted to the EcL for further analysis. Pathogenic isolates based on virulence gene determination were tested by the disk-diffusion (Kirby-Bauer) assay for antimicrobial resistance and those resistant to ceftiofur were selected for this study. The ceftiofur-resistant *E. coli* isolates were mainly recovered from pigs with diarrhea, respiratory problems, or oedema disease from 85 separate samples from 74 different cases on 41 distinct farms (Table 1 in the supplemental material). For DNA preparation, individual colonies on MacConkey agar plates were inoculated into 5 ml Luria Bertani (LB-Difco, USA) broth and incubated overnight at 37°C. DNA templates were prepared from *E.coli* isolates by boiled cell lysis for examination by PCR, as described previously (Maluta et al. 2014).

Detection of virulence genes and determination of pathotypes

The data of virulence genotyping were extracted from the EcL collection (1997-2007) and APZEC database (2008-2012) (http://www.apzec.ca). Briefly, the virulence gene typing was carried out at the EcL by PCR or colony hybridization using radioactively labeled (^{32}P) DNA probes for virulence genes which define the *E. coli* pathotypes commonly found in pigs: enterotoxigenic *E. coli* (ETEC) (*eltB, estA,* and *estB*), enteropathogenic *E. coli* (EPEC) (*eae*), shiga toxin-producing *E. coli* (STEC) (*stxA, stx2A*) and extraintestinal pathogenic *E. coli* (ExPEC) (*cnf, papC, iucD,* and *tsh*) and for the genes *faeG*, *fedA*, *fanC*, *fasA, f41, paa*, *aidA*, *astA,* which permit the identification of extended pathovirotypes. PCR and colony hybridization procedures for detection of these genes

were performed according to the protocol of the EcL available in the APZEC website (http://apzec.ca/en/Protocols), and Table 2 in the supplemental material, and as described previously (Harel et al. 1991).

Antimicrobial susceptibility testing

E. coli isolates were examined for susceptibility to the same 15 antimicrobial agents examined in the CIPARS surveillance program in Canada (CIPARS 2008) using the disk-diffusion (Kirby-Bauer) assay as previously described (Jahanbakhsh et al. 2015). *E. coli* isolates were considered multidrug-resistant based on the definitions described by Magiorakos et al., an isolate being MDR if it is non-susceptible to at least 1 antimicrobial agent in 3 or more antimicrobial defined categories (Magiorakos et al. 2012). Furthermore, different levels of MDR were defined as follows; MDR4: non-susceptible to at least 1 agent in only 4 antimicrobial categories, MDR5: non-susceptible to at least 1 agent in only 5 antimicrobial categories, etc.

CTX-M-positive isolates were confirmed for ESBL production in the Minimum Inhibitory Concentration (MIC) method (CLSI- M100-S22) using V2AGNF and ESB1F plates in the Sensititre System (CLSI 2012).

β-lactamase genotyping

Isolates were screened for the presence of the $bla_{\text{CMY-2}}$, bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{SHV} and $bla_{\text{OXA-1}}$ genes by multiplex PCR, as described previously (Poirel et al. 2011; Mataseje et al. 2012). Isolates that were PCR positive for $bla_{\text{CTX-M}}$ were examined for the presence of $bla_{\text{CTX-M-15}}$, because of its importance in human medicine, using the primers CTX-M-15-F (5'-CACACGTGGAATTTAGGGA-3') and CTX-M-15-15-R (5'-GCCGTCTAAGGCGATAAAC-3') as described (Doi et al. 2009).

Plasmid replicon typing and determination of class 1 and 2-integrons

Plasmid incompatibility (Inc) grouping was conducted using PCR replicon typing as described by Carattoli et al. for 18 Incompatibility (Inc) groups (Carattoli et al. 2005). A multiplex PCR assay targeting class 1 and 2-integrons (*int1* and *int2*) was performed to

investigate the presence of integrons using previously described primers and conditions (Ruiz del Castillo et al. 2013).

Phylogenetic grouping

Phylogenetic grouping was performed using a multiplex PCR-based assay as described by Clermont et al. (Clermont et al. 2000).

O serotyping

O serotyping was determined by standard agglutination methods (Edwards and Ewing 1972) for 86 O serogroups associated with swine disease. O antisera were produced at the EcL according to standard methods (Edwards and Ewing 1972).

Pulsed-Field Gel Electrophoresis (PFGE)

E. coli isolates were analyzed for genetic relationships by PFGE using restriction enzyme XbaI as described by PulseNet (Ribot et al. 2006). PFGE patterns were analyzed using the BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were generated by the unweighted pair-group method using arithmetic averages, based on the Dice similarity coefficient (optimization 1%; tolerance 1.2%).

Statistical analysis

Statistical analysis was carried out using JMPv.10 (SAS Institute, Cary, NC, USA). The Chi-square test or Fisher's exact test, as appropriate, was used to compare the significance of differences in prevalence of traits (genetic and phenotypic) with respect to different factors (e.g. study time, pathovirotypes). Odds ratios (OR) (95% confidence intervals) were used to evaluate the associations between traits. Statistical significance for each comparison was considered at a P value of <0.05.

Results

E. coli pathovirotype, O serogroup, and phylogenetic group

The most prevalent pathovirotype among 85 ceftiofur-resistant *E. coli* isolates from diseased pigs was ETEC:F4 (40%) (Fig 1). ETEC:F4 isolated before 2006 were mostly of virotype LT:STa:STb:F4 encoded by the *eltB, estA, estB*, and *faeG* virulence genes respectively, whereas those isolates from 2006 or afterwards were mostly LT:STb:F4, based on the presence of the appropriate virulence genes. Most ETEC:F4 isolates were O149 and belonged to phylotype A. Five STEC:F18 isolates (8.2%) and two ETEC:STEC:F18 isolates were found, all belonging to O147 and phylotype A. Isolates possessing a combination of virulence genes defining two pathotypes were considered to belong to both pathotypes. The remaining isolates were ExPEC (16.5%), EPEC (7%), or of other *E.coli* pathotypes (e.g. ETEC, ETEC:F5) or non-typeable, being heterogeneous with respect to O serogroup and phylotype. Two of the ExPEC isolates demonstrated the virotype PapC:CNF and phylotype B2, but were of different O serotypes, O88 and O4 (Fig. 1).

Isolates from cases of diarrhea were most commonly ETEC:F4 (48.8%), whereas those from oedema disease were mainly STEC:F18 (66.6%) and those from respiratory disease mostly ExPEC (50%) (Table 3 in supplemental material).

Antimicrobial resistance profiles and distribution of β-lactamase genes and class 1 and 2-integrons

Overall, the most frequently observed resistance in ESC-resistant *E. coli* was to ceftiofur (100%), ceftriaxone (100%), ampicillin (100%), cefoxitin (96.5%), amoxicillin/clavulanic acid (96.5%), streptomycin (89.4%), tetracycline (89.4%), sulfisoxazole (82.3%), trimethoprim-sulfamethoxazole (68.2%), chloramphenicol (58.8%), kanamycin (50.6%), gentamicin (40%), and nalidixic acid (4.7%). All isolates were susceptible to amikacin and ciprofloxacin (data not shown). Furthermore, 22 antimicrobial resistance (AMR) patterns were distinguished, all being classified as MDR.

The predominant AMR patterns were GEN, KAN, STR, CRO, TIO, FOX, SXT, FIS, AMP, AMC, CHL, TET (n=17, from 1997 to 2011) and STR, CRO, TIO, FOX, SXT, FIS, AMP, AMC, TET (n=8 from 2006 to 2011), being found in the nursery and preweaning phases of pig production and different farms (Fig. 1). Moreover, two previously undetected AMR patterns with respect to resistance to β-lactam antibiotics were found as follows, KAN, STR, CRO, TIO, SXT, FIS, AMP, TET (n=1, 2011) and STR, CRO, TIO, SXT, FIS, AMP, CHL, TET (n=2, 2012), in isolates from pigs in the nursery phase and from different farms (Fig. 1 and Fig. 5). The latter three isolates were found to be ESBL producers.

bla$_{\text{CMY-2}}$ was the most frequently detected β-lactamase gene in ESC-resistant *E.coli* isolates (96.5%), followed by *bla*$_{\text{TEM}}$ (49.4%) and *bla*$_{\text{CTX-M}}$ (3.5%) (Table 1, Fig. 1). Notably, the three ESBL-producing isolates were *bla*$_{\text{CTX-M}}$-positive but negative for *bla*$_{\text{CMY-2}}$. Nevertheless, they were not the *bla*$_{\text{CTX-M-15}}$ variant. In addition, the frequency of the presence of *bla*$_{\text{TEM}}$ decreased significantly over the course of the study. Furthermore, *bla*$_{\text{SHV}}$ and *bla*$_{\text{OXA-1}}$ were not detected in any ESC-resistant *E. coli* isolates (Fig. 2). The most prevalent β-lactamase genes were *bla*$_{\text{CMY2}}$ alone (48.2 %) and *bla*$_{\text{CMY2}}$:*bla*$_{\text{TEM}}$ in combination (48.2 %) (Table 1), the frequency of the combination *bla*$_{\text{CMY-2}}$:*bla*$_{\text{TEM}}$ decreasing with time from 81.8% in 1997-2002 to 35.2% in 2008-2012 (*P* value=0.0089 using Fisher's Exact test). Class 1-integrons were more frequently observed than class 2-integrons (Table 1) and there was no difference in frequency of class 1 and 2-integrons over time. Five isolates had both classes of integrons simultaneously. The presence of class 1-integrons was significantly associated with resistance to gentamycin, kanamycin, streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, chloramphenicol and tetracycline. In addition, no significant differences in resistance to antimicrobial agents and the frequency of class 1 and 2-integrons were found in relation to pathovirotypes (data not shown). There was no significant difference in the proportion of class 1 and 2-integrons among β-lactamase gene profiles (Table 1).

Different levels of multidrug resistance were observed, MDR8 (48%) being the most frequent, followed by MDR7 (26%). Interestingly, the frequency of MDR8 revealed a tendency to decrease over the time of the study, accompanied by a significant increase in the frequency of MDR7 (Fig. 3). Notably, the frequency of resistance to gentamicin

significantly decreased with time among ESC-resistant *E.coli,* from 81.8% in 1997-2002 to 40% in 2003-2008 (*P* value=0.0158 using Fisher's Exact test) and 26.5% in 2008-2012 (*P* value=0.0018 using Fisher's Exact test). In addition, resistance to kanamycin and chloramphenicol significantly decreased similarly to the gentamicin pattern over the study time. Likewise, the frequency of resistance to both gentamicin and chloramphenicol significantly differed with respect to production phase, as it was significantly higher in the nursery phase than the preweaning phase (P value = 0. 0135) (Fig.1).

