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Environmental Reservoir of Antimicrobial Resistance in Poultry Production Facilities and Processing Plants in Alberta

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Environmental Reservoir of Antimicrobial Resistance in Poultry Production Facilities and Processing Plants in Alberta

by

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Abstract

This study was conducted to determine the role of environmental bacteria in maintaining antimicrobial resistance genes in poultry barns and poultry processing plants in Alberta. Aerobic, non-fastidious, fast growing bacteria were isolated from environmental samples. Approximately half of the bacteria were gram-negative and half were gram-positive. Resistance to clinically important antibiotics such as third generation cephalosporins was observed in 20-35% of the gram-negative isolates. Sixty percent of all bacteria assayed were considered multi-drug resistant, having resistance to antibiotics in three or more drug classes. Ten of 14 *E. coli* isolates were resistant to 1st, 2nd and 3rd generation cephalosporins, three of which harboured the *bla*CMY-2 gene, which encodes a plasmid borne AmpC cephalosporinase enzyme. Bacterial species expressing a variety of resistance phenotypes were tested for their ability to form a biofilm *in vitro* and for their ability to withstand exposure to commonly used disinfectants while residing in biofilm. In conclusion, multi-drug resistant environmental bacteria present in poultry barns and processing facilities can serve as reservoir of antimicrobial resistant genes, which could potentially be transferred to pathogenic bacteria.

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Dedication

This thesis is dedicated to my mother Gauri Thapa from whom I learnt to become strong, resilient and optimistic during the struggle period of our lives. Her constant love, support and encouragement throughout helped me achieve my goals in my life.

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Symbols	Definition
^{0}C	degree centigrade
AGP	Antibiotic Growth Promoter
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
bp	base pairs
CAMHB	Cation Adjusted Mueller Hinton Broth
CFIA	Canadian Food Inspection Agency
CFU	Colony Forming Unit
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLSI	Clinical and Laboratory Standard Institute
cm	centimeters
CVMA	Canadian Veterinary Medical Association
DNA	Deoxy Ribonucleic Acid
EDTA	Ethylene Diamine Tetracetic Acid
ELDU	Extra Level Drug Use
ESBL	Extended Spectrum β-Lactamase
FAO	Food And Agriculture Organization
GDP	Gross Domestic Product
GNID	Gram Negative Identification
GPID	Gram Positive Identification
kb	kilo base pairs
kg	kilogram
LB	Luria Bertani
MBEC	Minimum Biofilm Eradication Concentration Assay
MDR	Multidrug Resistant
MIC	Minimum Inhibition Concentration
mL	milliliters
mm	millimeters
NCBI	National Center for Biotechnological Institute
ng	Nanogram
PCR	Polymerase Chain Reaction
QAC	Quaternary Ammonium Compound
rpm	revolutions per minute
RT	Room Temperature
TAE	Tris Acetate EDTA
TE	Tris EDTA
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VDD	Veterinary Drug Directorate
WHO	World Health Organization

µg	microgram
µL	microliters

Chapter One: **REVIEW OF LITERATURE**

1.1 General Introduction

Poultry production is a growing industry, contributing approximately $6.5 billion to the Canadian Gross Domestic Product (GDP) (1). Alberta is the fourth-largest chicken producing Province in Canada with 239 farmers and 73 processors, contributing $830 million to the national GDP (1). Alberta shares approximately nine percent of the total chicken production of Canada (2). The Canadian per-capita consumption of chicken meat has increased by 30 kg during the last two decades, while consumption of pork and beef have declined (3).

The poultry industry is well regulated by federal and provincial level organizations in order to ensure Canadians from safe poultry products through food safety regulations. At the federal level, the Canadian Food Inspection Agency (CFIA) works with provincial and local government through its Food Safety Enhancement Program to ensure consumers get high quality products free from potential foodborne pathogens, chemicals and physical food safety hazards from farm to fork (4). At the provincial level, organisations such as Alberta Agriculture and Rural Development regulates food safety, animal health and welfare (5). At the industry level, various commodity groups such as Chicken Farmers of Canada, Turkey Farmers of Canada, Egg Farmers of Canada and Alberta Chicken Producers (Alberta) collaborate with government to provide safe food to Canadians.

Despite the efforts to provide high quality food products to Canadians, bacterial contamination of food products result frequently in foodborne illness and food product re-call. It

is estimated that four million people suffer from foodborne illness every year in Canada. The five most common pathogens that cause foodborne illness in Canada are: Norovirus, *Clostridium perfringens*, *Campylobacter* spp., non-typhoidal *Salmonella*, and *Escherichia coli* 0157:H7 (6, 7). The bacterial pathogens *E. coli* 0157:H7, *Salmonella* Typhimurium, *S.* Heidelberg, *S.* Enteritidis (eggs), and *Campylobacter jejuni* are most commonly associated with chicken meat and egg food products in Canada (7-9)

The dissemination of foodborne pathogens is of great concern for the food safety and public health sectors. The increasing prevalence of antibiotic resistance among foodborne pathogens is also of concern. Increasingly antibiotic-resistant bacterial infections lead to higher morbidity and mortality, higher medical costs and longer hospital stays (10). The World Health Organization (WHO) recently declared that we are moving towards the post antibiotic era where a minor injury would be life threatening due to antimicrobial resistant infections (11).

The uses of antimicrobials in animals are hypothesized to be one of the drivers of antimicrobial resistant bacterial infections in humans. The European Food Safety Authority (EFSA) indicated food animals are the possible reservoir of cephalosporinase producing gram-negative bacteria (12). Such bacteria can spread from food animals to human via food products and the environment (13). Considering the fact that antimicrobial resistance is a global problem, following recommendations from the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), the WHO categorized antimicrobials into three categories based on their importance in human medicine: critically important, very important and important. The purpose of this categorization was to guide public health and animal health authorities,

medical and veterinary professionals, and related stakeholders in the prudent use of critically important antimicrobials both in human and animals (13). In order to minimise the development of resistance and preserve the effectiveness of life saving antibiotics both in humans and animals the Veterinary Drugs Directorate (VDD) under Health Canada has categorized antimicrobials into four categories based on their importance in human medicine: Category I- Very high importance, Category II- High importance, Category III- Medium importance and Category IV- Low importance (14). Based on this categorization, the Canadian Veterinary Medical Association (CVMA) developed an antimicrobial prudent use guideline for poultry, beef cattle, dairy cattle and swine. The purpose of this document is to guide veterinarians in antimicrobial selection decision-making process (15).

Since antimicrobial resistance is a multi-species, multi-drug, and multi-national problem, a collaborative effort among the public health organizations and stakeholders is necessary to mitigate the risk of spread of antimicrobial resistance in order to protect human and animal health. A co-ordinated intervention to manage the proper drug selection, dose, interval and route of administration would help improve the overall health of the patients including animals. This kind of intervention would limit the selection pressure for antibiotic resistance and spread of antibiotic resistant bacterial infections (16). In order to provide meaningful data for use in creating guidelines for antimicrobial usage, an efficient surveillance system is required to monitor regularly the use of antimicrobials and the occurrence of antimicrobial resistant bacteria. The Canadian Integrated Program for Antimicrobial Resistance (CIPARS) monitors trends of antimicrobial use and antimicrobial resistance in selected bacterial species such as generic *E. coli*, *Campylobacter* spp. *Enterococcus* spp. and *Salmonella enterica* from animal farms, animal

feed, abattoirs, retail meat, and human and animal clinical samples (17). The Health Canada uses the surveillance data in order to develop appropriate risk management strategies regarding the antimicrobial uses and resistance, and formulate policies and regulations (18).

1.2 Definition of Antimicrobials

An *antibiotic* is defined as a low molecular weight substance produced by microorganisms that inhibits or kills other microorganisms at low concentrations (19). An *antimicrobial agent* is a broader term that includes natural, synthetic or semisynthetic substance that kills or inhibits the growth of microorganisms causing little or no damage to the hosts (19). The term *antimicrobial* is used synonymously with the term *antibiotic* throughout this thesis.

1.3 Mechanisms of Resistance

Bacteria possess both intrinsic and acquired mechanisms, which confer resistance to antimicrobial compounds. These mechanisms include: enzymatic inactivation of antibiotic compound, modification of antibiotic target, expression of efflux pumps and changes in outer membrane permeability.

Bacteria produce enzymes that *inactivate* the antibiotic molecule rendering them ineffective. One of the best examples of this mechanism are the β-lactamases produced by bacteria, which cleave the β-lactam ring of penicillin and cephalosporin antibiotics (20). Another example of this resistance mechanism is the aminoglycoside and chloramphenicol inactivating

enzymes. These bacterial enzymes structurally modify the aminoglycoside and chloramphenicol antibiotics so that they fail to bind to the recognition site of the ribosome (21).

Another mechanism of antimicrobial resistance is the *modification of the target* such that antimicrobials are unable to bind to the target site. Modifications on the bacterial penicillin binding proteins (PBP) involved in cross-link peptidoglycan layer during cell wall synthesis results in ineffective binding of β-lactam antibiotics. For example, in methicillin resistant *Staphylococcus aureus*, the *mecA* gene mediates the production of alternate penicillin binding proteins (PBP2a) with lower affinity to β-lactam drugs (20, 21). Resistance to fluoroquinolone is achieved by mutation in the DNA gyrase and DNA topoisomerase IV. This modifies the binding site for the antibiotic, such that the fluoroquinolone is unable to interfere with DNA replication. (21).

In addition to enzymatic inactivation and target modification, bacteria resist the antimicrobial actions through *efflux pumps*. Efflux pumps are membrane proteins that pump antimicrobials out of the cell and maintain the intracellular concentrations at low levels (21). The efflux proteins may be drug specific for tetracyclines or macrolides; or non-specific that can export structurally unrelated drugs, thus conferring multi-drug resistance phenotypes as observed in *Pseudomonas aeruginosa* (21, 22).

Bacteria limit the penetration of antibiotic into the cytoplasm by altering the outer *membrane permeability* of the cell. Antimicrobials enter into the cytoplasm of a cell via diffusion (aminoglycosides) or via porin channels (β-lactams, fluoroquinolones and chloramfenicols)

present in the cell membrane of gram-negative bacteria. Mutations in the porin proteins leading to loss, decrease in size or permeability will limit the penetration of antimicrobials into the bacterial cells (21). For example, in *P. aeruginosa*, the loss of outer membrane protein OprD results in imipenem resistance (22).

1.4 Dissemination of Antimicrobial Resistance

Antimicrobial resistance in bacteria is disseminated by both vertical transfer and horizontal transfer of genetic materials. In vertical transfer or clonal spread, offspring acquire resistance genes from the parent with the chromosome or on extra-chromosomal DNA when the bacteria divide into daughter cells. The resistance to antibiotics may occur when chromosomal mutations occurred in the parents are transferred to their progeny via cell division. One study in Europe showed that multi-drug resistant clones of *Salmonella* Infantis of broiler chicken were found in multiple European countries (23). The authors found that the strain had same multidrug resistant pattern of nalidixic acid-streptomycin-sulfonamide-tetracycline, and harboured *aadA1* gene in a 1 kb class 1 integron located on a >168 kb conjugative plasmid. These isolates were widely distributed in Austria, Hungary and Poland, and were closely related to Hungarian clones, suggesting a common ancestor that had disseminated from Hungary to other countries. Similarly, a study by Coque *et al*. (24) found the clonal dissemination of *bla*CTX-M-15 harbouring human clinical *E. coli* isolates in Canada, France, Switzerland, Kuwait and India. These *E. coli* belonged to sequence type ST131 with multi-drug resistant IncFII plasmid.

Horizontal gene transfer is the exchange of extra-chromosomal DNA between the same or different bacterial species. This mechanism of gene transfer is considered the most common

means of dissemination of antimicrobial resistance genes among bacteria. Once the bacteria acquire transferrable resistance genetic elements such as plasmids, transposons or integrons, they can be transferred to other bacteria by the mechanisms of conjugation, transformation and transduction.

Conjugation is mediated by cell-to-cell contact and a pilus through which genetic materials are transferred from the donor to recipient cells. Conjugation is generally associated with transfer of plasmids which may contain antibiotic resistance genes, transposons or integrons (25). A study has shown that plasmid harbouring β-lactamase enzymes such as *bla*CMY-2 have been transferred from *S. enterica* isolated from ground beef to *E. coli* 0157:H7 by conjugation (26).

During the process of *transformation,* bacteria acquire naked DNA from the extracellular environment and incorporate into their own genome. In order to take up extracellular DNA, the bacteria must be 'competent' (ability to take up extracellular DNA), which involves various structural proteins that helps to uptake DNA from the extracellular space into the cytoplasm. The naked DNA in the environment may be the result of bacterial autolysis, phage lysis and in the laboratory settings by a chemical extraction procedure. The DNA fragments may contain resistance genes (25).

During the *transduction* process, bacteriophage transfers DNA from donor to recipient cells. At the infection phase, the bacteriophage integrates into the bacterial genome and is replicated with the host genome. During the lytic phase, the bacteriophage lyses the host cell and

progeny phages are released in the external environment. After lysis, the phage infects new bacterial host where the acquired DNA recombine to the recipient host genome The phage may get the resistance gene from the lysed cells if present in the genome (27). A study conducted by Colomer-Lluch *et al*. (28) found that bacteriophages isolated from cattle, pig and poultry fecal waste harboured *bla*CTX-M genes suggesting that phages could be the vehicle for transmission of antibiotic resistant genes in the environment.

1.5 Integrons in Gram-negative Bacteria

In many gram-negative bacteria, the mobile genetic element, integrons are responsible for horizontal transfer of antimicrobial resistance genes among bacteria. An integron is described as a mobile genetic element that is able to promote integration and expression of genes present in gene cassettes. (29). The Class 1 integron is commonly present on plasmids and in transposon Tn21, and therefore transferred by bacterial conjugation (30). The class 1 integron consists of 5' and 3' conserved regions separated by variable region. The variable region may have integrated gene cassettes. The 5' conserved region is usually of 1.4 kb and consists of an integrase gene *(intI)* that encodes a site specific integrase enzyme *(IntI)*; attachment site *attI* at which the gene cassettes are inserted or excised catalysed by *IntI*; and the Promoter *(Pc)* sequence that facilitates the expression of gene cassettes when inserted into *attI* (31). The 3'conserved region is usually of 2 kb size and consists of genes *sul1*, which encodes resistance to sulphonamides, a truncated *qac*Δ*E1*, that has been shown to increase the minimum inhibitory concentration of benzalkonium chloride (32). A gene cassette consists of a resistance gene and an attachment site *attC* downstream of the resistance gene. The *attI* of integron and *attC* of gene cassette consists of a core sequence where the site-specific recombination takes place during the integration process.

The gene cassette is a non-replicating mobile genetic element lacking its own promoter and is expressed from the promoter on the integron. Therefore, gene cassettes which are near to the promoter are expressed strongly than the gene cassettes away from the promoter (29). However, an integron may carry number of different gene cassettes up to 8 cassettes resulting in multidrug resistant integrons (33). The Class 2 integrons consist of a defective integrase gene and lack 3'conserved region, which attributes to the low diversity of integrated gene cassettes (29).

1.6 Extended Spectrum β-Lactamase and AmpC β-Lactamase Enzymes

The β-lactamase enzyme is produced by bacteria, which inactivate the β-Lactam antibiotics such as penicillins, cephalosporins and carbapenems. All β-lactam enzymes fall under two categories based on their active (functional) sites: serine or metallobactams. The metallo-β-lactamases require metal ion cofactors such as zinc, iron and manganese for activation. An example of metallo-β-lactamase enzyme is New Delhi Metallo-β-lactamase (NDM) initially found in *K. pneumonia* (34). This NDM enzyme hydrolyses the carbapenem antibiotics (34). The serine β-lactamases catalyse the hydrolysis of β-lactam antibiotics via acyl enzyme formed between substrate and the active site serine. Based on the comparison of amino acid sequences, the serine group of β-lactamases belong to one of the three Ambler classes: Class C, A, and D. Enzymes that belong to class A are encoded by genes such as *bla*TEM, *bla*SHV and *bla*CTX-M, while oxacillinase enzymes that belong to class D are encoded by *bla*OXA. The AmpC enzymes belong to class C and are encoded by the *ampC* gene. (34). Extended Spectrum β-Lactamases (ESBL) are the serine enzymes that are formed as a result of mutations in the early β-lactamases such as TEM-1, TEM-2 and SHV-1, which differ in as few as one amino acid, resulting in

substantial change in enzymatic activity. The ESBL enzymes are able to hydrolyse all penicillins, imino-penicillins, and cephalosporins but not cephamycins and carbapenems because of the structural difference in their functional site. These ESBL enzymes are inhibited by β-lactamase inhibitors such as clavulanic acid and sulbactam (35). When a β-lactam antibiotic in combination with clavulanic acid is given against the bacteria, the clavulanic acid binds with the β-lactamase enzyme produced by the bacteria thereby allowing the β-lactam antibiotic to act on the target site. During this process, clavulanic acid, which is itself a β-lactam is inactivated (also called as 'suicide inhibitor') (36). The class C AmpC enzymes can hydrolyse penicillins, first, second and third generation cephalosporins; and cephamycins. In contrast to ESBL, AmpC enzymes are not inhibited by clavulanic acid and monobactams but are inhibited by cloxacillin and boronic acid (37). Genes encoding ESBL enzymes are commonly located on conjugable plasmids. Plasmids may also carry resistant genes of other antibiotic classes such as aminoglycosides, fluoroquinolones, and sulphonamides (38). A plasmid becomes multi-drug resistant when it harbours multiple resistant genes of different drug classes. Spread of multi-drug resistant plasmids among bacteria in the environment may lead to emergence of antibiotic resistant infections in human and animals if they cause infection.

The *ampC* genes that are present on the chromosome are induced by β-lactams. The release of muropeptides from the damaged cell wall by β-lactams displaces the UDP-muropeptides from the AmpR regulatory protein in the cytoplasm. As a result, *ampC* gene is transcribed. The AmpC enzyme enters into the periplasm and binds to the oncoming β-lactams thereby inactivating the drug (39). In *E. coli,* the *ampC* gene is not inducible because of lack of

the regulatory gene *amp*R and is regulated by a weak promoter and the production of AmpC enzyme is constitutively low (40). Increased production of AmpC enzyme is associated with mutation at various positions in the promoter sequence, conferring resistance to early generation cephalosporins. The common mutations occur in the two conserved sequences of the promoter region: the -35 and the -10 box. Mutations also occur in the attenuator region that can destabilize the hairpin structure allowing increased transcription rate of AmpC production (41). Insertion of insertion elements (ISE) in the promoter region has been found to create a new stronger promoter where RNA polymerase strongly binds for overexpression of AmpC enzymes (42). Likewise, amino acid insertions or substitution of the chromosomal cephalosporinase enzyme have been shown to confer resistance to extended spectrum cephalosporins including cefepime and cefpirome. The amino acid alteration in the enzyme exposes more active sites for wider substrates (43, 44). Besides chromosomal AmpC enzymes, plasmid mediated AmpC enzymes are equally important in conferring resistance to extended spectrum cephalosporins. The plasmid mediated AmpC cephalosporinases were originated from chromosomal *amp*C genes that were transferred to plasmid by recombination (45). Among the many families of plasmid mediated AmpC genes, the *bla*CMY-2 gene is most commonly found in *Enterobacteriacae*. The *bla*CMY-2 genes have been isolated from *E. coli* found in retail chicken meat, feedlot cattle, and human clinical isolates in Canada (42, 46, 47). The plasmids harbouring *bla*CMY-2 genes also carry resistance genes for other antibiotic classes such as aminoglycosides, tetracyclines, sulphonamides, and fenicols. A study by Guo *et al*. (48) showed that *qnrS1* and *florR* genes were co-transferred with the *bla*CMY-2 gene on the same plasmid among *E. coli* isolates. The presence of multi-drug resistant plasmid harbouring *ampC* genes or ESBL genes in bacteria is increasingly important in horizontal dissemination of multidrug resistant bacteria. The *bla*CMY

harbouring plasmids usually belong to IncI1 or IncA/C incompatibility type. The IncI1 type have been commonly isolated in *E. coli* and *S.* Typhimurium of poultry origin and IncA/C from both cattle and poultry associated *E. coli* (46, 48, 49). The IncA/C plasmids have been shown to harbour multi-drug resistance genes and can be transferred horizontally. The IncI1 plasmid has an operon for type IV pilus that is used for adherence and invasion of *E. coli* into eukaryotic cells. It seems that, IncA/C plasmid are responsible for dissemination of multi-drug resistance genes while IncI1 are associated with pathogenic *E. coli* (48, 49). The transfer of multi-drug resistant ESBL or AmpC bearing plasmids among poultry and human *E. coli* through direct contact or via food chain may limit the availability of treatment of serious infections in both humans and animals.

1.7 Biofilm Formation and Antimicrobial Resistance

The term biofilm was coined by J. William Costerton in 1978. It is a structured community of microorganisms enclosed in a self-produced polymer matrix that is attached to an abiotic or biotic surface (50). The polymer matrix (or extracellular polymeric substance, EPS) is comprised of polysaccharides, proteins, phospholipids, teichoic acids and nucleic acids (51). Microorganisms that form biofilms include bacteria, fungi, and protozoa (52). Among the common foodborne pathogens that can make biofilm are: *E. coli* 0157:H7, *S.* Typhimurium, and *L. monocytogens* (51). The bacterial community in the biofilm may be single and/or multiple species and form single, multilayer or complex three-dimensional structures. A mature biofilm is a highly organised ecosystem with water channels running inside that provide passage for exchange of nutrients, metabolites and waste products (51). Most bacteria can grow in a biofilm and are ubiquitous in every ecosystem including natural, man-made engineered and pathogenic

settings (50). An example of natural biofilm formation is in the human gut consisting of commensal microflora attached to the gut epithelial cell, which acts as barriers for foodborne pathogens (53). In clinical settings, biofilms are associated with device related infections such as infections related with the use of urinary catheter and artificial heart valves (54).

A biofilm is a survival strategy of bacteria against harsh environmental conditions such desiccation, pH, temperature, ultraviolet light and starvation (50). Bacteria in biofilm possess a number of resistance mechanisms, which help them to survive against antibiotics and disinfectants as compared to their planktonic counterparts. One of the resistance mechanisms is slow penetration and enzymatic degradation of antibiotic at the biofilm matrix. A study in *P. aeruginosa* biofilm showed that *P. aeruginosa* produced β-lactamase enzyme when exposed to β-lactam antibiotics. The enzymes accumulated at the biofilm matrix which hydrolysed the antibiotic and protected the bacterial cells further below the matrix surface (55). Bacteria in biofilm undergo adaptive stress response in the presence of antimicrobial exposure. The stress response helps the bacteria in biofilm to tolerate antibiotics. Inside the biofilm matrix, there is a substrate gradient of oxygen and nutrients in the matrix so that bacteria near to the attached surface are slow growing or in metabolically inactive state. Thus, antibiotics such as β-lactams which act only on rapidly growing cells are not effective in killing the slow growing cells in the biofilm (56). Antibiotic exposure does not kill all the bacteria in the biofilm. A fraction of the bacterial population becomes unaffected despite the continued exposure of antibiotics. These surviving cells are known as persister cells. When the antimicrobial concentration is removed from the biofilm, the persister cells can cause re-infection or re-contamination of the processing plants (57, 58). Within the biofilm, mobile genetic elements such as conjugative plasmids,

transposons or integrons mediate horizontal gene transfer between resident bacteria (59). A study conducted by Savage *et al*. (60) showed that an antibiotic resistant plasmid was transferred in between *S. aureus* strains when grown in biofilm. Bacteria may also acquire resistance genes present in the EPS matrix by transformation. The resistance genes in the matrix may be the result of bacterial lysis in the biofilm.

Biofilm infections in human and animals are difficult to treat. It has been reported that bacteria in biofilms are 10-1000 times more resistant to antimicrobial agents compared to free living cells (61). Olson *et al*. (62) showed that *S. aureus* isolated from bovine mastitis was more than 500 times more resistant to penicillin G, streptomycin, cloxacillin and tetracycline in biofilm when compared to planktonic form. *S. aureus* has been shown to live in biofilms within the alveoli and lactiferous ducts (63).

