Université de Montréal

Potential pathogenicity and antimicrobial resistance of *Escherichia coli* from pig and poultry feces on-farm and carcasses at the abattoir in Vietnam

par THU MINH PHAM

Département de pathologie et microbiologie,

Faculté de médecine vétérinaire

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SUMMARY

Résumé

E. coli avec potentiel zoonotique pourrait éclore dans les réservoirs porcins et avicoles. Cette étude consiste à examiner la présence de souches *E. coli* porteuses de gènes virulents associés aux STEC (*E. coli* producteurs de Shiga-toxines), EPEC (*E. coli* entéropathogène), et ExPEC (*E. coli* pathogène extra-intestinal) chez les porcs et volailles élevés au Vietnam. Des prélèvements d'excréments et de carcasses ont été effectués dans des fermes et abattoirs porcins et avicoles sélectionnés où les animaux ont été suivis de l'élevage à l'abattage. Un total de 13,1% des souches, toutes sources confondues, ont été catégorisées comme potentiellement contaminées par ExPEC, possédant un ou plusieurs gènes de virulence *iucD*, *tsh*, *papC* et *cnf*. Peu d'isolats d'autres pathotypes ont été observés. Tous les gènes de virulence ExPEC, à l'exception de *cnf,* ont été identifiés plus fréquemment dans les isolats de fèces et carcasses avicoles que dans les isolats porcins. Même constatation pour le groupe du phylogénétique D. Une multirésistance aux médicaments a été régulièrement observée chez les deux isolats ExPEC. Les isolats de fèces de volailles ont souvent été associés à une résistance à l'acide nalidixique et à la ciprofloxacine ($P<0.05$), de même qu'au gène bla_{TEM}, alors que les gènes *qnr* et *aac(6')-Ib* ont peu été rencontrés des deux côtés. Cette étude démontre que les isolats ExPEC avicoles sont potentiellement plus pathogèniques que ceux porcins et que les isolats ExPEC de carcasses porcines et avicoles peuvent provenir de leurs excréments par la contamination associée au processus d'abattage. Ainsi, la volaille, particulièrement, serait un facteur de transmission de souches ExPEC zoonotiques.

Mots clé : résistance antimicrobienne, gènes virulents, porcs et volailles, fermes, abattoirs, carcasses, ExPEC, PCR.

Summary

Zoonotic potential pathogenic *Escherichia coli* could arise from poultry and pig reservoirs. The aim of this study is to investigate the occurrence of *E. coli* strains carrying virulence genes associated with STEC (Shiga toxin-producing *E. coli*), EPEC (Enteropathogenic *E. coli*), and ExPEC (Extraintestinal pathogenic *E. coli)* in pigs and poultry on-farm and at abattoirs in Vietnam. Samples of feces and carcasses were collected at selected pig and poultry farms and abattoirs, in which animals were traced from farms to the abattoir. A total of 13.1% strains from all sources were classified as potential ExPEC, possessing one or more virulence genes *iucD*, *tsh*, *papC* and *cnf*. Few isolates of other pathotypes were observed. All ExPEC virulence genes, except *cnf*, were more frequently found in isolates from poultry than in isolates from pigs. A higher proportion of ExPEC isolates belonging to phylogenetic group D was observed in poultry. Multi-drug resistance was frequently observed in ExPEC isolates from both pigs and poultry. Nalidixic acid and ciprofloxacin resistance were significantly associated with poultry feces isolates ($P<0.05$). bla_{TEM} gene was more frequently associated with poultry isolates, whereas *qnr* and *aac(6')-Ib* genes were present at low prevalence in pig and poultry isolates. This study demonstrates that poultry ExPEC isolates are potentially more pathogenic than pig ExPEC isolates, and ExPEC isolates in pig and poultry carcasses may originate from pig and poultry feces, due to contamination associated to slaughtering process. Thus, meats particularly from poultry, might be a vehicle for transmission of zoonotic ExPEC strains.

Key words: antimicrobial resistance, virulence genes, pigs and poultry, farms, abattoirs, carcasses, ExPEC, PCR.

TABLE OF CONTENTS

SUMMARY ii
Résumé iii
Summary iv
TABLE OF CONTENTS v
LIST OF TABLES viii
LIST OF ABBREVIATIONS ix
ACKNOWLEDGEMENTS xiii
INTRODUCTION 1
LITERATURE REVIEW 4
1. Classification of *E. coli* 5
1.1. Commensal *E. coli* 5
1.2. Intestinal pathogenic *E. coli* 5
1.2.1.Enteropathogenic *E. coli* (EPEC) 6
1.2.2. Shiga toxin-producing *E. coli* (STEC) 7
1.2.3. Enterotoxigenic *E. coli* (ETEC) 8
1.2.4. Enteroaggregative *E. coli* (EAEC) 8
1.3. Extraintestinal pathogenic *E. coli* (ExPEC) 9
2. Diseases caused by ExPEC in humans 10
2.1. Urinary tract infections 10
2.2. Neonatal meningitis 11
2.3. Sepsis 11
3. Virulence factors of pathogenic *E. coli* 12
3.1. Adhesins 12
3.1.1. Type 1 fimbriae 13
3.1.2. P fimbriae 13
3.1.3.Temperature sensitive hemagglutinin 15
3.2. Capsules (K antigen) 16
3.3. Lipopolysaccharide 16
3.4. Iron acquisition systems 17
3.5. Cytotoxin: 18

3.5.1.Hemolysin 18
3.5.2 Cytotoxic necrotizing factor 18
4. Association among virulence factors and phylogenetic groups 19
4.1. Associations among virulence factors 19
4.2. Association of phylogenetic groups and virulence factors 20
5. Antimicrobial resistance (AMR) of *E. coli* 21
5.1. Mechanisms of antimicrobial resistance 22
5.2. Antimicrobial use and emergence of antimicrobial resistance in food animals 23
5.2.1. Antimicrobial use in animal production 23
5.2.2.The trend in antimicrobial resistance in *E. coli* isolates from food-producing animals 24
5.3. Resistance of *E. coli* to category I antimicrobials 25
5.3.1. Resistance to fluoroquinolone 25
5.3.2. Resistance to cephalosporins 26
5.4. Resistance of *E. coli* to category II antimicrobials 27
5.4.1. Resistance to penicillins 27
5.4.2. Resistance to trimethoprim/sulfamethoxazole 27
5.5. Resistance to category III antimicrobials 28
5.5.1. Resistance to tetracyclines 28
5.5.2. Resistance to streptomycin 29
6. Multi-antimicrobial resistance among *E. coli* 29
6.1. Multidrug resistance associated with ESBLs 29
6.2. Multi-resistance associated with PMQR 30
7. Association between virulence and antimicrobial resistance of ExPEC 30
8. Food animals as a reservoir of extraintestinal pathogenic *E. coli* in humans 31
8.1. ExPEC in poultry 32
8.2.ExPEC in pigs 34
8.3 Prevalence of ExPEC in food derived from animals 35
9. Molecular methods for determination of pathogenic *E. coli* 37
9.1. General information on PCR 37
9.2. Multiplex PCR for identification of E. coli pathotype 37
9.2.1. Multiplex PCR for identification of intestinal pathogenic *E. coli* 37
9.2.2. Multiplex PCR for identification of ExPEC 38

9.3. Multiplex PCR for determination of the *E. coli* phylogenetic group 38
9.4. Other techniques for identification of pathogenic E. coli 39
10. Rationale and objectives of the study 40
METHODOLOGY AND RESULTS 41
ARTICLE IN PREPARATION 43
DISCUSSION 65
CONCLUSION 76
REFERENCE LIST 79
ANNEXES 95
Annex 1 List of samples of poultry feces and carcasses collected in Vietnam i
Annex 2 List of samples of pig feces and pig carcasses collected in Vietnam ii
Annex 3. Association of virulence genes and antimicrobial resistance among 132 potential ExPEC isolates iii
Annex 4. Association of phylotypes and antimicrobial resistance among 132 potential ExPEC isolates iv
Annex 5. Association of phylotypes and antimicrobial resistance genes among 77 potential ExPEC isolates from different sources v

LIST OF TABLES

ARTICLE IN PREPARATION

Table I. List of primers used in the PCR, single PCR conditions, and control strains 58

Table II. Virulence genes (VGs) associated with pathotypes of *E. coli* from pigs and poultry on farms and at abattoirs.. 60

Table III. Distribution according to phylogenetic group and virulence gene profile of ExPEC from pigs and poultry on farms and at abattoirs.. 61

Table IV. Antimicrobial resistance of potential ExPEC *E. coli* isolates from pigs and poultry on farms and at abattoirs.. 62

Table V. Frequency of antimicrobial resistance genes and association of AMR genes and VGs among 77 potential ExPEC isolates from pigs and poultry on farms and at abattoirs ... 63

Table VIa. Association of ciprofloxacin-resistance and PQMR genes 64

Table VIb. Association of ampicillin and streptomycin-resistance and AMR genes………..64

LIST OF ABBREVIATIONS

A/E	Attaching and effacing
Aac3	Aminoglycoside acetyltransferase
Aada	Aminoglycoside resistance protein
AAF	Aggregative adherence fimbriae
AMC	Amoxicillin/clavulanic acid
AMK	Amikacin
APEC	Avian pathogenic *Escherichiacoli*
AMR	Antimicrobial resistance
AMP	Ampicillin
Bla	Beta-lactamase
BMEC	Brain microvascular endothelial cells
CF	Colonization factor
CIP	Ciprofloxacin
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CHL	Chloramphenicol
Cnf	Cytotoxic necrotizing factor
ColV	Colicin V
CRO	Ceftriaxone

CLSI	Clinical and Laboratory Standards Institute
CT	Cholera toxin
DAEC	Diffusely adherent *Escherichia coli*
Dfr	Dihydrofolate reductase
EAEC	Enteroaggregative *Escherichia coli*
E. coli	*Escherichia coli*
ECOR	*E. coli* reference collection
EHEC	Enterohemorragic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBLs	Extended-spectrum beta-lactamases
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extra-intestinal pathogenic *Escherichia coli*
Fim	Fimbriae
FIS	Sulfisoxazole
FOX	Cefoxitin
GEN	Gentamicin
GyrA	DNA gyrase (type II topoisomerase), subunit A
GyrB	DNA gyrase (type II topoisomerase), subunit B

Iss	Increased serum survival
Iuc	Iron- Uptake Chelate
Iut	Iron-Uptake Transport
KAN	Kanamycin
kDA	Kilodalton
LEE	Locus of enterocytes effacement
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
NAL	Nalidixic acid
OMP	Outer membrane protein
PAI	Pathogenicity island
Pap	Pyelonephritis associated fimbriae
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMQR	Plasmid-mediated quinolone resistance
ST	Heat-stable enterotoxin
STEC	Shiga-toxin producing *Escherichia coli*

Stx	Shiga-toxin
STR	Streptomycin
SXT	Trimethoprim-sulphamethoxazole
TET	Tetracycline
TIO	Ceftiofur
Tsh	Temperature-sensitive hemagglutinin
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
VF	Virulence factor
VG	Virulence gene

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INTRODUCTION

Escherichia coli (E. coli) is a common micro-organism in the gastrointestinal tract of humans and animals. Most *E. coli* are harmless; however, some are pathogenic and can cause disease in humans and animals (Johnson, 2003; Kaper, Nataro, & Mobley, 2004). From a genetic and clinical perspective, *E. coli* strains of biological significance to humans can be broadly categorized as (1) commensal strains, (2) intestinal pathogenic (i.e. enteric or diarrheagenic) strains, and (3) extraintestinal pathogenic *E. coli* (ExPEC) strains (Russo & Johnson, 2003). In addition, *E. coli* are classified into phylogenetic groups A, B1, B2, and D (Clermont et al., 2000). Intestinal pathogenic *E. coli* such as enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) may cause gastrointestinal disease, mostly consisting of more or less severe diarrhea, in humans and animals. EPEC is considered as one of leading causes of infant diarrhea in developing countries, whereas STEC is one of the most common pathogens associated with outbreaks of foodborne illness, causing bloody diarrhea and hemolytic-uremic syndrome, particularly in North America, Japan and European countries (Kaper et al., 2004). ExPEC strains do not produce enteric disease; however, they may cause diverse extraintestinal infections, including urinary tract infections (UTIs), meningitis and septicemia, in both humans and animals (Smith et al, 2007). ExPEC express a variety of virulence-associated genes instead of having a common virulence mechanism (Johnson et al, 1991, Smith et al 2007). Typical virulence factors of ExPEC include diverse adhesins, siderophores for iron acquisition, capsules, toxins, proteases, invasins, and serum resistance proteins (Kaper et al, 2004). ExPEC isolates from urinary tract infections in humans most commonly belong to phylogenetic groups B2 or D.

Animal-derived raw foods are commonly contaminated with *E. coli* and have long been known as an important vehicle for transmitting diarrheagenic *E. coli*, but their role in spreading ExPEC has not been explored extensively (Xia et al., 2011). ExPEC can asymptomatically colonize the animal and human intestinal tract, thus could be transmitted to humans through food consumption (Bélanger et al., 2011; Fairbrother & Nadeau, 2006). Recent studies indicate retail meat may be an important vehicle for transmission of ExPEC that share characteristics with human clinical ExPEC strains (Johnson et al., 2003a, 2005b; Jakobsen et al., 2010; Vincent et al., 2010). In

addition, it was suggested that meat consumption was epidemiologically associated with infection by antimicrobial-resistant ExPEC that causes UTI (Johnson, et al., 2005b). These findings suggest the hypothesis that potentially zoonotic ExPEC in contaminated meat could arise from poultry and pig reservoirs.

There have been no published studies on the prevalence of STEC, EPEC, and ExPEC in pigs and poultry on-farm in Vietnam. In addition, few current data are available on the prevalence of antimicrobial-resistant *E. coli* from animals at the farm level and from carcasses in abattoirs in this country. The aim of this study is to investigate the prevalence of *E. coli* strains carrying virulence genes associated with STEC, EPEC, and ExPEC in pigs and poultry on farms and at abattoirs, and to characterize these potentially pathogenic strains for phylogenetic group and antimicrobial resistance profile.

LITERATURE REVIEW

1. Classification of *E. coli*

1.1. Commensal *E. coli*

Commensal *E. coli* constitute part of the gastrointestinal microflora in humans, mammals, and birds. The commensal strains are generally benign, do not cause intestinal tract disease, and can be beneficial to the host (Russo and Johnson, 2003). However, commensal strains may cause illness if the host is compromised immunologically or medically (Picard et al., 1999; Russo and Johnson, 2003).

Commensal *E. coli* generally lack the specialized virulence traits that enable intestinal and extraintestinal *E. coli* to cause disease within or outside the gastrointestinal tract, respectively. However, commensal *E. coli* can participate in extraintestinal infections when an aggravating factor is present, such as a foreign body (e.g. urinary catheter), host compromise (e.g. local anatomical or functional abnormalities such as urinary or biliary tract obstruction, or immunocompromise), or a high or a mixed bacterial species inoculum (e.g. with fecal contamination of the peritoneal cavity).

Generally, human commensal *E. coli* strains belong to phylogenetic groups A and B1 and typically lack the specialized virulence determinants found in pathogenic strains that cause intestinal or extraintestinal diseases (Picard et al., 1999; Russo and Johnson, 2000).

1.2. Intestinal pathogenic *E. coli*

Intestinal pathogenic *E. coli* have evolved a special ability to cause gastrointestinal disease, mostly consisting of more or less severe diarrhea in humans and animals. Intestinal pathogenic *E. coli* are currently classified in six categories based on virulence attributes that help bacteria to cause diseases, including: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004). EHEC is also known as the pathogenic

subgroup of Shiga toxin-producing *E. coli* (STEC) that cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Nataro & Kaper, 1998)

1.2.1.Enteropathogenic *E. coli* (EPEC)

EPEC is considered as one of leading causes of diarrhea in developing countries (Kaper et al, 2004). The central mechanism of EPEC pathogenesis is a lesion called attaching and effacing (A/E), which is 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 (Kaper et al., 2004). Ability to produce A/E lesions has also been detected in strains of Shiga toxin–producing *E. coli* (Nataro & Kaper, 1998).

The genetic determinants for the production of A/E lesions are located on the locus of enterocyte effacement (LEE), a pathogenicity island that contains the genes encoding intimin, a type III secretion system, a number of secreted (Esp) proteins, and the translocated intimin receptor named Tir. Intimin, a 94-kDa outer membrane protein encoded by the *eae* gene, is responsible for the intimate adherence between bacteria and enterocyte membranes. The Esp molecules (EspA, B, and D) are involved in the formation of a translocon that delivers effector molecules to the host cell and disrupts the cytoskeleton, subverting the host cell functions. Tir, which is one of the EPEC translocated proteins, is inserted into the host cell membrane, where it acts as a receptor to intimin (Kaper et al., 2004; Trabulsi et al., 2002)

Typical EPEC strains possess a plasmid of 70–100 kb called the EAF (EPEC adherence factor) plasmid, whereas atypical EPEC do not contain the EAF plasmid (Trabulsi et al., 2002). This plasmid encodes a type IV pilus called the bundle-forming pilus (BFP), which mediates interbacterial adherence and possibly adherence to epithelial cells (Kaper et al., 2004). Thus far, typical EPEC strains are identified by the presence of both *eae* and *bfp*, whereas atypical EPEC strains have been defined as those which possess only *eae* (Hernandeset al., 2009). Atypical EPEC serotypes have been isolated from different animal species suggesting that atypical EPEC may be a potential zoonotic cause of human diarrhea (Trabulsi et al, 2002).

Atypical enteropathogenic *E. coli* (EPEC) were identified among *E. coli* isolates from pork, indicating that pigs may also be potential reservoirs for the pathogen (Xia et al., 2010). Analysis of phenotypic and genotypic markers of atypical EPEC isolated from diarrheic and non-diarrheic dogs demonstrated that isolates of serotypes O4:H16 and O51:H40 were similar to those found in human disease (Almeida et al., 2012).

1.2.2. Shiga toxin-producing *E. coli* (STEC)

Shiga toxin-producing *E. coli* refers to those strains of *E. coli* that produce at least one member of a class of potent cytotoxins called Shiga toxin, also called verocytotoxin (Gyles, 2007). STEC have been characterized by a variety of methods, including serotyping. Isolates of O157:H7 serotype are the most common STEC pathogens associated with outbreaks of foodborne illness in North America, but several other serotypes, particularly those of the O26 and O111 serogroups, can also cause disease and are more important than O157:H7 in other countries (Kaper et al., 2004). It is well established that cattle are a major reservoir of STEC O157:H7 in North America but in countries such as Australia, sheep are of greater significance (Gyles, 2007).

Shiga toxin is the key virulence factor in STEC diseases. Currently, Shiga-like toxins are categorized into two immunologically distinct groups: Stx1 and Stx2, which show approximately 55% sequence homology (Kaper et al., 2004). 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 subdivided into distinct genetic variants which differ in their biological activity and association with disease (Gyles, 2007).

Adherence to intestinal epithelial cells is an early feature of STEC infection and two patterns of attachment and interaction have been observed, associated with *eae*-positive and *eae*-negative STEC isolates. The *eae*-positive STEC possess a pathogenicity island called the locus of enterocyte effacement (LEE), which encodes the bacterial proteins necessary for formation of the AE lesion (Kaper et al., 2004;

Nataro & Kaper, 1998). The combined presence of the *eae* and *stx_2* genes has been indicated as an important predictor of HUS (Ethelberg et al., 2004).