Detection of plasmid replicon types

Plasmid replicon typing identified 15 different plasmid types, all isolates except one carrying one or more plasmids. The most predominant Inc groups were IncFIB (81.2%), IncI1 (57.6%), IncA/C (49.4%) and IncFIC (41.2%) (Table 1), forty-nine replicon type patterns being observed (Fig. 1). Over the course of the study, the frequency of the presence of IncA/C in isolates significantly decreased, whereas the proportion of IncI1 in isolates significantly increased, and there were no significant differences in the frequency of IncFIB and IncFIC (Fig. 4). The frequency of IncA/C significantly differed between $bla_{\text{CMY-2}}$ and $bla_{\text{CMY-2}}$:bla_{TEM} carrying *E. coli* isolates, 29.7% and 73.2%, respectively (Table 1). The presence of IncA/C demonstrated a positive association with resistance to gentamicin, kanamycin, streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, chloramphenicol, tetracycline, MDR8, and the presence of bla_{TEM} and *Int1*, although there was a negative association with MDR7 (Table 3). In contrast, IncA/C was not detected in $bla_{\text{CTX-M}}$ positive *E. coli* isolates. The frequency of IncFIB, IncI1 and IncFIC did not differ among β-lactamase gene combinations (Table 1).

Furthermore, a positive association between IncFIB and IncA/C was observed [OR (95%CI) =3.67 (1.07-12.54), *P*=0.0302], whereas the presence of IncI1 showed negative association with IncA/C [OR (95% CI) =0.14 (0.055-.38), *P*<0.0001]. The presence of IncFIC and IncFIB was highly associated with ETEC:F4 isolates whereas a negative association between IncFIC and ExPEC and EPEC was seen. In addition, a positive or negative association between various other pathovirotypes and Inc plasmids was observed (Table 2). The presence of IncFIB positively associated with resistance to kanamycin, streptomycin, trimethoprim-sulfamethoxazole, tetracycline, MDR8 and bla_{TEM}. The

presence of IncI1 demonstrated negative association with resistance to gentamicin and MDR8 (Table 3).

Clustering analysis of ESC-resistant *E. coli* isolates based on tested genes

Clustering analysis based on tested genes revealed 74 unique patterns (Fig. 1). Isolates were divided into 32 clusters at 70% similarity and seven of these clusters were singletons. Approximately 32% of isolates were grouped in the major cluster, which represents ETEC:F4, O149 isolates belonging to phylotype A. Gene clustering analysis separated ESC-resistant *E. coli* isolates according to O serogroup, most O149 strains being differentiated from the other detected O serogroups (Fig. 1).

PFGE analysis of ESC-resistant *E. coli* isolates

In order to study the epidemiological genetic relationships of the ESC-resistant *E.coli,* 45 representative isolates were further analyzed by XbaI-PFGE. PFGE revealed 36 unique patterns each representing a clone and isolates were grouped into 15 clusters (I to XV) at 75% similarity, five of these clusters containing a single isolate (Fig. 5). Moreover, 45% (n=20) of isolates were grouped in the major cluster III at 75% similarity which represented ETEC:F4. In addition, isolates in the major cluster at similarity ≥ 90% were grouped in 4 subclusters, III-1, III-2, III-3 and III-4. Isolates of the subcluster III-1 possessed the *faeG, paa, eltB, estA, estB, astA* gene pattern (virotype LT:STa:STb:F4) except for isolate EC37 which was negative for *astA* whereas isolates in subclusters III-2, III-3 and III-4 possessed the *faeG, paa, eltB, estB, astA* gene pattern, all being negative for *estA* (virotype LT:STb:F4).

There was a correlation between clonal type and time of isolation, as closely related clusters (Similarity ≥ 90%) were separated by years, subcluster III-1 being found from 2001 to 2006, subcluster III-2 in 2006 and 2007, III-3 from 2006 to 2011, and III-4 from 2009 to 2012. Identical clones, with a few exceptions, revealed close relationships by presenting similar features with respect to virulence and β-lactamase genes, phenotypic AMR patterns, replicon types, class 1 and 2-integrons, phylotypes and O serogroups (Fig. 5). IncA/C was mostly found prior to 2006 in III-1 isolates, such as EC37 from farm 16. IncA/C re-emerged in three III-4 isolates from the same farm in 2009, 2011, and 2012.

Cluster III contained isolates from different farms and years. Indistinguishable PFGE patterns were present in isolates from multiple farms and years, such as the isolates in subclusters III-1 (EC7, EC22 and EC37), and III-2 (EC43, EC49) and cluster XI (EC73, EC84). Also, isolates EC60 and EC63 belonging to an identical clone were found in different years in the same farm and for isolates (EC19 and EC20) and (EC36 and EC40), each pair revealed an identical pattern in the same year from different farms, indicating a circulation of clones between farms. Further, an indistinguishable PFGE pattern was found from 2001 to 2006 (isolates EC7, EC22, EC23 and EC37) indicating the persistence of the clone with time.

The three *bla*$_{\text{CTX-M}}$ carrying isolates (EC67, EC77 and EC82) were ETEC:F4, all belonging to O149 and phylotype A, but were not clonally related and originated from different farms.

Discussion

In this study, clonal analysis by PFGE showed high diversity among ESC-resistant clinical *E. coli* isolates from pigs although half of the isolates belonged to a single cluster. Similarly, our previous study on healthy pigs on a commercial farm showed that ESC-resistant *E.coli* isolates belonged to several different clones with diverse AMR patterns, certain clones being predominant (submitted article). This is also in good agreement with previous studies which showed ESC-resistant *E. coli* from cattle were highly polyclonal (Donaldson et al. 2006; Daniels et al. 2007).

Our finding that identical ETEC:F4 clones were found in different years on the same farm suggest persistence of certain clones between batches. In addition, some clones were observed from multiple farms which suggested potential circulation among the farms. Also, these clones may have originated from a single source (e.g. same breeding pig farm), as observed by Agerso et al, (2014) who reported that the same *E. coli* clones possessing *bla*$_{\text{CMY-2}}$ were found in imported grandparents and in all levels of the Danish broiler production, indicating spread of ESC-producing *E. coli* clones and plasmids from imported parent animals to broilers and to broiler meat (Agerso et al. 2014). In our study,

transmission may have occurred because of imported and exported pigs, and also by the movement of water and wild animals and/or through contaminated food (Yamamoto et al. 2014). It is interesting to note that presently only 44% of farms in Quebec are «farrow-to-fattening», that is, on a single site, as compared to 33% fattening and 23% farrowing farms which together constitute multisite farms. Transport of pigs between these sites would result in a more widespread spreading of clones (Lacroix and Morin 2015). Likewise, in line with previous studies, *E. coli* may be transmitted from farm to farm by vehicles such as transport trucks and visitors on farms (truck drivers, veterinarians, producers, technicians) or by farm workers (Lim et al. 2010; Abraham et al. 2015).

Heterogeneity among ESC-resistant isolates was found, suggesting that their spread is not only due to the dispersion of successful *E. coli* clones, and horizontal gene transfer may be contributing to the emergence of ESC-resistance in pigs, as has been suggested by others (de Been et al. 2014). A notable finding was that, among ETEC:F4, O149 *E. coli* isolates, the more virulent clones with the virotype LT:STa:STb:F4 were mostly seen prior to 2006 (subcluster III-1) whereas from 2006 to 2012 most clones displayed the virotype LT:STb:F4 (subclusters III-2,3,4). In accordance with previous studies, the changes observed in O149 clones with time probably reflect the changes in the pathogenic population, in that an overall predominance of LT:STa:STb:F4 isolates was found in early 2000s (Noamani et al. 2003; Fairbrother et al. 2005).

Interestingly, the plasmid IncA/C was mostly seen prior to 2006 in ETEC:F4 O149 clones of subcluster III-1 although it re-emerged in 2009-2012 clones (III-4) on one particular farm. The presence of IncA/C was significantly associated with resistance to gentamicin, kanamycin, streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, chloramphenicol, tetracycline, as described previously (Doublet et al. 2012). Also, IncA/C plasmid-carrying *bla*$_{\text{TEM}}$ and *Int1* and genes conferring resistance to the above mentioned antimicrobials have been demonstrated (Fernandez-Alarcon et al. 2011). The decrease in resistance to gentamicin, kanamycin and chloramphenicol in ESC-resistant *E. coli* could be a result of less administration of these antimicrobials in pig production. The ban on the use of chloramphenicol in food animals in Canada since the 1980s could contribute to the decrease in resistance to chloramphenicol over time. Similarly, based on anecdotal evidence from local authorities and veterinarians, a tendency to less prescribing of

gentamicin has recently been seen. On the other hand, kanamycin is not used in pig production in Canada. The emergence and prevalence of antimicrobial resistance in bacteria are likely to be linked to administration of antimicrobials in animals through cross-resistance or co-resistance (Harada and Asai 2010). It can also be associated with disinfectant use and administration of metals like copper and zinc as feed additives (Bednorz et al. 2013).

In the present study, we observed a relationship between certain pathotypes and Inc plasmid types. Our finding that ETEC:F4 was positively associated with IncFIB and IncFIC but negatively with IncB/O and IncP confirms the observations in a recent study of pig isolates in Australia (Abraham et al. 2014). In addition, ExPEC showed a positive association with the presence of IncB/O and negative association with the presence of IncI1 and IncFIC groups. Johnson et al. described a similar trend in human ExPEC strains (Johnson et al. 2008). Future studies will further examine the relative role of pathogenic clones and the various plasmids in the circulation of the *bla*$_{\text{CMY-2}}$ gene.

This study demonstrated the recent emergence of multidrug-resistant CTX-M-producing ETEC:F4 *E. coli* isolates in pigs in 2011 and 2012. It seems that the spread of *bla*$_{\text{CTX-M}}$ may have occurred by horizontal transmission as *bla*$_{\text{CTX-M}}$ was detected in distinct clones. To our knowledge, this is the first report of *bla*$_{\text{CTX-M}}$-positive, ESLB-producing pathogenic *E. coli* isolates from pigs in Quebec, Canada.