1.8 Biofilm and Food Safety

In food processing plants, the nature of solid surface is important for initial cell attachment. Bacteria readily attach to hydrophilic surfaces such as stainless steel and glass and form biofilms. In addition, presence of organic matters on the surface such as carbohydrates, and proteins increases the rate of bacterial cell attachment (51). This might be true in case of meat processing plants where most of the equipment are made up of stainless steel and chances of having a thin layer of organic matters on the surface are high if those surfaces are not sanitized effectively. For example, shackling hooks and eviscerator, the joints of the equipment, or surface under the conveyor belts, are difficult to clean. Likewise, frequent scrubbing of the equipment may make the surface uneven allowing the bacteria to get trapped and not removed properly by

sanitation. As a result, biofilms may form on such surfaces. As the biofilm gets older, it gets more difficult to remove. A recent study showed that commonly used disinfectants such as sodium hypochlorite, sodium hydroxide and benzalkonium chloride were not able to eradicate a seven day old biofilm of *Salmonella enterica* from food contact surface even for 90 minutes contact time (64). Bacterial contamination of the food products in the processing plants decreases the product shelf-life and also results in foodborne illness if consumed without proper cooking (53). Therefore, more effective disinfection procedures may be needed to eliminate the bacteria from food contact surfaces in order to minimize the risk of foodborne disease outbreak.

1.9 Antimicrobial Resistance in Poultry Production

The issue of antimicrobial resistant bacteria in animals is not only related to the antimicrobial use for treatment purpose but also for growth promotion and mass medication (prophylaxis and metaphylaxis). This is especially true in poultry where antimicrobials are given for these purposes and individual medication is impossible. Antimicrobial growth promoters (AGP) are thought to increase the production yield by slowing down the bacterial metabolism inside the gut that leads to increased availability of nutrients and intestinal absorption (65).

In Canada, various classes of antimicrobials are used for therapeutic purpose in poultry including penicillins, aminoglycosides, macrolides, sulfonamides, tetracyclines and phenicols (15). Use of antimicrobials through feed or water may provide selection pressure to the commensal microorganisms in the gut (66). If the antimicrobial is given for therapeutic purposes and goes into the bloodstream, it can impose selection pressure on bacteria all over the organs. Antibiotics given for treatment through waterlines will also expose bacteria in the waterline to

selection pressure. Likewise, antibiotics excreted in the faeces in active form will exert selection pressure on the litter microbiota. Thus the use of antibiotics in the poultry house may impose selection pressure in the poultry production environment. The selective pressure may select resistant bacteria and ultimately be transferred to humans via direct contact or via food products (67, 68).

A number of studies have demonstrated that antimicrobial resistant bacteria are found in healthy poultry birds and retail chicken meat in Canada (47, 69). Sheikh *et al*. (47) found that *E. coli* from the retail chicken meat had higher percentage of resistance to amoxicillin-clavulanic acid (31%) and ceftriaxone (26%) compared to *E. coli* from other meat types. The authors also showed that *bla*CMY-2 gene were frequently associated with the β-lactam antibiotic resistant *E. coli* of chicken meat origin. This could reflect the use of β-lactams in chickens. These results suggest that chickens are more likely to have antimicrobial resistant determinants than other food animals and may serve as a source of antimicrobial resistant bacteria for transmission to human via direct contact or via food. Surveillance data collected by CIPARS in Canada (8) showed that ceftiofur resistant *S*. Heidelberg in human isolates consistently rose from 13% to 31%, and in retail chicken meat samples from 6% to 24%, during the year 2006-2013. In Quebec, the surveillance data showed that there was an increasing trend of ceftiofur resistant *E. coli* and *S. Heidelberg* being isolated from retail chicken meat and in *S. Heidelberg* isolates from human clinical cases. After the voluntarily removal of ceftiofur use *in ovo* in hatcheries in 2005, there was a sharp decline in both ceftiofur resistant *E. coli* and *S.* Heidelberg from retail chicken meat and ceftiofur resistant *S.* Heidelberg from human samples. When the *in ovo* use of ceftiofur in poultry was re-introduced in hatcheries in 2007, the numbers of ceftiofur resistant *E. coli* and *S.*

Heidelberg from chicken and ceftiofur resistant *S*. Heidelberg from human cases began to rise again. This surveillance data suggests that use of ceftiofur in hatcheries and the ceftiofur resistant *E. coli* and *S.* Heidelberg isolated from retail chicken meat and from human cases are associated and, that the human clinical infections were probably ceftiofur resistant *S*. Heidelberg acquired from poultry and egg consumption (70). A study in The Netherlands found that Dutch patients, broiler chicken and retail meat shared the same strain of *E. coli*, plasmid and ESBL genes suggesting that humans might have acquired *E. coli* from food (71). Several studies have also shown that bacterial pathogen can be transferred from human to animals. Price *et al*. (72) showed that livestock associated *S. aureus* clonal complex CC398 originated from human *S. aureus* clonal complex CC398 which was susceptible to methicillin antibiotic. During the transfer from human to livestock, the human CC398 strain acquired a tetracycline resistance gene, the *tetM*, and the Staphylococcal Cassette Chromosome *mec* element (SCC*mec*) that carry *mecA* gene responsible for methicillin resistance. A similar study showed that *S. aureus* clonal complex CC5 in poultry was originated from the human *S. aureus* sequence type ST5 in Poland. In the poultry host, the *S. aureus* clonal complex CC5 lost their virulence gene such as *spA* necessary for colonization in human lung epithelium and developed resistance to neutrophils (heterophils) from the poultry. The neutrophils are the primary defense cells against bacterial infections (73). These studies suggest that human bacterial pathogens can also be transferred to livestock and poultry and are able to adapt to new host with loss of virulence gene functions that are necessary in humans. The bacteria are able to resist the host immune system and may also acquire antibiotic resistant genes from the environment, which can cause serious disease outbreak.

Farming practices (organic or conventional) may also alter the prevalence of antimicrobial resistant bacteria in the farm. Alali *et al*. (74) showed that the prevalence of *Salmonella* isolated from organic broiler farms were very low (4.3%) compared to *Salmonella* from conventional broiler farms (29%). Similarly, resistance to β-lactam antibiotics in the *Salmonella* from organic broiler farms were lower than in the *Salmonella* isolates from conventional farms. A similar study by Sapkota *et al*. (75) showed that multi-drug resistant (≥3 drug classes) *Salmonella* Kentucky was less prevalent in organic broiler farms compared to conventional farms. Antibiotics are not used in organic poultry farming which may have led to low antibiotic selection pressure that may have resulted in lower prevalence of antibiotic resistant bacteria.

The third generation cephalosporins are in the very high important drug category in human medicine (14). Ceftiofur is the only third generation cephalosporin used in an extra-label manner in chickens in Canada until April 2014 (76). The CIPARS surveillance data showed that there was an increasing trend of ceftiofur resistant *S.* Heidelberg isolated from retail chicken meat and humans indicating that the use of ceftiofur in hatcheries and the ceftiour resistance in human *S*. Heidleberg isolate are associated (8). Therefore, in order to mitigate the risk of spread antibiotic resistant bacteria in humans, the chicken farmers of Canada decided to withdraw the use of category I antibiotics including ceftiofur from chicken farms starting May 2014 (76). In order to minimize the risk of development and spread of antimicrobial resistance due to the use of antimicrobials in food animals and veterinary medicine, the government of Canada announced to ban all kinds of growth promotion claims of medically important antimicrobials in food animals by 2016 (77).

The occurrence and spread of antimicrobial resistance is the result of human activities both on farms and hospitals (or community) providing a suitable environment to the bacteria to select for resistance and adapt into new hosts. This led to the evolution of newer antibiotic resistant genes. One particular example is of *bla*CTX-M gene, which was originated from the chromosomal gene *bla*Klu of *Klyuvera* spp. The naturally present chromosomal *bla*Klu gene is closely related to cefotaximases family but offers susceptibility to cefotaxime. Following mobilization of chromosomal *bla*Klu into plasmid in *Klyuvera* by recombination, the gene got transferred horizontally into *E. coli*, and evolved, giving rise to a new CTX-M gene, the *bla*CTX-M-1. The CTX-M-1 enzyme conferred resistance to cefotaxime antibiotic (78). During the course of time, the new variants of *bla*CTX-M genes emerged due to mutations that led to resistance activity for a wider variety of substrates. For instance, a single amino acid change in CTX-M-3 resulted in a new CTX-M type, the CTX-M-15 with ability to hydrolyse ceftazidime. Similarly, recombination events between the two varieties of *bla*CTX-M genes resulted in the evolution of a new CTX-M enzyme. For example, the CTX-M-64 enzyme was the result of recombination of CTX-M-14 and CTX-M-15 enzyme (79). To date, there are 169 variants of CTX-M enzymes updated in Lahey's database (http://www.lahey.org/Studies/). The *bla*CTX-M genes are commonly present in the mobile genetic elements such as conjugable plasmids transposons and integrons. These genes are disseminated among variety of bacterial species such as *K. pneumoniae*, *S. enterica*, *P. mirabilis*, *P. aeruginosa, and E. coli*, thereby disseminating cephalosporin resistance genes world wide (79).

1.10 Hypothesis and Study Objectives

Hypothesis:

Bacteria present in poultry production environments can serve as a persistent reservoir of antibiotic resistance genes that may be transferred to foodborne pathogens and pose a risk to food safety. If these resistant bacteria reside in biofilms that are difficult to remove, then these bacteria are even more persistent in the environment and pose an even greater risk to food safety.

Study Objectives:

1. To isolate and identify bacteria from different types of equipment and surfaces in broilers farms, layer farms, hatcheries and poultry processing plants.
2. To determine the phenotypic resistance of bacteria against different classes of antibiotics.
3. To characterize antimicrobial resistant genes and mobile genetic elements present in antimicrobial resistant bacteria.
4. To determine if the bacteria isolated from the environment can form biofilm *in vitro*.
5. To determine if the resistance against antibiotics and disinfectants is greater in bacteria living in biofilm than in planktonic culture.

Chapter Two: **MATERIALS AND METHODS**

2.1 Study Design

An observational study was performed in order to find out the occurrence and distribution of antimicrobial resistant genes in environmental bacteria in poultry farms and processing plants in Alberta. Aerobic, non-fastidious and fast growing bacteria were isolated and identified. Phenotypic analysis was conducted to determine the antimicrobial resistance patterns. Genotypic analysis was performed to determine the presence of antimicrobial resistant genes in order to evaluate poultry farms and processing plants as a reservoir of antimicrobial resistant bacteria and consequently of AMR genes.

2.2 Study Period and Location

The study was conducted between November 2012 and January 2015. Samples were collected from poultry farms and processing plants located in southern part of Alberta between November 2012 and January 2013. Sample processing and Minimum Biofilm Eradication Concentration (MBEC) assay was conducted in the Poultry Health Services laboratory, Airdrie, Alberta by study grant collaborators. Identification of the bacteria, antimicrobial sensitivity tests by Sensititre® system and genetic analysis were performed in the laboratory of Faculty of Veterinary Medicine, University of Calgary. Nucleotide sequencing was performed in the DNA sequencing facility at University of Calgary.

2.3 Sample Collection and Sample Processing

Samples were collected from 12 broiler farms, seven layers farms, two hatcheries and three processing plants located in Southern Alberta (Table 2.1). One broiler farm was sampled

after cleaning and sanitation and during down-time between flocks. Samples were collected and processed according to the procedures described in Appendix 1. Briefly, surface samples of 100 cm^2 (10 cm x 10 cm) were taken by swabbing up/down, left/right and diagonally with a sterile cotton swab moistened with sterile Phosphate Buffered Saline (PBS). Then the swab sample was placed in a sterile bag for transport. For liquid samples, 50 mL were taken in a sterile sampling tube, labelled and taken to the laboratory. Samples were processed as previously described (80). Briefly, swab samples were placed in a sterile vial containing 500 µL sterile neutralizer media that contained one gram L-histidine, one gram L-cysteine and two gram reduced glutathione. The vial was sonicated for 30 minutes using VWR B2500A-MT Ultrasonic cleaner (VWR International. Edmonton, AB) at 42 Hz. A 100 µL of each sample was placed into the first 12 empty rows of a 96 well microtitre plate (Nuclon Delta Surface, Thermo Fisher Scientific, Roskilde. Denmark). A serial dilution of 10^0-10^{-7} was made by transferring 20 µL down each of the 12 rows that contained 180 µL of 0.9 % sterile PBS. From each well, 20 µL were removed and spot-plated on Tryptic Soy Agar (Becton, Dickinson and Company, MD. USA), MacConkey agar (Becton, Dickinson and Company, MD, USA) and Manitol Salt Agar (Becton, Dickinson and Company, MD. USA). Bacteria were incubated at 36^0C for 24 hours. Individual colonies were collected aseptically and sub-cultured on the media they were originally isolated from and incubated at 36^0C for 24 hours. Following incubation, one to two colonies were taken to perform gram staining. A few colonies were taken to inoculate the cryo-preservatives in the cryogenic bead stock following manufacturer's instructions (Prolab Diagnostics, ON, Canada). The cryogenic beads were stored at -80^0C. For water samples, 200 µL of the water was placed into the first 12 empty wells of the first row of a 96 well microtitre plate. A serial dilution of 10^0-10^{-7}

was made as described above. Incubation and preservation of bacterial isolates were performed as described above. A total of 149 samples were taken and processed.

Table 2.1 Sampling scheme for equipment and material surfaces in poultry farms and poultry processing plants in Alberta.

Broiler (no=12)	Layer (n=7)	Hatchery (n=2)	Processing plants (n=3)
Drinking nipple (n=15)	Drinking nipple (n=8)	Humidifier water reservoir (n=3)*	Scald tank swab (n=3)
Drinking nipple water (n=3)	Drinking nipple water (n=4)	Water source line to vaccine preparation room (n=2)*	Scald tank water (n=1)
Feed pan(n=12)	Feed pan(n=8)	Bronchitis vaccine solution (n=1)	Chilled tank swab (n=3)
Feed auger(n=12)	Feed auger(n=5)	Water out of humidifier reservoir (n=1)	Chilled tank water (n=1)
Water (n=5)	Water (n=2)	Humidifier blade (n=1)	Stunning bath (n=1)
Total : 47	Total: 27	Tom turkey blade (n=1)	De-footer blade (n=1)
		Turkey toe trimmer (n=1)	Picker finger (n=2)
		Turkey injector port (n=1)	Escelatory to de-featherer (n=1)
		Counter top in vaccine prep room (n=1)	Eviscerator (n=2)
		Hatch baskets (n=4)	Saw guard (n=1)
		Chick processing belts (n=4)	Cutter on "custom cut" (n=1)
		Bronchitis tubing in vaccine (n=1)	Holder on "custom cut" (n=1)
		Bronchitis tubing connection to syringe (n=1)	Gut trough (n=1)
		Bronchitis vaccine tubing (n=1)	Vent cutter rubber hose (n=1)
		Chick separator drop sheet (n=3)	Drain metal (n=1)
		Incubator (n=2)	Shackle (n=1)
		Humidification unit (n=1)	Oil gland remover (n=1)
		Total: 34	Light switch (n=1)
			Hand washing sink (n=1)
			Floor drain (n=3)
			Meat hook in cooler (n=1)
			Cooler door handle (n=1)
			Foot cutter blade (n=1)
			Scald tank drain (n=2)
			Band saw wheel (n=1)
			Old unused plucker cord (n=1)
			Plucker trough (n=1)
			Drain water sample (n=2)
			Plastic sheet for feather shield (n=1)
			Meat grinder (n=1)
			Meet cutting blade (n=1)
			Total : 41

Note: no = number of farms/processing plants selected for sampling; n = number of samples taken.

* Both water sample and swab sample were taken.

2.4 Identification of Bacterial Species

The bacterial species were identified using the Sensititre® system (Trek Diagnostic Systems, OH. USA). The identification is based on the principle of various biochemical reactions mediated by bacteria in Sensititre® 96 wells gram-positive (GPID) and gram-negative (GNID) identification plates. The plate is divided into three sections, each section containing 32 biochemical compounds. Bacterial enzymes utilize the substrates that result in fluorescence. The Sensititre® OptiRead™ detects the fluorescence pattern and compares this to a database to determine the probability of species identification. The most common aerobic gram-negative and gram-positive bacteria can be identified by this method.

2.4.1 Experimental Procedure

The bacterial isolates preserved at -80^{0}C in cryogenic beads were thawed on ice. One bead was taken off the preservative tube and inoculated in one mL Brain Heart Infusion (BHI) broth and incubated at 36^{0}C for 24 hours. Ten uL was then streaked onto Columbia Blood Agar with 5% Sheep blood (Becton, Dickinson and Company, MD, USA), and incubated at 36^{0}C for 24 hours. Gram-negative isolates were sub-cultured on MacConkey Agar plates (Becton, Dickinson and Company, MD, USA), and gram-positive isolates were sub-cultured on Tryptic Soy Agar plates (Teknova, CA, USA) at 36^{0}C for 24 hours. After incubation, an individual colony from the overnight culture was streaked onto the Columbia Blood Agar with 5% sheep blood and incubated as described above. Subsequently, one or two isolated colonies from this blood culture plate were suspended in five mL Sensititre® demineralized water. The suspension was adjusted to 0.5 McFarland Standard using Sensititre® Nephlometer. Fifty µL of the

suspension was loaded into the Sensititre® plates: GNID for gram-negatives and GPID for gram-positives. Isolates, which were not grown at 36^0C, were incubated at RT at 21^0C for next 24 hours. The stepwise procedures are mentioned in Appendix 4. The assays generating a probability of identity of more than 80% were accepted and recorded as bacterial species identified. The assays generating a probability of identity of less than 80 % were re-tested once. Isolates that were not identified were also re-tested one more time. The control strains used were: *E. coli* American Type Culture Collection (ATCC) 25922, *P. aeruginosa* ATCC 1015, *K. pneumoniae* ATCC 70603, *S. aureus* ATCC 29213, *E. fecalis* ATCC 29212.

2.5 Antimicrobial Susceptibility Testing

2.5.1 Sensititre® Method

Antimicrobial resistance of planktonic bacteria was determined using the Sensititre® automated microbroth dilution method (Trek Diagnostic Systems, OH. USA) on the standard CMV2AGNF (gram-negative) and CMV3GPF (gram-positive) plate. The system can provide both qualitative (resistance or susceptible) and quantitative-Minimum Inhibitory Concentration (MIC) result. The assay is based on the principle that bacteria utilize fluorogenic substrates added in the susceptibility plate that gives fluorescence. The Sensititre® OptiRead™ system detects the amount of fluorescence, which is directly related to the amount of bacterial growth (81).

2.5.1.1 Experimental Procedure

One or two colonies from an overnight culture on Columbia Blood Agar with 5% sheep blood (Becton, Dickinson and Company, MD. USA) were suspended in five mL of Sensititre® Demineralized Water and adjusted for McFarland turbidity standard 0.5 using Sensititre® Nephlometer. Ten µL of this suspension was added to 11 mL Sensititre® Cation Adjusted Mueller Hinton Broth to give an inoculum of approximately $1x10^8$ CFU/mL. The suspension was vortexed and 50 µL was placed in each well of 96 well Sensititre® plates: CMV2AGNF or CMV3GPF using Sensititre® Auto-Inoculator™. The bacteria were incubated at 36^0C for 24 hours. Following incubation, the plate was read using the Sensititre® OptiRead™ and interpreted for MIC values. The protocol for this experiment is described in Appendix 5. Antimicrobials assayed in the gram-negative plate were: amoxicillin-clavulanic acid, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamycin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline and trimethoprim-sulfamethoxazole. The antimicrobials assayed in the gram-positive plate were: chloramfenicol, ciprofloxacin, daptomycin, erythromycin, gentamycin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, streptomycin, synercid, tetracycline, tigecycline, tylosin and vancomycin. The concentrations of these antimicrobials and the plate layout are presented in Appendix 6. *E. coli* ATCC 25922, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 10145 were used as control strains. Results were interpreted according to the guidelines established by the Clinical and Laboratory Standard Institute (82). Minimum Inhibitory Concentration (MIC) of ceftiofur for *Enterobacteriacae* were interpreted using cut-off values established by Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS): Short Report-2011

(17).

2.5.2 Disc Diffusion Assay

The detail procedures of disc diffusion assay are explained in Appendix 7.

2.5.2.1 Screening of ESBL Phenotype *E. coli*

Based on the Sensititre® results, which showed resistance to cefoxitin and ceftriaxone, 10 *E. coli* were subjected to a disc diffusion assay in order to determine if they were Extended Spectrum β-Lactamase (ESBL) producing phenotypes, following the guidelines set by CLSI (82). Before performing the experiment, *E. coli* were cultured on a Columbia Blood Agar Plate with 5% sheep blood (Becton, Dickinson and Company, MD. USA) at 36^0C for 24 hours. One or two colonies of overnight culture was suspended into five mL Sensitire® Demineralized Water and adjusted to make 0.5 McFarland turbidity standard using Sensititre® Nephlometer. With the help of a sterile cotton swab, the inoculum was spread on Mueller Hinton Agar plate. Eight antimicrobial discs were placed on a 150 mm plate using a sterile forceps. The forceps was sterilised in 70 % alcohol and placed on a flame before each antimicrobial disc was placed. The plate was incubated at 36^0C for 18 hours. The inhibition zone diameter was read in millimetres and was interpreted as sensitive, intermediate or resistant according to the guidelines established by CLSI (82). The antimicrobial discs used are mentioned in Appendix 8.

2.5.2.2 Screening of AmpC phenotype *E. coli*

Based on the screening result of the disc diffusion assay for ESBL phenotype, all of the 10 isolates were further screened for possible AmpC phenotype using the Mast D69C disc assay (Mast Group Inc. UK). The Mast D69C disc assay consists of three discs: discA = Cefpodoxime

(10 µg) + AmpC inducer, discB = Cefpodoxime (10 µg) + AmpC inducer + ESBL inhibitor, discC = Cefpodoxime (10 µg) + AmpC inducer + ESBL inhibitor + AmpC inhibitor. The disc diffusion assay was performed as described above on Mueller Hinton Agar plates. The inhibition zone diameter was measured in millimeters. A difference in zone diameter of ≥ 5 mm between discC and discA; and/or discC and discB, was interpreted as AmpC positive. The difference in zone diameter of ≤ 3 mm was interpreted as negative according to the guidelines established by the Mast Group Inc. UK. *Escherichia coli* ATCC 25922 was used as the control strain.

2.5.2.3 Screening of Methicillin Resistant *Staphylococcus* spp.

All 36 *Staphylococci* species isolates were screened for *mecA* mediated methicillin resistant phenotype by disc diffusion assay using a cefoxitin disc assay (30 µg) similar to the assay described above. The isolates were incubated at 35^0C for 24 hours. The inhibition zone diameter was interpreted according to the CLSI guidelines (82). *S. aureus* ATCC 29213, and ATCC 25923 were used as control strains.

2.6 Genomic and Plasmid DNA Extraction and Polymerase Chain Reaction of Targeted Gene Sequences

Polymerase Chain Reaction (PCR) followed by DNA sequencing was performed in order to identify Class 1 and Class 2 integrons in all 48 gram-negative isolates. PCR amplification and sequencing was performed to detect the chromosomal *ampC* promoter mutations in all 14 *E. coli* isolates. PCR amplification and amplicon sequencing was also performed to identify plasmid mediated *bla*CMY-2 genes in all the β-Lactam antibiotic resistant *E. coli* isolates, and for plasmid mediated *bla*CTX-M -1 group genes in one *E. coli* isolate.

Genomic DNA was extracted by the boiling method using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems Inc., ON. Canada) following manufacturer's instructions as described in Appendix 9. Bacterial isolates were cultured on Columbia Blood Agar with 5% sheep blood (Becton, Dickinson and Company, MD. USA) and incubated at 36^0C for 24 hours. Then, one or two bacterial colonies were suspended in one mL BHI broth and incubated at 36^0C for 24 hours on standing condition. The culture tube was centrifuged at 14,800 revolutions per minute (rpm) for two minutes at RT. Supernatant was removed carefully and bacterial pellets were mixed with 100 µL of PrepMan® Ultra Sample Preparation Reagent and vortexed well. Then the suspension was boiled for 10 minutes using a heat block at 100^0C. The sample was then allowed to cool for two minutes and centrifuged at 14,800 rpm for two minutes at RT. Finally, the supernatant containing DNA was collected in a sterile microcentrifuge tube, quantified by measuring optical density (OD) 260/280 using NanoDrop® Spectrophotometer ND-1000 and stored at 4^0C.