1.2.3. Enterotoxigenic *E. coli* (ETEC)

ETEC is a pervasive cause of diarrhea in children and travellers in developing countries (Kaper et al, 2004). ETEC produce one or both of two enterotoxins, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) which are major virulence factors. LT was found to be very similar physiologically, structurally, and antigenically to cholera toxin (CT) and to have a similar mode of action (Svennerholm, 2011). The molecular mass (84 kDa) and the subunit structure of the two toxins were essentially identical, with an active (A) subunit surrounded by five identical binding (B) subunits. LT can stimulate prostaglandin synthesis and stimulate the enteric nervous system; both of these activities can also lead to stimulation of secretion and inhibition of absorption (Kaper et al, 2004). STs are low-molecular-weight peptides, that are classified into two unrelated classes — STa and STb — which differ in both structure and mechanism of action. Only toxins of the STa class have been associated with human disease whereas the STb toxin is associated with animal disease (Kaper et al, 2004).

Other important virulence factors in ETEC include one or more colonization factors (CFs), which usually are fimbriae. More than 25 CFs have been recognized on human ETEC so far, and additional ones are likely to be recognized (Svennerholm, 2011). The CFs promote colonization of ETEC in the small bowel, thus allowing expression of either or both LT and ST in close proximity to the intestinal epithelium (Quadri et al., 2005). ETEC are also an important cause of diarrhoeal disease in animals and these animal strains express fimbrial intestinal colonization factors, such as K88 and K99, which are not found in human ETEC strains.(Kaper et al, 2004)

1.2.4. Enteroaggregative *E. coli* (EAEC)

EAEC is an emerging pathogen which is increasingly recognized as a cause of persistent diarrhea in adults and children. EAEC are characterized by the ability to

aggregate intimately with each other, adhere to human HEp-2 cells, and also attach to abiotic surfaces when grown in tissue culture plates (Okhuysen & Dupont, 2010). EAEC pathogenesis is determined by the organism's ability to adhere to intestinal cells, produce enterotoxins and cytotoxins, and induce inflammation. A number of studies described several virulence factors associated with EAEC pathogenesis. These are (i) heat stable toxin-1 (EAST-1), (ii) aggregative adherence fimbriae I and II (AAF/I and AAF/II) and AAF/III. In addition, different pathogenicity islands have been identified within the EAEC group, including *Shigella she* pathogenicity island, containing enterotoxin and mucinase genes and *Yersinia* high-pathogenicity island, containing the yersiniabactin siderophore gene (Weintraub, 2007)

1.3. Extraintestinal pathogenic *E. coli* (ExPEC)

E. coli strains that induce extraintestinal diseases are termed extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000). In humans, ExPEC strains are often found in the normal intestinal flora and do not cause gastroenteritis. ExPEC strains are phylogenetically and epidemiologically distinct from intestinal pathogenic strains. They do not produce enteric disease; however, they can asymptomatically colonize the human intestinal tract and may be the predominant *E. coli* strains in 20% of normal individuals (Johnson and Russo, 2002; Russo and Johnson, 2000, 2003). ExPEC may cause diverse infections in both humans and animals, including urinary tract infections (UTIs), meningitis and septicemia (Smith et al, 2007). The sub-set of ExPEC causing UTI has been designated uropathogenic *E. coli* (UPEC).

Most of the human ExPEC strains are found in the B2 and D phylogenetic groups and have acquired various virulence genes such as genes encoding attachment factors (*pap*, *sfa*, and *afa*), transmembrane protein involved in neonatal meningitis (*ibe10*), and alpha-hemolysin (*hly*) that allow them to induce extraintestinal infections in both normal and compromised hosts (Picard et al., 1999). The majority of the virulence factors present in ExPEC strains are distinct from those found in the intestinal pathogenic strains; Russo and Johnson, 2000, 2003).

ExPEC were defined by Johnson et al. as *E. coli* isolates containing two or more of the following virulence markers as determined by multiplex PCR: *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) (Johnson et al., 2003a). Other virulence markers are associated with ExPEC status, including *fimH* (type I fimbriae), *ibe* (outer membrane protein promoting invasion), and *hly* (hemolysin) (Kaper et al, 2004).

2. Diseases caused by ExPEC in humans

2.1. Urinary tract infections

Extraintestinal pathogenic *E. coli* are a frequent cause of urinary tract infections (UTIs), which are among the most common bacterial infections found in humans (Johnson and Russo, 2002, 2005). In fact, UTIs are a heterogeneous group of disorders, which are classified by the site of infection, the bladder (cystitis), kidney (pyelonephritis) and urine (bacteriuria) (Foxman, 2002). Risks of UTIs are increased in specific groups including infants, pregnant women, the elderly and compromised patients (Foxman, 2002).

Since the major means of transmission of UTI-causing ExPEC to the urinary tract is the ascending route from the fecal site into the bladder (Smith et al., 2007), the cause of ExPEC contamination in human has been debated. Johnson et al. indicate that meats, particularly poultry, can be an important source of ExPEC contamination (Johnson et al., 2003a, 2005a, 2005b).

Treatment of UTIs usually consists of administration of antimicrobials, including beta-lactams, quinolones, trimethoprim in combination with sulfamethoxazole, and nitrofuranes (Wagenlehner et al., 2005). Notably, resistance of *E. coli* to commonly prescribed antimicrobial agents used in the treatment of UTIs has been significantly increasing (Smith et al., 2007).

2.2. Neonatal meningitis

Bacterial neonatal meningitis is an inflammation of the membranes of the brain or spinal cord and consists of a purulent exudate of the membranes, perivascular inflammation, and brain edema. In the United States, more than 50% of cases of neonatal meningitis caused by Gram-negative enteric organisms were due to ExPEC, and approximately 80% of these cases were caused by strains carrying the K1 capsular antigen (Pong and Bradley, 1999).

The pathogenesis of ExPEC-induced neonatal meningitis occurs in several steps: bacteremia, binding of bacteria to the surface of brain microvascular endothelial cells, bacterial invasion of brain microvascular endothelial cells, and invasion of the meninges (membranes that surround the brain and spinal cord) and the central nervous system (Smith et al., 2007).

Many of the virulence genes associated with ExPEC neonatal meningitis strains are present on pathogenicity islands (PAIs) (Bonacorsi et al., 2003). For example, the genes involved in facilitating blood-brain barrier penetration *sfaS* (fimbrial adhesin S), *ibeA* (invasin IbeA) and *cnf1* (cytotoxic necrotizing factor) are located on PAIs (Bonacorsi et al., 2003).

Holt et al. reported that the most common antimicrobial regimes used for neonatal meningitis treatment based on third generation cephalosporins such as cefotaxime in a combination with a penicillin or ampicillin with or without an aminoglycoside (Holt et al., 2001). The use of third generation cephalosporins decreased mortality but not morbidity (Harvey et al., 1999).

2.3. Sepsis

Sepsis can be caused by a microbial infection that originates from the kidneys (UTI), bowel (peritonitis), skin (cellulitis), or lungs (pneumonia). Russo and Johnson estimated that *E. coli* is the cause of 17% of the cases of severe sepsis (dysfunction of at least one organ system) in the United States (Russo and Johnson, 2003). They

estimated there were 127,500 cases of *E. coli*-induced severe sepsis, with 40,000 deaths, in 2001; the mortality rate was approximately 30%.

Individuals at the extremes of age are the most susceptible to bacterial-induced, community acquired septicemia; *E. coli* was found to be the most frequent cause of septicemia in infants up to 1 year of age and in individuals aged 65 or greater in North America (Diekema et al., 2002). Putative virulence factors for septicemia-inducing strains of *E. coli* serogroups O2 and O78 include iron uptake systems (aerobactin, yersiniabactin, and IroN receptor), serum resistance, and adhesins (type 1 fimbriae, curli, and P fimbriae). Non-fimbrial adhesins were present only in strains of serotype O78, and the K1 capsule was present only in O2 (Mokady et al., 2005).

Hyde et al. noted that among the infants with early-onset sepsis, the proportion of *E. coli* infections that were resistant to ampicillin increased during the surveillance period (1998-2000) and mortality was higher with ampicillin-resistant *E coli* strains than with ampicillin-sensitive strains (Hyde et al., 2002).

3. Virulence factors of pathogenic *E. coli*

ExPEC strains have acquired genes encoding diverse extraintestinal virulence factors that enable them to cause infections outside of the gastrointestinal tract. Characteristic virulence traits that are present in most ExPEC include various adhesins (e.g. P and type I fimbriae), factors to avoid or subvert host defense systems (e.g. capsule, lipopolysaccharide), mechanisms for nutrient acquisition (e.g. siderophores), and toxins (e.g. hemolysin, cytotoxic necrotizing factor 1).

3.1. Adhesins

The adherence step contributes to extra intestinal virulence by promoting colonization and by facilitating bacterial interactions with host cells and matrix elements. Most *E. coli* adhesins are fimbrial (ie, discrete hair-like structures), but some are amorphous fibers or are capsule-like. Irrespective of morphology, most adhesins contain defined molecular regions that interact with specific host receptor

epitopes in a lock-and-key or lectin-like fashion (Johnson, 2003). The diverse adhesins of ExPEC are categorized primarily according to receptor specificity. The broadest functional division is between mannose-sensitive and mannose-resistant adhesins, reflecting the early observation that mannose blocks adherence for a subset of *E. coli* isolates that attaches to erythrocytes or epithelial cells (Johnson, 1991).

3.1.1. Type 1 fimbriae

Type 1 fimbriae are non-flagellar, filamentous appendages of bacteria, were classified into type I depending on haemagglutination and sensitivity activity with D-mannose (Antao et al., 2009)

Type 1 fimbriae are produced by more than 80% of all UPEC (Kucheria et al., 2005). The role of type 1 fimbriae in attachment of UPEC strains to host cells was demonstrated through *in vitro* and animal studies. Type 1-fimbriae mediated for specific binding of one *E. coli* strain to monkey kidney cells (Salit & Gotschlich, 1977). Further research showed that type 1-fimbriae accounted for binding of *E. coli* strains to human urinary tract epithelial cells (Eden & Hansson, 1978).

Type 1 fimbriae are encoded by the chromosomally located *fim* gene cluster, including genes for a structural subunit (FimA), an adhesin (FimH) andseveral accessory proteins involved in subunit transport and assembly, and regulatory proteins (Connell et al, 1996). *FimH* was found to be the gene responsible for the monomannose-containing receptors shown to be adaptive for UPEC which results in increased adhesion to vaginal epithelial cells (Antao et al., 2009).

Type 1 fimbriae also play an important role in the infection process is the adherence of APEC to the epithelium of the trachea. This fimbriae in colibacillosis has been associated with mucus adherence, colonization of the trachea and the intestinal tract, and interactions with lung epithelial cells (de Pace et al., 2010)

3.1.2. P fimbriae

The P fimbriae family is the best known group of mannose-resistant adhesins, so named because of their binding specificity for the Gal(α 1-4) Gal- β disaccharide

galabiose, which is present in the antigens of the human P blood system (Johnson, 2003). P fimbriae are important in the pathogenesis of UTI, primarily because they mediate Gal-Gal-specific bacterial adherence to epithelial cells within the human urinary tract, thereby permitting bacterial colonization and stimulating inflammation (Johnson, 1991).

P fimbriae are composed of different polymerized subunits, with one major subunit, PapA, constituting the bulk of the fimbria. Three minor adherence-related fimbrial subunits (PapE, PapF, and PapG) are present in minor amounts at the fimbrial tips. These fimbrial proteins, as well as a number of accessory proteins, are encoded by a chromosomal gene cluster termed *pap* or pyelonephritis-associated fimbriae genes since these were typical of strains isolated from human urinary tract infections (Johnson, 1991). The adhesion of the fimbriae is conferred by the adhesin *papG* located at the distal end fimbriae. There are three alleles of adhesins *papG* (I, II, and III). Molecular epidemiological studies have shown that allele III of PapG is usually the predominant variant among *E. coli* isolates from women and children with cystitis, whereas the PapGII variant is associated with pyelonephritis in humans (Johnson et al., 2000). Several other proteins are important in P-fimbrial synthesis. PapD is present in the periplasmic space and may complex with fimbrial subunits, stabilizing them during translocation across the periplasmic space to the outer membrane prior to assembly. PapC assists in the transport of subunits out of the cell and in their assembly into complete fimbriae (Johnson, 1991). The *papC* gene was observed at a high prevalence among *E. coli* strains associated with pyelonephritis, as determined by PCR (Le Bouguenec et al, 1992).

The P fimbriae are not restricted to uropathogenic *E. coli* (UPEC) causing UTI and newborn meningitis-causing *E. coli* (NMEC), but are also prevalent in avian pathogenic *E. coli* (APEC) strains. In poultry, P fimbriae are primarily associated with colibacillosis strains, but P fimbriae are also found in healthy poultry. *PapC* has been found at a low prevalence in *E. coli* from healthy chickens in Denmark (Jakobsen et al., 2010), whereas the prevalence of *papC* in APEC strains was greater than 20% (Ewers et al., 2007; Rodriguez-Siek et al., 2005). Until now, studies on the

frequency of P fimbriae in healthy pigs are scarce. However, some previous studies have suggested that P fimbriae-positive *E. coli* are more frequent in diseased pigs than healthy pigs (Jakobsen et al., 2010; Tan et al., 2012).

3.1.3.Temperature sensitive hemagglutinin

The mannose-resistant haemagglutinin of an avian pathogenic *E. coli* (APEC) isolate, which was best expressed at low temperatures, was first reported in 1994 (Provence & Curtiss, 1994). This temperature-dependent haemagglutination phenotype was termed Tsh for temperature sensitive haemagglutinin (Provence & Curtiss, 1994). Tsh and Tsh/Pap (P fimbriae)/ Iuc (aerobactin) pathotypes were suggested as important virulence factors of APEC (Ngeleka, et al., 2002).

Tsh is synthesized as a 140-kDa precursor protein, whose processing results in a 106-kDa passenger domain (Tsh_s) and a 33-kDa β-domain (Tsh_β). The presence of a conserved 7-amino-acid serine protease motif within Tsh_s classifies the protein in a subfamily of autotransporters, known as serine protease autotransporters of the *Enterobacteriaceae* (Kostakioti & Stathopoulos, 2004). The passenger domain (Tsh_s) is secreted into the extracellular environment, being temporarily in the outer membrane of the bacteria where it is capable of adhering to red blood cells, hemoglobin, and the extracellular matrix proteins fibronectin (Antao et al., 2009; Kostakioti & Stathopoulos, 2004)

Tsh is encoded by the gene *tsh* which is located on a ColV-type plasmid in many of the APEC strains. It has also been shown that *tsh* is more frequently observed in high-lethality isolates than in low-lethality isolates among APEC (Dozois et al., 2000). In another study, it was shown that the *tsh* gene was present in more than 50% of APEC, 4.5% UPEC and 11.5% NMEC isolates tested (Ewers et al., 2007). In pigs, *tsh* was found significantly more frequently in highly virulent ExPEC strains belonging to group B2, which might indicate that *tsh* is involved in the pathogenic mechanisms of ExPEC (Picard et al., 1999; Tan et al., 2012).

3.2. Capsules (K antigen)

Over 80 polysaccharide capsules (K antigen) have been described in *E. coli* (Orskov & Orskov, 1992). The capsules of most ExPEC strains belong to group 2 and 3 polysaccharides which are characterized by low-molecular-weight and high-charge density (Johnson, 1991; Johnson & O'Bryan, 2004). The capsule protects ExPEC bacteria against phagocytosis and complement-mediated killing, thereby contributing to extraintestinal virulence (Burns & Hull, 1999; Russo, Liang, & Cross, 1994). K1 capsule is the most commonly encountered capsular type among both urinary and fecal strains in humans (Johnson, 1991). APEC strains possessing the K1 capsule also showed greater resistance to serum and ability to colonize the internal organs in poultry than K1-negative mutants (Mellata et al., 2003).

Group 2 and group 3 capsules are encoded by *kps* operons which share moderately to highly conserved regions (e.g., *kpsDMTE*) encoding transport and assembly functions. Therefore, Johnson & O'Bryan developed a rapid technique for detection of K antigen in *E. coli* based on PCR amplification of the *kpsM* gene (Johnson & O'Bryan, 2004). The *kpsM* has been designated as one of virulence markers for identification of ExPEC in epidemiological studies (Johnson et al., 2005b).

3.3. Lipopolysaccharide

Lipopolysaccharide (LPS) is a structural component of the outer membrane of *E. coli* (Johnson, 1991). LPS, also known as endotoxin, consists of three components: the hydrophobic membrane anchor lipid A region, which is associated with the toxicity of the LPS and is well conserved among Gram-negative bacteria, the distal O-antigen polysaccharide region that is exposed to the surface and the core polysaccharide region that connects the two LPS components. The lipid A is largely responsible for stimulation of the innate immune system (DebRoyet et al., 2011). LPS is recognized by the Toll-like receptor 4 (TLR4) present on the surface of monocytes, macrophages, neutrophils and dendritic cells, cells of the innate immune system (Johnson, 2003). O antigens may contribute to the virulence of the organism, since certain O groups are associated with with specific diseases (DebRoy et al., 2011). A

study on *E. coli* O18:K1 mutants lacking either the lipopolysaccharide O antigen showed that loss of the O antigen resulted in the strain becoming more sensitive to the bactericidal effects of the classical complement pathway (Pluschke et al., 1983).

3.4. Iron acquisition systems

Uptake of iron in Gram-negative bacteria is achieved primarily through the synthesis, export, and uptake of small iron-chelating molecules termed siderophores. Recognized siderophore systems include the catecholates enterobactin, salmochelin, the hydroxamate aerobactin, and yersiniabactin (Henderson et al., 2009; Johnson, 2003). Enterobactin is efficient at sequestering iron *in vitro*; however, it is less effective than aerobactin and salmochelin in competing for iron during infection, as it is inhibited by serum albumin and specifically binds to the host innate defense protein neutrophil lipocalin (Caza et al., 2008).

The salmochelin-encoding system comprised of two divergently transcribed sets of genes, *iroBCDE* encoding the enzymes accounting for salmochelins production, and *iroN* encoding the outer membrane siderophore receptor. The specific roles of *iroBCDE* genes in salmochelins production as well as in contribution to APEC virulence have been identified (Caza et al., 2008). The *iroN* gene did not effect salmochelin production, however, it was required for the colonization of uropathogenic ExPEC (Russo et al., 2002), and the bacteremic step of *E. coli* neonatal meningitis (Negre et al., 2004).

The aerobactin system is encoded by a five-genes operon, with four genes *iucABCD* encoding the enzymes needed for aerobactin synthesis and a fifth gene *iutA* encoding the outer membrane receptor protein. In *E. coli*, this operon is found both on plasmids and on the bacterial chromosome, with the chromosomal location predominating among human clinical isolates (Johnson, 1991). Due to the high prevalence of aerobactin-encoding genes among isolates from poultry with colibacillosis, the *iucD* gene was selected as one of virulence markers for the rapid detection and characterization of APEC (Ewers et al., 2005).

3.5. Cytotoxin:

3.5.1.Hemolysin

There are two common types of this toxin, α-haemolysin and β-haemolysin. The α - haemolysin is commonly produced by strains isolated from cases of human UTI and other extra-intestinal infections. It is a heat-labile extracellular protein and its production can be plasmid or chromosomally determined. It is a pore forming cytolysin, and lyses erythrocytes by causing dissipation of transmembrane ion gradients. β-haemolysin is a cell associated haemolysin with a similar range of haemolytic activity (Johnson, 1991).