Three distinct phylotype B2 clones were detected among ESC-resistant *E.coli* which were ExPEC O4 and O88 and ETEC O98. *E. coli* of phylotype B2 and D, possessing virulence factors PapC and CNF and serogroup O4 is associated with disease such as urinary tract infections in humans (Johnson et al. 2005) suggesting a zoonotic potential. Similarly, a recent Australian study reported human associated zoonotic clones in pigs and cattle (Abraham et al. 2015). The results we observed in this study are based on our collection that includes 91% pathogenic *E.coli* from clinical cases. However, the discussed patterns could be different in commensal *E.coli* as was reported by Boerlin et al (2005) who showed that AMR differs significantly between pathogenic and commensal *E. coli* isolates (Boerlin et al. 2005).

In conclusion, our findings reveal that ESC-resistance was mostly associated with the *bla*$_{\text{CMY-2}}$ gene and that the *bla*$_{\text{CTX-M}}$ gene has recently emerged. ESC-resistant *E. coli* belonged to several different clones with diverse AMR patterns and often carried plasmids of several Inc groups. However, there was no persistent and predominant clone over the study time. Further, we confirmed that some clonal ESC-resistant isolates were present at multiple farms in the same and different years. Our results suggest that the *bla*$_{\text{CMY-2}}$ gene spreads both horizontally and clonally in this study and the spread of *bla*$_{\text{CTX-M}}$ gene may have occurred by horizontal transmission. Resistance to gentamicin, kanamycin, chloramphenicol, and the frequency of *bla*$_{\text{TEM}}$ and IncA/C plasmid significantly decreased over the period of time. In contrast, IncI1 and MDR7 significantly increased over the study time. To our knowledge, this is the first study describing characteristics of ESC-resistant *E.coli* isolated over a long period. In addition, we believe this to be the first report of *bla*$_{\text{CTX-M}}$ positive, ESLB-producing pathogenic *E. coli* strains from pigs in Canada. Our findings underline the importance of monitoring *E. coli* isolates from pigs for the presence of *bla*$_{\text{CTX-M}}$ gene in the future since they could be transmitted to humans via the food chain or by direct contact. Widespread occurrence of antimicrobial resistance plasmids in ESC-resistant *E. coli* from pigs is a concern for animal and public health and warrants further attention.

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Tables and figures

Table 1. Distribution of plasmid Inc groups and Integrons in relation to selected β-lactamase genes in ESC-resistant E. coli isolated from pig in Quebec, Canada from 1997-2012

Selected β-lactamase gene combinations	No. of positive isolates (%)	No. of positive isolates for (%)					
		Plasmid Inc groups				Integron classes	
		FIB	I1	A/C	FIC	1	2
bla$_{CMY-2}$[a]	41(48.2)	29 (70.7)	22 (53.6)	12 (29.7)	16 (39)	26 (63.4)	5 (12.2)
bla$_{CTX-M}$	2 (2.3)	2 (100)	2 (100)	0	2 (100)	2 (100)	1 (50)
bla$_{CMY-2}$:*bla*$_{TEM}$	41 (48.2)	37 (90.2)	24 (58.5)	30 (73.2)	16 (39)	33 (80.5)	5 (12.2)
bla$_{CTX-M}$:*bla*$_{TEM}$	1 (1.2)	1 (100)	1 (100)	0	1 (100)	1 (100)	0
Total	85 (100)	69 (81.2)	49 (57.6)	42 (49.4)	35 (41.2)	62 (72.9)	11 (12.9)

[a] Overall the frequency of *bla*$_{CMY-2}$ gene was 96% (41+41/85) and the frequency of *bla*$_{TEM}$ gene was 49.4% (41+1/85)

Table 2. Statistically significant association between pathovirotypes and major Inc groups.

E. coli pathovirotype	Associated Inc groups	OR[c]	95%CI[b]	*P* value[a]
ETEC:F4	FIB	NAp[d]	NA	0.0003
	FIC	43.5	12.15-155.71	<0.0001
	B/O	0.072	0.009-0.58	0.0022
	P	0.28	0.07-1.08	0.0448
STEC:F18	FIB	0.13	0.02-0.68	0.0068
	A/C	NAn	NA	0.0063
EXPEC	I1	0.23	0.06-0.81	0.016
	FIC	NAn	NA	0.0006
	B/O	4.57	1.3-16	0.0118
	N	19.5	4.92	<0.0001
ETEC:F5	I1	NAp	NA	0.0179
EPEC	I1	NAn	NA	0.0168
	A/C	NAp	NA	0.0382
	FIC	NAn	NA	0.0865

[a] Only statistically significant (P<0.05) associations are reported using Fisher's Exact test . A total of 85 isolates were examined.

[b]95%CI-95% confidence interval

[c] Odds ratio (OR) above 1 represents positive associations, and below 1 represents negative associations.

[d] Na: statistically highly significant positive (NAp) or negative (NAn) associations but calculation of odds ratios not applicable.

Table 3. Statistically significant association between major Inc groups, and antimicrobial resistance phenotypes and determinants

Major Inc groups	Associated traits	OR[c]	95%CI[b]	*P* value[a]
FIB	Kanamycin	10.25	2.15-48.65	0.0007
	Streptomycin	4.26	0.99-18.22	0.0376
	Trimethoprim-sulfamethoxazole	5.09	1.61-16.11	0.0034
	Tetracycline	7.38	1.71-31.86	0.0029
	MDR8	3.46	1.01-11.82	0.039
	bla_{TEM}	3.67	1.07-12.54	0.0302
I1	Gentamicin	0.32	0.12-0.78	0.0121
	MDR8	0.4	0.16-0.97	0.041
A/C	Gentamicin	37.57	9.64-146.4	<0.0001
	Kanamycin	9.3	3.47-24.96	<0.0001
	Streptomycin	9.37	1.11-78.64	0.0151
	Trimethoprim-sulfamethoxazole	3.36	1.26-8.94	0.0128
	Sulfisoxazole	5.03	1.3-19.41	0.0121
	Chloramphenicol	11.53	4.07-32.65	<0.0001
	Tetracycline	9.37	1.11-78.64	0.0151
	MDR7	0.14	0.04-0.48	0.0007
	MDR8	9.3	3.46-24.94	<0.0001
	bla_{TEM}	6.45	2.51-16.60	<0.0001
	Int1	2.96	1.06-8.21	0.0331
FIC	Tetracycline	6.47	0.77-54.35	0.0364
P	Streptomycin	NA[d]	NA	0.0457

[a] Only statistically significant (P<0.05) associations are reported using Fisher's Exact test . A total of 85 isolates were examined.

[b] 95%CI-95% confidence interval

[c] Odds ratio (OR) above 1 represents positive associations, and below 1 represents negative associations.

[d] Na: statistically highly significant positive associations but calculation of odds ratios not applicable.

Supplementary Table 1. Descriptive information of 85 ceftiofur-resistant clinical *E. coli* isolated from pig in Quebec-Canada from 1997-2012

Characteristics	Number (% of total)
Production phase (age)	
Preweaning (0-21 days)	25 (29)
Nursery (22-89 days)	52 (61)
Growing-finishing (90-240 days)	5 (6)
Unknown	3 (4)
Sample origin	
Intestinal	79 (93)
Extera-intestinal	6 (7)
Clinical sign	
Diarrhea	41 (48)
Oedema	3 (4)
Respiratory problem	14 (16)
Other clinical signs	11 (13)
Unknown	16 (19)
Three categories of study time (1997-2012)	
1997-2002	11 (12.9)
2003-2007	40 (47.1)
2008-2012	34 (40)
Farm (n=41)	
Total No. of samples and isolates=85	

Supplementary Table 2. List of primers used in the PCR and colony hybridization by DNA probe, PCR conditions, and control strains.

Virulence factor	Gene	Primer [a] (5'⟶ 3')	Amplicon size (bp)	Annealing temperature	Control strain	Reference
F6	*fasA*	For ATGAGAATGAAAAAATCCGCA Rev CGAATAGTCATTACTGCACT	333	60°C	ECL2316	[1]
F5	*fanC*	For TGCGACTACCAATGCTTCTG Rev TATCCACCATTAGACGGAGC	450	60°C	ECL13316	[2]
F18	*fedA*	For GTGAAAAGACTAGTGTTTATTTC Rev CTTGTAAGTAACCGCGTAAGC	510	60°C	ECL1033	[3]
F41	*f41*	For GAGGGACTTTC TCTTTTAG Rev AGT CCA TTC CAT TTA TAG GC	431	60°C	ECL13316	[2]
AIDA	*aidA*	For ACAGTATCATATGGAGCCA Rev TGTGCGCCAGAACTATTA	585	60°C	ECL1033	[4]
Paa	*paa*	For ATGAGGAACATAATGGCAGG Rev TCTGGTCAGGTCGTCAATAC	360	60°C	ECL7805	[5]
EAST1	*astA*	For TCGGATGCCATCAACACAGT Rev GTCGCGAGTGACGGCTTTGTAG	125	60°C	ECL7805	[4]

[a] The other primers used in the PCR and colony hybridization by DNA probe are available at http://apzec.ca/en/Protocols

Supplementary Table 3. Distribution of major clinical signs among *E. coli* pathovirotypes

Major clinical sign	No. of *E. coli* pathovirotype (%)					Total
	ETEC:F4	ExPEC	STEC:F18	EPEC	Others [a]	
Diarrhea	20(48.8)	1 (2.4)	4(9.7)	4 (9.7)	12(29.2)	41
Oedema	1 (33.3)	0	2(66.6)	0	0	3
Respiratory problem	4 (28.6)	7 (50)	0	1 (7.1)	2(14.2)	14

Others include other *E.coli* pathotypes (e.g. ETEC, ETEC:F5) or nontypeable.