Plasmid DNA of *E. coli* was isolated using Qiagen® Plasmid Mini kit (Qiagen Inc., Toronto, ON) following manufacturer's instructions. One or two colonies of overnight cultured *E. coli* isolates were suspended in three mL Luria-Bertani (LB) broth and incubated at 37^0C for eight hours in an orbital shaker at approximately 300 rpm. Six µL of the suspension was added to three mL LB broth and incubated at 37^0C for 16 hours at 300 rpm. The culture was then was centrifuged at 6,000 x g at 4^0C for 15 minutes. Lysis buffer was added to the resuspension buffer to make final concentration of 100 µg/mL. The bacterial pellet obtained after centrifugation was resuspended with 300 µL resuspension buffer followed by 300 µL precipitation buffer. Then the

tube was allowed to cool on ice for five minutes. The suspension was then centrifuged at 14,800 rpm for 10 minutes at 4^0C and supernatant was applied to Qiagen® Tip-20 and allowed to pass through. The DNA, bound to the resin in the Tip-20, was washed with two mL wash buffer and by 800 µL elution buffer. Eluted DNA was collected in a 1.5 mL microcentrifuge tube and precipitated by adding 100% molecular biology grade isopropanol. Then centrifugation was carried out at 14,800 rpm for 30 minutes at room temperature and supernatant was removed carefully. Then the pellet was washed with 70% ethanol and centrifuged at 14,800 rpm for ten minutes at room temperature and supernatant was removed carefully. Plasmid DNA pellet was air dried and re-dissolved in 40 µL Tris EDTA (TE) buffer pH 8.5, quantified by measuring OD 260/280 using NanoDrop® Spectrophotometer ND-1000 and stored at 4^0C. The details of the procedures are explained in Appendix 9.

PCR mastermix was prepared using Taq PCR Core Kit (Qiagen Inc., ON. Canada). The amount of 1x PCR buffer with $MgCl_2$, 200 µM each dNTP, 0.5 µM each primers and 2.5 units Taq polymerase was calculated and mixed in a sterile mastermix tube. The mastermix was aliquoted equally into seven PCR tubes. One tube was allocated for positive control and one tube was allocated for No Template Control. One µL template DNA (approximately 50 ng genomic DNA or 10 ng plasmid DNA) was added to each tube except for the tube allocated for No Template Control. Nuclease free water was added to each tube to make a final volume of 22 µL. The composition of PCR mastermix and the procedures are described in Appendix 9. The amplification of targeted genes, their respective primers, expected product size and annealing temperatures are mentioned in table 2.2. PCR was performed in Veriti® 96 well Thermal Cycler (Applied Biosystems Inc., ON. Canada) under the conditions as described in Appendix 9.

Table 2.2 Primer pairs used for PCR amplification of Class 1 and Class 2 integrons, the *ampC* promoter sequence, *bla*CTX-M-1 group genes and *bla*CMY-2 gene.

PCR Target	Primer Name	Primer sequence (5'-3')	Annealing temperature (^{0}C)	Product size (bp)	Reference
Class1 integron (5'CS-3'CS)	5' CS 3'CS	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	56	variable*	(83)
Class2 intergron (*attI*2-*orf*X)	Hep74 Hep51	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	55	variable*	(84)
ampC promoter	AB1 ampC2	GATCGTTCTGCCGCTGTG GGGCAGCAAATGTGGAGCAA	57	271	(85)
*bla*CMY-2	CIT-A CIT-B	ATGCAGGAGCAGGCTATTC TGGAGCG TTTTCTCCTGAAC	58	750	(42)
*bla*CTX-M-1 group	C1-F C2-R	GGACGTACAGCAAAAACTTGC CGGTTCGCTTTCACTTTTCTT	58	624	(86)

* the size of the integron depends upon the presence or absence of gene cassettes.
Abbreviation: bp, base pair; CS, conserved sequence

2.7 Plasmid Profiling of *E. coli* Isolates

Plasmids of ten β-Lactam antibiotics resistant *E. coli* isolates were electrophoresed on 0.7% agarose at 60 volts for two hours at room temperature. 1kb Plus Ladder (Qiagen Inc., ON, Canada) was used for comparing the position of the plasmids in the gel. The approximate size of the plasmid was calculated by plotting the migration of plasmid (in mm) on the gel, against the log10 fragment size of the DNA ladder. The plasmids from the agarose gel were extracted using QIAEX® II Gel Extraction kit as described in Appendix 12 following manufacturer's instructions. Extraction procedures were as follows: The gel was placed on an UV trans-

illuminator and visible bands were cut with a scalpel. The gel slice was placed in a microcentrifuge tube and mixed with mixing buffer and 10 µL binding buffer. Then the tube was placed in a water bath at 50^0C for 10 minutes to dissolve the gel slice. The solution was then centrifuged at 13,000 rpm for one minute at room temperature and the supernatant was removed. Next, the pellet was washed with 500 µL high salt wash buffer and centrifuged at 13,000 rpm for one minute at room temperature to remove the residual agarose. Following centrifugation, the supernatant was removed and mixed with 500 µL ethanol wash buffer to remove salt contaminants. The tube was centrifuged as described above and pellet was air dried before adding 20 µL TE buffer. Then the tube incubated on water bath at 50^0C or at room temperature. Following incubation, the tube was centrifuged and supernatant containing purified DNA was collected in a 1.5 mL microcentrifuge tube.

All the extracted plasmids were used as template for PCR to detect the following antimicrobial resistant genes in the *E. coli* isolates: *bla*CMY-2 in all the ten isolates and *bla*CTX-M-1 group gene in one isolate. An *E. coli* strain harbouring plasmid mediated *bla*CMY-2 gene (courtesy of Dr. Doug Morck, University of Calgary) and one *E. coli* strain possessing the *bla*CTX-M-15 gene (courtesy Calgary Laboratory Services, Calgary AB) were used as positive controls.

2.8 Agarose Gel Electrophoresis

An agarose gel of 0.7 to 1.2 % was prepared depending upon the expected PCR product size. Gel Pilot® LE Agarose powder (Qiagen Inc., Toronto ON. Canada) was dissolved in 1x Tris base, Acetic acid and EDTA (TAE) buffer in a microwave until clear solution was observed.

The solution was cooled to approximately 65^0C before ethidium bromide (0.5µg/mL) was added. The gel was poured into a gel-casting tray after placing a gel comb. Then the agarose gel was allowed to solidify for half an hour at room temperature and the gel comb was removed. The gel was then placed in an electrophoresis tank and 1x TAE running buffer was poured. Next, eight µL PCR product was loaded into the wells after adding two µL Gel Pilot® Loading dye (Qiagen Inc. Toronto, ON. Canada). Appropriate DNA ladder was placed on the first well in the gel. Electrophoresis was performed under different conditions as described in Appendix 10.

2.9 PCR Amplicon Purification and Nucleotide Sequencing

The PCR amplicons were purified by using QIAquick® PCR purification kit (Qiagen Inc., ON. Canada) as described in Appendix 11. Binding buffer was added in 5:1 ratio to the PCR product and the mixture was applied to Qiaquick® Spin Column and centrifuged at 13,000 rpm for one minute at RT. The supernatant was discarded and column was washed with wash buffer and centrifuged at 13,000 rpm for one minute at RT. The purified DNA in the column was eluted in TE buffer pH 8.5 by centrifuging at 13,000 rpm for one minute at room temperature. Approximately 80 ng PCR template per one kb PCR product size was added to 3.2 picomoles of each PCR primer in a sterile PCR tube. Two tubes for each PCR product were allocated for forward and reverse primer separately. Nuclease free water was added to each tube to make final volume of 12 µL. The steps in preparing sample for sequencing are described in detail in Appendix 13. The PCR products were sent for sequencing to University of Calgary, DNA sequencing facility. The DNA sequences obtained from the University of Calgary sequencing facility were compared with the reference database in the National Center for Biotechnology Information (NCBI).

2.10 *In vitro* Biofilm Formation

Eleven bacterial isolates of interest were assayed for the ability to form biofilm *in vitro* in MBEC™ device (Innovotech Inc. AB, Canada) These bacterial isolates were: *Bacillus subtilis, Pseudomonas stutzeri, Pseudomonas aeruginosa, Chryseomonas luteola, Escherichia coli* (n=3), *Staphylococcus xylosus, Enterobacter cloacae, Bacillus thuringiensis, and Aeromonas caviae.* Experimental procedures were followed as previously described (87, 88) with some modifications. The bacterial isolates preserved at -80^{0}C were thawed on ice. The first sub-culture was streaked on TSA (Becton, Dickinson and Company, MD. USA) and incubated at 36^{0}C for 24 hours. From the first sub-culture, a second sub-culture was streaked on TSA (Becton, Dickinson and Company, MD. USA) and incubated at 36^{0}C for 24 hours. An isolated colony from the second sub-culture was inoculated in 100 mL Tryptic Soy Broth (Becton, Dickinson and Company, MD. USA). The broth culture was placed on an orbital shaker in a humidified incubator and incubated at 36^{0}C for 24 hours at 110 rpm. Following incubation, 100 µL of the culture was transferred to 100 mL Tryptic Soy Broth (Becton, Dickinson and Company, MD, USA) to adjust the inoculum to an approximate cell density of 10^5 CFU/mL. One hundred µL of the diluted organism was used for an inoculum check by serially diluting and spot plating. Serial dilutions of 10^0-10^{-7} were prepared and spot-plated on TSA (Becton, Dickinson and Company, MD. USA). One hundred fifty µL of the diluted organism was placed in each well of Nunc™ 96-Well Polysterene MicroWell™ plate. A lid of the 96 pegs MBEC™ device was placed on the bottom plate containing the organism. The plate was then placed on an orbital shaker in a humidified (approx. 95%) incubator at 37^{0}C for 24 hours at 110 rpm. Following incubation, the pegs were detached using a sterile plier, and fixed with 2.5% gluteraldehyde in Phosphate-

Buffered Saline air-dried overnight, and visualised using Scanning Electron Microscope. To assure sample sterility and to check biofilm growth, pegs corresponding to sterility control and the growth wells were detached using a sterile plier and placed in the wells of 96 well plate containing 200 µL neutralizer solution which contained one gram L-histidine, one gram L-cysteine and two gram reduced glutathione mixed in double distilled water. The pegs were sonicated using VWR B2500A-MT Ultrasonic cleaner (VWR International. Edmonton, AB) at 42 Hz for 30 minutes and serially diluted (10^0-10^{-7}) and spot-plated on TSA (Becton, Dickinson and Company, MD, USA). The details of these procedures are explained in Appendix 14.

2.11 Minimum Biofilm Eradication Concentration Assay

Antimicrobial sensitivity plates were prepared for each isolate by reconstituting antimicrobial agents in 96-well AVIAN1F plate (Trek Diagnostic Systems OH, USA), by adding 50 µL Cation Adjusted Mueller Hinton Broth (CAMHB) according to the manufacturer's recommendation. Two hundred µL of the test organism at an inoculum density of 10^5 CFU/mL was added in each well except for sterility and growth control wells. The control wells were filled with CAMHB. The peg lids on which biofilm were grown from the overnight incubation was placed on a rinse plate containing 200 µL saline for two minutes to remove planktonic cells from the biofilm. After rinsing the pegs, the pegs were transferred to the antimicrobial sensitivity plate. Then the plate was placed on an orbital shaker at 110 rpm in a humidified incubator and incubated at 36^0C for 24 hours. After incubation, the peg lid was first placed on the rinse plate, then on the recovery plate containing 200 µL CAMHB and neutralizer solution. The plate was then sonicated for 30 minutes to dislodge bacteria from the biofilm matrix. Then the recovery plate was incubated at 36^0C for 24 hours. Visible growth or no growth of the dislodged bacteria

in the recovery wells was recorded as + or - respectively. Clear wells indicated biofilm eradication by the antimicrobial agent. The MBEC value was determined as the minimum concentration of antibiotic at which bacteria released from biofilm fail to regrow. Planktonic MIC was determined by incubating the challenge plate overnight and assessing visually to determine the growth of the bacteria. The experiment was repeated three times to confirm the results. *P. aeruginosa* ATCC 15442 was used as the control strain. The detailed protocol, antibiotics used, and their concentrations are mentioned in Appendix 14.

2.12 Disinfectant Resistance Assay

The disinfectant assay was performed as described in detail in Appendix 14. A stock solution of 10x, 2x, 1x (1x = manufacturer's recommended concentration) and 0.1x of the following disinfectants were prepared by diluting in sterile water: Peracetic-hydrogen peroxide, chlorine, iodine compound, hydrogen peroxide, potassium monopersulfate, aldehyde and quaternary ammonium compound. These are CFIA approved disinfectants for use in poultry barns and food processing industries. For each isolate, a sensitivity plate was prepared with the dilutions mentioned above for each disinfectant. Biofilm were grown on pegs of MBEC™ device as described and placed into the disinfectant sensitivity plate. The plate was incubated at RT for 10 minutes. The MBEC values were determined as described above in section 2.11. *P. aeruginosa* ATCC 15442 was used as the control strain.

2.13 Statistical Analysis

The Fisher Exact test was used for pairwise comparison between groups by building 2x2 contingency table. Pairwise comparison was made for the following groups of data.

- Distribution of *E. coli,* Pseudomonads and *S. xylosus* between feed pan, feed auger, and drinking nipple samples. Samples were collected most commonly from these sample equipment. *E. coli*, Pseudomonads and *S. xylosus* were the most commonly isolated bacteria from our samples in the study.
- Distribution of *E. coli*, Pseudomonads and *S. xylosus* between the samples collected from different surface types: plastic surface, steel surface, concrete surfaces and rubber surfaces.
- Distribution of gram-negative and gram-positive bacteria in the samples collected from different surface types.
- Distribution of gram-negative and gram-positive bacteria in the samples collected from feed pans, feed augers, and drinking nipples
- Distribution of gram-negative and gram-positive bacteria in the samples collected from broiler farms, layer farms, hatcheries and processing plants.
- Antimicrobial resistance pattern between the isolates collected from broiler farms, layer farms, hatcheries and processing plants.
- Multi-drug resistance between the gram-negative and gram-positive bacteria.
- Multi-drug resistance bacteria between the samples collected from different surface types.
- Multi-drug resistance bacteria between the broiler, layer, hatcheries and processing plants.

Isolates resistant to three or more drug classes were considered multidrug resistant bacteria. IBM SPSS version 21.0 (IBM SPSS Corp., Armonk, NY) was used for the statistical analysis. A P value <0.05 was considered statistically significant.

Chapter Three: **RESULTS**

3.1 Isolation and Identification of Bacteria

A total of 149 samples were collected from a variety of equipment and surfaces in broiler barns, broiler hatcheries, layer barns and poultry processing plants in Alberta during the summer 2012. The surfaces sampled were: stainless steel (n=59), plastic (n=51), rubber (n=11) and concrete surface (n=3). Twenty-five water/liquid samples were also collected. Out of 149 samples processed, 117 bacteria were isolated. One hundred and one of these isolates were identified by the Sensititre® method, among which 48% (n=48) were gram-negative bacteria and 52% (n=53) were gram-positive bacteria. Twelve isolates could not be identified by the method used in the study, and four did not survive on subsequent cultures. Among the gram-negative bacteria, *Enterobacteriaceae* were predominant, 48% (n=23) followed by *Pseudomonadaceae*, 25% (n=12) (Figure 3.1). *Enterobacteriaceae* included *Enterobacter cloacae*, *Escherichia coli* and *Pantoea agglomerans*; Pseudomonadaceae included *Chryseomonas luteola*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas mendocina* and *Pseudomonas stutzeri*. *Enterobacteriacae* were commonly isolated from plastic surfaces (n=10) and stainless steel surfaces (n=7) whereas, *Pseudomonadacae* were commonly isolated from stainless steel surfaces (n=6). *Acinetobacter* spp. and *Aeromonas caviae* accounted for 8% (n=4) and 6% (n=3) of the identified gram-negative isolates, respectively. Thirteen percent of other gram-negative bacteria identified were: *Moraxella osloensis*, *Riemerella anatipestifer*, *Achromobacter xylosoxidans*, and *Stenotrophomonas maltophilia*.

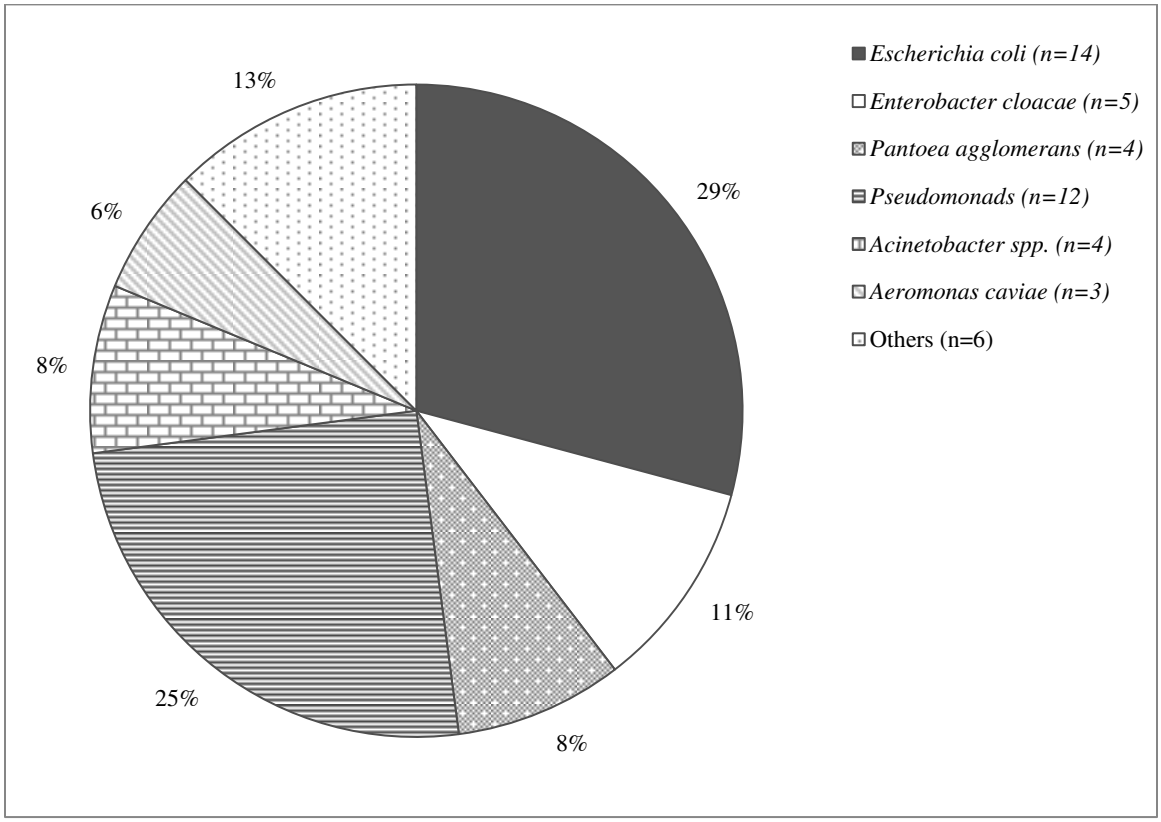

Figure 3.1 Gram-negative bacterial species isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Among the gram-positive bacteria, *Staphylococcus xylosus* was predominant, 53% (n=28) (Figure 3.2). *S. xylosus* was isolated mostly from plastic surfaces (n=19) followed by stainless steel surfaces (n=8) Other *Staphylococci* included: *S. aureus*, *S. saprophyticus*, and *S. warneri*. The species of three Coagulase Negative *Staphylococci* and one *Corynebacterium* sp. could not be determined by the method used in the study. *Kocuria rosea* accounted for 8% (n=4) of the total number of gram-positive bacteria. *Aerococcus viridans* and *Bacillus* spp. constituted 7% (n=4) of the gram-positive bacteria. *Streptococcus agalactiae* (n=1) and *Streptococcus dysgalactiae* (n=2) were also identified in our sample (Figure 3.2).

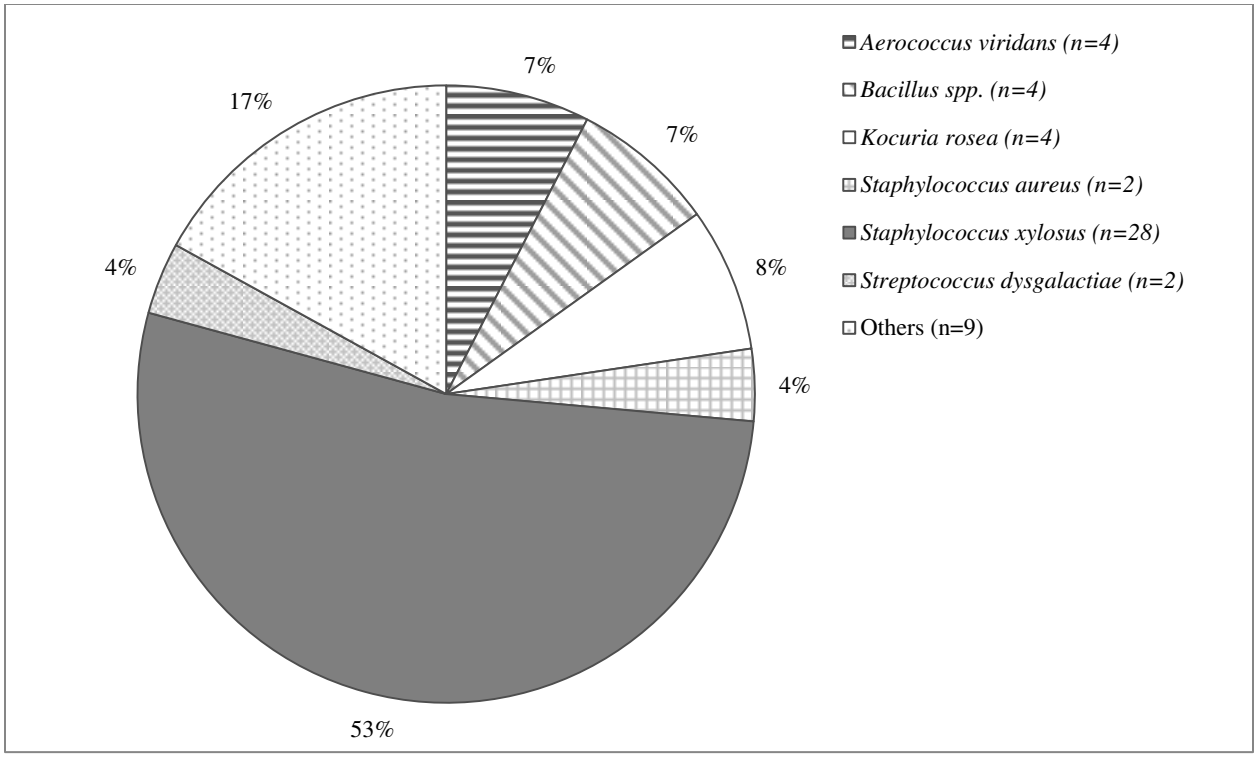

Figure 3.2 Gram-positive bacterial species isolated from environmental samples collected in poultry farms and processing plants in Alberta.

Among the 47 bacteria isolated from the plastic surface samples, 64% were gram-positive and 36% were gram-negative bacteria. Among the 38 bacteria isolated from steel surface samples, 53% were gram-positive and 47% were gram-negative bacteria. Out of five bacteria isolated from rubber samples, 40% were gram-positive and 60% were gram-negative bacteria. From the concrete samples, only two gram-positive bacteria were isolated. The distribution of gram-negative and gram-positive bacteria in plastic surface, steel surface, rubber surface and concrete surface samples was not significantly different ($p>0.05$). *E. coli*, *S. xylosus* and Pseudomonads were commonly isolated from plastic and steel surface samples. The distribution

of these bacteria in between plastic and steel surface samples was not significantly different ($p>0.05$).

Table 3.1 shows the different bacterial species isolated from the different sample types collected from the broiler farms, layer farms, hatcheries and processing plants in our study. The drinking nipples, feed augers and the feed pans from broiler and layer farms were the most common sample equipment from which bacteria were isolated. Among the 22 isolates from the drinking nipple samples, *S. xylosus* was predominant (n=7) followed by bacteria that belong to Pseudomonads (n=4). Among the less commonly isolated species from the drinking nipple samples were: *A. viridans* (n=2), unspeciated Coagulase Negative *Staphylococci* (n=2), *A. lwoffii* (n=1), *S. maltophilia* (n=1), *E. clocae* (n=1), *M. osloensis* (n=1), *Corynebacterium* sp. (n=1), *K. rosea* (n=1) and *B. cereus* (n=1). Among the 23 bacterial isolates collected from the samples from the feed pan, *S. xylosus* was predominant (n=10) followed by *E. coli* (n=4). Among the other bacterial species isolated from the feed pan samples were: *P. agglomerans* (n=1), *E. cloacae* (n=1), *R. anatipestifer* (n=1), *A. viridans* (n=1), unspeciated Coagulase Negative *Staphylococci* (n=1), *K. rosea* (n=1), *S. warneri* (n=1) and *S. aureus* (n=1). Among the 18 bacteria isolated from the samples collected from the feed auger, *S. xylosus* was predominant (n=9). Among the less commonly isolated bacterial species from the feed auger were: *E. coli* (n=2), *S. dysgalactiae* (n=2), *A. lwoffii* (n=1), *S. maltophilia* (n=1), *C. luteola* (n=1), *P. agglomerans* (n=1) and *S. saprophyticus* (n=1).