In addition to lysing erythrocytes, hemolysin is toxic to a range of host cells, probably contributing to inflammation, tissue injury, and impaired host defenses. Hemolysin production is encoded by a four-gene operon termed *hly* (Johnson, 1991). Plasmid and chromosomal *hly* regions differ with respect to flanking and regulatory sequences and to the precise sequence of *hlyA*, the gene encoding the structural hemolysin protein.

3.5.2 Cytotoxic necrotizing factor

Cytotoxic necrotizing factor type 1 (CNF1), presents in certain diarrheagenic *E. coli* and UPEC, is a member of a family of bacterial toxins that target the Rho family of small GTP-binding proteins in mammalian cells (Johnson, 1991). The role of CNF1 in the pathogenesis of UPEC-mediated urinary tract infection has been demonstrated in several studies. In a mouse model of ascending urinary tract infection, a CNF1-expressing UPEC, compared to its isogenic *cnf1* mutant, caused a greater acute inflammatory response in the bladder, colonized the bladder more extensively in a coinfection experiment, and survived better when coincubated with human neutrophils (Rippere-Lampe et al., 2001). Epidemiological data showed that *cnf1* gene is significantly associated with clinical strains related to cystitis, pyelonephritis and prostatitis in humans (Yamamoto, 2007). Strains of UPEC that produce CNF1 also often produce two established urovirulence determinants, α-hemolysin and P

fimbriae. The *cnf1* gene encoding cytotoxic necrotizing factor and the genes encoding these other virulence factors are typically linked on the same pathogenicity island in certain UPEC strains (Mills et al,, 2000).

4. Association among virulence factors and phylogenetic groups

4.1. Associations among virulence factors

Although several important ExPEC virulence factors and their role during pathogenesis have been described (Johnson, 1991; Smith et al., 2007), many ExPEC cannot be unambiguously distinguished from commensal *E. coli* based on a set of discriminatory virulence factors (Kohler & Dobrindt, 2011). Nevertheless, ExPEC classification has been proposed based on the detection of two virulence-associated genes typical of the specific pathotype (Johnson and Russo, 2005), and two virulence-associated genes for non-host samples such as food samples (Johnson et al., 2005b).

Certain VFs commonly occur together in patterns suggesting either co-selection or direct genetic linkage. A major virulence pattern, which was characterized by the presence of F1 variant fimbriae; S fimbriae; IbeA; and the aerobactin system was identified in 75% of the O18:K1 ExPEC isolates of human origin and avian origin (Moulin-Schouleur et al., 2006). Chromosomally located virulence-associated genes such as iron acquisition genes, for example the chromosomal *sitD* variant*, chuA, fyuA*, and *irp2*, were frequently found in APEC and also among UPEC and NMEC (Ewers et al., 2007). These results demonstrated that very closely related clones can be recovered from extraintestinal infections in humans and poultry.

Currently recognized putative VFs of ExPEC include adhesins, siderophores, toxins, protectins, and invasins, has and have been demonstrated within pathogenicity-associated islands (PAIs) and on plasmids. Ewers et al. suggested that ColV plasmids, commonly found in APEC, and might be the source of the plasmid in human UPEC and NMEC strains, were associated with virulence genes *tsh, iss*, and the episomal *sit* locus. (Ewers et al., 2007). Rodriguez-Siek et al. suggested that a

typical member of the APEC pathotype is likely to contain several iron transporter-encoding genes, like *irp2, fyuA, iutA, iroN,* and *sitA*, and plasmid-associated genes, including cvi/cvaC, tsh, and iss (Rodriguez-Siek et al., 2005b). Several PAIs and genetic islands harbouring *ireA* and pap operon genes have been identified in APEC including PAI I (APEC-O1) (Kariyawasam et al., 2006). PAI I (APEC-1)-related genes were observed in strains belonging to the APEC pathotype and also in UPEC and NMEC (Ewers et al., 2007).

4.2. Association of phylogenetic groups and virulence factors

Phylogenetics is the study of genetic distance among groups of organism. Phylogenic relationships have been traditionally studied based on morphological data. An initial phylogeny, obtained through cluster analysis of multilocus enzyme electrophoresis (MLEE) data for 35 enzyme-encoding loci of the standard reference collection of 72 *E. coli* strains (ECOR), defined six major phylogenetic groups, designated A, B1, B2, C, D and E (Tenaillon et al., 2010, Lindstedt et al., 2007). This phylogeny was reconstructed within 4 phylogenetic groups A, B1, B2, and D with a subsequent analysis of the ECOR strains based on polymorphisms of 38 loci (Tenaillon et al., 2010; Herzer et al., 1990).

Among human *E. coli* isolates, the phylogenetic B2 group and, to a lesser extent, the phylogenetic D group strains have been shown to exhibit numerous extraintestinal virulence determinants compared to phylogenetic A and B1 strains (Clermont et al., 2000). Among ECOR strains, the most common pattern of phylogenetic distribution exhibited by the various VFs, including *papG allele III, sfa/foc, sfaS, focG, hlyA, cnf1, cdtB, kpsMT-III, rfc*, and *ibeA*, was associated with group B2. Other VFs were sufficiently found in other phylogenetic groups, but were still significantly associated with phylogenetic group B2 (e.g., "*K5," fyuA, ompT*, and the PAI marker), or with both groups B2 and D (e.g., *papAH, papC, papEF, papG,and kpsMTII*) or with group D only (e.g., *papG allele II*, *iha*, K1, and *iutA*) (Johnson et al., 2001). Johnson et al. reported most of *E. coli* isolates from bacteremic patients (65.7%) belonged to phylogenetic group B2, whereas 11.6%,

10.5%, and 12.2% belonged to groups A, B1, and D, respectively. More than half (97/181, 53.6%) of the bacteremic-inducing *E. coli* strains were of urinary or pulmonary tract origin; 78/97 (80.4%) of these strains belonged to group B2 (Johnson et al., 2002a). Picard et al. reported that strains of the B2 phylogenetic group were highly virulent, killing in mice, and possessed the greatest number of virulence determinants. In contrast, commensal strains belong mainly to phylogenetic groups A and B1, were devoid of virulence determinants, and did not kill mice (Picard et al., 1999). Moulin et al. reported that most avian and human strains belonging to phylogenetic subcluster B2-1 expressed the K1 antigen and presented no significant differences concerning the presence of other virulence factors (Moulin-Schouleur et al., 2007). Johnson et al. reported that group B2 neonatal meningitis isolates had a significantly higher prevalence of many VFs than even the group B2 ECOR strains (Johnson et al., 2002b).

However, the phylogenetic distribution of the various ExPEC virulence factors varied among ExPEC strains from animals and retail food. Among all ExPEC isolates recovered from retail meat, *papC* was significantly positively associated with phylogenetic group B1 and negatively associated with group B2, whereas *kpsMTII* was negatively associated with groups B1 and A and positively associated with group B2. Other virulence genes, including *afa/dra, sfa/foc, iutA, papA,* were widely dispersed and were not significantly associated with any phylogenetic group (Xia et al., 2011). Phylogroup B2 and D isolates from pigs, pork, broiler poultry and broiler poultry meat carried more virulence genes in comparison with phylogroup A and B1 isolates (Jakobsen et al., 2010). A significant association was observed between the presence of *sitA* and group D and also the presence of *tsh* and group D in the APEC isolates (Wang et al., 2010).

5. Antimicrobial resistance (AMR) of *E. coli*

In recent years, antimicrobial resistance in bacteria of animal origin and the potential for transmission of resistant bacteria from animals to humans have become a public health concern (de Jong et al., 2012). *E. coli* is a candidate vehicle for such transfers because of its diversity and also because it constitutes part of the microflora in the

gastrointestinal tracts of both humans and animals. It is sensitive to selection pressure exerted by antimircrobial use and carries genetic mobile elements to achieve such transmission (Hammerum & Heuer, 2009). Thereby, *E. coli* of animal origin may act as a donor of antimicrobial resistance genes for other pathogenic bacteria. Johnson et al. concluded that some drug-resistant human fecal *E. coli* isolates may originate from poultry (Johnson et al., 2007a). Acquired antimicrobial resistance is particularly problematic when it occurs in ExPEC, the distinctive *E. coli* strains that possess the specialized virulence factors (VFs) required for extraintestinal disease (Johnson & Russo, 2002).

5.1. Mechanisms of antimicrobial resistance

Bacteria may acquire resistance to antimicrobials through several mechanisms. First, resistance can be mediated by chromosomally located genes encoding enzymes that inactivate the antimicrobial agent before it can have an effect (Tenover, 2006). For example, beta-lactamases play a major role in intrinsic and acquired resistance to beta-lactams in bacteria, predominantly in Gram-negative bacteria. Chromosomal beta-lactamases were found in *E. coli* that showed significant resistance to penicillins, monobactams and cephalosporins (Li et al, 2007) Second, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect (Tenover, 2006). For example, the efflux pump system AcrAB-TolC confers intrinsic resistance to quinolones in *E. coli* (Li, 2005). Third, bacteria may acquire mutations that change the binding site of the antimicrobial agents. Mutations on *gyrA* encoded DNA gyrase and *parC* encoded topoisomerase IV, which are the targets of the quinolone drugs, were associated with resistance to quinolones in *E. coli* isolates (Chen et al, 2001).

Bacteria also develop resistance through the acquisition of mobile genetic elements such as resistance plasmids including those transposon- and integron-associated (Li, Tenover). This provides an efficient mechanism for rapid horizontal and vertical dissemination of antimicrobial resistance determinants among bacteria. The well-known plasmid-mediated mechanisms have led to resistance to almost every class of

clinically important antimicrobials, such as beta-lactams, aminoglycosides, macrolides, quinolones, tetracyclines, chloramphenicols, sulphonamides and trimethoprim (Li, 2005, Tenover, 2006, Li et al., 2007, Skold, 2001).

5.2. Antimicrobial use and emergence of antimicrobial resistance in food animals

5.2.1. Antimicrobial use in animal production

Antimicrobial agents have been used for the prevention and treatment of bacterial infections in animals and are also still used for growth promotion in animal husbandry in many countries (Fabrega et al., 2008). The use of antimicrobial agents, however, can cause the emergence and dissemination of antimicrobial resistance genes in bacteria (Harada & Asai, 2010).

Because of the concern for the spread of antimicrobial-resistant bacteria from the large reservoirs in food animals, European countries have banned the use of antimicrobials as growth promoters by January 2006 (Hammerum & Heuer, 2009). To assist the microbiological safety assessment of pre- and post-market evaluation of veterinary antimicrobials, Health Canada has categorized antimicrobial agents based on the indication and the availability of alternative antimicrobials for the treatment of infections in human medicine. According to this categorization, antimicrobials are classified into 4 categories as i) very high importance, ii) high importance, iii) medium importance and iv) low importance in human medicine (http://www.hc-sc.gc.ca/dhp-mps/vet/antimicrob/amr_ram_hum-med-rev-eng.php).

For *E. coli*, first- and second-generation cephalosporins and sulfonamides are listed as highly important antimicrobial agents for the treatment of *E. coli* infection in humans (Hammerum & Heuer, 2009). Several cephalosporins are widely used in veterinary clinical therapy. Sulfonamides are commonly used for the treatment of un-complicated urinary tract infection in humans. Sulfonamides, in combination with trimethroprim, are also commonly used for treatment of diarrhea in weaner pigs. Fluoroquinolones are broad-spectrum antimicrobial agents that are highly effective for the treatment of a variety of infections in humans and animals. Legal restrictions

aimed at reducing the use of fluoroquinolones in food animals have been introduced in certain countries such as the United States and Denmark. In 2005, The US Food and Drug Administration withdrew the fluoroquinolones sarafloxacin and enrofloxacin, which were used to treat *E. coli* infections in poultry (www.gpo.gov/fdsys/pkg/FR-2005-08-01/html/05-15224.htm). In Canada, antimicrobial use via feed was the most common route of exposure. The most common classes of antimicrobials administered via feed were macrolides/lincosamides and tetracyclines (Deckert et al., 2010). Fluoroquinolones are not licensed for use in pigs or chickens, but since October 2012, injectable enrofloxacin is licensed in Canada for treating swine respiratory disease (http://www.swinehealth.ca/newsarchives.php).

In Vietnam, few data are available on antimicrobial use as veterinary drugs and feed additives, but it is known to be widespread. Antimicrobials are the most common registered drugs, accounting for 70% of all drugs included in the list of veterinary drugs approved for circulation (http://www.cddep.org/sites/cddep.org/files/publication_files/VN_Report_web_1.pdf?issuusl=ignore). Currently, there are eleven groups of antimicrobials used in animals, including: β-lactams, aminoglycosides, macrolides, tetracycline, quinolones and fluorquinolones, phenicols, polymyxins (colistin), pleuromutilins, lincosamides, sulfamides and trimethoprim. Results of a survey on antimicrobial use in pig farms in South Vietnam showed that amoxicillin, gentamicin, enrofloxacin, norfloxacine, tetracycline, ampicillin, and florfenicol are the most commonly used antimicrobials.

5.2.2.The trend in antimicrobial resistance in *E. coli* isolates from food-producing animals

The prevalence of antimicrobial resistance in *E. coli* isolated from food-producing animals has been monitored in several countries. The prevalence of resistance in *E. coli* to ampicillin, sulfisoxazole and tetracycline was reported similarly by Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) Retail Meat Program (http://www.phac-aspc.gc.ca/cipars-picra/2008/) and National Antimicrobial Resistance Monitoring System (NARMS) in the United States

(http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm293578.htm). Resistance in *E. coli* isolates to ampicillin, streptomycin, chloramphenicol and tetracycline was reported at higher rates in CIPARS Farm results in compared with than the results from European surveillance systems (Deckert et al., 2010). Resistance to fluoroquinolones which are regarded as critically important antimicrobials in human and animal medicine has been broadly observed in animals and meat, thus, the trends of their resistance would need continuous and careful observation (Hammerum & Heuer, 2009).

Although the use of chloramphenicol in food-producing animals has been banned since the 1990s, current monitoring data showed that chloramphenicol resistance is still prevalent in *E.coli* isolates from animals in China and Japan (Harada & Asai, 2010; Wang et al., 2010). The use of thiamphenicol and florfenicol, which belong to the same family as chloramphenicol, may be one of the factors causing the persistence of chloramphenicol resistance in food-producing animals.

In Vietnam, a high prevalence of antimicrobial resistance in *E. coli* isolates from poultry, poultry meat, beef and pork was reported in 2008 (Van et al., 2008). The rates of multi-resistance in *E. coli* isolates which showed resistance to three or more different classes of antimicrobials were up to 89.5% in poultry meat, 95% in poultry faeces and 75% in pork isolates. Resistance in *E. coli* was most frequently observed to tetracycline, sulfafurazole, ampicillin, amoxicillin, trimethoprim, chloramphenicol, streptomycin, nalidixic acid and gentamicin. Additionally, *E. coli* isolates also displayed resistance to fluoroquinolones, with poultry isolates showing the highest rates of resistance.

5.3. Resistance of *E. coli* to category I antimicrobials

5.3.1. Resistance to fluoroquinolone

Quinolones and fluoroquinolones are groups of antimicrobial compounds that are commonly used for the treatment of many bacterial infections. However, resistance to fluoroquinolones has increased globally, particularly in members of the

Enterobacteriaceae (Li, 2005). Quinolones target the DNA gyrase and topoisomerase IV enzymes of the bacteria, thus preventing DNA replication (Higgins et al., 2003). The main resistance mechanisms to quinolones are mutations in the *gyrA* and *parC* genes that alter the conformation of target amino acid residues within the protein (Jacoby, 2005).

Plasmids can also directly produce resistance to quinolones due to the presence of plasmid-mediated quinolone resistance (PMQR) genes (Jacoby, 2005). The first PMQR gene to be described was named *qnrA* and encodes a 218 aa pentapeptide repeat protein that is capable of protecting the DNA gyrase from the activity of quinolones (Robicsek et al., 2006a). Since the discovery of *qnrA*, two related PMQR proteins have been described; these proteins are thought to act in a manner comparable to *qnrA*, as they share 40 and 59 % amino acid similarity and have been named *qnrB* and *qnrS*, respectively (Jacoby et al., 2006). The *qnr* determinants were identified in ciprofloxacin-resistant *E. coli* strains isolated from clinical cases in China (Wang et al., 2003). A high prevalence of *qnr* determinants was also identified, particularly the *qnrS* gene, in both *E. coli* community- and hospital-associated strains in Vietnam (Le et al., 2009).

An additional PMQR determinant has also been described, which has resistance mechanisms distinct from that of the *qnr* genes. The *aac(6')-Ib-cr* gene, which encodes a variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, have been emerged more recently, but might be even more prevalent than the *qnr* genes (Robicsek et al., 2006b). The *aac(6')-Ib-cr* gene was detected in *E. coli* and *K. pneumoniae* from both hospital and community strains in Vietnam, albeit at a comparatively higher frequency in clinical strains (Le et al., 2009).

5.3.2. Resistance to cephalosporins

Extended-spectrum β-lactamases (ESBLs) are bacterial enzymes that degrade oxyimino-cephalosporins such as cefotaxime and ceftazidime (Paterson & Bonomo, 2005). Genes encoding extended-spectrum β -lactamases (ESBL) are often located

on conjugative plasmids (Pitout & Laupland, 2008). These most common plasmid-mediated ESBLs include the TEM-, SHV- or CTX-M-type. The CTX-M enzymes, named due to the potent hydrolytic activity of these β -lactamases against cefotaxime, have become the most prevalent family of ESBLs among *Enterobacteriaceae* (Hammerum & Heuer, 2009). To date, more than 80 CTX-M enzymes have been isolated (Paterson & Bonomo, 2005). Of the different CTX-M–type ESBLs, CTX-M-15 was the most common ESBL associated with human ExPEC strains isolated from UTI (Coque et al., 2008).

Many studies have demonstrated the presence of the genes encoding ESBLs in *E. coli* isolates from animals and meat. The genes bla_{TEM-1}, $bla_{CTX-M-14}$, $bla_{CTX-M-1}$, $bla_{CTX-M-2}$ were found in ESBLs-producing *E. coli* isolates from swine and poultry (Tang et al., 2011, Giufre et al., 2012). bla_{TEM-52} and bla_{CMY-2} were found in *E. coli* recoverd from retail meats (Jensen et al., 2006, Yan et al., 2004).

5.4. Resistance of *E. coli* to category II antimicrobials

5.4.1. Resistance to penicillins

A variety of beta-lactamases have been identified in bacteria derived from food-producing and companion animals. Beta-lactamase are classified on the basis of their primary structure into four molecular classes A through D (Jacoby & Munoz-Price, 2005). Gram-negative bacteria usually produce chromosomal AmpC-type beta-lactamases belonging to the Ambler class C (Li et al., 2007) . Other plasmid-encoded b-lactamases in *Escherichia coli* and other *Enterobacteriaceae* were identified such as TEM-1 or SHV-1 belonging to class A enzymes (Jacoby and Munoz-Price, 2005). Bla_{TEM} was found frequently in *E. coli* derived from pigs and poultry in Vietnam and China (Van et al,. 2007, Tang et al., 2011). Substitution of amino acid sequences at TEM-1 or SHV-1 resulted in TEM or SHV-type ESBLs (Li et al., 2007).