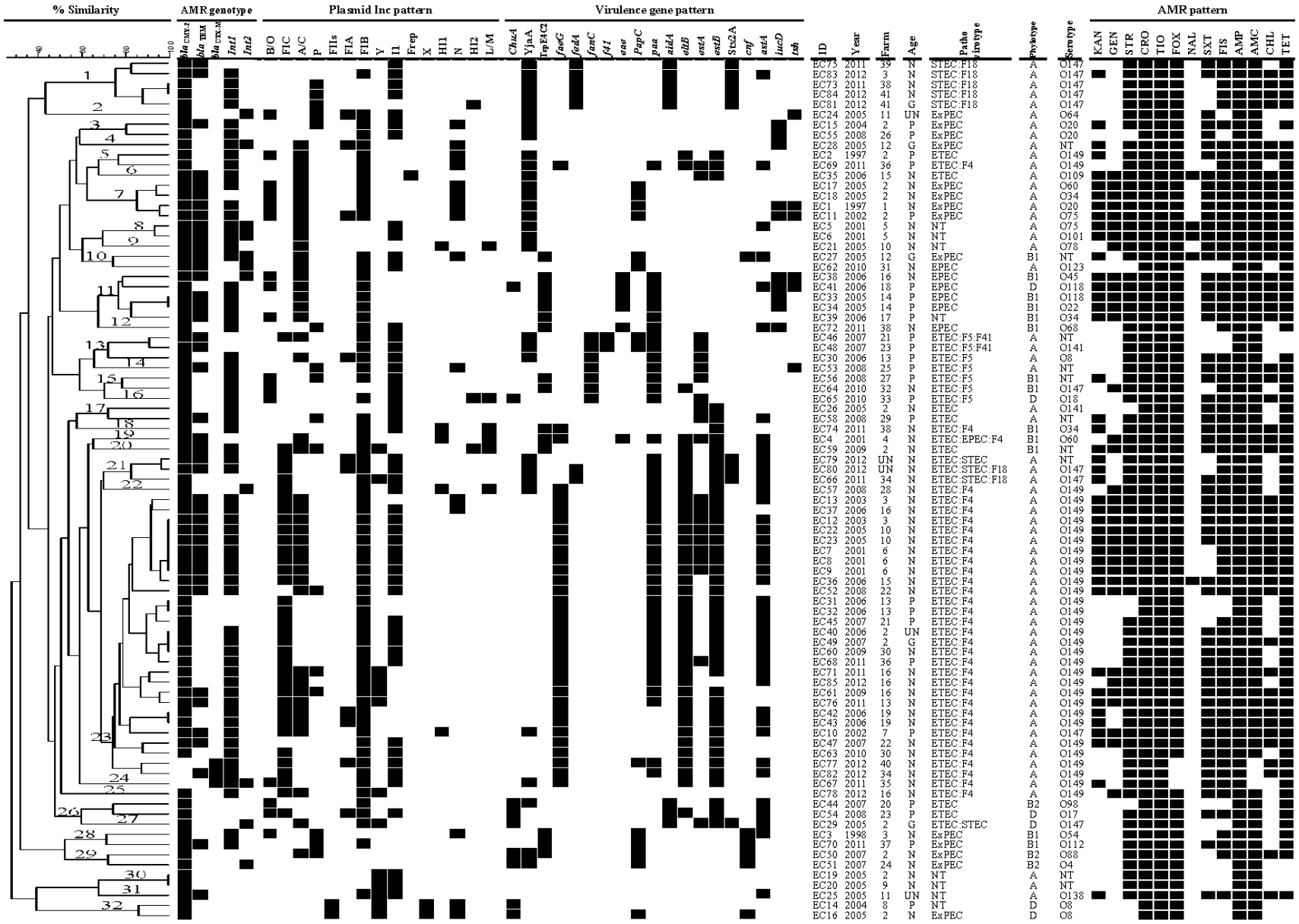
% Similarity
AMR genotype
Plasmid Inc pattern
Virulence gene pattern
AMR pattern
ID
Year
Farm
Age
Pathotype
Phylotype
Serotype
KAN
GEN
STR
CRO
TIO
FOX
NAL
SXT
FIS
AMP
AMC
CHL
TET

Figure 1. Clustering analysis of genetic variation of 85 ESC-resistant clinical *E. coli* isolates from 1997 to 2012.

The dendrogram was generated using the binary patterns (0, 1) of tested genes, Dice coefficient and unweighted pair group method with arithmetic mean. According to a similarity index of ≥70%, ESC-resistant *E. coli* isolates are separated into 32 clusters. Abbreviations for antimicrobials are as follows: kanamycin (KAN), gentamicin (GEN), streptomycin (STR), ceftriaxone (CRO), ceftiofur (TIO), cefoxitin (FOX), nalidixicacid (NAL), trimethoprim-sulfamethoxazole (SXT), sulfisoxazole (FIS), ampicillin (AMP), amoxicillin/clavulanicacid (AMC), chloramphenicol (CHL), tetracycline (TET). Black fields with "+" indicate the presence of genes or the resistance to described antimicrobial agents. UN-Unknown. NT-Non typeable.

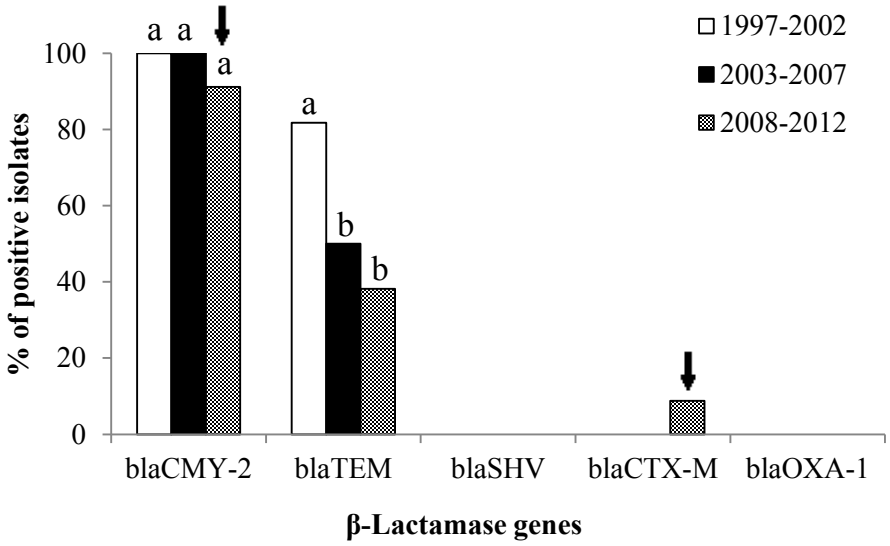

Figure 2. Frequency of β-lactamase genes among 85 ESC-resistant clinical *E. coli* isolates from pigs in Quebec, Canada over study time.

For each β-lactamase gene the bars marked by different letters were significantly different (P<0.05). Arrows show that the *bla*$_{\text{CTX-M}}$-positive isolates were negative for *bla*$_{\text{CMY-2}}$.

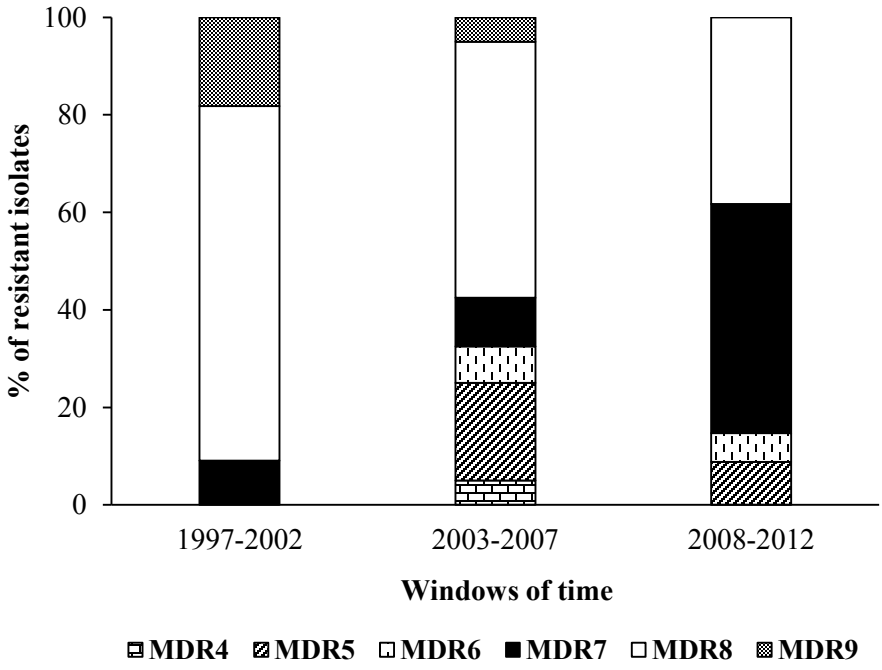

Figure 3. Proportion of different levels of MDR in 85 ESC- resistant clinical *E. coli* isolates from pigs in Quebec, Canada over study time.

MDR4: non-susceptible to at least 1 agent in only 4 antimicrobial categories, MDR5: non-susceptible to at least 1 agent in only 5 antimicrobial categories, etc.

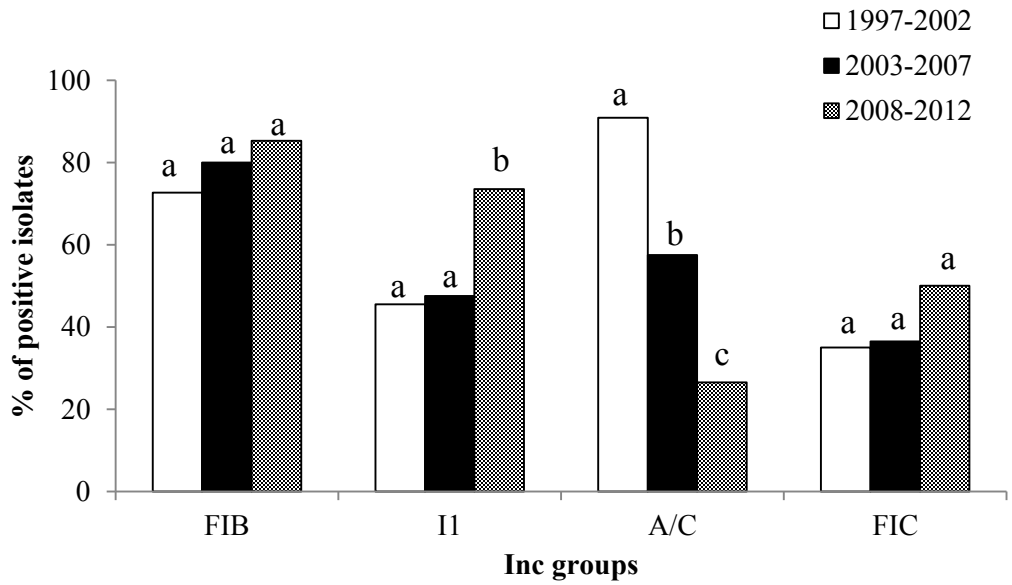

Figure 4. Frequency of the four most predominant Inc groups among clinical *E. coli* isolates from pigs in Quebec, Canada over study time.