Table 3.1 Bacterial species isolated from broiler farms, layer farms, hatcheries and processing plants in Alberta.

Bacterial species	Broiler (n=12)	Layer (n=7)	Hatchery (n=2)	Processing Plants (n=3)
Achromobacter xylosoxidans (1)	water (1)			
Acinetobacter baumannii (1)				scald tank water (1)
Acinetobacter lwoffii (3)	drinking nipple (1)	feed auger (1)	hatch basket (1)	
Aerococcus viridans (4)	drinking nipple (1)	drinking nipple (1), feed pan (1)		drain (1)
Aeromonas caviae (3)				floor drain (2), chilled tank (1)
Bacillus cereus (2)			hatch basket (1)	drain (1)
Bacillus subtilis (1)	drinking nipple (1)			
Bacillus thuringiensis (1)			bronchitis vaccine solution (1)	
Coagulase Negative *Staphylococci* *(3)	drinking nipple (1)	drinking nipple (1), feed pan (1)		
Corynebacterium sp.*(1)	drinking nipple (1)			
Cryseomonas luteola (2)	drinking nipple(1)	feed auger (1)		
Enterobacter cloacae (5)	drinking nipple (1), feed pan (1), water (1)		hatch basket (1)	drain (1)
Enterococcus fecalis (1)			turkey toe trimmer (1)	
Escherichia coli (14)	feed pan (3), feed auger (1)	feed pan (1), feed auger (1)	chick processing belt (2), bronchitis tubing (1), chick separator (1)	scald tank water (1), scald tank swab (1), drain water (1), drain (1)
Kocuria rosea (4)	feed pan (1)	drinking nipple (1)	chick separator sheet roller (1)	defooter blade (1)
Moraxella osloensis (2)	drinking nipple (1)			drain (1)
Pantoea agglomerans (4)	feed auger (1)	feed pan (1)	chick separator (1)	defooter blade (1)
Pseudomonas aeruginosa (5)	drinking nipple (2), water (2)	drinking nipple (1)		
Pseudomonas fluorescens (2)			humidifier reservoir (1), waterline to vaccine preparation room (1)	
Pseudomonas mendocina (1)	water (1)			
Pseudomonas stutzeri (2)			incubator (2)	
Riemerella anatipestifer (1)	feed pan (1)			

Bacterial species	Broiler (n=12)	Layer (n=7)	Hatchery (n=2)	Processing Plants (n=3)
Staphylococcus aureus (2)	feed pan (1)			scald tank swab (1)
Staphylococcus saprophyticus (2)	feed pan (1), feed auger (1)			
Staphylococcus warnerii (1)		feed pan (1)		
Staphylococcus xylosus (28)	drinking nipple (5), feed pan (6), feed auger (6)	drinking nipple(2), feed pan (4) feed auger (3),	chick processing belt (1)	sawguard (1)
Stenotrophomonas maltophilia (2)	drinking nipple (1), feed auger (1)			
Streptococcus agalactiae (1)			chick processing belt (1)	
Streptococcus dysgalactiae (2)	feed auger (2)			

n= total number of farms/processing plants selected.
* Species could not be further determined.
Numbers of species isolated are presented in parentheses.

E. coli, *S. xylosus* and Pseudomonads were commonly isolated from the feed pans, feed augers and drinking nipple samples from broiler and layers farm. The distribution of these bacteria in between the drinking nipple, feed pans and feed auger samples was not significantly different ($p>0.05$). Among 23 bacteria isolated from feed pan samples, 70% were gram-positive bacteria and 30% were gram-negative bacteria. Among 18 bacteria isolated from feed auger, 67% were gram-positive bacteria and 33% were gram-negative bacteria. Among 22 bacteria isolated from drinking nipple samples, 64% were gram-positive and 36% were gram-negative bacteria. There was no significant difference observed in between the distribution of gram-positive and gram-negative bacteria isolated from feed pans, feed augers and drinking nipples samples ($p>0.05$).

From the hatcheries, bacteria were isolated from the samples collected from different equipment such as: hatch baskets, bronchitis vaccine solution, bronchitis tubing, hatchery incubators, humidifier reservoir, chick processing belts and chick separators. *E. coli* was the predominant species isolated from the hatcheries (n=4). Among the samples collected from processing plants, bacteria were isolated from scald tank, scald tank water, chilled tank, drain water, sawguard and defooter blade. *E. coli* was the predominant species isolated from the processing plants (n=4). Bacteria were not isolated from drinking nipple water samples collected from the broiler and layer farms. Three bacterial species were isolated from the broiler farm that was in down-time period between the flocks after cleaning and sanitation. These bacterial species were: *E. coli* (n=1) and *S. dysgalactiae* (n=1) both from feed auger samples; *S. xylosus* (n=3) were isolated from drinking nipple, feed pan and the feed auger samples.

Among the 47 samples collected from the broiler farms, the commonly isolated bacterial species were, *S. xylosus*, 36% followed by *E. coli* 9%, *P. aeruginosa*, 9% and *E. cloacae* 6%. *S. maltophilia*, 4% *S. dysgalactiae*, 4% and *S. saprophyticus*, 4% were also identified from the samples collected from broiler farms (Figure 3.3).

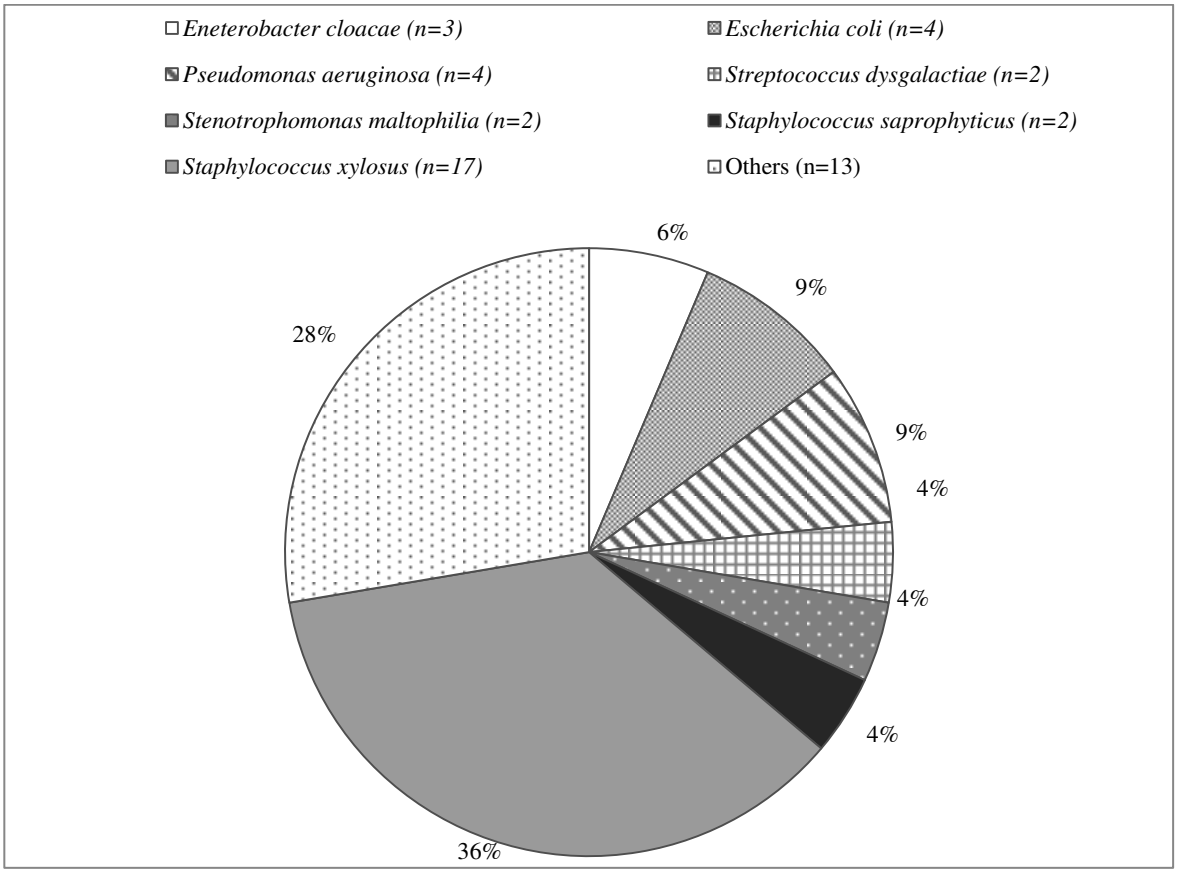

Figure 3.3 Bacterial species isolated from environmental samples collected in broiler farms in Alberta.

In layer farms, from the total of 21 isolates, 43% of the bacteria were identified as *S. xylosus* followed by *E. coli* (9%) and *A. viridans* (9%) (Figure 3.4). Other bacterial species isolated from layer farms were: *A. lwoffii*, *C. luteola, P. agglomerans*, *K. rosea*, *P. aeruginosa*, and *S. warneri*.

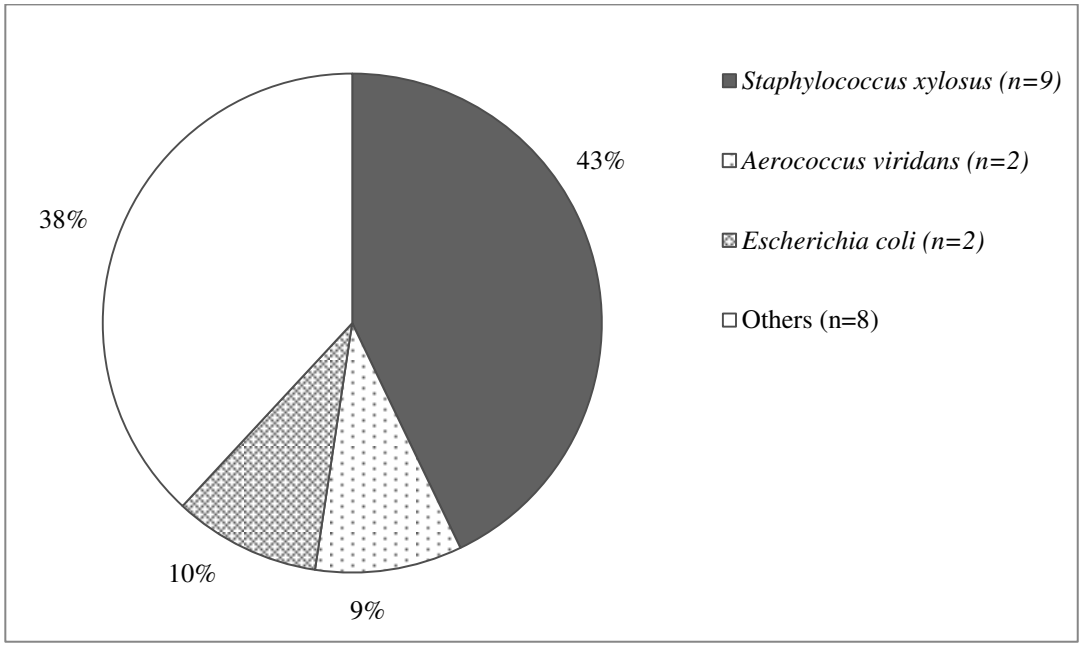

Figure 3.4 Bacterial species isolated from environmental samples collected in layer farms in Alberta.

Among the 17 bacteria isolated from hatcheries, four isolates were identified as *E. coli* (23%) followed by two isolates of *P. fluorescens* (12%) and two isolates of *P. stutzeri* (12%) (Figure 3.5). The nine single bacterial species isolated from the hatcheries were: *A. lwoffii*, *B. cereus*, *B. thuringiensis*, *E. cloacae*, *E. fecalis*, *K. rosea*, *P. agglomerans*, *S. xylosus* and *S. dysgalactiae*.

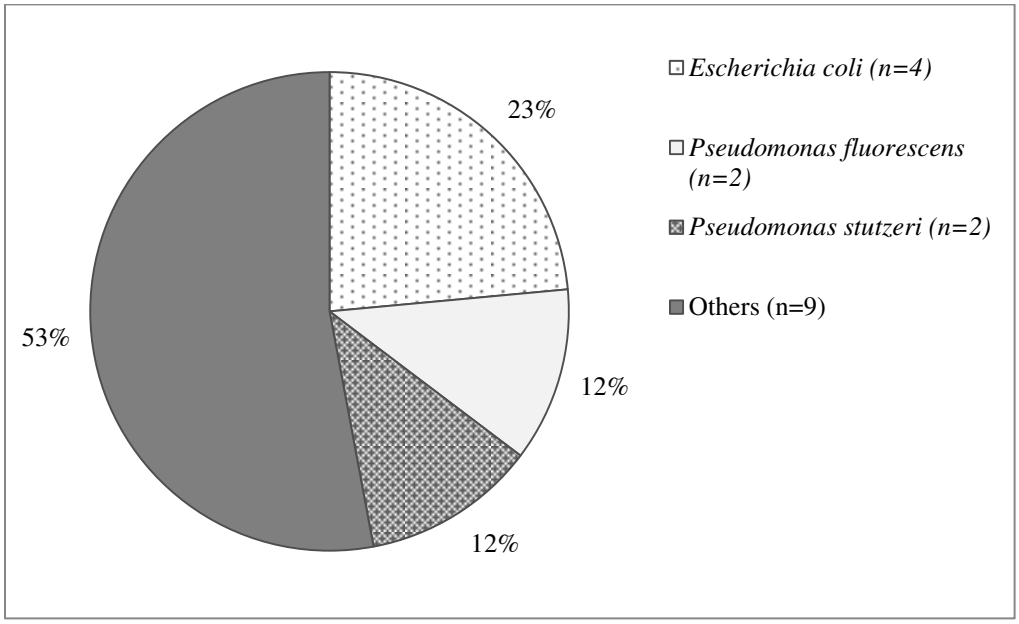

Figure 3.5 Bacterial species isolated from environmental samples collected in broiler hatcheries in Alberta.

Among the 16 bacteria isolated from the processing plants, four isolates were identified as *E. coli* (25%) followed by three *A. caviae* (19%). Nine single bacterial species from the processing plant samples were: *A. baumannii*, *A. viridans*, *B. cereus*, *E. cloace*, *K. rosea*, *M. osloensis*, *P. agglomerans*, *S. aureus*, and *S. xylosus* (Figure 3.6).

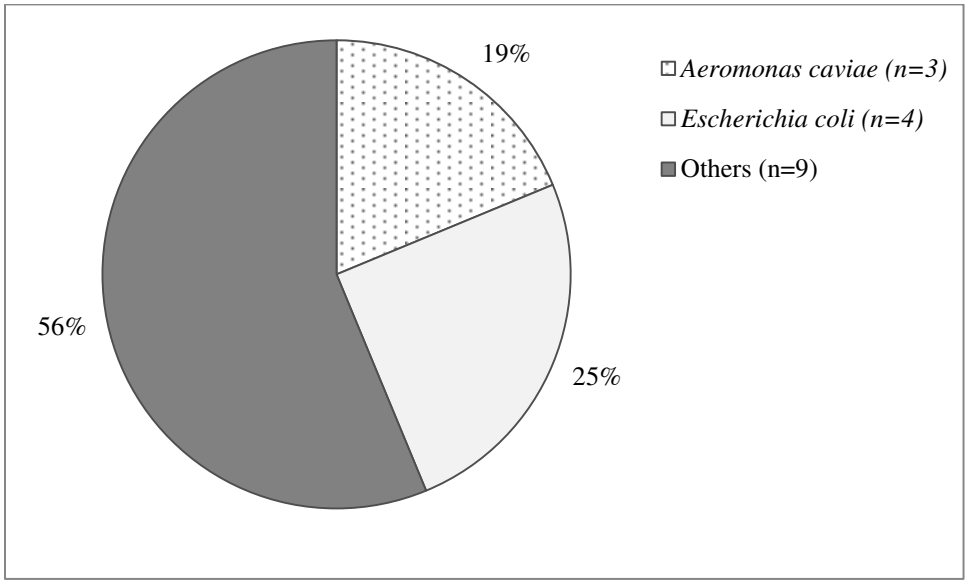

Figure 3.6 Bacterial species isolated from environmental samples collected in poultry processing plants in Alberta.

The distribution of gram-positive and gram-negative bacteria isolated from the samples of broiler farms, layer farms hatchery and processing plants was not significantly different ($p>0.05$).

3.2 Antimicrobial Susceptibility

3.2.1 *Antimicrobial Susceptibility Testing by Microbroth Dilution Method*

The antimicrobial susceptibility results of gram-negative and gram-positive bacteria are shown in tables 3.2 and 3.3 respectively. Among the gram-negative bacteria, ten of the 14 of *E. coli* were resistant to amoxicillin-clavulanic acid, ampicillin and cefoxitin; nine of 14 were resistant to ceftiofur, ceftriaxone and tetracycline. All of the 14 *E. coli* were sensitive to ciprofloxacin. All of five *E. cloacae* were resistant to amoxicillin-clavulanic acid and ampicillin, and sensitive to ciprofloxacin, kanamycin, tetracycline and sulfamethoxazole–trimethoprim.

Four *E. cloacae* were resistant to cefoxitin, ceftriaxone, and chloramphenicol. All 12 bacterial species of the *Pseudomonadaceae* were resistant to chloramphenicol (Table 3.2).

Table 3.2 Number of antibiotic resistance gram-negative bacteria isolated from environmental samples collected in poultry farms and processing plants in Alberta.

Bacterial species	AMC	AMP	FOX	*TIO	CRO	CHL	CIP	GEN	KAN	NAL	FIS	TET	SXT
E. coli (n=14)	10[a]	10	10	9	9	6	0	5	3	4	6	9	1
E. clocae (n=5)	5	5	4	1	4	4	0	3	0	1	1	0	0
P. agglomerans (n=4)	1	1	2	0	0	0	0	0	0	0	0	0	0
Pseudomonads (n=12)	8	NI	NI	NI	1	12	2	3	3	NI	5	6	6
A. caviae (n=3)	3	2	2	NI	1	0	0	1	1	NI	0	0	0
A. lwoffii (n=3)	0	NI	NI	NI	1	1	0	0	0	NI	0	2	0
M. osloensis (n=2)	0	NI	NI	NI	0	1	0	0	0	NI	0	0	0
S. maltophilia (n=2)	2	NI	NI	NI	NI	2	NI	2	2	NI	0	2	0
A. baumannii (n=1)	1	NI	NI	NI	0	1	0	0	0	NI	0	0	0
A. xylosoxidans (n=1)	1	NI	NI	NI	0	1	0	1	0	NI	0	0	0
R. anatipestifer (n=1)	0	NI	NI	NI	1	0	0	1	1	NI	0	0	0

*Ceftiofur resistance result was interpreted according to CIPARS short report-2011 (17).
n= total number of isolates.
[a] indicates number of isolates resistant to antibiotic.
Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin; KAN, kanamycin; NAL, nalidixic acid; FIS, sulfisoxazole, TET, tetracycline; SXT, sulfamethoxazole –trimethoprim; NI, no interpretation criteria available in CLSI guidelines (82).

Among the gram-positive bacteria, all 28 *S. xylosus* were resistant to gentamycin, kanamycin and tetracycline (Table 3.3). Twenty-seven *S. xylosus* were resistant to erythromycin (96%) and 21 were resistant to penicillin (75%). Ten *S. xylosus* were resistant to synercid antibiotic (36%). One isolate of *S. xylosus* was resistant to vancomycin. All four *K. rosea* were resistant to tetracycline and three isolates were resistant to erythromycin. Both isolates of the *B. cereus* were resistant to tetracycline and penicillin. One *S. dysgalactiae* isolated from the broiler farm in down-time period between flocks was resistant to chloramphenicol, erythromycin, penicillin and tetracycline antibiotic.

Table 3.3 Number of antibiotic resistance gram-positive bacteria isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Bacterial species	CHL	CIP	GEN	KAN	TET	DAP	ERY	LZD	PEN	SYN	VAN
S. xylosus (n=28)	1[a]	1	28	28	28	2	27	2	21	10	1
K. rosea (n=4)	0	NI	2	NI	4	NI	3	NI	NI	NI	0
A. viridans (n=4)	0	NI	NI	NI	3	NI	4	NI	NI	NI	0
Coagulase Negative *Staphylococci** (n=3)	0	0	NI	NI	3	0	3	0	NI	0	0
S. saprophyticus (n=2)	0	0	NI	NI	2	0	2	0	NI	1	0
S. aureus (n=2)	0	0	2	2	2	0	1	0	NI	1	0
S. dysgalactiae (n=2)	1	NI	NI	NI	1	NI	2	NI	2	NI	NI
B. cereus (n=2)	1	1	1	NI	2	NI	1	NI	2	NI	1
B. subtilis (n=1)	0	NI	NI	NI	0	NI	0	NI	NI	NI	0
B. thuringiensis (n=1)	0	0	NI	NI	0	NI	0	NI	1	NI	0
Corynebacterium sp.* (n=1)	0	0	NI	NI	1	0	1	0	1	0	0
E. fecalis (n=1)	0	NI	NI	NI	1	0	0	0	0	1	0
S. agalactiae (n=1)	0	NI	NI	NI	1	0	1	NI	1	NI	0
S. warneri (n=1)	0	0	1	1	1	0	1	0	0	0	0

n= total number of isolates.
[a] number of isolates resistant to antibiotic.
* species could not be further determined.
Abbreviations: CHL chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin, KAN, Kanamycin; TET, tetracycline; DAP, daptomycin; ERY, erythromycin; LZD, Linezolid; PEN, penicillin; SYN, synercid; VAN, vancomycin; NI, no interpretation criteria available in CLSI guidelines (82).

Table 3.4 shows the resistance pattern of gram-negative and gram-positive bacteria against medically important antimicrobials as classified by Veterinary Drug Directorate, Health Canada (14). Gram-negative bacteria were commonly resistant to category I drugs. The commonly detected resistance among the gram-negative bacteria were: amoxicillin-clavulanic acid 65% followed by ceftriaxone 35%.

Table 3.4 Resistance to medically important antibiotics among gram-negative and gram-positive bacteria isolated from environmental samples collected in poultry farms and processing plants in Alberta.

Antimicrobial Category*	Antimicrobial	Gram negative n=48 (%)	Drug	Gram positive n=53 (%)
I	Amoxycillin-Clavulanic acid	31[a] (64.6)	Ciprofloxacin	2 (3.8)
	Ceftiofur	10 (20.8)	Daptomycin	2 (3.8)
	Ceftriaxone	17 (35.4)	Linezolid	2 (3.8)
	Ciprofloxacin	2 (4.2)	Vancomycin	2 (3.8)
II	Ampicillin	18 (37.5)	Gentamycin	36 (68)
	Cefoxitin	19 (39.6)	Kanamycin	33 (62.3)
	Gentamycin	16 (33.3)	Erythromycin	46 (86.8)
	Kanamycin	10 (20.8)	Penicillin	40 (75.4)
	Nalidixic Acid	5 (10.4)	Synercid	14 (26.4)
	Sulfamethoxazole-Trimethoprim	7 (14.6)		
III	Chloramfenicol	28 (58.3)	Chloramfenicol	3 (5.6)
	Sulfisoxazole	12 (25)	Tetracycline	49 (92.5)
	Tetracycline	19 (39.6)		

*Category I: Very High Importance; II High Importance; III, Medium Importance, according to Veterinary Drug Directorate, Health Canada (14).
n= total number of isolates.
[a] number of isolates resistant to antibiotic.
Resistance to ceftiofur was interpreted according to CIPARS Short report -2011 (17).

Four percent gram-positive isolates were resistant to all the four category I drugs tested. Among the category II drugs, resistant to erythromycin was commonly detected in gram-positive bacteria, 89%. Resistance to gentamycin and kanamycin was also detected in 68% and 62% of gram-positive bacteria respectively. In gram-negative isolates, resistance to gentamycin and kanamycin was 33% and 21% respectively. Among the medium category III drugs, resistance to chloramphenicol was commonly detected in gram-negative bacteria (58%) compared to gram-positive bacteria (6%). Resistance to tetracycline was detected in 93% gram-positive bacteria and 40% gram-negative bacteria.

Among the gram-negative bacteria, there was a significant difference in resistance to chloramphenicol between the bacterial isolates collected from the broiler farms and the bacterial isolates collected from the processing plants ($p=0.028$). A significant difference was also observed for chloramphenicol resistance between the bacterial isolates collected from the hatcheries and the bacterial isolates collected from processing plants ($p=0.03$) (Table 3.5). There was no significant difference observed in the resistance of other antibiotics among gram-negative bacteria between the farms and processing plants ($p>0.05$).