5.4.2. Resistance to trimethoprim/sulfamethoxazole

Sulfonamide and trimethoprim have been used for treatment of *E. coli* infections in humans, and the presence of sulfonamide and trimethoprim resistance can lead to

treatment failure in urinary tract infection. Plasmid-mediated resistance to sulfonamide and trimethoprim is caused by drug-resistant variants of the chromosomal target enzymes dihydropteroate synthase and dihydrofolate reductase, respectively (Skold, 2001). Three genes, *sul1*, *sul2* and *sul3*, coding for sulfonamide-resistant dihydropteroate synthases have been identified (Hammerum & Heuer, 2009).

The combination of trimethoprim with sulfonamides increases its effectiveness against a broad spectrum of bacteria including *E. coli* and other Enterobacteriaceae. However, the resistance rates of *E. coli* to TMP-SMX have increased recently. In several regions of the United States, the resistance rate of UPEC strains to TMP-SMX was reported over 20% to 50% (Solberg et al., 2006). The highest rate of resistance of *E. coli* to TMP-SMX occurs most frequently in urinary tract infections in women. The class 1 integron which contains *sulI* gene was found in about 70% strains of *E. coli* resistant to TMP-SMX (Solberg et al., 2006, Fluit & Schmitz, 2004).

5.5. Resistance to category III antimicrobials

5.5.1. Resistance to tetracyclines

Tetracycline is one of the most widely used antimicrobials in animal production worldwide. The beneficial effect on growth rate and feed utilization of tetracyclines were first discovered in poultry, then observed in swine and cattle, leading to the development of both chlortetracycline and oxytetracycline as animal growth promoters (Chopra & Roberts, 2001). Resistance to tetracyclines has emerged in many commensal and pathogenic bacteria due to genetic acquisition of *tet* genes. Since *tet* genes can be located in integrons, the use of tetracyclines as growth promoters could result in selection and transfer of resistance to unrelated antimicrobials to humans in bacterial isolates whose resistance gene cassettes are also incorporated within the integron. A high prevalence of tetracycline-resistant *E. coli* was reported in most food producing animal species (Maynard et al., 2003;

Boerlin et al., 2005; Gyles, 2008). The prevalence of strains resistant to tetracycline among APEC sometimes exceeded 80% in certain studies (Gyles, 2008).

5.5.2. Resistance to streptomycin

The prevalence of streptomycin resistance in strains of *E. coli* were usually reported at high rates regardless of the animal species (Maynard et al., 2003, Boerlin et al., 2005, Gyles, 2008). The prevalence of streptomycin resistant strains was reported exceeding 60% for *E. coli* from pork and poultry strains in Vietnam (Van et al, 2008). Among streptomycin-resistant *E. coli,* the presence of the *strA*-*strB* gene pair and the *aadA* gene have been reported frequently (Sunde & Norström, 2005). The *aadA* gene cassettes, which are located within multiresistance integrons, are among the most prevalent gene cassettes in class 1 and class 2 integrons. These genes also encode for resistance to spectinomycin which is used therapeutically for bacterial respiratory and enteric infections in cattle, pigs and poultry (http://www.inchem.org/documents/jecfa/jecmono/v33je06.htm.)

6. Multi-antimicrobial resistance among *E. coli*

6.1. Multidrug resistance associated with ESBLs

The CMY- or CTX-M-encoding plasmids often contain other multiple resistance determinants and are also associated with transposons/integrons (Pitout & Laupland, 2008). The $bla_{CTX\text{-}M\text{-}15}$ gene, often associated with the $bla_{TEM\text{-}1}$, $bla_{OXA\text{-}1}$, and *aac(6)-Ib-cr* resistance genes, has been located mainly on plasmids belonging to the incompatibility group IncF, plasmids producing type F fimbriae susceptible to phage Ff (Carattoli, 2009). Multiresistance plasmids pEK499 and pEK516 belonged to IncFII plasmids harboring $bla_{CTX\text{-}M\text{-}15}$ and other resistance genes *catB4*, *tet*(A) were identified with a major role in the spread of CTX-M-15 ESBLs in *E. coli* (Woodford et al., 2009).

Considering the similar genetic organization of multidrug resistance determinant-integron/transposon elements, it is important to note the linkage of ESBL and fluoroquinolone resistance in Gram-negative bacteria. PMQR gene *qnrA* has been

found with a gene for β-lactamases CTX-M-9, CTX-M-14, whereas *qnrB* has been located on plasmids carrying genes for CTX-M-15, SHV-12, or SHV-30 (Robicsek et al., 2006). A survey in Canada also showed that *E. coli* strains producing CTX-M enzymes were substantially more resistant to ciprofloxacin than strains lacking *bla*$_{CTX-M}$ genes (Pitout et al., 2007).

6.2. Multi-resistance associated with PMQR

PMQR is associated with ESBLs on the same plasmid, and the spread of such multidrug resistance plasmids among *Enterobacteriaceae* strains has a potential impact on treating human infections (Nordmann & Poirel, 2005). The linkage of *qnrB4* and *qnrB6* genes with gene encoding for β-lactamases DHA-1, SHV-12 was found on IncF plasmids in *E. coli* and *K. pneumoniae* (Tamang et al., 2008). In the case of *qnrS*, the gene was co-located in *K. pneumoniae* possessed *bla*$_{SHV-12}$ but not in the same plasmid (Lavilla et al., 2008) and also found in *E. coli* possessed *bla*$_{TEM-1}$ (Ahmed et al., 2007). On the other hand, a number of studies described *qnr* genes in association with non-β-lactamase resistance genes, such as *aac(6')-Ib-cr*, *aadA1*, *aadA2*, *dfrA1*, *dfrA12*, and *catB2* (Rodríguez-Martínez et al., 2011).

7. Association between virulence and antimicrobial resistance of ExPEC

The genetic association between virulence genes and antimicrobial resistance and dissemination may be provided by the coexistence of different mobile genetic elements such as plasmids, integrons, transposons (Johnson, 1991; Johnson et al., 2004). A multiresistant *E. coli* K52 H1 strain carrying a plasmid similar to the Flme plasmids was found in an extensive outbreak of severe infections, including urosepsis, meningitis, and pneumonia (Phillips et al., 1988). Several of the genes encoding putative virulence factors, including *tsh*, a class 1 integron gene *intI1*, genes encoding aerobactin and CoIV-encoding operons, are found in a conjugative R plasmid isolated from an APEC isolate (Johnson et al., 2002c). Sequence analysis of a 30-kilobase (kb) region of this plasmid pTJ100 revealed the virulence genes, *iucC*

and *traT,* tetracycline resistance genes *tetA* and *tetR*, β-lactamase gene *bla*, and also two transposons, Tn21 and Tn3. The Tn21-like region included a gene of class 1 integrons, *intI1*, and several genes encoding antimicrobial resistance (*aadB, catB3, qacED1,*and *sulI*) (Johnson et al., 2004).

However, the association between virulence genes and antimicrobial resistance among ExPEC strains are not well understood, particularly in relation with other factors, such as distribution of phylogenetic groups. In a study on UTI isolates, Piatti et al (2008) reported phylogenetic group B2 accounted for a significantly higher prevalence among fluoroquinolone-susceptible strains than among resistant strain, whereas fluoroquinolone-resistant strains harbor fewer virulence factors than susceptible strains (Piatti et al., 2008). The lower prevalence of virulence genes was also observed among ciprofloxacin-resistant *E. coli* strains of both human and avian origin in comparison with susceptible strains, however no significant difference was observed between ciprofloxacin-resistant and susceptible strains in term of phylogenetic pattern (Graziani et al., 2009).

8. Food animals as a reservoir of extraintestinal pathogenic *E. coli* in humans

Much current research is investigating the contribution to human infections of food animal-associated ExPEC, particularly those strains that are resistant to therapeutically important antimicrobial agents. Based on existing evidence, the chicken is the food animal source most closely linked to human ExPEC. Poultry meat exhibits the highest overall levels of *E. coli* contamination, and these *E. coli* are often more frequently antimicrobial resistant than *E. coli* recovered from other meats. Poultry-associated *E. coli* also often possess virulence genes similar to those of human ExPEC, suggesting the potential to cause human disease. (Manges & Johnson, 2012). Contamination of pork with ExPEC has also been reported; however, fewer studies have been conducted to date (Jakobsen et al., 2010).

8.1. ExPEC in poultry

E. coli is considered as a member of the normal microflora of the poultry intestine, but certain strains which cause colibacillosis, an acute disease due to respiratory tract infectionare designated as avian pathogenic *E. coli* (APEC). APEC strains are a subset of extraintestinal pathogenic *E. coli* (ExPEC). It is becoming more and more apparent that the common presence of a set of virulence-associated genes among ExPEC strains as well as similar disease patterns and phylogenetic background indicate a significant zoonotic risk of avian-derived *E. coli* isolates (Ewers et al., 2007).

Many of the virulence genes detected in APEC strains such as *iroD, tktA* (transketolase), *fruA* (fructokinase) are also present in human ExPEC strains that cause human neonatal meningitis and UTIs, as well as in animal disease-causing ExPEC strains (Schouler et al., 2004). The distribution of some virulence factors was independent of the host, whether they originated from human or avian species, for example, the adhesion P-fimbriae were found in both O2 strains from UTI, NBM and neonatal sepsis, and avian septicemic O2 (Mokady et al, 2005; Ron, 2006). Rodriguez-Siek et al. reported two groups of 524 APEC and 200 UPEC isolates showed substantial overlap in terms of their serogroups, phylogenetic groups and virulence genotypes, including their possession of certain genes associated with large transmissible plasmids of APEC (Rodriguez-Siek et al., 2005a). The genome sequence of APEC O1:K1:H7 was found to be remarkably similar to that of three human UPEC strains, except only 4.5% of genome not found. Also, multilocus sequence typing showed that some of the sequenced human ExPEC strains were more like APEC O1 than were other human ExPEC strains (Johnson et al., 2007b). Ewers et al. found that an APEC O2:K1:H5 strain and an Ont:NM fecal strain belonged to sequence type complexes STC95 and STC69, respectively, which account for the majority of ExPEC pathotypes from humans, exclusively UTI strains (Ewers et al., 2009). These strong similarities of virulence gene profiles between ExPEC isolates from poultry meat, broiler poultry and UTI patient, community-dwelling human isolates were also identified by cluster analysis (Jakobsen et al.,

2010). Thus, these studies provide evidence that the potential APEC strains might act as human UPEC or as a reservoir of virulence genes for UPEC. These strains pose a zoonotic risk because either they could be transferred directly from birds to humans or they could serve as a genetic donor of ExPEC virulence genes and also potential reservoir of antimicrobial resistance genes for other bacteria.

APEC strains are often resistant to antimicrobials approved for use in poultry, including cephradine, tetracyclines, chloramphenicol, sulfonamides, amino-glycosides and β-lactam antimicrobials (Lutful Kabir, 2010). Recent studies suggest that avian *E. coli* commonly possess plasmid-mediated multidrug resistance (MDR), and might serve as reservoirs of MDR for human ExPEC (Johnson et al., 2007a; Manges et al., 2007; Jakobsen et al., 2010). However, it was found that APEC isolates had a higher frequency of MDR than human ExPEC isolates. Additionally, statistical analysis of data on antimicrobial susceptibility, phylogenetic group, and virulence gene content showed that ExPEC avian and human isolates segregated significantly according to host species and clinical status. Therefore, the actual frequency of such transmission relative to the entire human and avian ExPEC populations might be relatively low (Johnson et al., 2012).

Resistance to fluoroquinolones was reported within several years of the approval of this class of drugs for use in poultry. Several studies have suggested that fluoroquinolone-resistant strains of *E. coli* that infect humans probably emerged as a consequence of using fluoroquinolones in poultry. Two major ST clonal complexes CC10 and CC23 were found to be associated with ciprofloxacin resistance and multi-resistance were shared by human and avian isolates (Giufre et al., 2012) However, the hypothesis of an avian origin for the fluoroquinolone-resistant human ExPEC strains has yet to be determined. Based on analysis of phylogenetic backgrounds and virulence gene profiles, Graziani et al. concluded that ciprofloxacin-resistant strains in humans were clearly distinct from ciprofloxacin-resistant avian strains (Graziani et al., 2009).

8.2.ExPEC in pigs

Pigs, and consequently, pork may be a source of strains with these ExPEC related virulence genes in community-dwelling humans and UTI patients. Jakobsen et al. investigated similarities between 145 ExPEC isolates from healthy pigs with 102 human UTI isolates. Seven virulence genes were found commonly among UTI isolates and community-dwelling humans and ExPEC pig isolates (*kpsMII, iutA, papA, papC, hlyD, sfaS, focG*) (Jakobsen et al., 2010). Furthermore, 7% and 11% ExPEC isolates from pigs, respectively, belonged to phylogroups B2 and D which encompass potent human ExPEC isolates causing UTI, bacteremia and meningitis.

In comparing ExPEC strains causing human UTI (18 strains) or bacteremia (14 strains) with 19 strains causing bacteremia in calves and piglets, Girardeau et al. showed that these piglet strains shared virulence factors such as *iutA* and *iroN*, and also carry the same O antigens as human ExPEC strains (Girardeau et al., 2003). Maynard et al. compared 39 resistant ExPEC strains from animals (15, 8, 8, and 8 from swine, cattle, poultry, and pets, respectively) with 70 resistant human ExPEC strains. Fifty-one percent of the animal isolates and 50% of the human isolates were resistant to more than 3 antimicrobial compounds. Most of the animal strains (67%) belonged to phylogenetic groups A and B1; in contrast, 77% of the human strains belonged to groups B2 and D. Phylogenetic group B2 made up 54% of the human ExPEC strains and 88% of the pet strains (Maynard et al., 2004). Of 61 human-source ExPEC isolates, Johnson et al. demonstrated that all belonged to phylogenetic group B2 (66%) or D (34%). However, 28 ExPEC strains isolated from cattle and swine clinical cases belonged mostly to group A (82%) or B2 (11%). Certain ExPEC VFs including *papA, fyuA, iutA,* and *ompT* were found commonly associated (>50%) with ExPEC isolates from humans, swine, and cattle (Johnson et al, 2003a).

Recently, the frequency of porcine ExPEC was reported to increase from 3.1% in 2004 to 14.6% in 2007, suggesting that the prevalence of porcine ExPEC in China is increasing (Tan et al., 2012). The O2 and O78 antigens implicated in neonatal meningitis and UTI caused by ExPEC in humans

(Ron, 2006) were found among these porcine ExPEC. In addition, the higher frequency of ExPEC VFs, including *fimH, fyuA, iutA, iroN, kpsMT II, traT and sfa/foc*, among the porcine ExPEC isolates was also consistent with the previously demonstrated high frequency of these factors in UPEC and NMEC in humans (Bingen-Bidois et al., 2002; Johnson et al., 2002b; Sannes et al., 2004). Together, these findings suggest that porcine strains of *E. coli* may be potentially zoonotic to humans.

8.3 Prevalence of ExPEC in food derived from animals

Evidence showing that food can be a reservoir for extraintestinal *E. coli* includes 1) community-based outbreaks of extraintestinal infections caused by epidemic strains of *E. coli* causing UTIs (Manges et al., 2001) and other severe infections (Manges et al., 2004); 2) the determination that these epidemic strains share antimicrobial drug susceptibility patterns and genotypes with isolates from retail meat (Johnsonet al., 2005b); and 3) the epidemiologic association between retail meat consumption and intestinal acquisition of antimicrobial drug–resistant *E. coli* causing UTIs (Manges et al., 2007).

Vincent et al. compared antimicrobial susceptibility, O:H serotype and genotypes of ExPEC isolates from human UTI (n = 353); retail meat (n = 417); and restaurant/ready-to-eat foods (n = 74) in different geographic areas in Canada from 2005 to 2007. Two *E. coli* clonal groups from retail poultry O25:H4-ST131 and O114:H4-ST117 were found to be indistinguishable from *E. coli* from human UTIs. This study provides strong support for the role of food reservoirs or foodborne transmission in the dissemination of *E. coli* causing common community-acquired UTIs (Vincent et al., 2010).

In a study of 169 raw cut-up poultry samples obtained from retail grocery stores in the Minneapolis–St. Paul area, USA during 2000s, Johnson et al. isolated *E. coli* from 150 (88.8%) samples. Nalidixic acid-resistant *E. coli* was recovered from 62 (41%), and nalidixic acid-susceptible *E. coli* was recovered from 143 (95%), of the 150 *E. coli*-positive samples. Of a total of 110 strains that were analyzed for ExPEC

VFs, 23 strains were identified as ExPEC. Most of the ExPEC strains (73.9%) derived from phylogenetic groups B2 or D. O antigens O7 and O78, related to UTI in humans, were determined as the major O types among ExPEC isolates. These findings indicated that nalidixic acid–resistant *E. coli* and ExPEC are common in retail poultry meat (Johnson et al., 2003b).

A survey of 346 food products (222 vegetable, 74 fruit, and 50 raw meat items) purchased in Minneapolis–St. Paul retail establishments in 1999–2000 revealed that ExPEC was only isolated from turkey meat (28/50 meat samples) (Johnson, et al,, 2005a). Of 12 ExPEC strains isolated from the turkey meat, 8 strains derived from either phylogenetic group B2 (5 strains) or D (3 strains). 85.7% turkey samples contained *E. coli* strains that were resistant to at least one of 10 antimicrobials tested. This survey showed that retail turkey meat is a source of antimicrobial-resistant *E. coli* and ExPEC.

Johnson et al. also surveyed 1648 retail food items collected in the Minneapolis–St.Paul area during 2001–2003 for the presence of *E. coli*, including antimicrobial resistant *E. coli*, ExPEC, and urinary tract infection (UTI)-causing *E. coli* (Johnson et al., 2005b). Most (180/195) of the raw poultry samples were contaminated with *E. coli*. Only 25.6% of the miscellaneous food product samples contained antimicrobial resistant *E. coli*; however, resistant strains were present in most of the raw meat products. Approximately 17% of the resistant *E. coli* strains in beef and pork were resistant to 5 antimicrobials, whereas approximately 55% of the poultry samples contained strains resistant to 5 antimicrobials. The investigators suggest that the presence of resistant *E. coli* in these food items developed in the farm environment. The presence of ExPEC strains was particularly high in poultry products, whereas O-UTI (UTI associated with specific *E. coli* O antigens) *E. coli* strains were more evenly distributed. Seventeen of the food-derived ExPEC strains, from phylogenetic groups B2 and D, exhibited virulence traits consistent with potential causation of human disease (Johnson et al., 2005b). Together, the studies by Johnson et al. indicate that meats, particularly poultry, can be an important source of resistant ExPEC strains.

9. Molecular methods for determination of pathogenic *E. coli*

Typing methods for discriminating pathogenic *E. coli* that examine the relatedness of isolates at a molecular level have been successfully applied for many years. Several methods based on this approach are currently popular, including: polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

9.1. General information on PCR

PCR is a revolutionary method for identification of bacterial pathogens (Bartlett &Stirling, 2003). PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the targeted template strand. PCR has been used for detection of virulence genes because because it is specific and sensitive (Johnson & Russo, 2005).