For each Inc group the bars marked by different letters were significantly different ($P<0.05$).

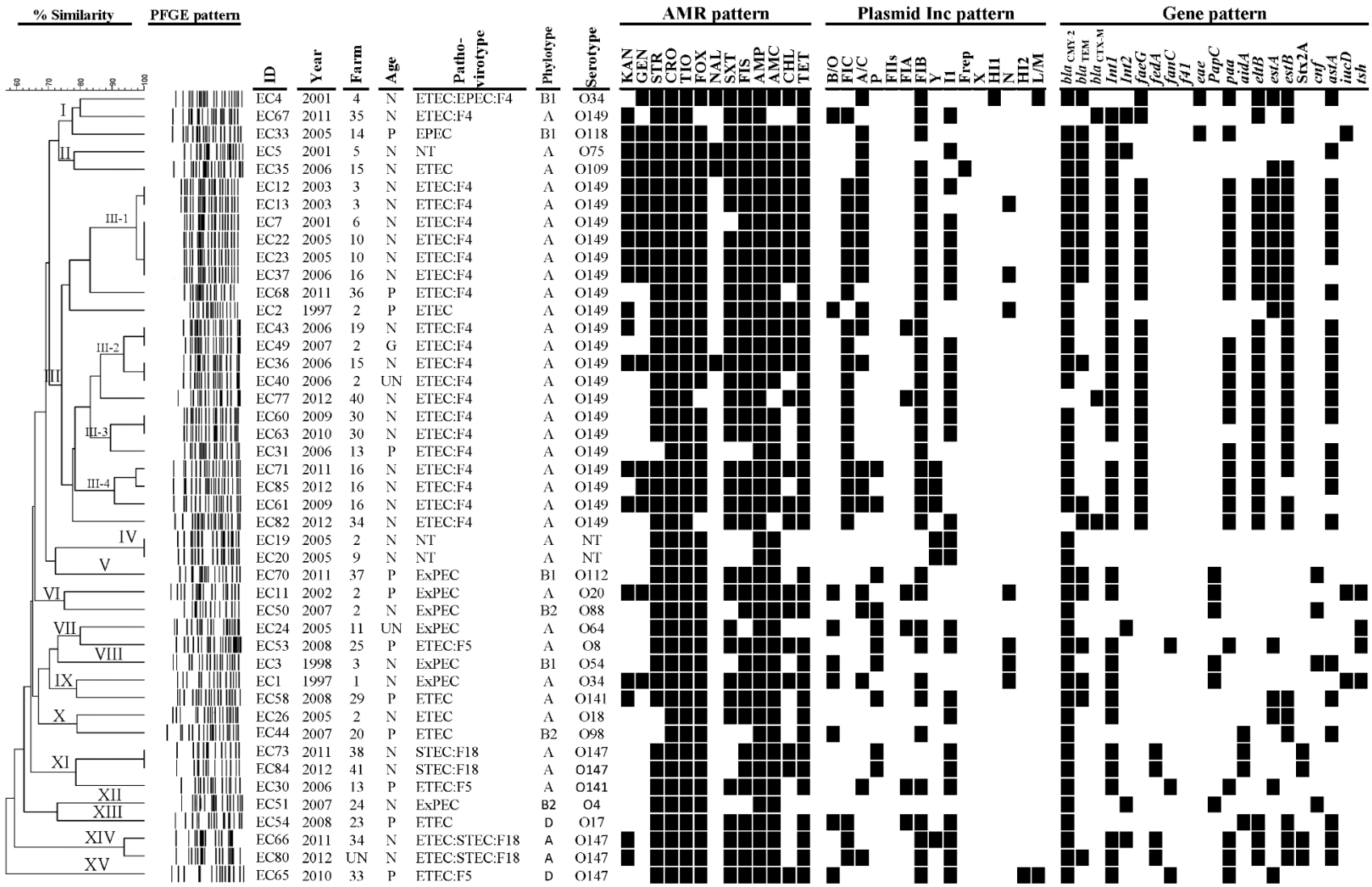
% Similarity
PFGE pattern
ID
Year
Farm
Age
Patho-virotype
Phylotype
Serotype
AMR pattern
KAN GEN STR CRO TIO FOX NAL SXT FIS AMP AMC CHL TET
Plasmid Inc pattern
B/O FIC A/C P FIIs FIA FIB Y I1 Frep X HI1 N HI2 L/M
Gene pattern
bla CMY-2 bla TEM bla CTX-M Int1 Int2 faeG fedA fanC f41 eae PapC pta aidA eltB estA estB Stx2A cnf astA iucD tsh
I II III III-1 III-2 III-3 III-4 IV V VI VII VIII IX X XI XII XIII XIV XV
EC4 2001 4 N ETEC:EPEC:F4 B1 O34
EC67 2011 35 N ETEC:F4 A O149
EC33 2005 14 P EPEC B1 O118
EC5 2001 5 N NT A O75
EC35 2006 15 N ETEC A O109
EC12 2003 3 N ETEC:F4 A O149
EC13 2003 3 N ETEC:F4 A O149
EC7 2001 6 N ETEC:F4 A O149
EC22 2005 10 N ETEC:F4 A O149
EC23 2005 10 N ETEC:F4 A O149
EC37 2006 16 N ETEC:F4 A O149
EC68 2011 36 P ETEC:F4 A O149
EC2 1997 2 P ETEC A O149
EC43 2006 19 N ETEC:F4 A O149
EC49 2007 2 G ETEC:F4 A O149
EC36 2006 15 N ETEC:F4 A O149
EC40 2006 2 UN ETEC:F4 A O149
EC77 2012 40 N ETEC:F4 A O149
EC60 2009 30 N ETEC:F4 A O149
EC63 2010 30 N ETEC:F4 A O149
EC31 2006 13 P ETEC:F4 A O149
EC71 2011 16 N ETEC:F4 A O149
EC85 2012 16 N ETEC:F4 A O149
EC61 2009 16 N ETEC:F4 A O149
EC82 2012 34 N ETEC:F4 A O149
EC19 2005 2 N NT A NT
EC20 2005 9 N NT A NT
EC70 2011 37 P ExPEC B1 O112
EC11 2002 2 P ExPEC A O20
EC50 2007 2 N ExPEC B2 O88
EC24 2005 11 UN ExPEC A O64
EC53 2008 25 P ETEC:F5 A O8
EC3 1998 3 N ExPEC B1 O54
EC1 1997 1 N ExPEC A O34
EC58 2008 29 P ETEC A O141
EC26 2005 2 N ETEC A O18
EC44 2007 20 P ETEC B2 O98
EC73 2011 38 N STEC:F18 A O147
EC84 2012 41 N STEC:F18 A O147
EC30 2006 13 P ETEC:F5 A O141
EC51 2007 24 N ExPEC B2 O4
EC54 2008 23 P ETEC D O17
EC66 2011 34 N ETEC:STEC:F18 A O147
EC80 2012 UN N ETEC:STEC:F18 A O147
EC65 2010 33 P ETEC:F5 D O147

Figure 5. Clustering analysis of 45 ESC-resistant clinical *E. coli* isolates from 1997 to 2012 based on PFGE patterns obtained by digestion of bacterial genomic DNA with XbaI.

The dendrogram was generated using the Dice coefficient and UPGMA. According to a similarity index of ≥75%, ESC-resistant *E. coli* isolates are separated into 15 PFGE clusters (I to XV) and closely related groups of isolates (Dice similarity value ≥90%) are subclusters III-1, III-2, III-3 and III-4 as well as cluster XIV. Abbreviations for antimicrobials are as follows: kanamycin (KAN), gentamicin (GEN), streptomycin (STR), ceftriaxone (CRO), ceftiofur (TIO), cefoxitin (FOX), nalidixicacid (NAL), trimethoprim-sulfamethoxazole (SXT), sulfisoxazole (FIS), ampicillin (AMP), amoxicillin/clavulanicacid (AMC), chloramphenicol (CHL), tetracycline (TET). Black fields with "+" indicate the presence of genes or the resistance to described antimicrobial agents. UN-Unknown. NT-Non-typeable.

Chapter 4: General discussion

General discussion

Administration of antimicrobials in food animal production has been monitored over the past two decades since the emergence of multidrug resistance in bacteria has become one of the most important problems in animal and human medicine. Development of antimicrobial resistance increases the risk of failure of antimicrobial treatment in animals and humans. In particular, the emergence and prevalence of ESC-resistant *E. coli* in food producing animals is of great concern since third generation cephalosporins (e.g. ceftriaxone, ceftiofur) are clinically important β-lactam antimicrobials in human and animal medicine. In *Enterobacteriaceae*, ESC-resistance has been associated with production of AmpC β-lactamases (e.g. CMY-2) and ESBLs (e.g. CTX-M and some OXA) encoded by genes on transferable plasmids. The CTX-M family are important ESBLs in human medicine and have become a threat to public health (Li et al. 2007).

Antimicrobial use as growth promoters, or as preventive or therapeutic agents during animal production can result in the development of AMR. These resistant bacteria and/or resistance genes may spread to humans directly or through food chain, resulting in potential public health risks (Johnston 2001; Wegener 2003). Also, the resistant bacteria are shed into the environment where they may persist for long periods (Chantziaras et al. 2014). Therefore, animal feeds may be supplemented with feed additives as an alternative for the use of antimicrobials or a complement to the antimicrobials. For instance, the clay mineral, clinoptilolite, has been used in an attempt to improve performance and health but also as an alternative to the use of antimicrobials or together with antimicrobials for prevention of post-weaning diarrhea in piglets. However, little is known about the mechanism of action of clinoptilolite. A previous *in vitro* study showed that minerals induce bacterial mutation and increase genetic diversity of bacteria (Yoshida et al. 2004), DNA bound to clay minerals resist more to degradation by DNase I (Romanowski et al. 1991). It has been demonstrated in *in vitro* studies that clay minerals boost horizontal gene transfer of AMR genes in different bacterial species (Lotareva and Prozorov 2000; Rodriguez-Beltran et al. 2013). These data imply that clay minerals may modulate the frequency of AMR and virulence genes of bacteria in the animal intestine. In weaned pigs, antimicrobials are administrated in feed mainly as a medication to prevent disease and thus

reduce mortality and morbidity (Cromwell 2002). Penicillin and tetracycline are among the used in-feed antimicrobials in pigs and often in mixture (Akwar et al. 2008). In-feed antimicrobial use in pigs has been associated with raised resistance of fecal *E. coli* within and between antimicrobials classes (Kim et al. 2005; Akwar et al. 2008).