Table 3.5 Percentage of antimicrobial resistance among gram-negative bacteria isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Antibiotics	Broiler[a] n=20 (%)	Layer[b] n=6 (%)	Hatchery[c] n=11 (%)	Processing Plant[d] n=11 (%)
AMP	8 (40.0)	1 (16.7)	4 (36.4)	5 (45.5)
AMC	15 (75.0)	3 (50.0)	6 (54.5)	7 (63.6)
FOX	8 (40.0)	2 (33.3)	4 (36.4)	5 (45.5)
TIO	4 (20.0)	1 (16.7)	4 (36.4)	1 (9.1)
CRO	9 (45.0)	1 (16.7)	5 (45.5)	2 (18.2)
CHL	15 (75.0)[d]	3 (50.0)	8 (72.7)[d]	2 (18.2)[a,c]
CIP	1 (5.0)	1 (16.7)	0 (0)	0 (0)
GEN	9 (45.0)	2 (33.3)	3 (27.3)	2 (18.2)
KAN	8 (40.0)	1 (16.7)	0 (0)	1 (9.1)
NAL	1 (5.0)	1 (16.7)	3 (27.3)	0 (0)
SUL	4 (20.0)	3 (50.0)	3 (27.3)	2 (18.2)
TET	9 (45.0)	3 (50.0)	5 (45.5)	2 (18.2)
SXT	3 (15.0)	2 (33.3)	1 (9.1)	1 (9.1)

a,b,c,d: statistical pairwise comparison categories by Fisher exact ($p < 0.05$).
n= total number of isolates.
Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin; KAN, kanamycin; NAL, nalidixic acid; FIS, sulfisoxazole, TET, tetracycline, SXT, sulfamethoxazole –trimethoprim.

Among the gram-positive bacteria, there was a significant difference in resistance to kanamycin between the isolates collected from the broiler farms and hatcheries (p=0.016), with more frequent resistance (74%) in isolates in the broiler farms (Table 3.6). A significant difference was also observed in resistance to erythromycin in the gram-positive isolates collected from hatcheries and broilers farms (p=0.031) with significantly higher resistance (93%) in the isolates from the broiler farms. There was also a significant difference in resistance to penicillin

between the isolates collected from the broiler farms and layer farms (p=0.03) with statistically more frequent resistance (81%) in the isolates collected from broiler farms.

Table 3.6 Percentage of antimicrobial resistance among gram-positive bacteria isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Antibiotics	Broiler[a] n=27 (%)	Layer[b] n=15 (%)	Hatchery[c] n=6 (%)	Processing Plants[d] n=5 (%)
CHL	1 (3.7)	1 (6.7)	0	1 (20.0)
CIP	0	1 (6.7)	0	1 (20.0)
DAP	1 (3.7)	1 (6.7)	0	0
ERY	25 (92.6)[c]	13 (86.7)	3 (50.0)[a]	5 (100.0)
GEN	20 (74.1)	10 (66.7)	2 (33.3)	4 (80.0)
KAN	20 (74.1)[c]	10 (66.7)	1 (16.7)[a]	2 (40.0)
LZD	1 (3.7)	1 (6.7)	0	0
PEN	21 (81.4)[b]	4 (26.7)[a]	4 (66.7)	2 (40.0)
SYN	8 (29.6)	2 (13.3)	2 (33.3)	2 (40.0)
TET	25 (92.6)	14 (93.3)	5 (83.3)	5 (100.0)
VAN	1 (3.7)	0	0	1 (20.0)

a,b,c,d: statistical pairwise comparison categories by Fisher exact ($p<0.05$)
n= total number of isolates.
Abbreviation: CHL chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin, KAN, Kanamycin; TET, tetracycline; DAP, daptomycin; ERY, erythromycin; LZD, Linezolid; PEN, penicillin; SYN, synercid; VAN, vancomycin

Tables 3.7 and 3.8 show the resistance patterns in antibiotic drug class combinations among gram-negative and gram-positive bacteria. Among the 48 gram-negative and 53 gram-positive isolates, six gram-negative (*E. coli* (n=3), *M. osloensis* (n=1) and *P. agglomerans* (n=2)) and one gram-positive isolate (*B. subtilis*) were sensitive to all the drug classes tested. Among the gram-negative bacterial species, 13 were resistant to only one drug class tested, eight were resistant to two drug classes, three were resistant to three drug classes, ten were resistant to four drug classes, six were resistant to five drug classes and two were resistant to six drug classes

tested. The *E. coli* isolated from the broiler farm that was in down-time period between the flocks was resistant to four drug classes tested (β-lactams, fenicol, tetracycline and sulphonamide). The common drug classes that the gram-negative bacteria resistant to were: β-lactams, aminoglycosides, fenicol and tetracycline (Table 3.7).

Table 3.7 Resistance patterns in antibiotic drug classes combination observed in gram-negative bacterial species isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

No of drug classes	β-lactams	Aminogly-cosides	Fenicols	Tetracyc-lines	Folate Pathway Inhibitors	Quino-lones	Number of isolates	Bacterial species
0							6	*Escherichia coli* (3), *Moraxella osloensis, Pantoea agglomerans* (2)
1	CRO						1	*Acinetobacter lwoffii*
				TET			2	*Acinetobacter lwoffii, Escherichia coli*
	AMC						1	*Aeromonas caviae*
	AMC-AMP-FOX						3	*Aeromonas caviae, Enterobacter clocae, Pantoea agglomerans*
			CHL				5	*Chryseomonas luteola, Moraxella osloensis, Pseudomonas mendocina, Pseudomonas stutzeri* (2)
	FOX						1	*Pantoea agglomerans*
2			CHL	TET			1	*Acinetobacter lwoffii*
	AMC		CHL				1	*Acinetobacter baumani*
	AMC-AMP-FOX-CRO	GEN-KAN					1	*Aeromonas caviae*
	AMC-AMP-FOX-CRO		CHL				1	*Enterobacter cloacae*
	AMC-AMP-FOX				SUL-SXT		1	*Escherichia coli*
	AMC-AMP-FOX-TIO-CRO	GEN-KAN					1	*Escherichia coli*
	AMC-CRO		CHL				1	*Pseudomonas fluorescens*
	CRO	GEN-KAN					1	*Riemerella anatipestifer*
3	AMC	GEN	CHL				1	*Achromobacter xylosoxidans*
	AMC-AMP-FOX-CRO	GEN	CHL				1	*Enterobacter cloacae*
	AMC-AMP-FOX-TIO-CRO			TET	SUL		1	*Escherichia coli*
4	AMC	GEN	CHL		SUL-SXT		1	*Chryseomonas luteola*
	AMC-AMP-FOX--CRO	GEN	CHL		SUL		1	*Enterobacter cloacae*
	AMC-AMP-FOX-TIO-CRO	GEN	CHL			NAL	1	*Enterobacter cloacae*

No of drug classes	β-lactams	Aminogly-cosides	Fenicols	Tetracyc-lines	Folate Pathway Inhibitors	Quino-lones	Number of isolates	Bacterial species
	AMC-AMP-FOX-TIO-CRO	GEN	CHL	TET			1	*Escherichia coli*
	AMC-AMP-FOX-TIO-CRO		CHL	TET	SUL		1	*Escherichia coli*
	AMC-AMP-FOX-TIO-CRO	KAN		TET		NAL	1	*Escherichia coli*
	AMC-AMP-FOX-TIO-CRO		CHL	TET		NAL	1	*Escherichia coli*
	AMC		CHL	TET	SXT		1	*Pseudomonas aeruginosa*
	AMC	GEN-KAN	CHL	TET			2	*Stenotrophomonas maltophilia* (2)
5	AMC-AMP-FOX-TIO-CRO	GEN	CHL	TET	SUL		1	*Escherichia coli*
	AMC	GEN-KAN	CHL	TET		CIP	1	*Pseudomonas aeruginosa*
	AMC		CHL	TET	SUL-SXT	CIP	1	*Pseudomonas aeruginosa*
	AMC	KAN	CHL	TET	SUL-SXT		2	*Pseudomonas aeruginosa* (2)
	AMC	GEN	CHL	TET	SUL-SXT		1	*Pseudomonas fluorescens*
6	AMC-AMP-FOX-TIO-CRO	GEN	CHL	TET	SUL	NAL	1	*Escherichia coli*
	AMC-AMP-FOX-TIO-CRO	GEN-KAN	CHL	TET	SUL	NAL	1	*Escherichia coli*

Number of isolates are indicated in parentheses.

Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin; KAN, kanamycin; NAL, nalidixic acid; FIS, sulfisoxazole, TET, tetracycline, SXT, sulfamethoxazole–trimethoprim.

Among the 53 gram-positive bacteria, three were resistant to one drug class, nine were resistant two drug classes, 15 were resistant to three drug classes, 10 were resistant to four drug classes and 12 were resistant to five drug classes tested (Table 3.8). Nine gram-positive bacteria were resistant to penicillin (β-lactams), gentamycin, kanamycin (aminoglycosides), tetracycline (tetracycline) and erythromycin (macrolide). These bacteria were: *S. xylosus* (n=8) and *S. saprophyticus* (n=1). Eleven bacteria were resistant to one additional drug class streptogramin. These 11 bacteria were *S. saprophyticus* (n=1) and *S. xylosus* (n=10). The three *S. xylosus,* which were isolated from the broiler farm that was in down-time period between flocks, were all resistant to four or five drug classes (β-lactams, aminoglycosides, tetracycline, macrolide and streptogramin). The gram-positive bacteria that were commonly resistant to the drug classes were: β-lactams, aminoglycosides, tetracyclines and macrolides.

Table 3.8 Resistance patterns in antibiotic drug classes combination observed in gram-positive bacterial species isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

No of Drug Class	β-lactams	Amino-glycosides	Feni-cols	Tetra-cyclines	Quino-lones	Macro-lids	Lipopep-tides	Strepto-gramins	Oxazo-lidones	Glyco-peptides	No of isolates	Bacterial species
0											1	*Bacillus subtilis*
1						ERY					1	*Aerococcus viridans*
	PEN										1	*Bacillus thuriengensis*
				TET							1	*Kocuria rosea*
2				TET		ERY					6	*Aerococcus viridans* (3), *Coagulase Negative Staphylococci* (2), *Kocuria rosea*
	PEN			TET							1	*Bacillus cereus*
				TET				SYN			1	*Enterococcus fecalis*
	PEN					ERY					1	*Streptococcus dysgalactiae*
3	PEN			TET		ERY					3	*Corynebacterium sp, Streptococcus agalactiae, Coagulase Negative Staphylococci*
		GEN		TET		ERY					2	*Kocuria rosea* (2)
		GEN-KAN				ERY		SYN			1	*Staphylococcus aureus*
		GEN-KAN		TET		ERY					7	*Staphylococcus warnerii, Staphylococcus xylosus (6)*
	PEN	GEN-KAN		TET							2	*Staphylococcus aureus, Staphylococcus xylosus*
4	PEN	GEN-KAN		TET		ERY					9	*Staphylococcus saprophyticus, ,Staphylococcus xylosus* (8)
	PEN		CHL	TET		ERY					1	*Streptococcus dysgalactiae*
5	PEN	GEN-KAN		TET		ERY		SYN			11	*, Staphylococcus saprophyticus, Staphylococcus xylosus* (10)
	PEN	GEN-KAN		TET		ERY				VAN	1	*Staphylococcus xylosus*
6	PEN	GEN-KAN		TET		ERY	DAP		LZD		1	*Staphylococcus xylosus*
7	PEN	GEN	CHL	TET	CIP	ERY				VAN	1	*Bacillus cereus*
≥8	PEN	GEN-KAN	CHL	TET	CIP	ERY	DAP	SYN	LZD		1	*Staphylococcus xylosus*

Number of isolates are indicated in parentheses

Abbreviation: CHL chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin, KAN, Kanamycin; TET, tetracycline; DAP, daptomycin; ERY, erythromycin; LZD, Linezolid; PEN, penicillin; SYN, synercid; VAN, vancomycin.

Figure 3.7 shows the various multi-drug resistance (resistance ≥3 drug classes) observed in the gram-positive and gram-negative bacteria isolated in this study. Thirteen of 48 gram-negative bacteria (27%) and 24 of 53 gram-positive bacteria (45%) were resistant to three to four drug classes tested. Twenty-one gram-negative bacteria (44%) and 12 gram-positive bacteria (23%) showed resistance to one to two drug classes tested. Resistance to five or more drug classes were observed in less than ten gram-negative (17%) and in 16 gram-positive bacteria (30%). Six gram-negative (13%) and one gram-positive bacteria (2%) were sensitive to all the drug classes tested. There was no significant difference observed in between the multi-drug resistance gram-negative and gram-positive bacteria for three to four drug classes or more than five drug classes tested ($p>0.05$).

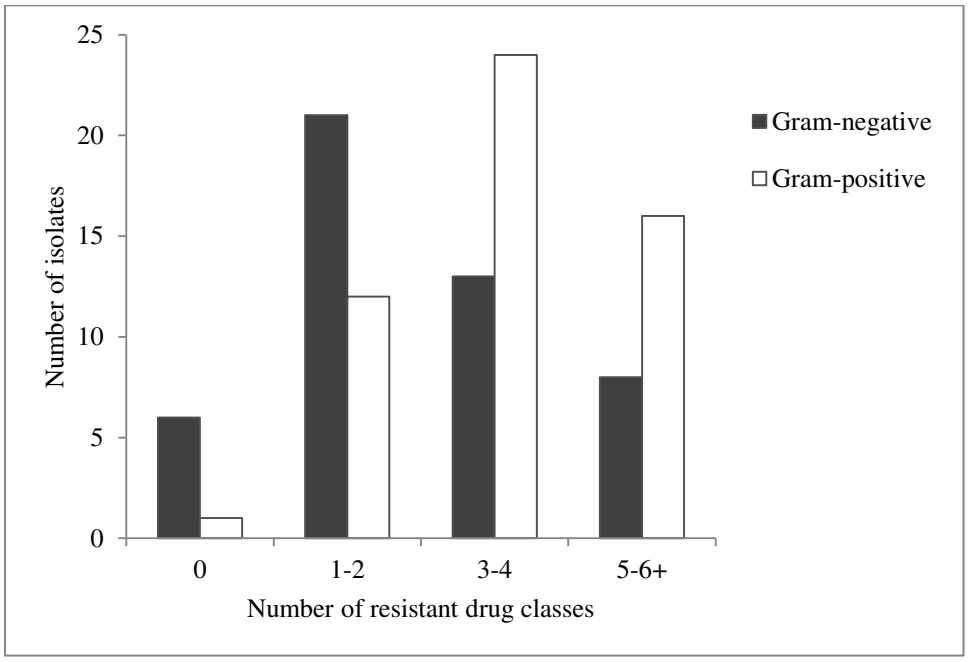

Figure 3.7 Multi-drug resistance (resistance ≥3 drug classes) observed among gram-negative and gram-positive bacteria isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Multi-drug resistance was observed in the bacterial isolates from the samples collected in all the farms and processing plants (Figure 3.8). Seventy-two percent of the bacterial isolates collected from the samples of the broiler farms were multi-drug resistant followed by the bacterial isolates collected from the samples of layer farms (67%). Forty-seven percent of the bacterial isolates collected from hatcheries and 31% of bacterial isolates collected from the processing plants were also multi-drug resistant. Among three to four drug classes tested, a statistically significant difference was observed between the multi-drug resistant bacterial isolates collected from the samples of broiler farms and processing plants (p=0.034). The samples collected from the broiler farms had significantly higher number of multi-drug resistant bacteria (n=22) compared to that of processing plants.

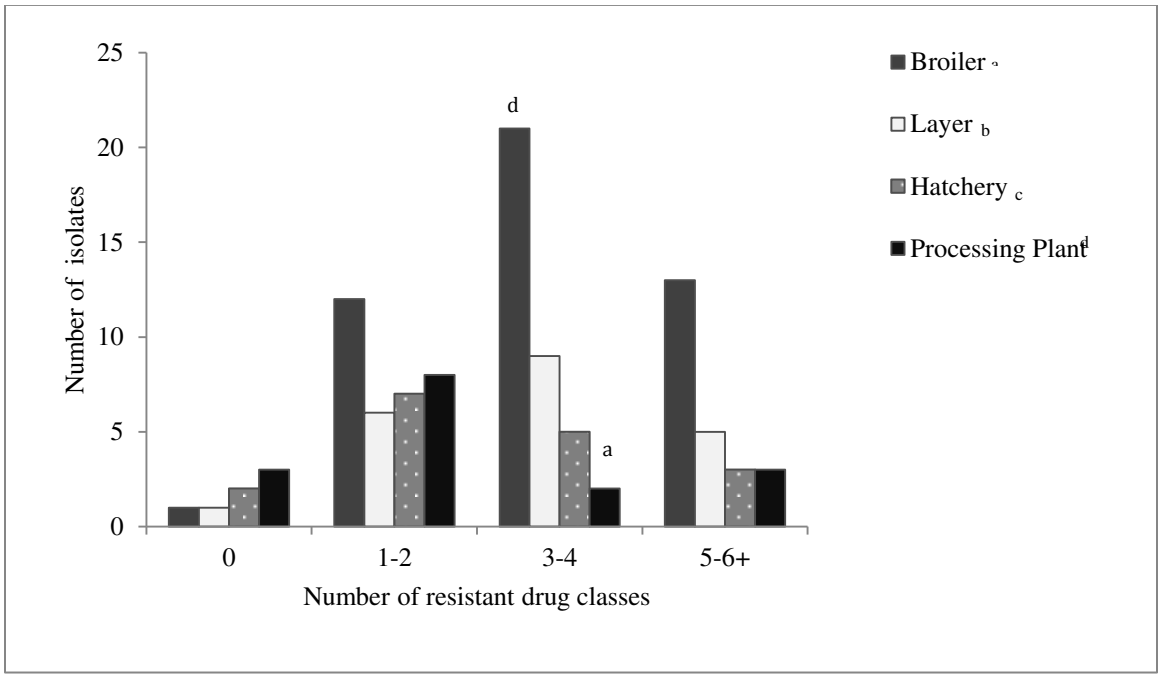

Figure 3.8 Multi-drug resistance (resistance ≥3 drug classes) observed in bacteria isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

a,b,c.d: statistical pairwise comparison categories by Fisher exact test ($p<0.05$)

Among three to four drug classes tested, we observed a significant difference in the number of multi-drug resistant bacterial isolates collected from steel surfaces and those collected from plastic surfaces (p=0.024). A significantly higher number of multi-drug resistant bacteria were detected from the plastic surface samples (n=22) compared to the bacterial isolates collected from steel surface samples (n=9) (Figure 3.9).

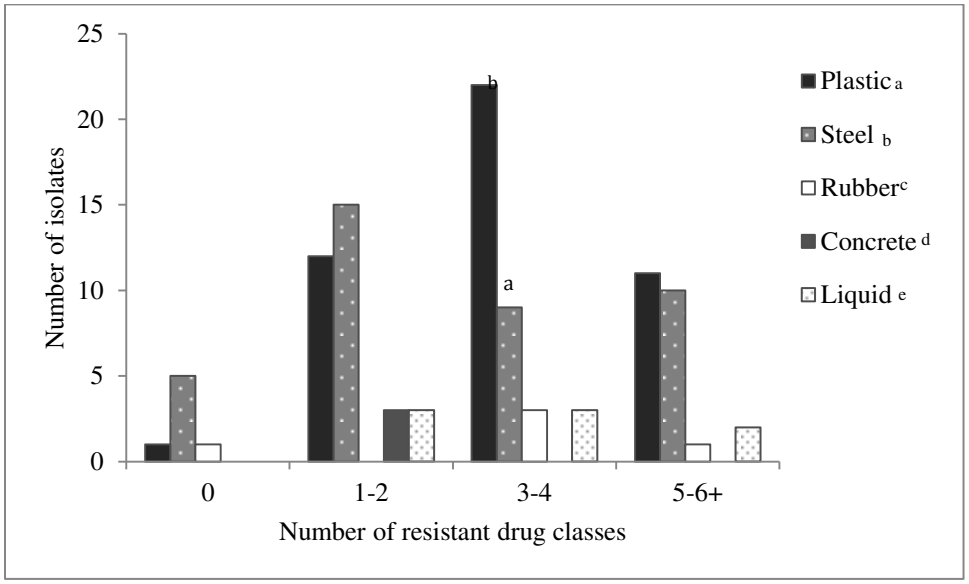

Figure 3.9 Multi-drug resistance (resistance ≥3 drug classes) observed in bacteria isolated from material surface types present in poultry farms and poultry processing plants in Alberta.

a, b, c, d: statistical pairwise comparison categories by Fisher exact ($p<0.05$).

Resistance to same drug classes of medically important antibiotics in Canada was observed among our gram-negative and gram-positive isolates from poultry production (Table 3.9). Resistance to ampicillin not recommended for use in poultry species was observed in *A. caviae* and *Enterobacteriacae* isolates. Resistance to ceftiofur (used as extra label manner *in ovo* in hatcheries) and ceftriaxone was observed in *Enterobacteriacae* isolates. Resistance to erythromycin and penicillin was observed in *Staphylococcus* spp., *Streptococcus* spp., *Bacillus*

spp., *K. rosea* and *A. viridans.* Resistance to tetracyclines, which are recommended for use in broilers and turkeys against gram-positive and gram-negative pathogens was noted in bacterial species that are not poultry pathogens. Resistance against chloramfenicol was observed in other gram-negative bacterial species such as *E. coli*, *P. aeruginosa*, *E. cloacae* and *A. baumannii*. *mecA* mediated methicillin resistant *Staphylococci* were not detected in our study.