The tremendous sensitivity of PCR allows target genes to be detected when present in extremely low concentrations, thereby permitting the detection of a minor bacterial subpopulation within a complex mixed flora without the need for isolation of the organisms of interest (Johnson, 2000). However, for screening the presence of virulent markers, the use of PCR is limited by cost and sometimes the availability of adequate test sample volume. To overcome these shortcomings, multiplex PCR, a modification of the test in which more than one target sequence is amplified using more than one pair of primers, has been developed. By targeting multiple genes at once, multiplex-PCR is applied successfully for identification of pathogenic *E. coli.*

9.2. Multiplex PCR for identification of E. coli pathotype

9.2.1. Multiplex PCR for identification of intestinal pathogenic *E. coli*

Multiplex PCR for identification of *E. coli* pathogens has been developed since the 1990s. China et al. developed a multiplex PCR which detects genes including *eaeA* gene encoding intimin and *slt* genes encoding the Shiga-like toxins. The pairs of primers used in the multiplex PCR were chosen on the basis of two criteria: first, their amplification products had different sizes, and second, their optimal annealing

temperatures were closed. In parallel, the pathotypes of these *E. coli* strains were determined by colony hybridization with *slt* specific probes. The multiplex PCR was proved to be fast and specific and in perfect correlation with the hybridization method (China et al., 1996). A multiplex PCR for identification of ETEC and STEC was developed by amplifying genes encoding K99 and F41 fimbriae, intimin, and Stx1 and Stx2. (Franck et al., 1998). Other multiplex PCR assay was developed, enabling simultaneous detection of STEC O103, O91, O113, O145, O111, O157, and O26 in beef carcass swabs, beef trim, and ground beef (Valadez et al., 2011).

9.2.2. Multiplex PCR for identification of ExPEC

The basis for the establishment of a multiplex PCR for identification of ExPEC were incidence and distribution of virulence-associated genes in ExPEC isolates. A multiplex PCR protocol for identification of ExPEC has been developed in the Reference laboratory for *E. coli* (EcL) based on detection of *tsh*, *papC*, *iucD* and *cnf* genes simultaneously (http://www.ecl-lab.ca). Another multiplex PCR has been applied for identification of ExPEC by screening of 5 virulence markers, including *papA* and *papC, sfa/foc afa/dra* (Dr binding adhesions), *iutA*, and *kpsM* II (group 2 capsules). In this method, the criterion of "presence of 2 of the five markers," was used to differentiate ExPEC and non-ExPEC isolates (Johnson et al., 2003a).

9.3. Multiplex PCR for determination of the *E. coli* phylogenetic group

Phylogenetic characterization of *E. coli* strains on the basis of a very few phenotypic or genotypic features initially appeared to be very difficult. In the past, phylogenetic grouping can be done by multilocus enzyme electrophoresis or ribotyping but both of these reference techniques are complex and time-consuming and also require a collection of typed strains. (Tenaillon et al., 2010; Chaudhuri & Henderson, 2012) Clermont et al developed a multiplex PCR for determining the phylogenetic groups of *E. coli* strains based on detection of these specific DNA fragments including: (i) *chuA*, a gene required for heme transport in enterohemorrhagic O157:H7 *E. coli*; (ii) *yjaA*, a gene initially identified in the recent complete genome sequence of *E. coli* K-12; and (iii) an anonymous DNA fragment designated TSPE4.C2 (Clermont et al.,

2000). According to this method, the *chuA* gene was present in all strains belonging to groups B2 and D and was absent from all strains belonging to groups A and B1. This result was used to separate groups B2 and D from groups A and B1. In the same way, the *yjaA* gene allowed perfect discrimination between group B2 and group D. Finally, clone TSPE4.C2 was present in all but absent from all group A strains. This simple and rapid phylogenetic grouping technique has been applied broadly for determination of links between phylogenetic group and virulence.

9.4. Other techniques for identification of pathogenic E. coli

Molecular typing techniques such as PFGE and MLST that achieve high levels of discrimination of pathogenic *E. coli* have been applied successfully in investigation of outbreaks and epidemiological surveillance (Maiden et al., 1998, Sabat et al., 2013).

PFGE is a fingerprinting technique involving digestion of the genomic DNA with a restriction endonuclease that recognizes specific sequences in the bacterial genome (Sabat et al., 2013) The resulting restriction fragments are then separated on an agarose gel by using an alternating voltage gradient system known as pulsed-field gel electrophoresis, resolving into a pattern based on molecular size **(**Herschleb et al., 2007). PFGE has been considered as the 'gold standard' among molecular typing methods for a variety of clinically important bacteria (Sabat et al., 2013). The network for foodborne bacterial disease surveillance (PulseNet) has developed PFGE standard protocols for molecular subtyping of *E. coli* O157:H7, *Salmonella enterica* serotype Typhimurium*, Listeria monocytogenes, and Shigella species* (Swaminathan et al., 2001).

MLST has become the method of choice for bacterial typing recently (Sabat et al., 2013). In MLST, internal sequences (of approximatively 450–500 bp) of several housekeeping genes are amplified by PCR and sequenced. For each locus, unique sequences (alleles) are assigned arbitrary numbers and, based on the combination of identified alleles (i.e. the 'allelic profile'), the sequence type is determined. By using MLST technique, *E. coli* ST23 was identified as a major type among APEC O78

isolates in Japan (Ozawa et al., 2010). The other sequence type *E. coli* ST131 was found broadly disseminated among CTX-M-15 extended-spectrum beta-lactamase-producing *E. coli* isolated from human and also detected in animals and foods (Rogers et al., 2011).

10. Rationale and objectives of the study

Previous studies show the occurrence of STEC, EPEC and ExPEC in pigs and poultry on farms and at abattoirs. Several studies show a general trend of increasing prevalence of pathogenic and antimicrobial resistant *E. coli* strains of meat and meat products. Furthermore, several studies also show that virulence genes and antimicrobial resistance genes are sometimes carried by the same genetic elements, which suggests a possible association between these genes. Antimicrobial resistance is of increasing concern due to the overuse of antimicrobials in animal husbandry. It is therefore necessary to study the presence of these virulence genes and antimicrobial resistance among potentially pathogenic *E. coli*.

The objectives of this study are as follows:

- To investigate the occurrence of *E. coli* strains carrying virulence genes associated with STEC, EPEC, and ExPEC in pigs and poultry on farms and in abattoirs in Vietnam,
- To characterize these potentially pathogenic strains for phylogenetic group, and antimicrobial resistance profile.
- To evaluate the prevalence of AMR genes among potentially pathogenic *E. coli* strains from pigs and poultry on farms and in abattoirs.
- To determine the associations between the presence of AMR genes and virulence genes of *E. coli* strains from pigs and poultry on farms and in abattoirs.

METHODOLOGY AND RESULTS

My contribution to this work

Sampling of feces and carcasses.

Carrying out of PCR on fecal samples and carcass samples for detection of virulence genes of *E. coli* (article in preparation).

Carrying out of PCR of *E.coli* isolates for detection of virulence genes and antimicrobial resistance genes (article in preparation).

Carrying out of PCR of ExPEC isolates for determination of phylogenetic groups (article in preparation).

Carrying out of antimicrobial susceptibility testing of ExPEC isolates

Statistical analysis of results of the prevalence and associations of virulence genes and antimicrobial resistance of ExPEC isolates

Writing the article: Potential pathogenicity and antimicrobial resistance of *Escherichia coli* from pigs and poultry on farm and at the abattoir in Vietnam

Writing the literature review and discussion of results.

ARTICLE IN PREPARATION

Potential pathogenicity and antimicrobial resistance of *Escherichia coli* from pigs and poultry on-farm and at the abattoir in Vietnam

Thu M. Pham[1]; Ann Letellier[2,]; John M. Fairbrother *[1]

(1) Reference Laboratory for *Escherichia coli* (EcL), Faculty of Veterinary Medicine , Université de Montréal, 3200, Sicotte street, Saint-Hyacinthe (Québec), J2S 2M2

(2) Research Chair in meat safety, Faculty of Veterinary Medicine,

Université de Montréal, 3200, Sicotte street, Saint-Hyacinthe (Québec), J2S 2M2

*Corresponding author address: John M. Fairbrother, Reference Laboratory for *Escherichia coli (*EcL), Faculty of Veterinary Medicine, 3200, Sicotte street, St-Hyacinthe (Québec), J2S 2M2. Tel. 450-773-8521, ext. 8234

Key words: antimicrobial resistance, virulence genes, pigs and poultry, farms, abattoirs, carcasses, ExPEC, PCR.

ABSTRACT

Zoonotic potential pathogenic *Escherichia coli* could arise from poultry and pig reservoirs. The aim of this study is to investigate the occurrence of *E. coli* strains carrying virulence genes associated with STEC (Shiga toxin-producing *E. coli*), EPEC (Enteropathogenic *E. coli*), and ExPEC (Extraintestinal pathogenic *E. coli)* in pigs and poultry on-farm and at abattoirs in Vietnam. Samples of feces and carcasses were collected at selected pig and poultry farms and abattoirs, in which animals were traced from farms to the abattoir. A total of 13.1% strains from all sources were classified as potential ExPEC, possessing one or more virulence genes (*iucD*, *tsh*, *papC* and *cnf)*. Few isolates of other pathotypes were observed. All ExPEC virulence genes, except *cnf*, were more frequently found in isolates from poultry than in isolates from pigs. A higher proportion of ExPEC isolates belonging to

phylogenetic group D was observed in poultry. Multi-drug resistance was frequently observed in ExPEC isolates from both pigs and poultry. Nalidixic acid and ciprofloxacin resistance were significantly associated with poultry feces isolates ($P<0.05$). *bla*$_{TEM}$ gene was more frequently associated with poultry isolates, whereas *qnr* and *aac(6')-Ib* genes were present at low prevalence in pig and poultry isolates. This study demonstrates that poultry ExPEC isolates are potentially more pathogenic than pig ExPEC isolates, and ExPEC isolates in pig and poultry carcasses may originate from pig and poultry feces, due to contamination associated to slaughtering process. Thus, meats particularly from poultry, might be a vehicle for transmission of zoonotic ExPEC strains.

INTRODUCTION

Escherichia coli is a common micro-organism in the gastrointestinal tract of humans and animals. From a genetic and clinical perspective, *E. coli* strains can be broadly categorized as commensal strains, intestinal pathogenic strains, and extraintestinal pathogenic *E. coli* (ExPEC) (Russo & Johnson, 2003). Intestinal pathogenic *E. coli* such as enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) have been well recognized as causes of gastrointestinal disease, mostly consisting of more or less severe diarrhea in humans and animals. ExPEC strains do not produce enteric disease; however, they cause diverse infections, including urinary tract infections (UTIs), meningitis and septicemia, in both humans and animals (Smith et al., 2007). ExPEC express a variety of virulence-associated genes instead of having a common virulence mechanism (Johnson, 1991; Smith et al., 2007). Typical virulence factors of ExPEC include adhesins, siderophores for iron acquisition, capsules, toxins, proteases, invasins, and serum resistance proteins (Kaper et al., 2004). Recent studies indicate retail meat may be an important vehicle for transmission of ExPEC that share characteristics with human clinical strains (Jakobsen et al., 2010; Johnson et al., 2005a). In addition, it was suggested that meat consumption was epidemiologically associated with infection with antimicrobial-resistant ExPEC that causes UTI (Johnson et al., 2005b; Johnson et al., 2003b). These findings suggest the

hypothesis that potentially zoonotic ExPEC could arise from poultry and pig reservoirs following contamination of the meat in abattoirs or in retail-food.

There have been no published studies on the prevalence of STEC, EPEC, and ExPEC in pigs and poultry on-farm in Vietnam. In addition, few current data are available on the prevalence of antimicrobial-resistant *E. coli* on farms and in abattoirs in this country. The aim of this study is to investigate the occurrence of *E. coli* strains carrying virulence genes associated with STEC, EPEC, and ExPEC in pigs and poultry on farms and in abattoirs, and to characterize these potentially pathogenic strains for phylogenetic group, and antimicrobial resistance profile.

MATERIALS AND METHODS

Sample collection

Fecal samples were taken in 10 pig farms in the provinces of Ninh Binh, Thai Binh, Bac Giang, Bac Ninh and Hanoi, and 5 poultry farms located in the provinces of Hoa Binh, Thai Nguyen, Bac Giang in the North of Vietnam. Samples of carcasses were taken at the associated abattoirs (6 pig abattoirs and 2 poultry abattoirs) located in Hanoi, Vietnam, in which animals were tracked from the farms to the abattoir. On the farms, fecal swabs were randomly collected from five points on each pen floor using steriled cotton swabs (Nam Khoa Biotek Company, Ho Chi Minh city, Vietnam), then pooled to constitute samples per farm. At the pig abattoirs, swab samples were taken from 5-10 randomly selected carcasses. From each carcass, a total area of 3x10cmx10cm was swabbed after the final washing step, then swabs from 5 carcasses were pooled to constitute one sample. At poultry abattoirs, chicken carcasses were collected according to the Vietnam technical regulation QCVN 01 - 04: 2009/BNNPTNT (www.tcvn.gov.vn/media/66btnptnt2009.doc). Carcasses were collected in sterile plastic bags, put on ice in coolers, and sent to the laboratory for rinsing. Overall, there were 58 pooled samples of pig feces, 51 pooled samples of poultry feces, 21 pooled samples of pig carcasses, and 18 pooled samples of poultry

carcasses. (*Details of the farms, abattoirs and samples in Annex 1*). All animals were healthy both on farms and at abattoirs.

Sample processing

All samples were transported to the laboratory at National Veterinary Institute in Hanoi, Vietnam. After enriching in peptone water at 37^0C, overnight, samples were cultured on Mac Conkey agar, and incubated at 37^0C, overnight. All samples were kept at 4^0C until shipping to the EcL laboratory in Canada.

Identification of virotypes

Identification of virulence genes: The prevalence of ExPEC, STEC and EPEC virulence genes in samples were identified by multiplex PCR as described below. Then, PCR tests were carried out with samples consisting of 5 pooled colonies selected from cultures of PCR-positive samples. When the PCR reactions of pooled samples were positive, all isolates were examined for the presence of the appropriate virulence genes.

Template preparation: Each sample was inoculated in a tube of 5 ml of LB broth, then incubated at 37^0C overnight before extraction of the DNA. Briefly, 1 ml of each overnight enrichment was pelleted by centrifugation, re-suspended in 0.5 ml of sterile deionized water and boiled for 10 minutes. The boiled cell suspensions were centrifuged and the resulting lysates were used for PCR.

PCR reactions: The presence of virulence genes encoding for ExPEC, STEC and EPEC was determined by multiplex PCRs. The following genes encoding ExPEC virulence factors were tested: *papC* (P fimbriae assembly), *cnf* (cytotoxic necrotizing factor), *tsh* (temperature-sensitive hemagglutinin), and *iucD* (aerobactin). *stx1*, *stx2* genes were tested for STEC and *eae* gene was tested for EPEC. Two ExPEC strains, ECL 13421 and ECL 17088, and one STEC strain, ECL 6611, were used as positive controls. The PCRs were performed in 25 µl reaction mixtures containing 5 µl of DNA template, 1.0 µM each oligonucleotide primer, 200 µM concentrations of each of the four deoxynucleoside triphosphates (GE Healthcare UK Limited-Buckinghamshire HP7 9NA UK), 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.0 mM

MgCl2 (BioTools, B & M Labs, S.A-Madrid-Spain), and 1U of Taq polymerase (BioTools, B & M Labs,S.A-Madrid-Spain). PCR was performed for 30 cycles of denaturation at 94°C for 30s, annealing at 55°C or 60°C depending on the primer (Table I) for 30s, and extension at 72°C for 45s. PCR products were visualized by SYBR Green I staining after agarose gel electrophoresis.

Biochemical tests: All positive-ExPEC isolates were confirmed to be *E. coli* by biochemical tests, including Simmons citrate agar, mobility and indole.

Phylogenetic grouping: ExPEC isolates were subjected to phylogenetic grouping by a PCR-based method (Clermont et al., 2000) to detect *chuA*, *yjaA*, and DNA fragment TspE4.C2, using the primers listed in Table 1. The PCR conditions were 94°C for 3 min, 30 cycles at 94°C for 5s, 59°C for 10s, and a final 5 min extension at 72°C. Strain ECL2015 was used as positive control.

Antimicrobial resistance testing: Antimicrobial resistance was determined using the disk diffusion method ECL-PROC-055 based on Kirby-Bauer technique. Bacterial isolates grown overnight on blood agar were mixed in tubes containing 10 mL of sterile water to a turbidity of 0.5 McFarland Standard. The contents of the tubes were placed onto Mueller-Hinton agar plates using a sterile swab and the following disks were applied using aseptic precautions: amoxicillin + clavulanic acid (20 + 10 µg), ceftiofur (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), ampicillin (10 µg), cefoxitin (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), sulfisoxazole (0.25 mg) and trimethoprim + sulfamethoxazole (1.25 + 23.75 µg). The plates were incubated at 37°C for 24 h, and the diameters of the zones of complete inhibition were measured. The breakpoints used were those recommended by the Clinical and Laboratory Standards Institute (CLSI) as described in 2007 for most of the antimicrobials and in 2010 for ceftiofur.

Detection of antimicrobial resistance genes: the multi-resistant isolates were examined by uniplex PCR for antimicrobial resistance genes: including bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$, bla_{OXA} (extended-spectrum β-lactamases (ESBLs), *qnrA, qnrB, qnrS* and *aac(6)-lb* (fluoroquinolone) and *aadA1* (streptomycin). The PCRs were

performed in 25µl reaction mixtures as described above. PCR was performed for 30 cycles of denaturation at 94°C for 30s, annealing at 48°C, 53°C, 50°C or 55°C depending on the primer for 30s, and extension at 72°C for 45s.

RESULTS

Prevalence of ExPEC, STEC and EPEC in pig and poultry samples

ExPEC genes were frequently observed in samples of both pig and poultry feces and carcasses, whereas the EPEC gene *eae* occurred at much lower frequency, and STEC gene *stx1* was only observed in one sample (Table II). Among ExPEC VGs, *iucD* was more prevalent in feces and carcass samples from poultry than those from pigs. The VG *tsh* was most frequent in samples of pig feces but there was no difference of *tsh* frequency in pig and poultry carcasses. *papC* was more frequent in carcasses samples from pigs than those from poultry.

ExPEC from pigs and poultry differed in their virulence gene profiles

A total of 2505 isolates from samples positive for one or more of the ExPEC VGs were tested the presence of *iucD*, *tsh*, *papC* and *cnf* by PCR. Overall, 328 isolates (13.1%) from all sources were classified as potential ExPEC due to the possession of one or more of these VGs.

As observed at the sample level, *iucD* was less frequently detected in isolates from pigs than those from poultry. In contrast to the situation in samples, both *tsh* and *papC* were also more frequently detected in isolates from poultry feces and carcasses than those from pigs. Interestingly, *iucD, tsh* and *papC* were more frequently detected in isolates from feces than in isolates from carcasses, both in pigs and poultry.

Distribution of ExPEC isolates according to phylogenetic groups

In order to gain more insight into the pathogenic potential and relatedness of the ExPEC from the different animal species at the farm and abattoir levels, isolates were tested for phylogenetic groups by PCR. The distribution of phylogenetic groups

among isolates from pig feces and carcasses was similar. More than half (> 60%) of the ExPEC-positive isolates from pig feces and carcasses fell into group A, which were followed by group B1 and group D (Table III). Among pig isolates, the common profiles A-*iucD:tsh* and A-*iucD*:*papC* were observed both on farms and at abattoirs, Overall, the *tsh* virulence profile was more prevalent in pig isolates than in poultry isolates, and was most frequently observed in the isolates of group A.