To shed a light on how feed supplements influence the virulence and AMR dynamics of *E. coli* in the intestinal ecosystem of pigs, we designed a series of experiments and as a first step, we focused on the effect of feed supplementation with clay minerals. We examined the changes in AMR phenotype as well as virulence and AMR gene profiles in *E. coli* isolated from pigs in a commercial farm that received a diet containing chlortetracycline and penicillin G in therapeutic doses following weaning and we investigated the effect of feed supplementation with a clay mineral, clinoptilolite, on this dynamic.

Our results revealed that the frequency of resistant *E. coli* isolates to several antimicrobials (streptomycin, ceftriaxone, ceftiofur, cefoxitin, trimethoprim-sulfamethoxazole, sulfisoxazole, ampicillin, amoxicillin/clavulanic acid, chloramphenicol, and tetracycline) was increased time dependently. This could be a result of continuous usage of medicated feed with chlortetracycline and penicillin G. These results are in good agreement with the data of Akwar et al. (2008) and Kim et al. (2005), who demonstrated that administration of antimicrobials in feed in pigs, may apply a selection pressure resulting in increased resistance of fecal *E. coli* within and between classes of antimicrobials (Kim et al. 2005; Akwar et al. 2008). Furthermore, Looft et al. (2012) showed that use of chlortetracycline, penicillin, and sulfamethazine (known as ASP250) may increase the prevalence of resistance genes not only for the administered antimicrobial but also for antimicrobials of other classes that were not used, likely due to co-selection (Looft et al. 2012). Also, Funk et al. (2006), in a phenotypic resistance study, observed that adding of chlortetracycline in the pig diet has an effect on resistance to multiple antimicrobials, including ampicillin, ceftriaxone and tetracycline, in the gram negative fecal flora of the pig (Funk et al. 2006).

Risk factors for development of AMR in food animal production are complex and it has been demonstrated that antimicrobial use and selection or co-selection pressure may not be the only factors increasing AMR with time in farm conditions. Thus, other

mechanisms such as clonal transmission of resistant *E. coli*, horizontal gene transfer between bacteria, adaptation, maintenance of some sets of resistant strains, transmission from animal to animal and farm conditions could also have been responsible for the development of AMR with time in the present study (Hoyle et al. 2005; Blahna et al. 2006; Sharma et al. 2008; Nilsson et al. 2014; Mazurek et al. 2015).

Interestingly, in our study resistance to all antimicrobials except the cephalosporins and ciprofloxacin was observed in pigs at weaning, before they were given in-feed antimicrobials. The latter antimicrobials are not used in pigs in Canada, with the exception of injectable ceftiofur (CIPARS 2008). Even though the number of strains tested for antimicrobial susceptibility was relatively low, detection of resistance to ceftiofur, which was first found at day 7 and increased to day 28, was unexpected, as ceftiofur was not administrated in this batch of pigs. Further, we confirmed a positive association between ceftiofur-resistance and the presence of *bla*$_{\text{CMY-2}}$ gene in *E.coli* isolated from healthy pigs, as observed in previous investigations (Diarra et al. 2007; Mataseje et al. 2010).

Moreover in our study, the *bla*$_{\text{CMY-2}}$ gene was detected in the pen environment before introduction of pigs and administration of chlortetracycline may have effected subsequent expansion of the *bla*$_{\text{CMY-2}}$ gene. Similarly, in a recent 26 day metagenomic study in cattle, chlortetracycline use led to exacerbation of the number of *bla*$_{\text{CMY-2}}$ gene copies/gram of feces following ceftiofur therapy (Kanwar et al. 2014). Further, Volkova et al. (2015) showed that a low but stable fraction of ceftiofur-resistant *E. coli* isolates could persist in the absence of ceftiofur pressure, being transmitted by horizontal and vertical transfer of plasmids harbouring AMR genes, and ingestion of resistant bacteria (Volkova et al. 2012). In addition, our results showed that resistance to chloramphenicol increased up to 50% although this antimicrobial has not been used in pig production in Canada since the 1980s and florfenicol was not used in this batch. This is in consistent with Harada et al. who reported that co-selection with other registered antimicrobials in pig production, dihydrostreptomycin (DSM) and trimethoprim (TMP) apparently contributes to the selection and persistence of chloramphenicol resistant *E. coli* (Harada et al. 2006). Furthermore, it has been demonstrated that the gene conferring resistance to chloramphenicol (*catA1*, *cmlA*, *floR*) co-carried on the IncA/C plasmids with the genes encoding resistance to other antimicrobials that are approved for administration in food

animal such as *qacEΔ1-sul1*, *sul2*, *aadB-bla*$_{\text{OXA-21}}$, *aadA1*, *tetA*, *tetR*, *bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM}}$ and *Int1* genes (Fernandez-Alarcon et al. 2011; Carattoli et al. 2012; Doublet et al. 2012). Also, Diarra et al. (2007) reported that, in the absence of antimicrobial selection pressure, certain resistance genes can be conserved due to a link with the genes encoding resistance to other antimicrobials that are approved for administration in food animal (Diarra et al. 2007). In our study, almost all ESC-resistant *E. coli* strains were co-resistant to streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, amoxicillin/clavulanic acid, ampicillin, chloramphenicol, and tetracycline. The association on the same plasmid of *bla*$_{\text{CMY-2}}$ gene and genes conferring resistance to non-beta-lactam antimicrobials and co-transmission of resistance to non-beta-lactam antimicrobials with CMY-2 have been shown previously (Doublet et al. 2004; Blanc et al. 2008).

The second objective of our first step in this study was to provide insights regarding the effect of clinoptilolite, as a feed supplementation, on the dynamic of AMR phenotype, virulence and AMR gene profiles in *E. coli* from pigs receiving in-feed antimicrobial medication following weaning. According to our understanding, this is the first study to investigate the feed supplementation with clay minerals in controlling the spread of AMR and virulence genes in *E. coli in vivo* in pigs in farm conditions. We showed that feed supplementation with clinoptilolite was associated with an early temporary increase in the frequency of the gene responsible for ESC-resistance, *bla*$_{\text{CMY-2,}}$ in the supplemented group *E. coli* isolates. On the other hand, a delayed increase in the frequency of *bla*$_{\text{CMY-2}}$, or one of the virulence genes *iucD* and *tsh*, was found in the control group *E.coli* isolates. The initial temporary increase in frequency of isolates carrying *bla*$_{\text{CMY-2}}$ in the supplemented group is in agreement with data from Rodriguez-Beltran et al. (2013) who described that clay minerals (e.g. sepiolite) boost direct horizontal gene transfer (HGT) between bacterial species in *in vitro* conditions, which may be exacerbated by antimicrobial use as growth promoters. In addition, these authors demonstrated that the increase of HGT was due to plasmid transfer in certain conditions such as presence of septiolite (final concentration of 100 µg/ml) and friction forces. Friction forces may have been provided by peristalsis in the intestine of the pigs receiving continuously clay mineral as a feed supplementation (Rodriguez-Beltran et al. 2013). On the other hand, the subsequent significant decline in the proportion of *bla*$_{\text{CMY-2}}$-positive *E. coli* isolates, and the lack of increase in the frequency

of isolates carrying the plasmid-encoded virulence genes *iucD* and *tsh* in the supplemented group could be associated with accumulation of clinoptilolite in the gut of pigs over time after weaning which may lead to a decrease in the HGT as clay minerals effect on HGT is concentration depended. For instance, previous *in vitro* studies demonstrated that higher concentrations of clay minerals result in a decrease in the number of transformed cells, in other words, a reduction in HGT (Richaume et al. 1989; Lotareva and Prozorov 2000). Alternatively, the later decrease in the proportion of $bla_{\text{CMY-2}}$-positive isolates observed in the supplemented group could be due to a direct or indirect antimicrobial activity of clinoptilolite or an interaction between clinoptilolite and chlortetracycline or penicillin G. The potential of clay minerals to reduce the number of pathogenic bacteria has been shown *in vivo* and *in vitro* (Haydel et al. 2008; Vondruskova et al. 2010). Besides, Wu et al. (2013) reported that the total number of *Lactobacillus acidophilu*s bacteria was significantly increased from days 22 to 42 in broiler chickens fed with clinoptilolite and also they showed that small intestinal and cecal PH values in the treatment group were significantly lower than those in the control group, Besides, these authors showed that total viable counts of *E. coli* were significantly decreased by clinoptilolite (Wu et al. 2013). As probiotics containing L. acidophilus may eliminate growth of pathogenic bacteria by production of organic acids and antibiotic-like compounds (Lordelo et al., 2007; Vondruskova et al., 2010), an increase in L. acidophilus numbers due to the presence of clinoptilolite in the current study may have led to the later decrease in the proportion of $bla_{\text{CMY-2}}$, *iucD*, and *tsh*-positive isolates. Whereas the possible interaction of clay minerals and antimicrobials is controversial, nonetheless, a potential interaction between clinoptilolite and some antimicrobials has been described. For instance, chlortetracycline, tylosin, and metronidazole can be absorbed strongly to clinoptilolite or other clay minerals, in *in vitro* conditions. The interference is probably related to polarity of the antimicrobial molecules, since no interaction was confirmed with sulfamethoxazole and β-lactams, which are highly polar compounds (Papaioannou et al. 2005; Allaire et al. 2006).

So far, our findings showed an increased resistance to 10 antimicrobials, both within and between classes respecting to chlortetracycline or penicillin G used for feeding pigs following weaning. We observed that feed supplementation with clinoptilolite could decrease the frequency of *E. coli* carrying $bla_{\text{CMY-2}}$ and certain virulence genes at least in

the first month after weaning. However the mechanism(s) of such influence was still unclear. To clarify, we expanded our study and investigated the mechanisms leading to an increase in the prevalence of *bla*$_{CMY-2}$ conferring resistance to ceftiofur in a pig nursery barn in a batch of pigs receiving a feed medicated with chlortetracycline and penicillin G but not having received ceftiofur and studied the effect of clinoptilolite on this phenomenon. To do so, we characterized the *bla*$_{CMY-2}$-positive isolates from this batch of pigs to investigate the relative role of bacterial clonal lineages and plasmids in the spread of *bla*$_{CMY-2}$ gene in pigs and their environment.