Table 3.9 Antimicrobials used in poultry production in Canada, and resistance observed in bacterial species isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Drug Category[a]	Antimicrobials[b]	Organism (Diseases)	Poultry species	Resistance observed in antimicrobials[c] of same drug class	Resistant bacterial species isolated
I	Ceftiofur	*Eserichia coli* (air sacculitis)	Turkey	Ceftiofur, Ceftraixone	*Escherichia coli* (10), *Enterobacter cloacae* (5), *Pantoea agglomerans* (2)
II	Penicillin G	*Staphylococcus aureus* (arthritis) *Clostridium perfringens* (necrotic enteritis)	Brolier, Turkey Broiler	Penicillin	*Bacillus cereus* (2), *Bacillus thuringiensis* (1), *Corynebacterium sp* (1), *Staphylococcus aureus* (2), *Staphylococcus saprophyticus* (2), *Staphylococcus warneri* (1), *Staphylococcus xylosus* (28), *Streptococcus agalactiae* (1), *Streptococcus dysgalactiae* (2)
	Amoxicillin	*Escherichia coli* (air sacculitis, omphalitis, arthritis) *Staphylococcus aureus* (arthritis)	Broiler	Amoxycillin, Amoxycillin-Clavulanic acid Ampicillin	*Achromobacter xylosoxidans* (1), *Acinetobacter baumanii* (1), *Aeromonas caviae* (3), *Chryseomonas luteola* (1), *Enterobacter cloacae* (5), *Escherichia coli* (10), *Pantoea agglomerans* (1), *Pseudomonas aeruginosa* (5), *Pseudomonas fluorescens* (2), *Stenotrophomonas maltophilia (2)*
	Gentamycin Neomycin	*Escherichia coli* (omphalitis) *Escherichia coli* (air sacculitis) *Clostridium perfringens* (necrotic enteritis)	Broiler (ELDU if used *in ovo*) Turkey (ELDU if used *in ovo*) Broiler	Genamycin, Kanamycin	*Achromobacter xylosoxidans* (1), *Aeromonas caviae* (1), *Bacillus cereus* (1), *Chryseomonas luteola* (1), *Enterobacter cloacae* (3), *Escherichia coli* (6), *Kocuria rosea* (2), *Pseudomonas aeruginosa* (3), *Pseudomonas fluorescens* (1), *Riemerella anatipestifer* (1), *Staphylococcus aureus* (2), *Staphylococcus saprophyticus* (2), *Staphylococcus warneri* (1), *Staphylococcus xylosus* (2) *Stenotrophomonas maltophila* (2)
	Lincomycin-Spectinomycin	*Escherichia coli* (arthritis, omphalitis) *Clostridium perfringens* (necrotic enteritis) *Erysipelothrix rusiopathie* (erysepalas)	Broiler, Turkey Broiler Turkey	Spectinomycin resistance genes detected	*Escherichia coli* (5), *Enterobacter cloacae* (1), *Pseudomonas fluorescens* (1)

Drug Category[a]	Antimicrobials[b]	Organism (Diseases)	Poultry species	Resistance observed in antimicrobials[c] of same drug class	Resistant bacterial species isolated
	Erythromycin Tylosin	*Staphylococcus aureus* (arthritis) *Clostridium perfringens* (necrotic enteritis) *Escherichia coli* (peritonitis) *Mycoplasma synoviae* (mycoplasmosis)	Broiler, Turkey Broiler Layer Layer	Erythromycin	*Aerococcus viridans* (4), *Bacillus cereus (*1), *Coagulase Negative Staphylococci* (3), *Corynebacterium sp.* (1), *Kocuria rosea* (3), *Staphylococcus aureus* (1), *Staphylococcus saprophyticus* (2), *Staphylococcus warneri* (1), *Staphylococcus xylosus* (27), *Streptococcus agalactiae* (1), *Streptococcus dysgalactiae* (2)
	Trimethoprim-Sulfadiazine	*Escherichia coli* (air sacculitis, omphalitis, arthritis) *Staphylococcus aureus* (arthritis) *Clostridium perfringens* (necrotic enteritis)	Broiler, Turkey Turkey Turkey	Sulfaquinoxaline-Trimethoprim	*Chryseomonas luteola* (1), *Escherichia coli* (1), *Pseudomonas aeruginosa* (4), *Pseudomonas fluorescens* (1),
III	Florfenicol	*Pasteurella multocida* (fowl cholera), *Mycoplasma synoviae* (mycoplasmosis)	Broiler breeder, Turkey	Chloramfenicol	*Achromobacter xylosoxidans* (1), *Acinetobacter baumanii* (1), *Acinetobacter lwoffii* (1), *Chryseomonas luteola* (2), *Escherichia coli* (6), *Enterobacter cloacae* (2), *Moraxella osloensis* (1), *Pseudomonas aeruginosa* (5), *Pseudomonas fluorscens* (2), *Pseudomonas mendocina* (1), *Stenotrophomonas maltophilia* (2)
	Sulfamethazine/ Sulfaquinoxaline	*Escherichia coli* (air sacculitis, omphalitis, arthritis)	Broiler, Turkey	Sulfisoxazole	*Chryseomonas luteola* (1), *Escherichia coli* (6), *Enterobacter cloacae* (1), *Pseudomonas aeruginoas* (4)
	Tetracycline	*Escherichia coli* (air sacculitis) *Staphylococcus aureus* (arthritis) *Clostridium perfringens* (necrotic enteritis) *Mycoplasma synoviae* (Mycoplasmosis), *Pasteurella multocida* (fowl cholera)	Broiler, Turkey Broiler, Turkey Broiler Turkey	Tetracycline	*Acinetobacter lwoffii* (1), *Aerococcus viridans* (3), *Aeromonas caviae* (1), *Bacillus cereus* (2), *Coagulase Negative Staphylococci* (3), *Enterobacter cloacae* (1), *Escherichia coli* (9), *Pseudomonas aeruginosa* (5), *Pseudomonas fluorescens* (1), *Stahylococcus warneri* (1), *Staphyloccus xylosus* (28), *Staphylococcus aureus* (2), *Staphylococcus saprophyticus* (2), *Stenotrophomonas maltophilia* (2), *Streptococcus dysgalactiae* (1), *Streptococus agalactiae* (1)

Note: [a]Category I: Very High Importance; II High Importance; III, Medium Importance according to Veterinary Drug Directorate, Health Canada (14). [b]Antimicrobials allowed for use in Canadian poultry (15). [c] Antimicrobials tested in the Sensititre® panel. Numbers in the parentheses represents number of resistant isolates. ELDU, Extra label drug use

3.2.2 Characterization of ESBL/AmpC Phenotype *E. coli*

All 10 *E. coli* isolates screened for ESBL phenotypes were not inhibited by cefotaxime-clavulanic acid. Nine of them were not inhibited by ceftazidime clavulanic acid and only one *E. coli* isolate was inhibited by ceftazidime-clavulanic acid and indicated an ESBL phenotype. A disc diffusion assay consisting cefpodoxime and a cephalosporin-inhibitor combination showed that all the 10 of 14 *E. coli* in our study had the AmpC phenotype. An AmpC phenotype is defined as resistance to 1st, 2nd and 3rd generation cephalosporins including cephamycin such as cefoxitin and, third generation cephalosporin plus clavulanic acid.

3.3 Identification of Gene Cassettes in Class 1 and Class 2 Integrons of Gram-negative Bacteria

Only eight of the 48 gram-negative isolates harboured at least one class 1 integron, and one isolate harboured a class 2 integron (Table 3.10). Gram-negative bacteria identified as having class 1 integron were: *E. coli* (n=5), *E. cloacae* (n=1), *P. fluorescens* (n=1), *P. stutzeri* (n=1). Only one *E. coli* isolate harboured a Class 2 integron. Amplification and sequencing of the class 1 integron showed that *P. stutzeri* harboured an empty integron. A single gene cassette *aadA1* that confers resistance to streptomycin and spectinomycin was detected in four *E. coli*, one *E. cloacae* and one *P. fluorescens*. One *E. coli* isolate harboured two gene cassettes *aadA2* and *dfrA12* that confers resistance to streptomycin and trimethoprim respectively. The one *E. coli* which had a class 2 integron had *dfr*A12, *sat*2 and *aad*A1. The *sat*2 confers resistance to streptothricin.

Table 3.10 Identification of antibiotic resistance gene cassettes in Class 1 and Class 2 integrons present in multi-drug resistant gram-negative bacteria isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Species	Class 1	Class 2	Resistance Conferred	Phenotypic Resistance Observed
E. coli	*aad*A1	-	Streptomycin, Spectinomycin	AMC, AMP, FOX, TIO, CRO, CHL, GEN, KAN, NAL, FIS, TET
E. coli	*aad*A1	-	Streptomycin, Spectinomycin	AMC, AMP, FOX, TIO, CRO, CHL, GEN, TET
E. coli	*aad*A1	-	Streptomycin, Spectinomycin	AMC, AMP, FOX, TIO, CRO, CHL, FIS, TET
E. coli	*aad*A1	-	Streptomycin, Spectinomycin	AMC, AMP, FOX, TIO, CRO, FIS, TET
E. coli	-	*dfr*A12, *sat*2,*aad*A1	Trimethoprim, Streptothricin, Spectinomycin	AMC, AMP, FOX, TIO, CRO, GEN, KAN
E. coli	*dfr*A12, *aad*A2	-	Trimethoprim, Streptomycin,	AMC, AMP, FOX, FIS, SXT
E. cloacae	*aad*A1	-	Streptomycin, Spectinomycin	AMC, AMP, FOX, CRO, CHL, GEN
P. fluorescens	*aad*A1	-	Streptomycin, Spectinomycin	AMC, CHL, GEN, FIS, TET, SXT
P. stutzeri	(empty)	-	-	CHL

Abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; TIO, ceftiofur; CRO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamycin; KAN, kanamycin; NAL, nalidixic acid; FIS, sulfisoxazole, TET, tetracycline, SXT, sulfamethoxazole–trimethoprim.

3.4 Detection of *ampC* Promoter Mutations in *E. coli*

The genetic sequences of of *ampC* promoter region were compared with the *ampC* promoter sequence of the control strain *E. coli* ATCC 25922. Single Nucleotide Polymorphisms (SNP) was detected at the promoter sequence. In total, five SNP patterns (or mutation types) were detected (Table 3.11). The most common nucleotide changes were at positions -73 (T➔C) and at *ampC* coding region +81 (A➔G). Mutation type 1 was detected in *E. coli* isolates that were resistant to cephalosporins (n=2) and those that were sensitive to cephalosporins (n=3). These nucleotide changes were at positions -88 (C➔T), -82 (A➔G), -73 (T➔C), -18 (G➔A), -1 (C➔T), +58 (C➔T) and +81 (A➔G). Transition that occurred from G to A at position -18 at spacer region created a new -10 box (TATCGT). Mutation type 2 occurred in three isolates (one sensitive and two resistant) with common mutations at positions -73 (T➔C), +70 (C➔T), and +81 (A➔G). Mutation type 3 occurred in one resistant isolate had four nucleotide changes in the attenuator region at positions +22 (C➔T), +26 (T➔G), +27 (A➔T), and +32 (G➔A). Mutation type four occurred in three isolates that had mutations at positions -73 and +81 in the promoter region. Mutation type 5 occurred in one isolate and, that had mutations occurred at positions -73 (T➔C), +37 (G➔T), +70 (C➔T), and +81 (A➔G) in the promoter region. There were no mutations observed in the -35 box and -10 box. The size of one PCR amplicon of the *ampC* promoter-attenuator region of one *E. coli* isolate was approximately 1.5 kb, which was larger than the expected 271 bp sequence. When compared with the sequence contained in the NCBI database, the sequence matched with the *ampR* gene of *Citrobacter freundii*.

Table 3.11 Single nucleotide polymorphisms detected in the *amp*C promoter sequence in *E. coli* isolates, and the respective MIC values for cefoxitin and cetriaxone.

Mutation pattern	No of isolates	Cefoxitin MIC (µg/mL)	Ceftriaxone MIC (µg/mL)	Mutation position
				1 -88 -82 -76 -73 -42 -35 (-35 box) -30
E. coli ATCC 25922		=4	≤0.25	A T **C** C A C G T **A** C C T G C **G** G G **T** A A A T G G G T T T T C T A C G G T C T G G C T G C T A T C C T G A C A G T T G T C A C G C
1	5	(=4->32)	(≤0.25-=16)	T G C
2	3	(=2>32)	(≤0.25-=16)	C
3	1	>32)	=32	A C
4	3	≥32	(4-32)	C
5	1	>32	=32	C
				(attenuator region)
				65 (Spacer region) -18 -13 (-10 box) -8 -1 +1 +17 +22 +26 +27 +32 +37
E. coli ATCC 25922		=4	≤0.25	T G A T T G G T **G** T C G T T A C A A T C T A A C G C A T C G C C A A T G T A A A T C C G G C C **C** G C C **T** **A** T G G C **G** G G C C **G** T
1	5	(=4->32)	(≤0.25-=16)	A T
2	3	(=2>32)	(≤0.25-=16)	
3	1	>32)	=32	T G T A
4	3	≥32	(4-32)	
5	1	>32	=32	T
				129 +58 (*amp*C start codon) +70 +81
E. coli ATCC 25922		=4	≤0.25	T T T G T A T G G A A A C C A G A C C **C** T A T G T T C A A A A **C** G A C G C T C T G C **A** C C T T A T T A A T T A C C G C C T
1	5	(=4->32)	(≤0.25-=16)	T G
2	3	(=2>32)	(≤0.25-=16)	G
3	1	>32)	=32	T G
4	3	≥32	(4-32)	G
5	1	>32	=32	T G

Note: *amp*C promoter sequences were compared with that of control strain *E. coli* ATCC 25922. Mutations at -18 position create an alternate promoter. MIC values for cefoxitin ≤ 8µg/mL and ceftriaxone ≤1.0 µg/mL were considered sensitive (82).

3.5 Plasmid Profiling of *E. coli*

All 10 cephalosporin resistant *E. coli* harboured at least one plasmid. Eight *E. coli* isolates harboured more than one plasmids. The plasmid size ranged from approximately 1000 bp to 4000 bp estimated using a 1000 bp ladder. The most common plasmid size was approximately 4000 bp, which occurred in eight *E. coli* isolates. These isolates were from processing plant (n=2), hatchery (n=3), and broiler farms (n=5). Only two isolates, either from broiler-feed pan or hatchery-bronchitis tubing, had a single plasmid of 4000 bp. The rest of the *E. coli* isolates had two to five plasmids. Only one isolate from broiler-feed auger harboured of five plasmids, which ranged in size from approximately 1000 bp to 4000 bp (Figure 3.10).

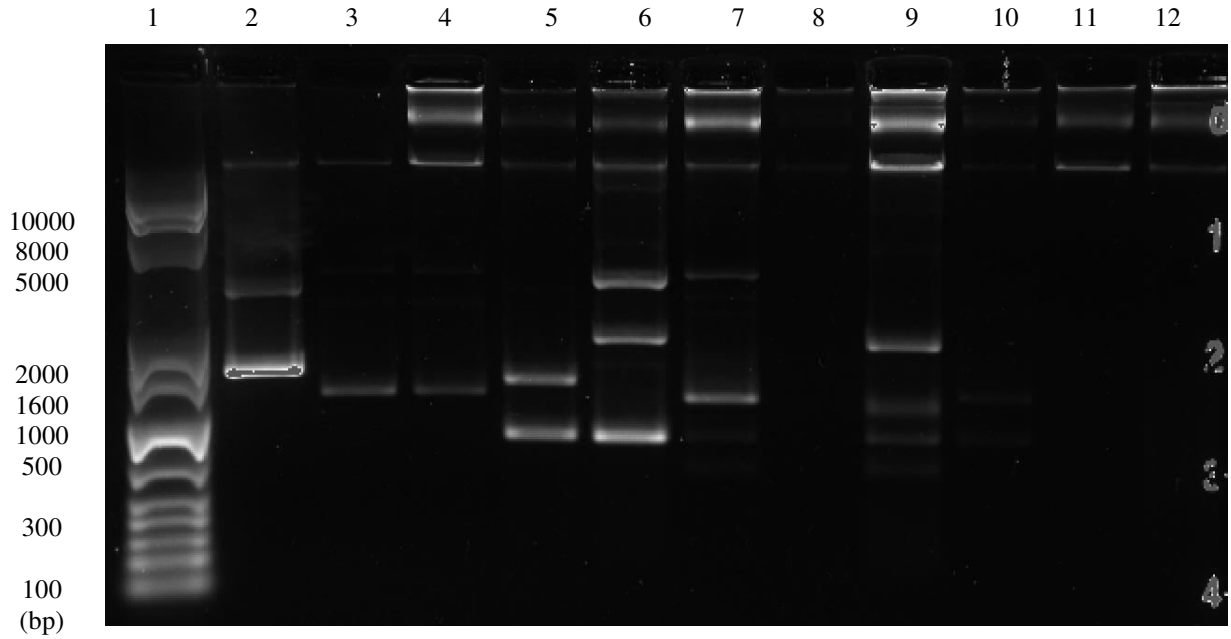

Figure 3.10 Plasmid profiles for *E. coli* possessing AmpC phenotype isolated from environmental samples collected from poultry farms and poultry processing plants in Alberta.

Lane 1, Gel Pilot 1 Kb plus ladder (Qiagen Inc. Toronto ON), Lane 2, plasmid positive *E. coli* DH5α.
Lane 3 to 12, plasmid positive *E. coli* isolates in this study.
Abbreviation: bp, base pairs.

3.6 Detection of Plasmid Mediated *bla*CMY-2 genes in *E. coli*

Out of 10 AmpC phenotype *E. coli,* plasmid mediated *bla*CMY-2 genes 1143 bp in length were detected only in three isolates (Figure 3.11). The isolates were from processing plant-drain water sample, hatchery-incubator and broiler-feed pan samples. All *bla*CMY-2 genes were located on a 4000 bp size plasmid. No *bla*CTX-M-1 group genes were detected by PCR in one ESBL *E. coli*.

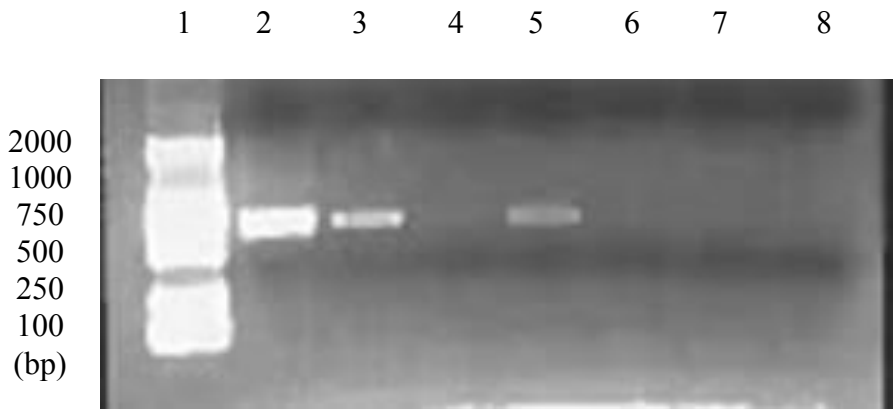

Figure 3.11 A gel picture of PCR amplicons of *bla*CMY-2 genes in plasmid of *E. coli* with ceftriaxone resistant phenotypes isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Lane 1, Base pair ladder; Lane 2, *bla*CMY-2 positive control of *E. coli*; Lane 3, and Lane 5, *bla*CMY-2 positive isolate; Lanes 4, 6 and 7, *bla*CMY-2 negative isolate; Lane 8, no template control.
Note: GelPilot Mid-Range Ladder (Qiagen Inc, Toronto, ON)
Abbreviations: bp, base pairs.

3.7 Minimum Biofilm Eradication Concentration Assay

Eleven isolates were chosen based on sample sites, prevalence and organisms of interest. These isolates were one *B. subtilis* (broiler-drinking nipple), one *P. stutzeri* (hatchery-incubator), one *C. luteola* (layer-drinking nipple), three *E. coli* (hatchery-bronchitis tubing, processing plant-scald tank water, and broiler-feed pan), one *S. xylosus* (layer-grain auger), one

E. cloacae (layer-feed pan), one *B. thuringiensis* (hatchery- bronchitis vaccine solution), one *P. aeruginosa* (broiler-drinking nipple) and one *A. caviae* (processing plant-chilled tank). These selected bacterial species were cultured to form biofilm *in vitro* using the MBEC™, for 24 hours, The *A. caviae* and *B. thuringiensis* isolates selected did not form a good biofilm on the pegs at the given experimental conditions (mean log10CFU/peg ≤0.5). The rest of the isolates formed biofilm on the pegs (mean log10CFU/peg >4.6) and were further tested for antimicrobial and disinfectant susceptibility. Interestingly, the *S. xylosus* isolated from the broiler farm after sanitation during the down-time period between flocks, were able to form biofilm on MBEC™ peg lids. Figure 3.12. shows the SEM picture of 24 hours *B. subtilis* biofilm grown on MBEC™ device.

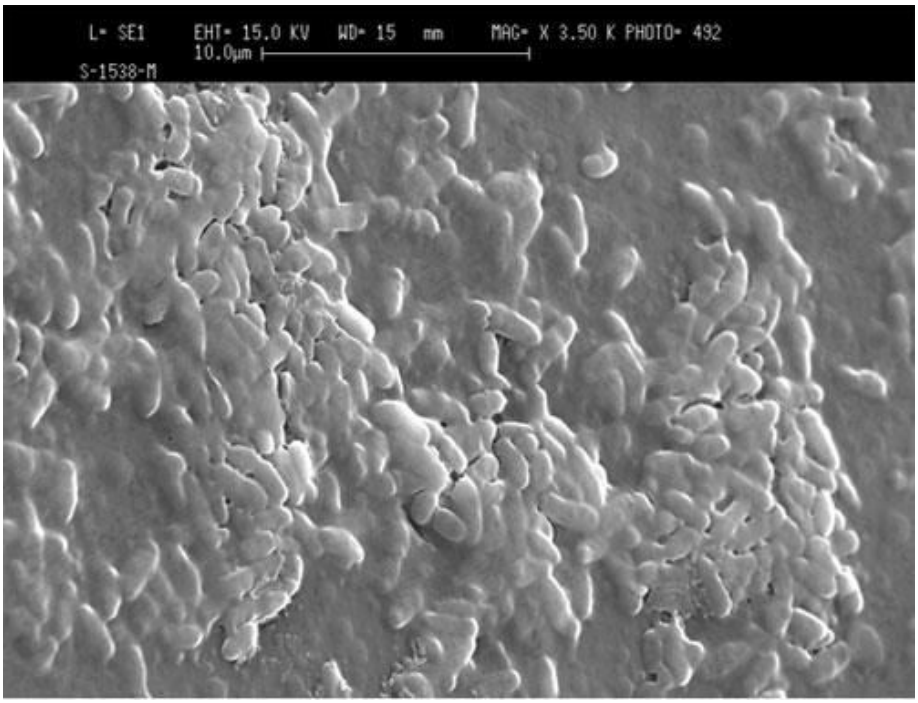

Figure 3.12 Scanning Electron Micrograph of *B. subtilis* biofilm cultured *in vitro* in the MBEC™ device after 24 hours incubation.

Bacteria tend to grow in micro-colonies and are mixed with exo-polysaccharide materials.

The MIC results of planktonic and biofilm bacteria are summarised in table 3.12. Breakpoints were reached for the following antimicrobials and the organisms were considered sensitive according to CLSI guidelines (89): enrofloxacin, gentamycin, neomycin and streptomycin. The bacteria sensitive to these antimicrobials were *B. subtilis, P. stutzeri, C. luteola, E. coli, S. xylosus* and *P. aeruginosa.* There were considerable variations in the resistance pattern of planktonic and biofilm bacteria against different drugs tested. Planktonic *B. subtilis* was sensitive to the lower concentrations of the drugs tested but was resistant to streptomycin, up to 128 fold more resistant in biofilm. *P. aeruginosa* biofilm bacteria were inhibited by the same concentration of drugs that inhibited planktonic form except for gentamycin, neomycin and streptomycin. This bacterium in biofilm was 16 fold more resistance to gentamycin and neomycin and; 128 fold resistant to streptomycin when compared to planktonic form. All three *E. coli* grown in biofilms were one to 17 fold more resistant to antimicrobials tested except for streptomycin (32 fold). These *E. coli* were resistant to antimicrobial classes such as aminoglycosides, β-lactams, fenicols, macrolides, sulphonamides and tetracyclines.

Table 3.12 Comparison of antimicrobial resistance of bacteria grown in planktonic culture versus in vitro biofilm culture. Bacteria were isolated from environmental samples collected in poultry farms and poultry processing plants in Alberta.

Bacteria	ENRO	GEN	TIO	NEO	ERY	OXY	TET	AMOX	SPE	SDM	SXT	FFN	STZ	PEN	STR	NOV	TYL	CLI
B. subtilis																		
MIC	<0.12	<0.5	<0.25	<2	<0.12	<0.25	<0.25	>16	32	<32	<0.5/9.5	<1	<32	>8	<8	<0.5	<2.5	<0.5
MBEC	>2	>8	>4	>32	>4	>8	>8	>16	>64	>256	>2/38	>8	>256	>8	>1024	>4	>20	>4
FIR	>17	>16	>16	>16	>33	>32	>32	1	>2	>8	>4	>8	>8	1	>128	>8	>8	>8
P. stutzeri																		
MIC	0.25	<0.5	2	<2	>4	1	2	8	32	>256	<0.5/9.5	>8	>256	4	16	>4	>20	>4
MBEC	0.25	1	>4	>32	>4	8	>8	>16	32	>256	>2/38	>8	>256	>8	128	>4	>20	>4
FIR	1	2	>2	>16	1	8	>4	>2	1	1	>4	1	1	2	8	1	1	1
C. luteola																		
MIC	0.25	<0.5	1	<2	>4	2	2	>16	32	>256	<2/38	>8	>256	>8	<8	>4	>20	>4
MBEC	>2	>8	>4	>32	>4	>8	>8	>16	>64	>256	>2/38	>8	>256	>8	>1024	>4	>20	>4
FIR	>8	>16	>4	>16	1	>4	>4	1	>2	1	>1	1	1	1	>128	1	1	1
E. coli																		
MIC	0.5	<0.5	>4	<2	>4	>8	>8	>16	>64	>256	<0.5/9.5	2	64	>8	32	>4	>20	>4
MBEC	0.5	2	>4	16	>4	>8	>8	>16	>64	>256	>2/38	>8	>256	>8	>1024	>4	>20	>4
FIR	1	4	1	8	1	1	1	1	1	1	>4	>4	>4	1	>32	1	1	1
E coli																		
MIC	<0.12	<0.5	1	<2	>4	>8	>8	8	<8	>256	<0.5/9.5	2	>256	>8	<8	>4	>20	>4
MBEC	>2	>8	>4	>32	>4	>8	>8	>16	>64	>256	>2/38	>8	>256	>8	>1024	>4	>20	>4
FIR	>17	>16	>4	>16	1	1	1	>2	>8	1	>4	>4	1	1	>128	1	1	1
S. xylosus																		
MIC	1	<0.5	<0.25	<2	>4	>8	>8	<0.25	>64	>256	<0.5/9.5	<1	>256	0.12	64	>4	<2.5	>4
MBEC	2	>8	>4	>32	>4	>8	>8	>16	>64	>256	>2/38	>8	>256	>8	>1024	>4	>20	>4
FIR	2	>16	>16	>16	1	1	1	>64	1	1	>4	>8	1	>67	>16	1	>8	1
E. cloacae																		
MIC	<0.12	<0.5	>0.5	<2	>4	>8	>8	<0.25	>64	>256	<0.5/9.5	2	>256	<0.06	>1024	>4	>20	>4
MBEC	>2	>8	>4	>32	>4	>8	>8	>16	>64	>256	>2/38	>8	>256	>8	>1024	>4	>20	>4

Bacteria	ENRO	GEN	TIO	NEO	ERY	OXY	TET	AMOX	SPE	SDM	SXT	FFN	STZ	PEN	STR	NOV	TYL	CLI
FIR	>17	>16	8	>16	1	1	1	>64	1	1	>4	>4	1	>133	1	1	1	1
E. coli																		
MIC	<0.12	>8	>4	>32	>4	>8	2	>16	>64	>256	<2/38	<1	>256	>8	256	>4	>20	>4
MBEC	>2	>8	>4	>32	>4	>8	>8	>16	>64	>256	>2/38	<1	>256	>8	>1024	>4	>20	>4
FIR	>17	1	1	1	1	1	>4	1	1	1	>1	1	1	1	>4	1	1	1
P. aeruginosa																		
MIC	>2	<0.5	>4	<2	>4	>8	>8	>16	>64	>256	<2/38	>8	>256	>8	<8	>4	>20	>4
MBEC	>2	>8	>4	>32	>4	>8	>8	>16	>64	>256	>2/38	>8	>256	>8	>1024	>4	>20	>4
FIR	1	>16	1	>16	1	1	1	1	1	1	>1	1	1	1	>128	1	1	1

Biofilm was grown in MBEC™ device for 24 hours.