In contrast, isolates from poultry feces and carcasses showed a substantial difference in phylogenetic distribution as compared to those from pigs. Phylogroup A was less prevalent in isolates from poultry, and group B1 was far more prevalent in isolates from poultry carcasses than in those from all other sources (Table III). In addition, a higher proportion of poultry isolates was assigned to group D. Only two poultry feces isolates belonged to group B2. Among all tested virulence genes, *iucD, tsh* and *papC* were associated more frequently with groups A and B1 whereas *cnf* was associated only with group B1. About half of the group B1 isolates from chicken carcasses possessed both the virulence genes *iucD* and *tsh*. As observed for pig isolates, isolates sharing the same combination of virulence genes and phylotypes were found both on farms and at abattoirs. For instance, the profiles B1-*iucD:tsh* and D-*iucD:papC* occurred in both poultry feces and carcass isolates.

ExPEC isolates from poultry were more frequently antimicrobial resistant and multidrug resistant than those from pigs

A total of 132 ExPEC-positive isolates, representative of the virotypes and phylotypes found among isolates from the different sources, were selected for antimicrobial susceptibility testing. Multi-drug resistance was far more frequently observed in isolates from both feces and carcasses in poultry than in those from pigs (Table IV). All poultry feces isolates were resistant to 4 or more antimicrobial classes. Most notably, poultry-feces isolates were significantly associated with resistance to both CIP and NAL ($P<0.05$), whereas isolates from feces and carcasses in pigs were less associated with CIP and NAL resistance than those in poultry. In contrast, frequency of resistance to TET, CHL, FIS, SXT and AMP was very high among isolates from all different sources. Frequency of resistance to cephalosporins

was very low in isolates from all different sources. However, two isolates from poultry carcasses, which were found on Mac Conkey agar containing ceftiofur, were resistant to both TIO and CRO. Interestingly, one poultry-feces isolate and one poultry-carcass isolate shared the same phylo-virotype D-*iucD* and the same multi-resistance pattern for ten antimicrobial agents (data not shown).

Frequency of AMR genes among ExPEC-positive isolates from different sources

A total of 77 isolates, including isolates from pig feces (n=25), pig carcasses (n=12), poultry feces (n=28) and poultry carcasses (n=12), which showed the highest degree of multi-resistance, were examined by uniplex PCR for plasmid-mediated ESBL and fluoroquinolone associated AMR genes. Among plasmid-mediated quinolone resistance (PMQR) genes, *qnrS* was the most frequently observed in pig-carcass isolates , *qnrB* was present only in pig-carcass isolates and *qnrA* was more frequent in poultry isolates (Table V). The *aac(6')-Ib* gene was detected only in one pig-feces isolate and two poultry-feces isolates. Nevertheless, PQMR genes were only observed in a small proportion of the ciprofloxacin-resistant isolates (Table VIa).

Among ESBL genes, bla_{TEM} was found to be significantly associated with poultry isolates ($P<0.05$) (Table V). In addition, bla_{TEM} were more frequently associated with AMP –resistant isolates from poultry feces and carcasses than those from pigs (Table 6b). Only a low prevalence was observed for the other ESBL genes in isolates of all origins (Table V). Interestingly, $bla_{CTX\text{-}M\text{-}15}$ was found in two poultry-carcass isolates recovered from Mac Conkey agar containing ceftiofur and possessing the virulence profiles *iucD:tsh:papC.* Most ampicillin-resistant ExPEC isolates from poultry possessed bla_{TEM} whereas only about half of the pigs ExPEC isolates possessed this gene (Table VIb). Most of the streptomycin resistant isolates possessed *aadA1*.

DISCUSSION

To our knowledge, this is the first study describing the prevalence of potential ExPEC isolated from pigs and poultry at farms and in abattoirs in Vietnam. Our study also shows the low prevalence of STEC and EPEC in pigs and poultry, which

need to be studied further to determine the potential risks of these food-borne pathogens in Vietnam.

In our study, 13.1% of strains from all sources were classified as potential ExPEC due to the possession of one or more virulent genes contributing to bacterial virulence and uropathogenesis in humans, including *iucD, tsh, papC* and *cnf*. For pig-feces isolates, low prevalence of *iucD* and *tsh* gene occurred in 6% (87/1460), and 2.5% (36/1460) respectively, whereas the occurrence of these genes varied among previous studies. Schierack et al. reported the occurrence of *iucD* and *tsh* was 11.8% and 5.9% in *E. coli* isolated from a small group of piglets (Schierack et al., 2008). Cortes et al. noted *iucD* gene was present in only one isolate (1/29) and *tsh* occurred in 24.1% (7/29) of *E. coli* isolates from pig farms (Cortes et al., 2010).

Interestingly, our data showed the presence of *cnf* in pig isolates, which were not detected in healthy pigs in other countries (Cortes et al., 2010; Wu et al., 2007). Overall, the prevalence of *iucD*, *tsh* and *papC* were respectively higher in poultry isolates than pig isolates. However, the specific prevalence of these virulence genes in poultry isolates were lower than reported in previous data (Cortes et al., 2010; Ewers et al., 2009). Our findings support the hypothesis that ExPECs are more predominant in poultry than in pigs (Jakobsen et al., 2010, Cortes et al., 2010).

The finding that ExPEC virulent factors were much more common in poultry carcasses than in pig carcasses, is consistent with previous studies (Johnson et al., 2005b; Xia et al., 2011). A similar prevalence of ExPEC was reported in *E. coli* isolated from poultry meat (Johnson et al., 2003b; Xia et al., 2011). Poultry has long been considered a source of *E. coli* strains with zoonotic potential (Bélanger et al., 2011; Fairbrother & Nadeau, 2006), therefore, our observations that ExPEC are common in poultry meat support the hypothesis of retail meats as a source of zoonotic pathogenic *E. coli* (Johnson et al., 2005b)

Analysis of phylogenetic groups and virulence profiles suggest that ExPEC isolates from poultry are potentially more pathogenic for humans than those from pigs. The prevalence of potential ExPEC strains belonging to phylogroup B2 in poultry in

Vietnam was much lower than observed in other studies (Cortes et al., 2010; Ewers et al., 2009). However, our data are consistent with previous studies in terms of the predominant prevalence of phylogroup D-ExPEC strains in poultry versus the prevalence in pigs (Cortes et al, 2010). The occurrence of phylogroup D isolates, particularly, the presence of B2 poultry isolates, implicate the emergence of ExPEC strains which may be related to human clinical ExPEC isolates. Furthermore, among these pig farms and associated abattoirs, we found *E. coli* strains sharing the phylo-virotype D-*iucD* and A-*iucD*. Among these poultry farms and associated abattoirs, we also found *E. coli* strains sharing the same virotypes including phylo-virotypes D-*iucD* and, *iucD:tsh*. These findings indicate that *E. coli* in pig carcasses may originate from pigs on-farm and *E. coli* in poultry carcasses may originate from poultry on-farm.

Our results showed that multidrug resistance is widespread among these potential ExPEC isolates from pigs and poultry. Overall, *E. coli* isolates recovered from carcasses exhibited less resistance to antimicrobials than *E. coli* from farms. These results were consistent with the high multidrug resistance in *E. coli* from retail meat reported in a previous study, which might be explained by the widespread use of antimicrobials, often without prescription, in food-producing animals in Vietnam (Van et al., 2008). Fluoroquinolones are critically important for the treatment of serious infections in humans, such as diarrhea in children (Nguyen et al, 2005). Our findings showed that poultry feces isolates were more significantly associated with CIP and NAL resistance than those from pigs ($P<0.05$). These findings were in broad agreement with other studies on high prevalence of resistance to CIP among *E. coli* of pig and poultry origin reported in other Asia countries such as China and Korea (Tang et al., 2011; Yang et al., 2004; Lee et al., 2005). In contrast, resistance to CIP in *E. coli* isolates from poultry was reported as either low or non-existent in United States, Canada and some European countries (Gyles, 2008). Moreover, our study showed that *E. coli* isolates from poultry carcasses also displayed high resistance to NAL and CIP. The association of CIP and NAL resistance was also reported in ExPEC isolated from poultry meat in US (Johnson et al., 2005b). Therefore,

fluoroquinolone resistance in *E. coli* induced by the use of these antimicrobials in food animals might be a major public health concern in Vietnam.

As observed in previous studies (Do et al., 2006; Van et al., 2008; Yang et al., 2004), most pig and poultry isolates were resistant to TET, STR, AMP, and SXT. It is important to note that the two latter antimicrobials are frequently prescribed for treatment of UTIs in humans (Talan et al., 2008). Also, more than 70% isolates from all sources were resistant to chloramphenicol. Since, chloramphenicol has been banned for use in food animals in Vietnam since 2002 according to the Decision No. 54/2002/QĐ-BNN (http://law.omard.gov.vn/TraC%E1%BB%A9uVB.aspx), the high prevalence of chloramphenicol resistance might be caused by the co-selection pressure due to use of other antimicrobials in the phenicol group, such as florfenicol, in animal production (Harada & Asai, 2010) or the persistence of chloramphenicol-resistant gene(s) among *E. coli* populations (Do et al., 2006). It is noteworthy that multi-resistance to AMP, CHL, and SXT was also reported in diarrheagenic *E. coli* strains isolated from children in Vietnam (Nguyen et al, 2005). Taken together, these results suggest food animals might be a reservoir, and consequently, meats might be a vehicle for transmitting antimicrobial resistant *E. coli* strains which may cause infections in humans.

In our study, the PCR results revealed that 26.2% quinolone-resistant isolates in pig and in poultry possessed a *qnr* gene. However, 62.5% isolates in pigs and poultry which possessed *qnr* genes were susceptible or intermediate to NAL and CIP. Our findings supported previous studies which showed PMQR determinants confer low-level resistance to quinolones and facilitate selection of higher level quinolone resistant bacteria (Jacoby et al., 2006; Tran et al., 2005). The *aac(6')-Ib* gene encodes a common aminoglycoside acetyltransferase responsible for aminoglycoside resistance (Ramirez & Tolmasky, 2010). A variant of the *aac(6')-Ib* gene, the *aac(6')-Ib-cr* has been indicated as the gene encoding for enzyme acetylates ciprofloxacin and norfloxacin (Poirelet al, 2012). In our study, *aac(6')-Ib* gene was detected in one isolate from pig farms and two isolates from poultry feces. The presence of *aac(6')-Ib* might be caused by the use of quinolone and aminoglycoside

at pig and poultry farms, that might gradually have generated selection pressure for these resistant strains. Overall, our results indicate that other as yet unknown PMQR determinants may be responsible for quinolone resistance in ExPEC isolates from pigs and poultry in this study.

In recent years, the dramatic increase in the incidence and prevalence of ESBLs from food animals has become a public health concern (Pitout & Laupland, 2008). The bla_{TEM-1}-type beta-lactamase gene is widely distributed in *E. coli* strains from food of animal origin (Li et al., 2007). Our findings showed that bla_{TEM} gene was associated significantly with potential ExPEC isolates from poultry feces and abattoirs ($P<0.05$). Interestingly, the detection in poultry carcasses of ExPEC carrying $bla_{CTX-M-15}$, encoding the most common ESBL associated with human ExPEC from UTI, indicates a possible source of this gene for transmission to humans. Further studies will elucidate the prevalence of this gene in pig and poultry isolates.

This study relied on a limited number of virulence genes and AMR genes, thus, the prevalence of potential pathogenic strains and AMR strains may be underestimated. Further comprehensive studies with epidemiological and genetic tools are needed to elucidate more fully the prevalence of pathogenic and AMR strains in food producing animals in Vietnam.

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TABLES

Table I. List of primers used in the PCR, single PCR conditions, and control strains

Virulence factor or antimicrobial	Gene	Primer	Amplicon size (bp)	Annealing TM	Control strain	Reference
Aerobactin	*iucD*	for 5’ AAGTGTCGATTTTATTGGTGTA rev 5’ CCATCCGATGTCAGTTTTCTG	778	60^0C	ECL17088	Herrero et al., 1988.
Tsh	*tsh*	for 5’ GGTGGTGCACTGGAGTGG rev 5’ AGTCCAGCGTGATAGTGG	640	55^0C	ECL17088	Dozois et al., 2000.
P (PapC)	*papC*	for 5' TGATATCACGCAGTCAGTAGC rev 5' CCGGCCATATTCACATAAC	501	60^0C	ECL 13421	Daigle et al., 1994.
CNF (multiplex)	*cnf*	for 5’TCGTTATAAAATCAAACAGTG rev 5’CTTTACAATATTGACATGCTG	446	60^0C	ECL 13421	Toth et al., 2003.
Stx1	*stx1*	for 5’ TTA GAC TTC TCG ACT GCA AAG rev 5’ TGT TGT ACG AAA TCC CCT CTG	531	60^0C	ECL6611	Woodward et al., 1992.
Stx2	*stx2*	for 5’ TTA TAT CTG CGC CGG GTC TG rev 5’ AGA CGA AGA TGG TCA AAA CG	327	60^0C	ECL6611	Woodward et al., 1992.
Eae (intimin)	*eae*	for 5’ CAT TAT GGA ACG GCA GAG GT rev 5’ ATC TTC TGC GTA CTG CGT TCA	791	60^0C	ECL6611	Beaudry et al., 1996.
chuA	*chuA*	for 5' GACGAACCAACGGTCAGGAT rev 5' TGCCGCCAGTACCAAAGACA	279	59^0C	ECL2015	Clermont et al., 2000
yjaA.1	*yjaA.1*	for 5' TGAAGTGTCAGGAGACGCTG rev 5'ATGGAGAATGCGTTCCTCAAC	211	59^0C	ECL2015	Clermont et al., 2000

Table I. List of primers used in the PCR, single PCR conditions, and control strains (continued)

Virulence factor or antimicrobial	Gene	Primer	Amplicon size (bp)	Annealing TM	Control strain	Reference
Tsp4C2.1	*Tsp4C2.1*	for 5’ GAGGTAATGTCGGGGCATTCA rev 5'CGCGCCAACAAAGTATTACG	152	59^0C	ECL2015	Clermont et al., 2000
Ampicillin	bla_{TEM}	for 5’ GAGTATTCAACATTTTCGT rev 5’ACCAATGCTTAATCAGTGA	857	55^0C	ECL3482	Maynard et al, 2003
Beta-lactamase	bla_{SHV}	for 5’TCGCCTGTGTATTATCTCCC rev5’CGCAGATAAATCACCACAATG	768	50^0C	PMON38	Maynard et al, 2003
Beta-lactamase	bla_{OXA-1}	for 5’GCAGCGCCAGTGCATCAAC rev 5’CCGCATCAAATGCCATAAGTG	198	50^0C	ECL12572	Maynard et al, 2003
Beta-lactamase	$bla_{CTX-M-15}$	for 5’GCCGTCTAAGGCGATAAACA rev 5’CACACGTGGAATTTAGGGACT	996	50^0C	SUR 255	Doi et al 2009
Streptomycin	*aadA1*	for 5’ CATCATGAGGGAAGCGGTG rev 5’ GACTACCTTGGTGATCTCG	786	50^0C	PMON38	Ecl Lab contact
Quinolone	*qnrA*	for 5’TCAGCAAGAGGATTTCTCA rev 5’GGCAGCACTATTACTCCCA	627	48^0C	J53pMG252	Yue et al, 2008
Quinolone	*qnrB*	for 5’GATCGGAAAGCCAGAAAGG rev 5’ACGATGCCTGGTAGTTGTCC	469	53^0C	J53pMG298	Yue et al, 2008
Quinolone	*qnrS*	for 5’ACGACATTCGTCAACTGCAA rev 5’TAAATTGGCACCCTGTAGGC	417	53^0C	J53pMG306	Yue et al, 2008
Fluoroquinolone	*aac(6’)-Ib*	for 5’TTGCGATGCTCTATGAGTGGCTA rev 5’CTCGAATGCCTGGCGTGTTT	482	55^0C	J53pMG298	Yue et al, 2008

Table II. Virulence genes (VGs) associated with pathotypes of *E. coli* from pigs and poultry on farms and at abattoirs

Source	Total no. of samples examined (n)	No. of *E. coli* samples (%) positive for pathotype VG							Total no. of *E. coli* isolates examined (n)	No. of *E. coli* isolates (%) positive for ExPEC VG				
		ExPEC				STEC and EPEC								
		iucD	*tsh*	*papC*	*cnf*	*stx1*	*stx2*	*eae*		*iucD*	*tsh*	*papC*	*cnf*	Total [a]
Pig feces	58	45 (77.6)	53 (91.4)	17 (29.3)	2 (3.4)	1 (1.7)	0	8 (13.8)	1460	87 (6.0)	36 (2.5)	4 (0.27)	3 (0.21)	104 (7.1)
Pig carcasses	21	16 (76.2)	16 (76.2)	11 (52.4)	0 (0)	0	0	0	250	30 (12.0)	12 (4.8)	4 (1.6)	0	39 (15.6)
Poultry feces	51	46 (90.2)	38 (74.5)	21 (36.2)	0 (0)	0	0	4 (7.8)	595	116 (19.5)	30 (5.0)	23 (3.9)	0	141 (23.7)
Poultry carcasses	18	15 (83.3)	14 (77.8)	7 (3.9)	0 (0)	0	0	6 (33.3)	200	44 (22.0)	15 (7.5)	12 (6.0)	0	44 (22.0)
Total									2505	277 (11.1)	93 (3.7)	43 (1.7)	3 (0.12)	328 (13.1)

[a]: Potential ExPEC isolates within each group

Table III. Distribution according to phylogenetic group and virulence gene profile of ExPEC from pigs and poultry on farms and at abattoirs

Source/ Phylogenetic group	No.of isolates (n)	Frequency of virotypes (%)								
		iucD	*tsh*	*papC*	*iucD:tsh*	*iucD:papC*	*tsh:papC*	*iucD:tsh: papC*	*iucD:cnf*	*iucd: tsh: papC:cnf*
Pig feces										
A	67 (64.4)	45 (67.2)	12 (17.9)	1 (1.49)	7 (10.4)	2 (3.0)	0	0	0	0
B1	33 (31.7)	15 (45.5)	3 (9.1)	0	12 (36.4)	0	0	0	2 (6.1)	1 (3.0)
D	4 (3.8)	3 (75)	1 (25)	0	0	0	0	0	0	0
Total	104	63 (60.1)	16 (15.4)	1 (1.0)	19 (18.3)	2 (1.9)	0	0	2 (1.9)	1 (1.0)
Pig carcasses										
A	24 (61.5)	12 (50)	7 (29.2)	0	1 (4.2)	2 (8.3)	0	2 (8.3)	0	0
B1	12 (30.8)	10 (83.3)	2 (16.7)	0	0	0	0	0	0	0
D	3 (7.7)	3 (100)	0	0	0	0	0	0	0	0
Total	39	25 (64.1)	9 (23.1)	0	1 (2.6)	2 (5.1)	0	2 (5.1)	0	0
Poultry feces										
A	69 (48.9)	36 (52.2)	10 (14.5)	8 (11.6)	7 (10.1)	0	6 (8.7)	2 (2.9)	0	0
B1	43 (30.5)	40 (93.0)	1 (2.3)	0	2 (4.7)	0	0	0	0	0
B2	2 (1.4)	2 (100)	0	0	0	0	0	0	0	0
D	27 (19.1)	18 (66.7)	0	0	2 (7.4)	7 (25.9)	0	0	0	0
Total	141	96 (68.1)	11 (7.8)	8 (5.7)	11(7.8)	7 (5.0)	6 (4.3)	2 (1.4)	0	0
Poultry carcasses										
A	8 (18.2)	8 (100)	0	0	0	0	0	0	0	0
B1	32 (72.7)	;17 (53.1)	0	0	5 (15.6)	0	0	10 (31.3)	0	0
D	4 (9.1)	2 (50)	0	0	0	2 (50)	0	0	0	0
Total	44	27 (61.4)	0	0	5 (11.4)	2 (4.5)	0	10 (22.7)	0	0

Table IV. Antimicrobial resistance of potential ExPEC *E. coli* isolates from pigs and poultry on farms and at abattoirs

Source	Total no. of isolates (*n*)	No. of isolates by no. of AM classes in the resistance pattern				% of resistant isolates by antimicrobial class[a] and antimicrobial[b]														
						(1)					*(2)*		*(3)*				*(4)*	*(5)*	*(6)*	*(7)*
		0-1	2-3	4-5	6-7	AMP	AMC	TIO	FOX	CRO	NAL	CIP	AMK	GEN	KAN	STR	SXT	CHL	FIS	TET
Pig feces	40	0	2	14	24	92.5	10	0	0	0	47.5	30	0	45	27.5	80	70	70	92.5	100
Pig carcasses	24	0	3	7	14	91.7	12.5	0	4.17	0	45.8	25	0	29.2	25	62.5	79.2	79.2	75	100
Poultry feces	47	0	0	3	44	91.5	23.4	0	0	0	89.4[c]	80.9[c]	2.1	68.1	74.5	83	100	93.6	97.9	100
Poultry carcasses	21	0	0	3	18	95.2	14.3	9.52	0	9.52	61.9	38.1	0	38.1	57.1	66.7	100	81	100	100

[a] Classes of antimicrobial agents: (1) β-Lactam; (2) Fluoroquinolones; (3) Aminoglycosides: (4) Sulfonamide combinations; (5) Phenicols; (6) Sulfonamides; (7) Tetracyclines.
[b] AMP, ampicillin; AMC, amoxicillin/clavulanic acid ; TIO, ceftiofur; FOX, cefoxitin; CRO, ceftriaxone; NAL, nalidixic acid; CIP, ciprofloxacin; AMK, amikacin ,GEN, gentamicin; KAN, kanamycin; STR, streptomycin; FIS, sulfisoxazole, CHL, chloramphenicol, TET, tetracycline , SXT, trimethoprim-sulphamethoxazole.
[c] $P < 0.05$ for comparison of indicated group with all other groups.