First we studied the genetic relationship of *bla*$_{CMY-2}$-positive *E. coli* isolates resistant by PFGE typing, phylotyping and O serogrouping. PFGE analysis revealed heterogeneity among the studied isolates and displayed seven distinct clonal lineage among the 36 *bla*$_{CMY-2}$-positive *E. coli* isolates examined based on a similarity of ≥90%. This is in accordance with previous studies which demonstrated CMY-2-producing *E. coli* from livestock to be highly polyclonal (Daniels et al. 2007; Hiki et al. 2013; Guo et al. 2014). Furthermore, PFGE patterns with 100% similarity between at least 2 isolates were found. This suggests that *bla*$_{CMY-2}$ spreads both horizontally and clonally in our study, and some clonal lineages are more common than others. Despite the number of tested isolates for clonal analysis by PFGE was relatively low, taken together, the phylotyping and PFGE results reveal that that the *bla*$_{CMY-2}$ -positive *E. coli* isolates were more clonally diverse in the control group than the supplemented group. In contrast, Bednorz et al. (2013) showed higher diversity of multi-resistant *E.coli* in zinc supplemented group in comparison with the control group (Bednorz et al. 2013). Moreover, *bla*$_{CMY-2}$-positive *E. coli* genotypes were diverse and affected by sampling time within both groups in our study , likely as a result of continuous use of chlortetracycline and penicillin G in the feed. This is in agreement with Alexander et al. (2009) who showed a clear effect of treatment and time on genetic diversity of resistant *E.coli* in fecal samples from cattle (Alexander et al. 2009). In the current study, phylotypes A, B1 and D were found among the *bla*$_{CMY-2}$-positive *E. coli* isolates as was also observed in a previous study in food animals (Guo et al. 2014). It has been shown that phylotype D *E.coli* is related to disease in humans such as urinary tract infections (Johnson et al. 2005; Jakobsen et al. 2010).

Moreover, our results showed that certain clonal lineages became predominant. The clonal lineage phylotype A predominated in the supplemented group, whereas the clonal lineages phylotype B1 predominated with time, especially in the control group, the latter often positive for the ExPEC-virulence gene *iucD*. Furthermore, in our initial study where we studied a large collection of *E. coli* isolates, we had seen an increase in the proportion of *iucD* gene with time, especially in the control group (Jahanbakhsh et al. 2015). This finding could support the speculation of Mathers et al. (2015) that some ExPEC virulence genes such as *sat*, *iutA*, *malX*, *usp*, *iha*, *hra*, and *ompT* may play a part in the fitness and adaptation of ST131 *E. coli* isolates (Mathers et al. 2015).

In our study, *bla*$_{\text{CMY-2}}$-carrying plasmids of Inc groups, I1, A/C and ColE were observed in transformants. Similarly, several studies have reported that IncI1 and IncA/C are the most frequent *bla*$_{\text{CMY-2}}$-positive plasmids in humans and animals worldwide (Carattoli 2009; Mataseje et al. 2010; Martin et al. 2012; Hiki et al. 2013). We showed a high frequency of IncI1 carrying *bla*$_{\text{CMY-2}}$ plasmids in transformants on all sampling days in the present study as described in a previous study over time in broilers (Agerso et al., 2014). We found that IncI1 plasmids carrying *bla*$_{\text{CMY-2}}$ confered resistance only to beta-lactam antimicrobials, including ceftiofur, ceftriaxone, cefoxitin, ampicillin, amoxicillin/clavulanic acid as previosly shown (Folster et al. 2011; Martin et al. 2012; Hiki et al. 2013). The high frequency and persistence of IncI1 *bla*$_{\text{CMY-2}}$-positive plasmids and resistance to ESCs in the absence of ceftiofur use could be a result of continuous administration of penicillin G in the feed. Similarly, Cameron-Veas et al. (2015) found that amoxicillin use resulted in an increase in the frequency of cephalosporin resistant-*E. coli* in pigs (Cameron-Veas et al. 2015) and Agerso et al. reported that administration of aminopenicillins affected the persistence and spread of ESC-producing *E. coli* in broiler production (Agerso et al. 2014).

Furthermore, a possible source of the *bla*$_{\text{CMY-2}}$ gene for a new batch of weaned pigs could be the presence of this gene in the pen environment before animal arrival and inadequate pen sanitation may allow the *bla*$_{\text{CMY-2}}$ gene to persist in the environment from previous batches. We recovered *E. cloacae* isolates, carrying *bla*$_{\text{CMY-2}}$ encoding IncA/C plasmids, from the pens before introduction of the pigs of this batch. Notably, Laanen et al. (2013) described and quantified a clear link between biosecurity measure and

antimicrobial treatment-related criteria in pig herds (Laanen et al. 2013). In addition, we confirmed the transferability of IncA/C plasmid harbouring *bla*$_{CMY-2}$ from *E. cloacae* to *E. coli* DH5α *in vitro* by conjugation, being a natural horizontal gene transfer between bacteria which thus could have occurred on this pig farm. Similarly, transfer of AmpC beta-lactamase plasmids and AMR genes between *E. coli* and *Salmonella* among humans and animals has been shown (Winokur et al. 2001; Blake et al. 2003). In addition, dissemination between sows and piglets has been demonstrated, sow treatment with ceftiofur may lead to development of resistant *E. coli* isolates which are transferred to piglets. The inability to determine resistance to ceftiofur at day 0 in this study could be due to the low level of this phenotype in piglets initially and effect of selection pressure of antimicrobials over time (Thompson et al. 2008; Schierack et al. 2009; Volkova et al. 2012). In the present study, the most frequently observed *bla*$_{CMY-2}$ encoding plasmid was IncI, while only IncA/C *bla*$_{CMY-2}$ encoding plasmids were detected in *E. cloacae* isolates from the pens before introduction of the pigs. There is posibility of mobilization of the plasmid-borne *bla*$_{CMY-2}$ gene from IncA/C to IncI1. Yassine et al. (2015) showed the critical role of IS1294b (IS91 family) in the mobilization of the *bla*$_{CMY-2}$ gene from IncA/C to IncI1 in *Enterobacteriaceae* and its contribution to the evolution of diverse incompatibility group plasmids (Yassine et al. 2015).

Seven AMR patterns were found among *bla*$_{CMY-2}$-positive *E. coli* isolates, all being multidrug resistant. Particular AMR patterns were found in certain clonal lineage, with those of phylotype D showing a unique AMR pattern. Similar AMR patterns were reported in a previous study (Mulvey et al. 2009). In our study, two main serogroups O83 and O101 were detected O83 in phylotype D which has been related with porcine ExPEC isolates and O101 in phylotype A which has been associated with ETEC and ExPEC isolates in pigs (Gyles and Fairbrother 2010). Likewise, serogroup O83 has been associated with urinary tract infections (Johnson et al. 1994; Yamamoto 2007; Abe et al. 2008) In addition, Girardeau et al. (2003) demonstrated that serogroup O101 isolates caused extraintestinal infections in humans and cattle (Girardeau et al. 2003).

A limiting factor in this part of our study was the fact that the number of tested *bla*$_{CMY-2}$-positive *E. coli* isolates for clonal analysis by PFGE was relatively low (n=36). All details and criteria for isolates selection were described in materials and methods

section in article 2. However, phylogenetic grouping was performed on 138 *bla*$_{CMY-2}$-positive *E. coli* isolates and our conclusions are based on 138 strains selected from the 400 *bla*$_{CMY-2}$-positive *E. coli* isolates found in over 7000 total isolates initially examined, as well as the 36 isolates which we examined in more detail. Also, this study has relied on phylogenetic grouping definition of *E. coli* as was described by Clermont et. al. (2000) via profiling three target genes (Clermont et al. 2000). However, the same group improved their definitions recently by using four target as well as some complementary genes (Clermont et al. 2013). According to older version that was the only available definition at the time of this study, *E. coli* isolates fall into four groups A, B1, B2 and D however the most recent definition categorizes the *E. coli* isolates into seven groups A, B1, C, E, D, F, B2. However, phylotype A and B1 that are implied in our report as important phylotypes to differ between control and supplemented groups, are not really different in definition with respect to old and new methods. Addintionally, we studied genetic relatedness of *E. coli* isolates by performing PFGE which is a gold standard fingerprinting method.

The second step of our study on healthy pigs of a commercial farm revealed that ESC-resistant *E.coli* isolates belonged to several different clonal lineages with diverse AMR patterns and possessed the *bla*$_{CMY-2}$-carrying plasmids of the Inc groups I1, A/C, and ColE. Our results suggest that the *bla*$_{CMY-2}$ gene spread both horizontally and clonally in these pigs and may have spread from previous batches of pigs via plasmids carried by *E. cloacae* and expanded in animals of the present batch in the presence of the selection pressure due to administration of chlortetracycline and penicillin G in the feed. Feed supplementation may have an effect on clonal diversity of *bla*$_{CMY-2}$ -positive isolates. Based on our results we infer that implementation of improved hygiene measures, decreased use of certain antimicrobials on farm, and feed supplementation with a clay mineral may limit antimicrobial resistance spread between and within batches of animal.

In the third step of our study, we studied *E. coli* clinical isolates in a wider range of farms over a 16-year period of time from 1997 when we first observed resistance to ceftiofur in clinical isolates. The aim of this step was to investigate the temporal characterization of ESC-resistant pathogenic *E. coli* with respect to pathogenic clonal lineages, plasmids and antimicrobial resistance.

In this study, PFGE clonal analysis demonstrated high diversity and revealed 36 distinct clonal lineages among the 45 ESC-resistant *E. coli* isolates examined and given a similarity of 75% isolates were grouped into 15 clusters. However, half of the isolates belonged to a single cluster which revealed the same pathovirotype, O serogroup and phylotype. Similarly, previous studies reported that ESC-resistant *E. coli* from livestock were highly polyclonal (Donaldson et al. 2006; Daniels et al. 2007; Hiki et al. 2013).