Abbreviations: ENRO, enrofloxacin; GEN, gentamycin; TIO, ceftiofur; NEO, neomycin; ERY, erythromycin; OXY, oxytetracycline; TET, tetracyclin; AMOX, amoxicillin; SPE, spectinomycin; SDM, sulfadimethoxine; SXT, sulfamethoxazole-trimethoprim; FFN, florfenicol; STZ, sulfathiazole; PEN, penicillin; STR, streptomycin; NOV, novobiocin; TYLT, tylosin tartarate; CLI, clindamycin; MIC, (Minimum Inhibitory Concentration): minimum concentration of antimicrobial that inhibited the growth of bacteria on liquid culture;

MBEC, (Minimum Biofilm Eradication Concentration): minimum concentration of antimicrobial that eliminated bacteria in biofilm culture; FIR (Fold Increased Resistance): Increase in MIC of bacteria in biofilm culture. MIC and MBEC are expressed in µg/mL

3.8 Disinfectant Susceptibility of *in vitro* Biofilm

Table 3.13 shows the growth inhibition of biofilm bacteria at different concentrations (1x, 2x, 10x and 0.1x) of gluteraldehyde, peracetic-hydrogen peroxide, chlorine, quaternary ammonium compound, iodine compound, hydrogen peroxide and potassium monopersulphate disinfectants tested. *C. luteola, P. aeruginosa and* one *E. coli* growth were inhibited by all the disinfectants at manufacturer's recommended concentration (1x). *P. stutzeri* biofilm growth was inhibited by all the disinfectants at one tenth of the recommended concentration. For two of the *E. coli*, the biofilm growth was inhibited by hydrogen peroxide at twice the recommended concentration (2x). The remaining six disinfectants inhibited the biofim growth of *E. coli* at the manufacturer's recommended concentration (1x). Ten times the recommended concentration of hydrogen peroxide (10x), and two times the recommended concentration of peracetic acid-hydrogen peroxide (2x) was required to inhibit the biofilm growth *S. xylosus* but five of the disinfectants inhibited its growth at manufacturer's recommended concentration (1x). The *B. subtilis* isolate was the most resistant to the action of disinfectants. This *B. subtilis* was inhibited only by gluteraldehyde at all the manufacturer's recommended concentration (1x). The remaining disinfectants failed to inhibit the growth of this organism at any of the tested concentrations at the tested contact time.

Table 3.13 Growth inhibition of bacteria grown in biofilm *in vitro* by different concentrations of disinfectants.

Bacterial species	Peracetic acid-hydrogen peroxide	Gluteral-dehyde	Chlorine	Quaternary ammonium compound	Iodine compound	Hydrogen peroxide	Potassium-monoper-sulfate
Bacillus subtilis	NI	1x	NI	NI	NI	NI	NI
Chryseomonas luteola	1x	1x	1x	1x	1x	1x	1x
Enterobacter cloace	1x	1x	2x	1x	1x	1x	1x
Escherichia coli	1x	1x	1x	1x	1x	1x	1x
Escherichia coli	1x	1x	1x	1x	1x	2x	1x
Escherichia coli	1x	1x	1x	1x	1x	2x	1x
Pseudomonas aeruginosa	1x	1x	1x	1x	1x	1x	1x
Pseudomonas stutzeri	0.1x	0.1x	0.1x	0.1x	0.1x	0.1x	0.1x
Staphylococcus xylosus	2x	1x	1x	1x	1x	10x	1x

Disinfectants were tested on biofilm grown in vitro for 24 hours in the MBEC™ device
1x, manufacturer's recommended concentration; 2x, twice recommended concentration; 10x, ten times recommended concentration; 0.1x, one tenth recommended concentration.
Abbreviation: NI, no inhibition of growth at the concentration tested.

Chapter Four: **DISCUSSIONS**

4.1 Identification of Bacteria from Poultry Farms and Processing Plants

In this study, a variety of gram-negative and gram-positive environmental bacterial species were isolated from broiler farms, layer farms, hatcheries and processing plants in Alberta. Swab samples and liquid samples were collected from different equipment. Some examples of such equipment were: feed pan, feed auger, drinking nipple, hatch basket, incubator, chick separator, vaccine tube, drains, carcass scald tank, and carcass chill tank. These pieces of equipment were made up of plastic, rubber, stainless steel, metal, or concrete materials. The isolation scheme was targeted for aerobic, non-fastidious and fast growing organisms. Samples were serially diluted and plated. Limited numbers of different colonies were picked from the culture plates. The fast growing bacteria will outcompete the slow growing and more fastidious ones. Therefore, these colonies have a much higher probability of being the ones picked. The Sensititre® system was used to identify the bacterial species in this study.

Among gram-negative bacteria isolated, we observed *Enterobacteriaceae* as the predominant bacterial family (48% of total), which included isolates of the species *E. cloacae*, *E. coli* and *P. agglomerans*. The use of MacConkey agar for plating samples favored the selection of *Enterobacteria* in the environmental samples collected. Gram-negative bacteria were isolated from feed pan and feed auger samples from broiler barns and layer barns; from hatch baskets and chick separators in the hatcheries; and from the scald tank and floor drain in the poultry processing plants. *E. coli* and *E. cloacae* are the normal flora of gastro-intestinal tract of poultry and food animals (90), suggesting that the presence of these bacteria on poultry farm equipment

might be of fecal origin or from contaminated feed (91). The presence of *E. coli* in the carcass scald tank and floor drain of the poultry processing plant suggests that they may have originated from the feces of the processed birds. Bacteria belonging to the *Pseudomonadacae* family was the second most predominant bacterial family observed among gram-negative isolates. These bacteria were most commonly isolated from the water samples, feed pans, feed auger, drinking nipples and the humidifier reservoir. Pseudomonads are naturally present in the environment in soil and water, therefore it was not surprising to see their abundance in the poultry production and processing environments.

Among the gram-positive bacteria isolated from our environmental samples, *S. xylosus* was the predominant species. *S. xylosus* has been commonly isolated from skins of healthy animals and humans, the bio-aerosol of poultry farms and from poultry litter, food surfaces and food contact surfaces (92-94). Consistent with previous studies (93, 95), *S. xylosus* was the predominant gram-positive bacterial species in our samples from broiler barns.

We isolated 11 different bacterial species from drinking nipple and feed pan samples; and seven bacterial species from feed auger samples collected in the broiler barns. The most common bacteria isolated from feed pans were *E. coli* and *S. xylosus*. *S. xylosus* was also commonly isolated from drinking nipple and feed auger samples. Among samples collected from the material surface types, 20 different bacterial species were isolated from swabs of plastic equipment, and nine species from swabs of stainless steel equipment. Very few bacterial species were isolated from rubber and concrete surface swab samples. Most of the plastic equipment sampled was from the broiler and layer barns. Swab samples of stainless steel equipment were

collected from poultry processing plants and broiler hatcheries. The reason so few bacterial species were isolated from stainless steel equipment in the processing plants might be due to the strict sanitation procedures followed during the sanitation shift during the daily processing cycle (96).

It was interesting to note that among the hatchery samples, *E. coli* was isolated from bronchitis vaccine tubing, *B. thuringiensis* from the bronchitis vaccine solution, and *P. stutzeri* from swab samples of the egg incubator (Table 3.1). The presence of such bacteria in hatchery environment suggests that sanitation processes may not have been effective in eliminating those bacteria. Thus, these bacteria may be carried by the chicks or by the transport box from the hatchery to the broiler farms and get disseminated into the flocks (97).

4.2 Antimicrobial Susceptibility

The use of antibiotics in food animals exerts selective pressure on the bacteria resident in and on the animals and in the production environment. As a result, antimicrobial sensitive bacteria are eliminated and resistant bacteria survive and proliferate. The resistant bacteria may create a large reservoir of antimicrobial resistance genes in the environment (98). A number of studies have shown that use of antibiotics in food animals is associated with resistance in bacteria isolated from animals and humans (70, 99). In Denmark, until 1995, avoparcin was used as a growth promoter in poultry and swine (99). Resistance to avoparcin confers cross-resistance to vancomycin drug, which is an important medicine for treating *Enterococcus* infection in humans (13). Acquired or intrinsic vancomycin gene clusters confer vancomycin resistance in Enterococci. For example, *vanA, vanB*, *vanD*, *vanE* and *vanG* are acquired gene clusters whereas

vanC is an intrinsic gene cluster (100). As a precautionary measure in order to reduce the observed reservoir of the antibiotic resistance genes in food animal bacteria, Denmark banned avoparcin use in poultry and pig in 1995 (101). Following the ban, there was a sharp decrease in Vancomycin Resistance *Enterococci* (VRE) in fecal samples of chicken and swine (99). A similar surveillance data in Canada showed that use of ceftiofur in broiler hatcheries was associated with the ceftiofur resistant *S*. Heidelberg isolated from retail chicken and human clinical samples (8).

In our study, we observed a high percentage of gram-positive and gram-negative bacteria resistant to many drug classes in the VDD categories I and II, which are important to human health. We observed that 20-35% of gram-negative bacteria were resistant to the third generation cephalosporins (Table 3.4). Category I drugs are those antibiotics that are used to treat serious bacterial infections in humans (14). Among the category I antibiotics, third generation cephalosporins are one of the top prioritized antibiotics for treating Salmonellosis in children (13). In Canada, ceftiofur, a third generation cephalosporin was used *in ovo* in broiler chicken hatcheries in an extra-label manner until April 2013. Due to the increased concern over antibiotics used in poultry and their associated antibiotic resistant bacteria in humans, chicken farmers voluntarily decided to ban ceftiofur use in hatcheries starting in May 2014 throughout Canada (102). Our samples were collected from the poultry farms and processing facilities before the ban took place. The use of ceftiofur *in ovo* may have imposed selective pressure on the microflora of the chicken, particularly in *E. coli* (97), thus we observed that nine of 14 *E. coli* isolates were resistant to ceftiofur in our study. Enrofloxacin is a fluorinated ciprofloxacin drug whose use has been limited or banned already in Australia, Europe and America in food animals

including poultry (103, 104). In Canada, this drug is not currently used in poultry, which could possibly explain the low frequency of resistance to ciprofloxacin observed in this study. We observed ciprofloxacin resistance only in two isolates of *P. aeruginosa.* This is probably due to the intrinsically present MexB-MexA-OprM efflux system that pumps ciprofloxacin out of the cell cytoplasm (22). Although chloramphenicol use in food animals was banned in 1985 by Health and Welfare Canada (105), resistance was observed in 58% of the gram-negative bacteria including *E. coli*, *E. cloacae* and Pseudomonads. The persistence of the chloramfenicol resistance in the environment might be due to co-selection of its resistance genes such as *cmlA* with other resistance genes such as *aadA1* and *aadA2.* These genes may be located on the same mobile genetic elements such as plasmids and integrons and can transfer horizontally among bacteria (106). The florfenicol resistance gene *floR* has been shown to confer cross-resistance to chloramphenicol (106). Florfenicol, a fluorinated chloramphenicol is used in broiler breeder and turkey for pasteurelosis and mycoplasmosis (15). This might be an additional reason for frequent resistance to chloramphenicol in broiler farms. We did not assay resistance against florfenicol in our study. Mainali *et al*. (69) found that *E. coli* isolates from broiler chickens at the time of slaughter were resistant to ampicillin, sulfonamides, streptomycin and tetracycline, and were also resistant to chloramphenicol (ASSuCT pattern). Similar ASSuCT pattern have been found in foodborne pathogens such as *S.* Typhimurium, suggesting the resistance genes responsible for this resistance pattern may move as a group on a mobile genetic element (8). Ampicillin, sulfonamides, tetracyclines, and aminoglycosides are allowed for use against different gram-positive and gram-negative infections in Canadian livestock and poultry (15). Chlortetracyclines are also used for growth promotion and prophylaxis in broilers (107). Thus the use of ampicillin, sulfonamides, aminoglycosides, and tetracycline in poultry production might have resulted in

selection pressure for developing the ASSuCT multi-drug resistance pattern.

Among the gram-positive bacteria we isolated from our environmental samples, 87% were resistance to erythromycin. Erythromycin, a macrolide class antibiotic, is an important WHO category I drug for treatment of *Campylobacter* infection in children (13). Erythromycin is also used to treat *Staphylococcal* arthritis in broilers and turkeys in Canada. Tylosin, which belongs to the same drug class, is also used in poultry for enteritis, peritonitis and mycoplasmosis (15). In our study, 27 of 28 *S. xylosus* isolates were resistant to erythromycin (Table 3.3). Although we did not assay for resistance against Tylosin, a possible reason for the high level of erythromycin resistance we observed could be the presence of the *ermA* gene in *Staphylococci* that confers resistance to the macrolide group of antibiotics. This *ermA* gene has been reported as the most common macrolide resistance gene in *Staphylococci* of poultry origin (108). Another potential explanation for our result could be due to cross-resistance between tylosin and erythromycin being conferred by the *ermC* gene present on plasmids (109, 110). Since we don't have the antimicrobial used in chickens at farm level, we assumed that ceftiofur or gentamycin could have been injected *in ovo* or into day old broiler chicks for prevention of omphalitis. We observed a high frequency of resistance (30 out of 35 *Stapyhlococci*) to kanamycin among isolates from environmental samples collected in broiler barns. This might be due to the presence of the *aacA-aphD* gene that confers resistance to both kanamycin and gentamycin (111). Penicillin G is used as growth promoter and for therapeutic purposes against gram-positive bacterial infections in broilers (15, 107). This may reflect the frequent resistance to penicillin observed among gram-positive bacteria isolated from samples collected in the broiler barns. The selection pressure may have selected for the β-lactamase genes such as

*bla*TEM and *bla*SHV, which confer resistance to penicillins (35).

Occurrence of multi-drug resistance (MDR) bacteria (resistance ≥3 drug classes) on poultry farms and on poultry meat has been frequently observed in Canada (47, 69). There are two possible reasons for multiple drug resistance to occur: Cross-resistance and co-resistance. Cross-resistance has the potential to occur when different antimicrobial agents attack the same target or initiate a common pathway to cell death, selecting for the resistance mechanism that confers cross resistance (112). Co-resistance occurs when the resistance genes for a particular drug are located with other resistant genes on the same mobile genetic elements such as plasmids, integrons, and transposons. Selection of one resistance gene also selects for resistance of other co-located genes (112). In Canada, about 17 antimicrobial drugs belonging to five different classes (aminoglycosides, beta lactams, macrolides, sulfonamides and tetracyclines) are allowed for therapeutic and prophylactic purposes in broiler chickens (15). It should be noted that antibiotics were not used for therapeutic purpose in any of the chicken flocks present during our sampling period (Dr. Thomas Inglis, personal communication). Despite the fact that there was no use of antibiotics for therapeutic purpose in the farms, we saw a significantly higher frequency (34 of 47 isolates) of MDR gram-positive and gram-negative bacteria isolated from the environment of the broiler barns. This could partly be due to the selection pressure posed by the use of antibiotic growth promoters on the farms (98). Additionally, the resistance genes selected may have been disseminated horizontally, thereby spreading to different bacterial species. We also observed significantly higher frequency of MDR bacteria from swab samples of plastic equipment collected in the broiler farms, layers and hatcheries. In poultry farms, many equipment types such as drinking nipples, feed pans, feed augers, waterlines, vaccine tubes,

hatch baskets, humidifier tanks are made up of plastic materials. Improper sanitation may not have eliminated all the bacteria harboured in or on such equipment. In addition, although the chickens are raised in the 'all in all out' system, improper cleaning and disinfection after the flocks have been sent for slaughter may leave bacteria behind to recolonize. These left over bacteria may be the source of contamination and spread to the oncoming flocks. One example from our study is the isolation of antibiotic resistant *S. xylosus* and *E. coli* from the feed auger in the broiler barn during the down-time period in between the flocks post cleaning and sanitation. Interestingly, this *S. xylosus* was able to form biofilm *in vitro* and was resistant to killing by β-lactams, aminoglycosides and hydrogen peroxide.

4.3 AmpC β-Lactamase Producing *E. coli*

In one ESBL *E. coli* isolate, plasmid mediated *bla*CTXM-1 group genes were not detected by PCR. The *bla*CTX-M family of genes are usually present on mobile genetic elements such as conjugable plasmids and integrons and are transferred horizontally among bacteria. We chose to look for *bla*CTX-M-1 group ESBL genes because they are most commonly found in *E. coli* isolated from food animals including poultry (113). The *bla*CTX-M-1 group genes are also found in *E. coli* isolated from humans suggesting that this gene could have been transferred from *E. coli* of poultry to the *E. coli* of humans (71, 114). The detection of ESBL phenotypes may be due to the presence of other ESBL genes such as *bla*CTX-M-9 group or *bla*TEM-52 that were not tested for in this study (71, 113).

In all *E. coli* with an AmpC phenotype, PCR was performed to amplify the chromosomal *ampC* promoter sequence located upstream of the chromosomal *ampC* gene. The chromosomal

ampC promoter sequence was compared with the chromosomal *ampC* promoter sequence of *E. coli* ATCC 25922. The sequence comparison revealed single nucleotide polymorphisms in the *ampC* promoter sequence. The increased MIC's for cephalosporin β-lactam resistance we observed could be due to: a) mutations in the *ampC* promoter region resulting in increased transcription, b) mutation in the attenuator region thereby destabilizing the hairpin structure so that RNA polymerase can more easily move along the *ampC* coding region to transcribe *ampC* gene (41), c) insertion of insertion elements (ISE) in the promoter leading to the formation of a stronger promoter (42), d) increase in the copy number of chromosomal *ampC* gene and e) expression of porin proteins that pump the antibiotics out of the cell (115). In addition, alteration of nucleotide coding sequence of chromosomal *amp*C gene, or amino acid deletion/insertion in the region coding for the cephalosporinase enzyme potentially results in expanded hydrolysis spectrum of AmpC enzymes to the oxyimino-cephalosporins cefotaxime, ceftriaxone and ceftazidime. Likewise, presence of plasmid mediated *ampC* genes such as *bla*CMY genes, and alteration in the coding sequence of plasmid encoded *ampC* genes results in a wider spectrum of cephlosporinase activity (116). In our study, all AmpC phenotype *E. coli* had mutations in the promoter sequence. In the promoter region, the consensus -35 box (TTGACA), -10 box (TATAAT) and the distance between these two boxes (spacer region) play an important role in the transcription rate of AmpC enzyme production (117). A previous study has shown that mutation at -88, -82, -18, -1, +58 resulted in increased MIC of cefoxitin (=256 µg/mL) and ceftriaxone (=256 µg/mL) in *E. coli* (41). In contrast, our mutation pattern type 1 (two cefoxitin-ceftriaxone resistant and three cefoxitin-ceftriaxone sensitive *E. coli*) had the same nucleotide changes in those positions, with additional mutations at positions -73 and in the *ampC* coding sequence +81 (Table 3.11). In these isolates, mutation at -18 (G→A) also resulted in an alternate -

10 box (TATCGT) (41, 115). It has been shown that formation an alternate promoter (TATCGT) due to transition of G to A at -18 resulted in increased MIC for cefoxitin (256 µg/mL) and ceftriaxone (256 µg/mL) (41). In our study, this same alternate promoter was formed in the promoter region of both cefoxitin sensitive (MIC 4 µg/mL) and ceftriaxone sensitive (MIC ≤0.25 µg/mL) *E. coli.* Therefore, we could not conclude that higher MIC of cefoxitin (>32 µg/mL) and ceftriaxone (16 µg/mL) was due to the formation of an alternate promoter. Two *E. coli* isolates (mutation pattern type 3 and 5) had mutations in the attenuator region. Mutations in this region result in destabilization of hairpin structure thereby increasing the *ampC* transcription rate (41). However, Tracz *et al*. (115) showed that mutation in the attenuator region had just 1-2 fold increase in *ampC* gene expression, which may not account for the increase in MIC we observed. In our study, one *E. coli* (mutation type 3) had mutations at attenuator region at positions +22, +26, +27 and +32. In addition, this *E. coli* isolate had mutations at the *ampC* coding region +70 and +81. The MIC of cefoxitin and ceftriaxone of this *E. coli* was >32 µg/mL and 32 µg/mL respectively. Further investigation would be required to confirm the cause of increased MIC of cefoxitin and ceftriaxone in this isolate. In one isolate, an *ampC* regulatory sequence *amp*R of 876 bp was detected. The *ampR* is a transcriptional regulator located upstream of *ampC* gene that activates *ampC* transcription, and would account for the increased MIC's observed in this isolate (118). The presence of *ampR* is not common in *E. coli*, but has been reported previously (119).

Two of the isolates that harboured plasmid *bla*CMY-2 genes had mutations in the *ampC* promoter region at positions -73, +70 and +81. Another plasmid-*bla*CMY-2 bearing *E. coli* had mutations in the *ampC* promoter region at positions -88, -82, -18, -1, +58. Thus, it can be suggested that the increase in MIC of cefoxitin and ceftriaxone in these plasmid bearing

*bla*CMY-2 *E. coli* may not be due to the mutations in the promoter sequence but could be due to the presence of *bla*CMY-2 genes that produced plasmid AmpC beta-lactamase enzymes.

It is well recognized that plasmids are the most common means of transfer of resistance genes among bacteria. The AmpC enzymes that belong to the CMY family are the most common plasmid mediated AmpC cephalosporinases in the *Enterobacteriacae* and are distributed world wide (37). The *bla*CMY bearing plasmids in *E. coli* are larger in size (>80 kb) and usually belong to IncI1 or IncA/C incompatibility type. IncI1 plasmid type is commonly present in *E. coli* and *Salmonella* Typhimurium from poultry origin and IncA/C in *E. coli* from both poultry and cattle origin (46, 48, 49). IncI1 plasmids have also been characterized by the presence of a type IV pilus that is used by *E. coli* to adhere and invade eukaryotic cells, suggesting that this particular IncI1 type are associated with pathogenic *E. coli* (49). These incompatibility plasmids can be transferred horizontally among different bacteria (46, 48). Zao *et al*. (26) showed that *E. coli* isolated from ground chicken and *S. enterica* from ground beef transferred *bla*CMY-2 plasmids into *E. coli* 0157:H7 by conjugation. Similarly, plasmid mediated co-transfer of *bla*CMY-2 genes with the genes of other non-β-lactam antibiotic class such as aminoglycosides, quinolones, tetracyclines and sulphonamides have been reported in *E. coli* (46, 48). The spread of multi-drug resistant plasmids among foodborne pathogens such as *E. coli* 0157:H7 and *S. enterica* serotypes is of great concern because dissemination of antibiotic resistant foodborne bacteria in humans may compromise the available treatment options. In our study, the *bla*CMY-2 harbouring *E. coli* was phenotypically resistant to fenicols, aminoglycosides, sulfonamides and tetracycline, similar to what has been seen in other studies (44, 45). Further investigations would

be required to confirm if the *bla*CMY-2 plasmid is multidrug resistant plasmid and is transferrable by conjugation.