Table V. Frequency of antimicrobial resistance genes and association of AMR genes and VGs among 77 potential ExPEC isolates from pigs and poultry on farms and at abattoirs

Source/ Virulence gene profile	Total No. of isolates (n)	No. (%) of isolates positive for AMR gene:								
		bla_{TEM}	*$bla_{CTX\text{-}M\text{-}15}$*	*bla_{SHV}*	*bla_{OXA}*	*qnrA*	*qnrB*	*qnrS*	*aac(6')-Ib*	*aadA1*
Pig feces	25	10 (40.0)	0	1 (4.0)	0	1 (4.0)	0	6 (24.0)	1 (4.0)	17 (68.0)
Pig carcasses	12	7 (58.33)	0	0	0	0	2 (16.7)	8 (66.7)	0	9 (75.0)
Poultry feces	28	24 (85.71)[a]	0	0	0	3 (10.7)	0	1 (3.57)	2 (7.1)	20 (71.4)
Poultry carcasses	12	10 (83.33)[a]	2 (16.67)	0	0	3 (25.0)	0	3 (25.0)	0	7 (58.3)
Total	77	51 (66.2)	2 (2.6)	1 (1.3)	0	7 (9.1)	2 (2.6)	18 (23.4)	3 (3.9)	53 (68.8)
iucD	53	35 (66.0)	0	1 (1.89)	0	6 (11.3)	1 (1.89)	12 (22.6)	2(3.8)	37 (69.8)
tsh	7	5 (71.4)	0	0	0	1 (9.7)	0	4 (57.1)	1(14.3)	6 (85.7)
iucD:tsh	8	4 (50.0)	0	0	0	0	0	1 (12.5)	0	5 (62.5)
iucD:papC	4	4 (100)	0	0	0	0	0	1 (25.0)	0	2 (50.0)
iucD:cnf	2	0	0	0	0	0	0	0	0	0
iucD:tsh:papC	3	3 (100)	2 (66.7)	0	0	0	1 (33.3)	0	0	3 (100)

[a] $P < 0.05$ for comparison of indicated group with all other groups.

Table VI1a. Association of ciprofloxacin-resistance and PQMR genes

Source	No. of -resistant isolates	No. (%) of ciprofloxacin -resistant isolates possessing PQMR gene:				
		qnrA	*qnrB*	*qnrS*	*aac(6')-Ib*	None[a]
Pig feces	12	0	0	0	1 (8.3)	11 (91.6)
Pig carcasses	5	0	1 (20.0)[b]	3 (60.0)[b]	0	2 (40.0)
Poultry feces	27	3 (11.1)	0	1 (3.7)	2 (7.4)	21 (77.8)
Poultry carcasses	5	2 (40.0)[c]	0	2 (40.0)[c]	0	2 (40.0)

[a]: No. (%) of CIP resistant isolates which are negative for all tested PQMR genes.
[b]: one CIP-resistant *E. coli* isolate from a pig carcass possessing both *qnrB* and *qnrS* genes.
[c]: one CIP-resistant *E. coli* isolate from a poultry carcass possessing both *qnrA* and *qnrS* genes.

Table VIb. Association of ampicillin and streptomycin-resistance and AMR genes

Source	No. of ampicillin - resistant isolates	No. (%) of ampicillin -resistant isolates possessing β-lactamase genes					No. of streptomycin-resistant isolates	No. (%) of streptomycin-resistant isolates possessing resistance gene:	
		bla_{TEM}	bla_{SHV}	bla_{OXA}	$bla_{CTX\text{-}M15}$	None[a]		*aadA1*	None[b]
Pig feces	25	10 (40.0)	1 (4.0)	0	0	14 (56.0)	24	17 (70.8)	7 (29.2)
Pig carcasses	11	7 (63.6)	0	0	0	4 (36.4)	10	8 (80.0)	2 (20.0)
Poultry feces	26	22 (84.6)	0	0	0	4 (15.4)	25	18 (72.0)	7 (28.0)
Poultry carcasses	11	9 (81.8)	0	0	2 (18.1)	2 (18.1)	8	6 (75.0)	2 (25.0)

AMR: antimicrobial resistance; AMP: ampicillin; STR: streptomycin.
[a]: No. (%) AMP-resistance isolates which are negative for all tested β-lactamase genes.
[b]: No. (%) STR-resistance isolates which are negative for *aadA1* gene.

DISCUSSION

To our knowledge, most studies have investigated the prevalence of ExPEC in retail meat (focusing mainly on poultry) and/or human isolates without regarding isolates from animal production (Johnson et al., 2005b, 2007a). This is the first study describing the potential ExPEC isolated from pigs and poultry at farms and in abattoirs in Vietnam. Our findings suggest correlations among strains isolated from pig and poultry feces and isolated from carcasses at abattoirs.

In our study, 13.1% (328/2505) of strains from all sources were classified as potential ExPEC due to the possession of one or more virulent genes contributing to bacteria virulence and uropathogenesis in humans, including *iucD*, *tsh*, *papC* and *cnf*. For pig-feces isolates, low prevalence of *iucD* and *tsh* gene occurred in 6% (87/1460), and 2.5% (36/1460) respectively, whereas the occurrence of these genes varied among previous studies. Schierack et al. reported the occurrence of *iucD* and *tsh* was 11.8% and 5.9% in *E. coli* isolated from a small group of piglets (Schierack et al., 2008). Cortes et al noted *iucD* gene was present in only one isolate (1/29) and *tsh* occurred in 24.1% (7/29) of *E. coli* isolates from pig farms (Cortes et al., 2010).

Interestingly, our data showed the occurrence of *cnf* in pig-feces isolates at a very low prevalence (0.21%), which were not detected in healthy pigs in other countries (Cortes et al., 2010; Wu et al., 2007). However, the occurrence of *papC* in pig-feces isolates was lower than reported in other study (Jakobsen et al., 2010). The specific prevalence of each of the virulence genes in poultry-feces isolates were lower than reported in previous data (Cortes et al., 2010; Ewers et al., 2009). However, the frequency of *tsh* in poultry-feces isolates in our study was consistent with the prevalence of *tsh* among fecal poultry isolates in Brazil (Delicato et al., 2002).

The distribution of the four ExPEC virulence genes was significantly different among pigs and poultry. All four virulence genes were detected among isolates from pigs but only three virulence genes were detected among poultry isolates. In contrast, a previous study of ExPEC isolates with the five virulence genes including *papC*, *sfa/foc*, *afa/dra*, *kpsM*, and *iutA* in retail meats found poultry possessing more virulence genes compared to pork (Johnson et al., 2005b). However, the prevalence of *iucD*, *tsh* and *papC* were respectively higher in poultry isolates than pig isolates.

Our findings support the hypothesis that ExPECs are more predominant in poultry than in pigs (Jakobsen et al, 2010, Cortes et al, 2010).

ExPEC virulent factors were more common in poultry-carcass isolates (22%) than those from pig carcasses (15.6%), which is consistent with previous studies (Johnson et al., 2005b; Xia et al., 2011). A similar prevalence (20 to 21%) of ExPEC was reported in *E. coli* isolated from poultry meat (Johnson et al., 2003; Xia et al., 2011). Poultry has long been considered a source of *E. coli* strains with zoonotic potential (Bélanger et al., 2011), therefore, our observation that ExPEC are common in poultry meat support the hypothesis of retail meats as a source of pathogenic *E. coli* capable of infecting both animals and humans (Johnson, et al., 2005b). Among these farms and abattoirs which are linked in the production chain, we found *E. coli* strains sharing the same virotypes including *iucD* and *tsh* in pigs and *iucD*, *iucD:tsh* and *iucD:tsh:papC* in poultry. These findings suggest that the *E. coli* in pig carcasses may originate from pigs on-farm and *E. coli* in poultry meat may originate from poultry on-farm due to cross contamination of fecal *E. coli* on carcasses during slaughter.

In addition to prevalence data, our study provides novel information on the phylogenetic classification of potential ExPEC strains from pigs and poultry. According to our understanding, this is the first study related to the distribution of phylogenetic groups among ExPECs isolates from pig and poultry in Vietnam. The majority of potential ExPEC strains belonged to phylogroups A and B1. However, isolates from poultry feces were associated more frequently with group A whereas isolates from poultry carcasses were associated substantially with group B1. As indicated in comparison of virulence profiles of potential ExPEC strains from poultry and pigs, the virulence potential of ExPEC strains from poultry was further supported by phylogenetic analysis of these strains. Our data show the existence of potential ExPEC strains belonging to phylogroup B2 (1.4%) in poultry feces and the significantly higher frequency of phylo D- ExPEC strains in poultry feces (19.1%) versus the frequency in pigs feces (3.8%). Studies in US demonstrated that virulent extraintestinal *E. coli* strains originated mostly from phylogenetic group B2, and to a

lesser extent from group D (Johnson et al., 2007a). Compared to other studies, the prevalence of potential ExPEC strains belonged to phylogroup B2 in poultry in Vietnam was lower than those from European countries (Cortes et al., 2010; Giufre et al., 2012). However, our data are consistent with previous studies in terms of the predominant prevalence of phylo D- ExPEC strains in poultry versus the prevalence in pigs (Cortes et al., 2010). These findings have shown the occurrence of potential pathogenic *E.coli* strains in food animals in Vietnam, particularly in poultry, which may be associated with clinical strains in humans.

Our analysis of 83 potential ExPEC isolates from pig carcasses and poultry carcasses revealed a small difference between the proportions of potential ExPEC belonging to group D in poultry strains (9.1%) and in pig strains (7.7%). This finding is likely inconsistent with the higher frequency of phylo D- ExPEC isolates in poultry implied that poultry might deserve greater attention than pigs, with respect to the possibility of transmission of ExPEC (Johnson et al., 2005b; Xia et al., 2011).

In our study, the phylogroups A and B1 *E.coli* strains, traditionally belonging to commensal strains in humans, possessed more investigated virulence genes than phylogroups B2 and D isolates. All investigated virulence genes were detected among phylogroups B1 isolates whereas only *iucD* gene was detected among isolates belonged to phylogroups B2. The *cnf* gene, which is involved in UTI in humans (Johnson et al., 1991), was not present in phylogroups B2 and D isolates. These findings were not in accordance with other studies in terms of the dominance of ExPEC virulence genes in B2 and D isolates (Jakobsen et al, 2010, Duriez et al., 2001). Thus, the virulence genes profiles may vary due to a limitation on the number of investigated virulence genes or the specificity of these primers used in our study

In the present study, most *E.coli* isolates that carried *iucD:tsh* belonged to group A and B1, whereas these strains carried *iucD:papC* were more frequently associated with group D. Those strains that carried the *iucD:tsh:papC* were associated positively with group A and B1 but negatively associated with group B2 and D. Thus, phylogroup A and B1 isolates should not be underestimated when assessing

their potential impact on human disease. Also, based on a poultry infection model, Ewers et al. demonstrated that pathogenicity of commensal poultry isolates might result in a source of strains capable of causing extraintestinal infections (Ewers et al., 2009).

Our results showed that multidrug resistance is widespread among these potential ExPEC isolates from pigs and poultry, not corresponding to geographical and/or sanitary conditions at farms and abattoirs. There were 95% and 94% isolates from pigs and poultry, respectively that showed resistance to four or more classes of antimicrobials. These results were consistent with the high multidrug resistance in *E.coli* from retail meat reported in a previous study in Vietnam (Van et al., 2008). The findings might be explained by the widespread use of antimicrobials, often without prescription, in animal production in Vietnam. Therefore, it is tempting to suggest that this is a primary source of selection for resistant organisms.

Overall, our findings showed a widespread resistance to quinolones and fluoroquinolones among *E. coli* from pigs and poultry. *E. coli* isolates from poultry were more significantly associated with ciprofloxacin and nalidixic acid resistance than those from pigs ($P<0.05$). These findings were in broad agreement with other studies on high prevalence of resistance to ciprofloxacin among *E. coli* of pig and poultry origin reported in other Asia countries such as China and Korea (Tang et al., 2011; Yang et al., 2004; Lee et al., 2005). In contrast, resistance to ciprofloxacin in *E. coli* isolates from poultry was reported as either low or non-existent in United States, Canada and some European countries (Gyles, 2008), perhaps due to restricted use of fluoroquinolones in animal husbandry in these countries. For instance, use of fluoroquinolones were not permitted in pigs in Canada, thus *E. coli* isolated from this source showed very little resistance to these antimicrobials (Boerlin et al., 2005). Fluoroquinolones are critically important for treating serious infections in humans, such as diarrhea in children (Nguyen et al., 2005). Therefore, the likelihood that resistance to fluoroquinolones in *E. coli* was induced by the use of these antimicrobials in food animals is a major public health concern in Vietnam.

Remarkably, *E. coli* isolates from poultry feces and carcasses displayed a significant association of resistance of nalidixic acid and ciprofloxacin, at the rate of 89.4% and 61.9% and 80.9% and 38.1% respectively. The associations of nalidixic acid and ciprofloxacin resistance were also observed in *E. coli* and ExPEC isolated from poultry meat in US (Johnson et al., 2005b). This might be explained due to contamination of animal carcasses with the host's fecal flora during slaughter and processing and with use of antimicrobial agents in food-animal production. Likewise, the associations of nalidixic acid and ciprofloxacin resistance with poultry and pigs correspond with the approved veterinary use of fluoroquinolones in Vietnam.

In this study, comparison of the resistance phenotypes of the potential ExPEC strains isolated from carcasses with those from pigs and poultry feces revealed that abattoir and farm isolates shared the major resistance patterns. The presence of overlapping resistance phenotypes between *E. coli* isolates from retail meat and farms has been previously described (Jacobsen et al, 2010). Strikingly, in this study, we found one ExPEC isolate from poultry feces and one isolate from poultry carcasses shared the same phylo-virotype D-*iucD*, and the same multi-resistance pattern of ten antimicrobials. The finding of ExPEC strains which share the same virotypes, phylotypes and antimicrobial resistance profiles among farms and abattoirs indicate the risk of contamination of potentially pathogenic *E. coli* from feces to carcasses during slaughter.

As observed in previous studies (Do et al., 2006; Van et al., 2008; Yang et al., 2004), most *E. coli* isolates from pigs and poultry were resistant to tetracycline, streptomycin, amoxicillin, and trimethoprim/sulfamethoxazole. It is important to note that the two latter antimicrobials are frequently prescribed for treatment of UTIs in humans (Talan et al., 2008). Also, more than 70% isolates from all sources were resistant to chloramphenicol. In fact, chloramphenicol is prohibited for use in food animals and chloramphenicol residue is rarely detected in retail food in Vietnam (http://www.cddep.org/sites/cddep.org/files/publication_files/VN_Report_web_1.pdf ?issuusl=ignore). Thus, the high prevalence of chloramphenicol resistance might be caused by the co-selection pressure due to use of other antimicrobials in the phenicol

group, such as florfenicol, in animal production (Harada et al., 2006). In the other way, the high prevalence of chloramphenicol -resistance might be caused by the persistence of chloramphenicol -resistant genes such as the *cmlA* gene located on transferable plasmids in linkage with other genes encoding resistance to antimicrobials that are currently approved for use in food animals (Bischoff et al, 2005). It is noteworthy that multi-resistance to ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole was also reported in diarrheagenic *E. coli* strains isolated from children in Vietnam (Nguyen et al., 2005). Taken together, these results suggest food animals might be a reservoir, and consequently, meats might be a vehicle for transmitting antimicrobial resistant *E. coli* strains which may cause infections in humans.

In our study, most isolates were susceptible to antimicrobials that belonged to the third-generation cephalosporin class, including cefoxitin, ceftiofur and ceftriaxone. Resistance to cefoxitin was detected in only one isolate from pig carcasses. Interestingly, resistance to ceftiofur and ceftriaxone and the presence of $bla_{CTX\text{-}M\text{-}15,}$ encoding the most common ESBL associated with human ExPEC from UTI, was found in poultry *E. coli* isolates recovered from Mac Conkey agar containing ceftiofur. In terms of public health impact, this observation suggested the risk of development and selection for extended spectrum-lactamases (ESBLs) that could be potentially transferred through the food chain in Vietnam.

On comparison of VFs and the antimicrobial resistance phenotype of our ExPEC isolates, we observed significant correlations. Among pig isolates from farms and abattoirs, these included correlations between nalidixic acid and ciprofloxacin resistance and possession of *iucD*; and ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole, and tetracycline resistance and possession of *iucD* or *tsh* (Annex 3). Among poultry isolates from farms and abattoirs, there were correlations between nalidixic acid and ciprofloxacin resistance and possession of *iucD*; and ampicillin, trimethoprim-sulphamethoxazole, and tetracycline resistance and possession of *iucD* or *iucD:tsh*. Correlations between VF and resistance profiles were reported among ExPEC isolates from poultry and humans, for

example,virulence genes were positively associated with multidrug resistance, including *ireA, ibeA, fyuA, cvaC, iss, iutA, iha,* and *afa* (Johnson et al., 2012). This is supportive of previous work suggesting that certain subsets of ExPEC are capable of zoonotic transfer (Johnson et al., 2003a, 2007). Nevertheless, poultry and pig isolates differed significantly in their assignments to phylogenetic groups and antimicrobial resistance phenotypes, with phylogroup A being more prevalent among nalidixic acid and ciprofloxacin- resistance pig isolates than among poultry isolates, whereas phylogroup D was associated with nalidixic acid and ciprofloxacin- resistance isolates of poultry origin (Cortes et al., 2010. This finding strengthens the hypothesis that poultry might be a source for at least ciprofloxacin-resistant group D strains which are considered as potential clinical strains in humans.