Identical clonal lineages of ETEC:F4 were found in different years in the same farm indicating the persistence of certain clonal lineages between batches. Several identical clonal lineages were seen from multiple farms, suggesting potential transmission among the farms. In addition, those clonal lineages may have originated from a single source (e.g. same breeding pig farm), Similarly, Agerso et al. (2014) and Nilsson et al. (2014) have shown that the same *bla*$_{\text{CMY-2}}$ –positive *E. coli* clones were observed in imported grandparent and in all levels of the Danish and Swedish broiler production, suggesting spread of ESC-producing *E. coli* clone and plasmids from imported parent animals to broilers and to broiler meat (Agerso et al. 2014; Nilsson et al. 2014). In our study, transmission may have happend because of imported and exported pigs, and also by the movement of water and wild animals and/or through contaminated food (Yamamoto et al. 2014). It is good to mention that currently only 44% of farms in Quebec are «farrow-to-fattening», that is, on a single site, as compared to 33% fattening and 23% farrowing farms which together constitute multisite farms. Transport of pigs between these sites would result in a more widespread spreading of clonal lineages (Lacroix and Morin 2015). Likewise, as previously described, *E. coli* could be transmitted farm to farm by vehicles such as transport trucks and visitors on farms (truck drivers, veterinarians, producers, technicians) or by farm workers (Lim et al. 2010; Abraham et al. 2015).

Heterogeneity among ESC-resistant isolates was observed, indicating that their spread is not only due to the dissemination of successful *E. coli* clonal lineages, and horizontal gene transfer may be contributing to the emergence of ESC-resistance in pigs as described by others (de Been et al. 2014). In our study a remarkable finding was that, among ETEC:F4, O149 *E. coli* isolates, the more virulent *E. coli* clonal lineages with the virotype LT:STa:STb:F4 were mostly observed prior to 2006 (subcluster III-1) whilst from 2006 to 2012 most clonal lineages showed the virotype LT:STb:F4 (subclusters III-2,3,4).

In agreement with previous studies the changes were observed in O149 clonal lineages with time in this study probably reflect overall changes in the pathogenic population, in that an overall predominance of LT:STa:STb:F4 isolates was found in the early 2000s (Noamani et al. 2003; Fairbrother et al. 2005; Leclerc et al. 2007).

Interestingly, the plasmid IncA/C was mostly observed prior to 2006 in ETEC:F4 O149 clonal lineages of subcluster III-1, however it re-emerged in 2009-2012 clonal lineages (III-4) on one particular farm. In 85 ESC-resistant *E. coli*, IncA/C was significantly associated with resistance to gentamicin, kanamycin, streptomycin, trimethoprim-sulfamethoxazole, sulfisoxazole, chloramphenicol and tetracycline, and the presence of bla_{TEM} and *Int1*(Johnson et al. 2012). Also, IncA/C plasmids carrying bla_{TEM} and *Int1* and genes conferring resistance to these antimicrobials have been demonstrated (Fernandez-Alarcon et al. 2011; Carattoli et al. 2012; Doublet et al. 2012). The decrease in resistance to gentamicin, kanamycin and chloramphenicol in ESC-resistant *E. coli* over the study time could be a result of less administration of these antimicrobials in pig production. The ban on the administration of chloramphenicol in food animals in Canada since the 1980s could result on the decrease in resistance to chloramphenicol over time. Likewise, a tendency to less prescribing of gentamicin has recently been observed based on anecdotal evidence from local authorities and veterinarians. On the other hand, kanamycin is not used in pig production in Canada. As previously shown, the emergence and prevalence of antimicrobial resistance in bacteria are likely to be linked to antimicrobial use in animals through cross-resistance or co-resistance (Harada and Asai 2010). It can also be related to administration of disinfectant and usage of metals like copper and zinc as feed additives (Bednorz et al. 2013).

In the current study, we found a relationship between certain pathotypes and plasmid Inc types. Our finding that ETEC:F4 was positively associated with IncFIB and IncFIC but negatively with IncB/O and IncP confirms the observations in a recent study of pig isolates in Australia (Abraham et al. 2014). Moreover, ExPEC showed a positive association with the presence of IncB/O and negative association with the presence of IncI1 and IncFIC groups. Johnson et al. described a similar trend in human ExPEC strains (Johnson et al. 2008). Future investigations will further look at the relative role of pathogenic clonal lineages and the various plasmids in the spread of the $bla_{\text{CMY-2}}$ gene.

This study showed the recent emergence of multidrug resistant CTX-M-producing ETEC:F4 *E. coli* isolates in pigs in 2011-2012. Since *bla*$_{\text{CTX-M}}$ was detected in distinct clonal lineages, the spread of *bla*$_{\text{CTX-M}}$ may have occurred by horizontal transmission. To our knowledge, this is the first report of *bla*$_{\text{CTX-M}}$- positive, ESLB-producing pathogenic *E. coli* isolates from pigs in Quebec, Canada. Although the presence of *bla*$_{\text{CTX-M}}$ gene in *E. coli* isolated from pigs has been reported from other countries (Hansen et al. 2013; Hu et al. 2013; Freire Martin et al. 2014).

In the current study, the ESC-resistant *E. coli* isolates belonged to phylotypes A, B1, B2 and D, phylotype A being the most predominant one, as with previous studies in food producing animals (Tan et al. 2012; Hu et al. 2013; Marchant et al. 2013; Rao et al. 2014). Three distinct phylotype B2 clonal lineages were found which were ExPEC O4 and O88 and ETEC O98. As previously described, phylotype B2 and D *E.coli* are related to disease in humans such as urinary tract infections (Johnson et al. 2005; Jakobsen et al. 2010). In addition, O4 serogroup is urinary tract infection (UTI)-associated (Johnson et al. 1994; Yamamoto 2007; Abe et al. 2008) suggesting a zoonotic potential (Tan et al. 2012).

Initially, 85 ESC-resistant *E. coli* isolates were clustered based on tested genes. Gene clustering analysis separated ESC-resistant *E. coli* isolates according to O serogroup and revealed similar comparison results to those of PFGE. Our findings suggested that the gene panel used in this study may be appropriate to study genetic relatedness in large numbers of isolates.

The results were seen in this study are based on our collection that includes 91% pathogenic *E.coli* from clinical cases. Nevertheless, the discussed patterns could be different in commensal *E.coli* as was described by Boerlin et al (2005) who showed that AMR differs significantly between pathogenic and commensal *E. coli* isolates (Boerlin et al. 2005).

We recognized that in this study the small sample size (compared to similar studies) may have limited our ability to subsequently generalize our results. However, in EcL collection of *E. coli* strains (our only source), we only identified 85 ceftiofur-resistant *E. coli* isolates over the study time (1997-2012) that all were included in this report. Among these isolates, some were from the same farm at the same year with the same patterns of tested traits, so we had to eliminate them before performing PFGE that further

reduced the number of isolates to almost 50% (n=45) of total which were representative for all years, pathovirotypes, phylotype, farms, production phase (age) etc. However, despite this limitation of the study, our findings provide a significant advance in our understanding of the temporal molecular epidemiology of ESC-resistant *E.coli*. Similarly to previous part of our study, the phylogenetic grouping method may be considered as another study limitation. However, we investigated genetic relationships of ESC-resistant *E. coli* isolates by PFGE.

In last step of our study, our findings reveal that ESC-resistance was mostly associated with the *bla*$_{\text{CMY-2}}$ gene and that the *bla*$_{\text{CTX-M}}$ gene has recently emerged. ESC-resistant *E.coli* are polyclonal with diverse AMR patterns and often carried plasmids of several Inc groups. However, there was no predominant clonal lineage over the study time. Further, we confirmed that some identical clonal lineage were present at multiple farms in the same and different years. Further, the *bla*$_{\text{CMY-2}}$ spreads both horizontally and clonally in this study and the spread of *bla*$_{\text{CTX-M}}$ gene may have occurred by horizontal transmission. Indeed, Resistance to gentamicin, kanamycin, chloramphenicol, and the frequency of *bla*$_{\text{TEM}}$ and IncA/C plasmid significantly decreased over the study of time. Whereas, IncI1 and MDR7 significantly increased over the period of time. To our understanding, this is the first study describing characteristics of ESC-resistant *E.coli* isolated over a long period. In addition, we believe this to be the first report of *bla*$_{\text{CTX-M}}$ positive, ESLB-producing pathogenic *E. coli* strains from pigs in Quebec, Canada. Our findings underline the importance of monitoring *E. coli* isolates from pigs for the presence of *bla*$_{\text{CTX-M}}$ in the future since they could be transmitted to humans via the food chain or by direct contact. The CTX-M family are important ESBLs in human medicine and a threat to public health. Widespread occurrence of antimicrobial resistance plasmids in ESC-resistant *E. coli* from the pig is a concern for animal and public health and warrants further attention.

Chapter 5: General Conclusion and Future perspective

General conclusion

In conclusion, the studies reported in this thesis reveal an increased level of resistance, lasting at least 4 weeks, to several antimicrobials in *E. coli* from weaned pigs with medicated feeding. It is also shown that feed supplementation with clinoptilolite decreases the proportion of fecally shed *E. coli* possessing certain virulence and antimicrobial resistance genes, at least in the first month after weaning. In addition, horizontal and clonal spread of $bla_{\text{CMY-2}}$ suggest that implementation of improved hygiene measures and decreased administration of certain antimicrobials on farm, and feed supplementation with certain ingredients may limit antimicrobial resistance spread between and within batches of animals. By studying the temporal characterization of ESC-resistant pathogenic *E. coli* with respect to pathogenic clonal lineages, plasmids and antimicrobial resistance in wider range of farms over 16 years, we have shown herein the importance of monitoring *E. coli* isolates from pigs for the presence of $bla_{\text{CTX-M}}$, with regards to CTX-M family as an important ESBL in human medicine, since their transmission to humans via the food chain or by direct contact is a threat to public health. It was also inferred that the widespread occurrence of antimicrobial resistance plasmids in ESC-resistant *E. coli* from pigs, as observed in our studies, is a concern for animal and public health and warrants further attention.

Future perspective

1- Regarding the impact of feed supplementation with clinoptilolite, since in farm conditions various factors may contribute, further studies involving more pigs from different farms and a longer time after weaning will be required to confirm our findings over the long term.

2- To gain insight into evolution of ESC- resistant *E.coli* over time, further investigations are needed using Multilocus sequence typing (MLST) and/or whole genome sequencing technology for phylogeny of ESC-resistant *E.coli* isolated from pig farms in Quebec, Canada to compare worldwide. Moreover, plasmid classification can be carried out by PCR-based plasmid replicon typing on transformants or conjugants and/or plasmid sequencing.

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