In our study, the presence of the AMR genes in correlation with antimicrobial resistance phenotype was investigated with 77 *E. coli* isolates which showed the highest degree of antimicrobial resistance. Although quinolone resistance results mostly from chromosomal mutations in *Enterobacteriaceae*, it may also be caused by plasmid-mediated genes. Several reports had shown a low prevalence of the *qnr* genes in food-producing animals (Yue et al., 2008). In our study, the PCR results revealed that 26.2% quinolone-resistant isolates in pig and in poultry possessed a *qnr* gene. However, 62.5% isolates in pig and poultry which possessed *qnr* gene were susceptible or intermediate with nalidixic acid and ciprofloxacin. Since *qnr* was found in both ciprofloxacin-susceptible and -resistant *E. coli* strains, *qnr* genes may promote further selection from low- to high-level resistance when quinolones are used (Robicsek et al, 2006). Our findings support the previous studies which showed PMQR determinants confer low-level resistance to quinolones and facilitate selection of higher level quinolone resistant bacteria (Tran et al., 2005; Jacoby et al., 2006, Yue et al, 2008). However, further studies are necessary to determine the factors contributing to the nalidixic acid and ciprofloxacinresistance phenotype in potential ExPEC isolates in our study.

In the present study, the simultaneous presence of *qnrA* and *qnrS* was detected in two strains from poultry carcasses, however only one strain showed full resistance to

nalidixic acid and ciprofloxacinwhereas the other strain was intermediate with nalidixic acid and susceptible with ciprofloxacin. The co-existence of *qnrB* and *qnrS* genes was also found in one nalidixic acid and ciprofloxacin-resistant *E. coli* strain from a pig carcass. In contrast, the only one *qnrB*-positive *E. coli* strain from pig carcasses was susceptible to both nalidixic acid and ciprofloxacin. These results suggest that the combination of these *qnr* genes did not have any additional effect on resistance to nalidixic acid.

Our data show the high prevalence of *qnr* genes in *E. coli* from food production animals, in particular in poultry meat and pork. In a previous study, a high prevalence of *qnr* genes, in particular the *qnrS* gene was observed in hospitalized patient *E. coli* strains as well as in *E. coli* community strains in Ho Chi Minh City, Vietnam (Le et al., 2009). These findings together suggest a potential public health concern about the spread of genetic resistance elements from food animal products to humans through the food chainin Vietnam. In addition, class 1 integrons are known to be associated with multiple-drug resistance among Gram-negative bacteria, and specifically have been shown to be associated with the presence of *qnr* gene which provides low-level quinolone resistance (Wang et al, 2003). The occurrence of *qnr* genes in fluoroquinolone-resistant *E. coli* isolates suggests the possibility that *qnr* genes which are present in some of our pig and poultry strains might be associated with the presence of class 1 integrons.

A variant of the *aac(6')-Ib* gene, the *aac(6')-Ib-cr* has been indicated as the gene encoding for enzyme acetylates ciprofloxacin and norfloxacin (Robicsek et al., 2006a). The co-transmission of *qnr* with *aac(6')-Ib-cr* genes which speeds up the formation of multidrug resistance in *E. coli* has been previously reported in China (Yue et al., 2008). In our study, *aac(6')-Ib* gene was present in only one isolate from pig feces and two isolates from poultry feces. The findings showed that currently there is no significant association between *aac(6')-Ib* gene and the quinolone resistance in pig and poultry farms in Vietnam. Our findings also showed that there is no association between *aac(6')-Ib* genes and other antimicrobial resistant genes. However, the presence of the potential ExPEC strains harbouring the *aac(6')-Ib*

might reflect a steady increase due to the use of quinolone and kanamycin on pig and poultry farms, that might gradually have generated selection pressure for these resistant strains.

The high correlation between genotype and phenotype of ExPEC strains with respect to resistance to ampicillin and streptomycin indicated that the resistance genes investigated had important roles in conferring resistance among the isolates. Our findings showed that bla_{TEM} was associated significantly with potential ExPEC isolates from poultry feces and poultry carcasses ($P<0.05$). The gene encoding resistance to streptomycin, *aadA1*, was also widely distributed at high frequency in pigs and poultry isolates. These results were consistent with previous studies which reported that bla_{TEM} and *aadA1* were the predominant genes associated with ampicillin and streptomycin resistance, respectively, in animal strains (Tang et al, 2011, Van et al, 2008).

To our knowledge, this study is the first to report on the occurrence of ESBL-producing isolates in pig feces and poultry meat in Vietnam. Previously, $bla_{CTX\text{-}M\text{-}15}$, the most common ESBL gene among human ExPEC strains isolated from both UTI and sepsis, had been detected in *Shigella spp* in Vietnam (Nguyen et al., 2010). In our study, the $bla_{CTX\text{-}M\text{-}15}$ gene was detected in poultry carcass *E. coli* isolates recovered from Mac Conkey agar containing ceftiofur. Interestingly, associations between virulence genes and antimicrobial resistance genes were observed in our study. For instance, bla_{TEM} was observed in association with *iucD* among pig and poultry-feces isolates.

Interestingly, it was observed that compared to other sources, *E. coli* in poultry showed a higher frequency of antimicrobial resistance, and of presence of antimicrobial resistance genes and virulence genes. Overall, it could be concluded that poultry meat is an important risk-associated food source in terms of antimicrobial resistance and virulence potential. The spread of antimicrobial agents given to poultry might be the reason for high rates of resistance and overcrowding in

poultry husbandry may contribute to the spread of antimicrobial and virulence genes between populations.

CONCLUSION

Our results confirm those of several other studies that meats, particularly poultry meat, and to a lesser extent, pork, might be a vehicle for transmission of potentially zoonotic ExPEC strains. This study suggests that ExPEC in pig and poultry carcasses may originate from pigs and poultry on-farm, respectively, due to fecal contamination during slaughter. This study also shows the low prevalence of STEC and EPEC in pigs and poultry, which need to be constantly monitored to determine the potential risks of these food-borne pathogenic *E. coli* in Vietnam. Phylogroup distribution and virulence profiles suggest that ExPEC isolates from poultry are potentially more pathogenic for humans than those from pigs. The occurrence of phylogroup D isolates in pigs and poultry and, particularly, the presence of group B2 poultry-isolates, implicate the emergence of ExPEC strains which may be related to clinical human ExPEC isolates.

Moreover, this study shows that antimicrobial resistance is widespread among *E. coli* from pigs and poultry in Vietnam, not corresponding to geographical areas. Additionally, our results provide evidence for the possibility of contamination of AMR strains from animals to carcasses during slaughter. The frequencies of resistances to several antimicrobials, and several relevant resistance genes, and the occurence of multidrug resistant ExPEC have been found to be substantially high in pigs and poultry. This indicates that a lack of control of antimicrobial use in food-producing animals in Vietnam has resulted in the emergence and dissemination of multidrug resistant isolates. The finding that PMQR and ESBL genes were present among *E. coli* isolates on farms and also in abattoirs, indicate a potential public health concern that food animals might be a reservoir for the dissemination of these genes from food animals to humans through the food chain. Further investigation is required to assess the risk of zoonotic transmission via the food chain. Based on the results of this study, it is suggested that the widespread use of quinolones and/or penicillin to food-production animals might select for β-lactamase and PMQR strains in animals.

Although the prevalence of ExPEC virulence genes is not very high in *E. coli* isolates on the populations of pigs and poultry on-farm, their presence in the microflora of

health animals and the association between virulence genes and antimicrobial resistance genes is a major public health problem. The results of prevalence of virulence gene *iucD* and its association with β-lactamase isolates are of major concern, considering the likelihood that these two genes are encoded on the same plasmid. Thus, future work should include further study of the genomic profile of strains of *E. coli* as well as of the genetic background of antimicrobial resistance.

Because this study relied on a limited number of virulence and AMR genes, the prevalence of potential ExPEC strains and AMR strains may be underestimated. Further comprehensive studies with appropriate epidemiological and genetic tools are needed to elucidate more fully the prevalence of ExPEC and AMR strains in food producing animals in Vietnam. In conclusion, demonstration of the occurrence of ExPEC and AMR strains in pigs and poultry on farms and in abattoirs in Vietnam, underlines the importance of restricting antimicrobial use and improving sanitary conditions during slaughter, in order to minimize public health risks associated with animal food in Vietnam.

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ANNEXES

Annex 1 List of samples of poultry feces and carcasses collected in Vietnam

No.	Farm	Number of fecal samples		No.	Abattoir	Number of carcass samples	
		Total	Code			Total	Code
1.	Poultry farm in Bac Giang province	8	FC1-FC8	1.	Phuc Thinh Company, Hanoi	3	CM1-CM3
2.	Poultry farm 1 in Thai Nguyên province	4	FC9-FC12				
				2.	Phuc Thinh Company, Hanoi	3	CM4-CM6
3.	Poultry farm 2 in Thai Nguyên province	4	FC13-FC16				
	Poultry farm 2 in Thai Nguyên province	8	FC17-FC24	3.	Phuc Thinh Company, Hanoi	3	CM7-CM9
4.	Poultry farm 3 in Thai Nguyên	12	FC25-FC36	4.	Minh Hien Company, Hanoi	9	CM10-CM18
5.	Poultry farm in Hoa Binh province	15	FC37-FC51				
	Total	**51**				**18**	

Annex 2 List of samples of pig feces and pig carcasses collected in Vietnam

No.	Farm	Number of fecal samples		No.	Abattoir	Number of carcass samples	
		Total	Code			Total	Code
1.	Pig farm 1 in Son Tay town, Hanoi	4	FP1-FP4	1.	Foodex Limited Company, Hanoi	2	PM1-PM2
2.	Pig farm 2 in Son Tay town, Hanoi (Herd 1)	5	FP5-FP9	2.	Foodex Limited Company, Hanoi	4	PM3-PM6
	Pig farm 2 in Son Tay town, Hanoi (Herd 2)	7	FP10-FP16				
	Pig farm 2 in Son Tay town, Hanoi (Herd 3)	8	FP17-FP24				
3.	Pig farm 3 in Son Tay town, Hanoi	2	FP25-FP26				
4.	Pig farm in Ninh Binh province	4	FP27-FP30	3.	Abattoir 1, Hanoi	1	PM11
5.	Pig farm 1 in Thai Binh province	4	FP31-FP34	4.	Abattoir 2, Hanoi	2	PM14-PM15
6.	Pig farm in Thanh Oai district, Hanoi	2	FP35-FP36	5.	Abattoir 3, Hanoi	1	PM7
7.	Pig farm 2 in Thai Binh province	2	FP37-FP38	6.	Abattoir 4, Hanoi	3	PM8, PM12-PM13
				7.	Abattoir 5, Hanoi	2	PM9-PM10
8.	Pig farm in My Duc district, Hanoi	10	FP39-FP48	8.	Abattoir 1, Hanoi	2	PM16-PM17
9.	Pig farm in Bac Giang province	4	FP49-FP52	9.	Abattoir 2, Hanoi	2	PM18-PM19
10.	Pig farm in Bac Ninh province	6	FP53-FP58	10.	Abattoir 3, Hanoi	2	PM20-PM21
	Total	**58**				**21**	

Annex 3. Association of virulence genes and antimicrobial resistance among 132 potential ExPEC isolates

Source/ Virotype	No. of isolates (*n*)	Percentage of resistance isolates														
		(1)					*(2)*		*(3)*				*(4)*	*(5)*	*(6)*	*(7)*
		AMP	AMC	TIO	FOX	CRO	NAL	CIP	AMK	GEN	KAN	STR	SXT	CHL	FIS	TET
Pig feces	40	92.5	10	0	2.5	0	47.5	30	0	45	27.5	80	70	70	92.5	100
iucD	21	95.2	14.3	0	4.76	0	66.7	47.6	0	52.4	42.9	85.7	76.2	76.2	95.2	100
tsh	7	85.7	14.3	0	0	0	0	0	0	28.6	28.6	42.9	85.7	71.4	100	100
iucD:tsh	8	87.5	0	0	0	0	37.5	0	0	25	0	87.5	50	50	87.5	100
iucD:papC	1	100	0	0	0	0	0	0	0	100	0	100	0	100	0	100
iucD:tsh:papC	1	100	0	0	0	0	0	0	0	0	0	100	0	0	100	100
iucD:cnf	1	100	0	0	0	0	100	100	0	100	0	100	100	100	100	100
Pig carcass	24	91.7	12.5	0	4.17	0	45.8	25	0	29.2	25	62.5	79.2	79.2	75	100
iucD	13	84.6	0	0	0	0	61.5	30.8	0	30.8	15.4	53.8	61.5	76.9	61.5	100
tsh	7	100	28.6	0	0	0	42.9	28.6	0	0	28.6	71.4	100	85.7	85.7	100
iucD:tsh	1	100	0	0	0	0	0	0	0	0	0	0	100	0	100	100
iucD:papC	2	100	0	0	50	0	0	0	0	100	50	100	100	100	100	100
iucD:tsh:papC	1	100	100	0	0	0	0	0	0	100	100	100	100	100	100	100
Poultry feces	47	91.5	23.4	0	0	0	89.4	80.9	2.1	66	74.5	83	97.9	93.6	97.9	100
iucD	34	91.2	14.7	0	0	0	91.2	85.3	0	70.6	76.5	88.2	97.1	91.2	97.1	100
tsh	4	100	75	0	0	0	75	50	0	75	75	25	100	100	100	100
iucD:tsh	6	83.3	33.3	0	0	0	100	83.3	0	33.3	66.7	83.3	100	100	100	100
iucD:papC	2	100	0	0	0	0	100	100	50	100	100	100	100	100	100	100
tsh:papC	1	100	100	0	0	0	0	0	0	0	0	100	100	100	100	100
Poultry carcass	21	95.2	14.3	9.52	0	9.52	66.7	38.1	0	38.1	57.1	71.4	100	81	100	100
iucD	14	92.9	7.14	0	0	0	78.6	50	0	50	57.1	85.7	100	78.6	100	100
iucD:tsh	3	100	0	0	0	0	33.3	33.3	0	33.3	0	33.3	100	66.7	100	100
iucD:papC	2	100	100	0	0	0	0	0	0	0	100	0	100	100	100	100
iucD:tsh:papC	2	100	0	100	0	100	100	0	0	0	100	100	100	100	100	100

Annex 4. Association of phylotypes and antimicrobial resistance among 132 potential ExPEC isolates

Source/ Phylotype	No. of isolates (*n*)	Percentage of resistance isolates														
		(1)					*(2)*		*(3)*				*(4)*	*(5)*	*(6)*	*(7)*
		AMP	AMC	TIO	FOX	CRO	NAL	CIP	AMK	GEN	KAN	STR	SXT	CHL	FIS	TET
Pig feces	40	92.5	10	0	2.5	0	47.5	30	0	45	27.5	80	70	70	92.5	100
A	24	87.5	12.5	0	4.2	0	33.3	29.2	0	41.7	33.3	70.8	66.7	75	87.5	100
B1	13	100	7.69	0	0	0	61.5	30.8	0	53.8	15.4	92.3	69.2	61.5	100	100
D	3	100	0	0	0	0	100	33.3	0	66.7	33.3	100	100	33.3	100	100
Pig carcass	24	91.7	12.5	0	4.17	0	45.8	25	0	29.2	25	62.5	79.2	79.2	75	100
A	15	93.3	20	0	6.67	0	40	26.7	0	26.7	26.7	60	93.3	73.3	86.7	100
B1	7	85.7	0	0	0	0	57.1	14.3	0	14.3	0	57.1	57.1	85.7	57.1	100
D	2	100	0	0	0	0	50	50	0	100	100	100	50	100	50	100
Poultry feces	47	91.5	23.4	0	0	0	89.4	80.9	2.1	66	74.5	83	97.9	93.6	97.9	100
A	15	93.3	46.7	0	0	0	80	73.3	0	66.7	66.7	80	100	100	93.3	100
B1	15	93.3	13.3	0	0	0	93.3	93.3	0	53.3	73.3	86.7	93.3	86.7	100	100
D	15	86.7	6.7	0	0	0	93.3	80	6.7	80	86.7	80	100	93.3	100	100
B2	2	100	50	0	0	0	100	100	0	100	50	100	100	100	100	100
Poultry carcass	21	95.2	14.3	9.52	0	9.52	66.7	38.1	0	38.1	57.1	71.4	100	81	100	100
A	4	75	0	0	0	0	100	25	0	50	75	75	100	75	100	100
B1	13	100	7.7	15.4	0	15.4	61.5	38.5	0	30.8	46.2	76.9	100	76.9	100	100
D	4	100	50	0	0	0	50	50	0	50	75	50	100	100	100	100

Annex 5. Association of phylotypes and antimicrobial resistance genes among 77 potential ExPEC isolates from different sources

Source/ Phylotype	Total[a] (n)	No. (%) of *E.coli* isolates[b]								
		bla_{TEM}	$bla_{CTX\text{-}M\text{-}15}$	bla_{SHV}	bla_{OXA}	*qnrA*	*qnrB*	*qnrS*	*aac(6')-Ib*	*aadA1*
Pig feces	25	10 (40.0)	0	1 (4.0)	0	1 (4.0)	0	6 (24.0)	1 (4.0)	17 (68.0)
A	13	6 (46.2)	0	0	0	1 (7.7)	0	3 (32.1)	1 (7.7)	10 (76.9)
B1	9	3 (33.3)	0	1 (11.1)	0	0	0	2 (22.2)	0	4 (44.4)
D	3	1 (33.3)	0	0	0	0	0	1 (33.3)	0	3 (100)
Pig carcasses	12	7 (58.3)	0	0	0	0	2 (16.7)	8 (66.7)	0	9 (75.0)
A	7	5 (71.4)	0	0	0	0	2 (28.6)	5 (71.4)	0	6 (85.7)
B1	3	0	0	0	0	0	0	1 (33.3)	0	2 (66.7)
D	2	2 (100)	0	0	0	0	0	2 (100)	0	1 (50.0)
Poultry feces	28	24 (85.71)	0	0	0	3 (10.7)	0	1 (3.57)	2 (7.1)	20 (71.4)
A	7	5 (71.4)	0	0	0	1 (14.3)	0	0	0	6 (85.7)
B1	10	8 (80.0)	0	0	0	2 (20.0)	0	0	2 (20.0)	5 (50.0)
D	9	9 (100)	0	0	0	0	0	0	0	8 (88.9)
B2	2	2 (100)	0	0	0	0	0	1 (50.0)	0	1 (50.0)
Poultry carcasses	12	10 (83.33)	2 (16.67)	0	0	3 (25.0)	0	3 (25.0)	0	7 (58.3)
A	3	2 (66.7)	0	0	0	1 (33.3)	0	1 (33.3)	0	2 (66.7)
B1	5	5 (100)	2 (40.0)	0	0	2 (40.0)	0	2 (40.0)	0	3 (60.0)
D	4	3 (75.0)	0	0	0	0	0	0	0	2 (50.0)
Total	77	51 (66.2)	2 (2.6)	1 (1.3)	0	7 (9.1)	2 (2.6)	18 (23.4)	3 (3.9)	53 (68.8)

[a]: Number of potential ExPEC isolates from different sources.

[b]: Percentages of ExPEC isolates which are positive with the AMR gene.