4.4 Integron Analysis in Gram-negative Bacteria

We tried to determine the contribution of integrons to the multi-drug resistance of our gram-negative bacterial isolates. Integrons are the mobile genetic elements that harbour antibiotic resistance genes and can be transferred horizontally among bacteria (30). Interestingly, we found only eight of 48 gram-negative bacteria with gene cassettes in class 1 and class 2 integrons in our study. In agreement with previous studies (84, 120), the most common bacteria harbouring class 1 integron was *E. coli* and the common gene cassette was *aadA1*, which confers resistance to streptomycin and spectinomycin. Since there was no MIC interpretation criteria for streptomycin available in CLSI guidelines, we could not co-relate the presence of *aadA1* and streptomycin resistance in our study. One *P. stutzeri* harboured a class 1 integron that contained no resistance gene cassettes. This suggests two possibilities: first, cassettes have been excised in absence of antimicrobial selection pressure. In this case, the integron may capture gene cassettes in presence of antimicrobial selection pressure and the bacteria would become phenotypically resistant to the particular antibiotic (121). Second, this bacterium may not have come in contact with other bacteria harbouring class 1 integrons. Only one isolate harboured a class 2 integron, which contained the resistance genes *sat1*, *dfrA12* and *aadA1,* a pattern previously reported (120). Studies have reported that integrons may harbour resistant genes of different drug classes such as quinolones, fenicols and β-lactams (78, 122). We did not detect any resistance gene cassettes that might account for their phenotypic resistance to β-lactams, chloramfenicols,

tetracyclines and sulfonamides. Thus, we hypothesized that these resistant determinants may be present on plasmids or on the bacterial chromosome.

4.5 Biofilm Bacteria Resistance to Antibiotics and Disinfectants

Biofilm formation by commensal and pathogenic bacteria and their resistance against different antibiotics has been previously reported (62, 123). Increased resistance to antibiotic and disinfectants during biofilm formation may be due to up-regulation of genes that make these bacteria phenotypically distinct from their planktonic forms (124). The expressions of genes such as *bap in S. xylosus, alg* in *P. aeruginosa* and *fliC* in *E. coli* help bacteria attach to surfaces, colonize, and produce exopolysaccharides that form the biofilm matrix (125-127). In agreement with a previous study (62), a higher concentration of antimicrobials was required to kill our isolates when growing in biofilms compared to the same bacteria living in planktonic culture. *E. coli* isolates have been shown to form biofilms on polysterene, steel, and glass surfaces and have increased resistance to antimicrobials and disinfectants (62, 128, 129). In our study, *E. coli* living in biofilm was resistant to the same concentration of antibiotics that inhibited their growth in planktonic culture. These antibiotic classes were: β-lactams, aminoglycosides, fenicols and trimethoprim-sulfonamide. It was interesting to note that two of the three *E. coli* that formed biofilm *in vitro* had AmpC phenotype. Biofilm forming ability of AmpC enzyme producing *E. coli* has been previously reported (130). The biofilm matrix may provide additional protection to the AmpC producing *E. coli* from the antibiotics thereby making it difficult to treat infections. In our study, all the three *E. coli* in biofilm were inhibited by peracetic acid- hydrogen peroxide, gluteraldehyde, chlorine, quaternary ammonium compound, iodine and potassium monopersulfate at the manufacturer's recommended concentration and contact time. This

indicates that cleaning and sanitation procedures against susceptible *E. coli* would also be effective against AmpC producing *E. coli*. However, the *E. coli* living in biofilm resistant to hydrogen peroxide at the manufactured recommended concentration (3000 ppm), possibly suggests the production of catalase enzymes could destroy the peroxide molecule (131).

Bacillus spp. are also considered potential foodborne pathogens and are associated with foodborne illness due to production of toxins. These bacteria can adhere and form biofilms on a variety of surfaces such as stainless steel, polystyrene and glass surfaces. Lee *et al*. (132) showed that *B. cereus* biofilm bacteria on a stainless steel surface were eliminated by using 200 ppm chlorine, 100 ppm iodophore, and 400 ppm quaternary ammonium compound at 10 minutes contact time. Ryu and Beuchat (133) suggested that biofilm and spores formation of *B. cereus* on stainless steel coupons were resistant to peroxy-acetic acid sanitizer. Our study showed that *B. subtilis* biofilm grown on MBEC^{TM} peg lids were killed only by penicillin and amoxicillin in our panel of 18 antibiotics, and at the same concentration required to kill the isolate grown in planktonic culture. In addition, gluteraldehyde was the only effective disinfectant to eliminate *B. subtilis* biofilm when used at the manufacturer's recommended concentration. The resistance to the antimicrobials and disinfectants we assayed could be due to the impermeability of the biofilm matrix or the formation of spores (133).

S. xylosus have been shown to attach to and form biofilm on polystyrene and stainless steel surfaces (94, 95). Studies have reported that *S. xylosus* isolated from skin of humans, animals including chicken were resistant to a number of antimicrobials such as: penicillins, tetracyclines, ampicillin, erythromycin, nalidixic acid and novobiocin (94, 95). Marino *et al*. (94)

reported that several *Staphylococcal* species were resistant to quaternary ammonium compounds. In our study, *S. xylosus* isolate from a broiler barn was able to form a biofilm in vitro, and showed higher resistance (increased MIC) to a number of antimicrobial drug classes we tested when compared to when grown in planktonic form, such as β-lactams, aminoglycosides and sulphonamide-trimethoprim (Table 3.12). It was interesting to note that this particular *S. xylosus* was isolated from the broiler barn that was in a down-time period between flocks after cleaning and disinfection. In contrast to the study by Marino *et al*., this particular *S. xylosus* isolate grown in biofilm was sensitive to Quaternary Ammonium Compound (QAC) when used at manufacturer's recommended concentration (2500 ppm), but highly resistant (increased MIC) to hydrogen peroxide and per acetic acid–hydrogen peroxide (Table 3.13). Co-resistance of QACs to antibiotics such as β-lactams, sulfonamides and aminoglycosides has been reported previously (134). Although *S. xylosus* isolate was phenotypically resistant to such antimicrobials, the susceptibility to QAC suggests that *qac* genes may not have been present in this bacterium. The resistance to hydrogen peroxide might be due to the production of catalase enzyme that inactivates the peroxide molecule (135). The ability to live in a resistant biofilm may explain why we were able to isolate this *S. xylosus* from the poultry barn after cleaning and sanitation. The ability to survive cleaning and sanitation means that this isolate will maintain its antimicrobial resistance genes in the poultry barn environment.

The presence of *S. xylosus* in the broiler farm even after the disinfection process indicates that the disinfection used may not have been effective in removing the bacteria from the farm. This particular *S. xylosus* was isolated from a swab of the feed auger. In poultry farms, equipment such as feed pan, feed auger, drinking nipple are not individually cleaned or replaced

until these equipment are non-functional or damaged. Such equipment and surfaces thus create a favourable environment for bacteria to attach and form biofilm. As the biofilm grows over time, it matures and becomes harder to remove by disinfectants (136). Thus, a strict cleaning and disinfectant protocol should be required in the farms and processing plants. One example of this process would be mechanical cleaning (water under pressure) followed by sanitation with disinfectants. A study about the sanitation effectiveness in broiler farms and broiler processing plants found that the total count of *E. coli*, *B. cereus* and *S. aureus* were far lower when vigorous mechanical cleaning was followed by sanitation (137). Extra attention may be required to those equipment and surfaces in poultry farms that are not regularly cleaned and disinfected in order to minimize the risk of maintaining antimicrobial resistant bacteria in the environment. Effective sanitation would help reduce the bacterial contamination in the farm thereby decreasing the incidence of diseases and foodborne bacteria. This would ultimately minimise the use of antibiotics and improve the overall health and welfare of the poultry flocks and improve food safety.

4.6 Limitations of the Study

In this study, enough samples were not collected from all the poultry houses and processing plants. The numbers of samples collected from each of the broiler farms, layer farms hatcheries and processing plants; and from the different surface types were not uniform. The sample processing was targeted to isolate aerobic, fast growing and non-fastidious bacteria. The samples were serially diluted and cultured. Due to the budget and time constraints, all the different colonies were not picked for study. The fast growing bacteria, which may outcompete the slow growing, and more fastidious bacteria were most likely picked from the culture plate.

Therefore, the bacteria isolated from those samples may not be the actual representation of the whole sample sites. Due to the lower sample size, statistical comparison was limited to selected bacteria only. Sensitire® system was used to identify the bacteria in our study. The limitation of this system is that the database may not contain all the bacterial isolated we had collected. This would probably be the reason that 12 of our isolates were not identified. In addition, there were no MIC interpretive criteria for some bacterial species for specific antibiotics in the CLSI guidelines. Therefore, the contribution of such bacteria in maintaining antibiotic resistance in the poultry production environment could not be evaluated. Although in nature, multi-species bacteria form biofilms, we chose to form a single bacterial species to form biofilm in MBEC^{TM} peg lids for accuracy and simplicity and also to generate sufficient biofilm for SEM analysis. This would reduce the time and effort for optimization with regard to inoculum density, conditioning of peg lids, growth medium, sonication and incubation parameters for multi-species biofilm formation (88).

Chapter Five: **CONCLUSIONS AND FUTURE DIRECTIONS**

This study isolated a variety of gram-negative and gram-positive bacterial species from environmental samples collected from broiler barns, layers barns, hatcheries and poultry processing plants in Alberta. These bacteria were isolated from different equipment and surfaces and were resistant to different antimicrobial drug classes such as β-lactams, aminoglycosides, tetracyclines, fenicols and sulphonamides. Multi-drug resistance patterns were observed both in gram-positive and gram-negative bacteria. *Enterobacteriacae* showed resistance to very high important category of drugs (VDD category I). In particular, the *E. coli* was resistant to third generation cephalosporin including cephalosporin-clavulanic acid combination, and to drugs in other classes (MDR). Plasmid mediated *bla*CMY-2 genes were also detected in cephalosporin resistant *E. coli* isolates. This result clearly indicate the risk of transfer of cephalosporin resistant *E. coli* into humans via direct contact or via food chain thereby challenging the treatment outcome of *E. coli* or *Salmonella* infections particularly in children. We also observed that *S. xylosus* had a high frequency of resistance against erythromycin. Erythromycin is classified as category II antibiotic by Veterinary Drug Directorate, but is considered Category I by the World Health Organisation (13, 14). This antibiotic is one of the top prioritized antibiotics in human medicine to treat *Campylobacter* infections in children (13). Transfer of erythromycin resistant bacteria such as *Staphylococci* or *Campylobacte*r from poultry to humans may limit the treatment options available for serious bacterial infections.

This study showed that some of the environmental bacteria were able to form biofilm *in vitro*. Biofilm formation is the survival strategy of bacteria to protect themselves against harsh environmental conditions including antimicrobial pressure. The biofilm formed on the peg lids of the MBEC^{TM} device were resistant to a number of antimicrobials used in poultry production for growth promotion or treatment purposes. The biofilm bacteria were also resistant to commonly used disinfectants in poultry barns and processing plants. Interestingly, *S. xylosus* isolated from the farm that was in a holding period between flocks were able to form biofilm and was resistant to antibiotics and disinfectants, which suggest that cleaning and disinfection may not have been effective to remove the bacteria from the barn. This multi-drug resistant isolate living in a biofilm will serve as a reservoir for antibiotic resistance genes in this environment that may be transferred to poultry pathogens and foodborne bacteria. This finding should alert poultry farmers and poultry processors to use effective disinfection procedures, and to pay extra attention to those equipment and surfaces where bacteria are not eliminated easily.

It was hypothesised that environmental bacteria in poultry farms and processing plants are the reservoirs of antimicrobial resistance determinants. This study clearly indicates the burden of antimicrobial resistance genes in the bacterial population in poultry farms and processing plants in Alberta. Antimicrobials given to food animals either at therapeutic or sub-therapeutic levels provide selective pressure on the gut microbiota. As a result, susceptible bacteria are killed and resistant bacteria survive. The resistant bacteria may be transferred to humans via direct contact or via food chain thereby causing serious foodborne outbreak. The multi-drug resistant bacteria may also transfer their resistant genes horizontally to zoonotic pathogens or human pathogens causing serious infections. In addition, disinfectant resistant

genes that are physically located with other antimicrobial resistant genes are also co-transferred thus challenging cleaning and disinfection process in poultry production and processing facilities. Improper cleaning and sanitation of poultry farms and processing plants may leave resistant bacteria behind to populate and disseminate. Therefore, this study suggests that environmental bacteria present in poultry farms and processing plants in Alberta may constitute an important reservoir of antimicrobial resistant determinants, which could contribute in selection, persistence, and emergence of antimicrobial resistant bacteria in the environment.

Based on our results, we would like to recommend to the poultry farmers and processors that proper cleaning and disinfection protocol is required in order to eliminate bacteria living in biofilm particularly in poultry houses. Extra attention is necessary to those equipment and surfaces where disinfectants are not reached and where bacteria may form biofilms such as feed pans and feed augers. These equipment should individually cleaned and disinfected. Vaccine solutions, vaccines tubes, incubators and hatch baskets should be cleaned and sterile all times. Alternative to antibiotics growth promoters such as probiotics, oligosaccharides, and enzymes may be used for enhancing growth of the poultry birds. Strict biosecurity measures and control of viral diseases is necessary to reduce secondary bacterial infections This would help reduce the need of antibiotics for treatment purposes thereby minimizing the risk of development of antibiotic resistance in poultry production and processing environments.

As a continuation of this study, transformation via electroporation, and conjugation studies of the MDR AmpC phenotype *E. coli* should be performed to determine the contribution

of multi-drug resistant plasmids in the dissemination of antimicrobial resistant determinants in the poultry production and processing environment.

Plasmid replicon typing should be performed in the AmpC phenotype *E. coli* so as to determine whether the *bla*CMY-2 gene bearing plasmids and the other plasmids identified in the isolates belong to specific compatibility types (IncA/C or IncI1 or any others) in order to support previous studies that IncA/C or IncI1 MDR plasmids are circulating in the poultry associated bacteria and are responsible for spread of cephalosporin resistance genes.

As a further study, detection of mutations in the chromosomal *ampC* gene, or detection of plasmid mediated AmpC gene families other than *bla*CMY-2 in *E. coli* could be performed. This would help to determine the possible cause of AmpC enzyme production.

Virulence typing and serotyping of *E. coli* could be performed to see if the bacteria are poultry pathogens (avian pathogenic *E. coli*), commensal microbiota, or potential foodborne pathogens such as *E. coli* 0157:H7. This would help to determine which kind of *E. coli* are important in maintaining antimicrobial resistance genes in poultry production and processing facilities.

In vitro transfer of resistance genes in the biofilm bacteria could also be performed. For instance, antibiotic resistant biofilm producing bacteria could be conjugated to biofilm bacteria, which are sensitive to antibiotics. This study would help determine the role of biofilm bacteria in dissemination of antimicrobial resistance genes among the bacteria in the biofilm matrix.

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1.

Appendix 1. Sampling Procedure and Sample Recovery

Sampling Procedure

1. Take a sterile swab out the swab bag and moisten it with Phosphate Buffered Saline if the swab is dry.
2. Take care not to touch the tip of the swab or the swab bag.
3. Then swab 100 cm^2 surface area (10 cm x 10 cm) in three directions: up/down; left/right; and diagonally, covering as much surface as possible.
4. Put the swab back into the swab bag and label it with sample code and date and place of collection
5. Collect the swab sample in each swab bag.
6. Keep the swabs cool and avoid freezing at any time, including during shipping.
7. Swab irregular surface in the same manner each time to allow for accurate comparisons.
8. Take a picture of the sampling area with labels and scale bars in place.
9. Send the samples to the laboratory as soon as the collection is finished.
10. For collecting liquid sample, aseptically take the liquid sample in a sterile 50 mL collection tube.
11. Label the tube with sample code, date and place of collection.
12. Keep the liquid sample cool and avoid freezing at any time, including during shipping.
13. Follow the steps 8 and 9.

Recovery of Swab Sample

1. Aseptically trim each of the swab and place into a sterile vial containing 500 µL of sterile neutralizer.
2. Sonicate the swab-containing vial for 30 minutes at 42 Hz in a VWR B2500A-MT Ultrasonic cleaner (VWR International. Edmonton, AB).
3. Following sonication, place 180 µL of 0.9% sterile saline on the 96 well microtitre plate (Nuclon Delta Surface, Thermo Fisher Scientific. Roskilde, Denmark) starting from the second row wells.
4. Then place 100 µL of each sample into the first 12 empty wells of the first row.
5. Pipette out 20 µL from the well of the first row and place into the corresponding wells of second row.
6. Pipette in an out in the wells to get uniform dilution in the wells.
7. Then from the wells of second row, pipette out and 20 µL and transfer into wells of third row.
8. Pipette in and out in the wells to get uniform dilution in the wells.
9. Transfer 20 µL from the wells of third row to fourth row.
10. Continue transferring 20 µL further down the wells of remaining rows.
11. In this way, a serial dilution of 10^{-0} to 10^{-7} is prepared.
12. From each well, take 20 µL and spot plate on Tryptic Soy Agar, MacConkey Agar and Manitol Salt Agar plate.
13. Incubate the plates at 36^{0}C for 24 hours.
14. Pick individual colony from the plate and sub-culture on the plates from which they were originally isolated.

15. Incubate the plates at 36^0C for 24 hours.
16. Take one or two colonies for gram staining purpose.
17. Take few colonies to inoculate the cryo-preservative in the cryogenic bead stock (Prolab Diagnostics ON. Canada) following manufacturer's instructions.
18. For liquid sample, transfer the liquid sample into a sterile container.
19. Place 180 µL of 0.9% sterile saline on the 96 well microtitre plate (Nuclon Delta Surface, Thermo Fisher Scientific. Roskilde, Denmark) starting from the second row wells.
20. Then transfer 200 µL of the liquid sample into the empty wells of the first row.
21. Transfer 20µL from the first row and make serial dilution of 10^{-0} to 10^{-7} as explained above.
22. Follow the steps 12-17.

Appendix 2. Preparation of Culture Media

Media used: Brain Heart Infusion (BHI) Broth (Becton Dickson and Company, MD, USA), Lauria-Bertani (LB) Broth (Becton Dickson and Company, MD, USA), Mueller Hinton Agar (Himedia Laboratores Pvt Ltd, Mumbai, India)

1. Calculate the amount of media required for making working stock. Per liter of distilled water, use MHA=38 gm, LB=20 gm and BHI=37 gm.
2. Mix the media powder with distilled water and boil in a conical flask on a magnetic stirrer heat plate.
3. After the clear solution is observed, autoclave at 121^0C and 17 psi for 40 minutes and allow cooling at room temperature.
4. Pour one to three mL media on sterile tubes and store at 4^0C.
5. For making Mueller Hinton Plate, after autoclaving, cool the media to approximately 65^0C.
6. Pour 25 mL on 100 mm plates and 60 mL on a 150 mm plates.
7. Allow the agar to solidify at room temperature for 2-3 hours and store at 4^0C.

Appendix 3. Preparation of Culture

1. Take the frozen stock of isolates in beads at -80^{0}C thaw on ice.
2. Take out one bead and place in one mL BHI broth.
3. Shake the tube to dislodge bacteria from the bead and take the bead out.
4. Incubate the culture in a standard aerobic incubator at 36^{0}C for 24 hours.
5. With a 10 µL sterile loop, streak the culture on to a MacConkey Agar Plate (Becton Dickson and Company, MD, USA) for gram negative isolates and TSA (Teknova, CA, USA) for gram positive isolates and incubate at 36^{0}C for 24 hours.
6. Take a single colony from the culture plate and sub-streak on to Columbia Agar Plate with 5% sheep blood (Becton Dickson and Company, MD, USA) and incubate as described above.
7. Use the fresh culture for subsequent experimental protocols.

Appendix 4. Bacterial Identification by Sensititre® Method

1. Suspend one to two colonies from an overnight blood culture plate on five mL Sensititre® demineralized water.
2. Adjust the suspension to 0.5 McFarland standard using Sensititre® Nephlometer.
3. Replace the lid of the tube with Sensititre® Dosing Head.
4. Take out the Sensititre® plates: GPID (for gram positive) and GNID (for gram negative) and place on the plate holder of Sensititre® Auto-Inoculator with its barcode facing outside.
5. Place the bacterial suspension in the tube inverted in the tube holder of the Sensititre® Auto-Inoculator and fix it properly.
6. Load 50 µL of the suspension in the first 32 wells by Sensititre® auto-inoculator.
7. Replace the tube with another isolate in the Sensititre® Auto-Inoculator and load the sample for the next 32 wells.
8. Load the last 32 wells well by third isolate.
9. All together, three isolates can be inoculated on a single plate.
10. Place mineral oil on wells: A1, A2, A5, A6 and A9, A10, covered with adhesive seal and incubated for 36^0C for 24 hours.
11. Read the plate with the Sensititre® Auto-reader and record the isolates that are identified.
12. Repeat the experiment once if the isolate cannot be identified.
13. Use the following control isolates:

E. coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 1015, *Kleibsiella pneumonia* ATCC 700603, *Staphylococcu aureus* ATCC 29213, *Enterococcus fecalis* ATCC 29212.

Appendix 5. Antimicrobial Sensitivity Testing by Sensititre® Method

1. Make 0.5 McFarland standard of the bacterial suspension as explained in appendix 5.
2. Transfer 10 µL to 11 mL Sensititre® Cation Adjusted Mueller Hinton Broth (CAMHB). For *Staphylococcus* spp., transfer 30 µL of the suspension.
3. Replace the lid of the CAMHB tube with Sensititre® Dosing Head and place on the tube holder of Sensititre® Auto-inoculator as described inappendix 5.
4. Load 50 µL of the suspension on all the wells of Sensititre® plate: CMV2AGNF (for gram negative isolates) and CMV3GPF (for gram positive isolates).
5. Seal the plate and incubate at 36^0C for 24 hours.
6. Read the plate with the Sensititre® Auto-reader and record the MIC values.
7. Use the following isolates as control.
8. *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 1015, *Kleibsiella pneumonia* ATCC 700603, *Staphylococcu aureus* ATCC 29213, *Enterococcus fecalis* ATCC 29212

Appendix 6. Sensititre Custom Plate Format for Gram-negative and Gram-positive Organisms

a) gram-negative plate, b) gram-positive plate

SENSITITRE CUSTOM PLATE FORMAT

2303101525

Plate Code: CMV2AGNF Date: 26-Oct-10

	1	2	3	4	5	6	7	8	9	10	11	12
A	FOX 32	AZI 8	CHL 16	AXO 64	AXO 0.25	CIP 2	GEN 16	NAL 16	XNL 2	FIS 32	KAN 64	AMP 2
B	FOX 16	AZI 4	CHL 8	AXO 32	AUG2 32/16	CIP 1	GEN 8	NAL 8	XNL 1	FIS 16	KAN 32	AMP 1
C	FOX 8	AZI 2	CHL 4	AXO 16	AUG2 16/8	CIP 0.5	GEN 4	NAL 4	XNL 0.5	SXT 4/76	KAN 16	STR 64
D	FOX 4	AZI 1	CHL 2	AXO 8	AUG2 8/4	CIP 0.25	GEN 2	NAL 2	XNL 0.25	SXT 2/38	KAN 8	STR 32
E	FOX 2	AZI 0.5	TET 32	AXO 4	AUG2 4/2	CIP 0.12	GEN 1	NAL 1	XNL 0.12	SXT 1/19	AMP 32	NEG
F	FOX 1	AZI 0.25	TET 16	AXO 2	AUG2 2/1	CIP 0.06	GEN 0.5	NAL 0.5	FIS 256	SXT 0.5/9.5	AMP 16	POS
G	FOX 0.5	AZI 0.12	TET 8	AXO 1	AUG2 1/0.5	CIP 0.03	GEN 0.25	XNL 8	FIS 128	SXT 0.25/4.75	AMP 8	POS
H	AZI 16	CHL 32	TET 4	AXO 0.5	CIP 4	CIP 0.015	NAL 32	XNL 4	FIS 64	SXT 0.12/2.38	AMP 4	POS

ANTIMICROBICS

FOX	Cefoxitin
AZI	Azithromycin
CHL	Chloramphenicol
TET	Tetracycline
AXO	Ceftriaxone
AUG2	Amoxicillin / clavulanic acid 2:1 ratio
CIP	Ciprofloxacin
GEN	Gentamicin
NAL	Nalidixic Acid
XNL	Ceftiofur
FIS	Sulfisoxazole
SXT	Trimethoprim / sulfamethoxazole
KAN	Kanamycin
AMP	Ampicillin
STR	Streptomycin
NEG	Negative Control
POS	Positive Control

PLATE TYPE:	MIC
REQUIRED FOR (system):	SWIN
READ METHOD:	ARIS/AUTOREAD/VIZION/SENSITOUCH/MANUAL
PRODUCT LABELLING - ORGANISM:	Susceptibility testing of veterinary isolates
SUBSTRATE IN WELLS:	GRAM NEGATIVE
RECONSTITUTION VOLUME:	50µl
INOCULUM CONCENTRATION:	1 x 10^5 cfu/ml
PRODUCT LABELLING - USE:	FOR VETERINARY USE ONLY
LABEL COLOUR:	RED
SUPPLY FORMAT PADS:	N/A
PLATE EXPIRY:	12 Months
PACK INSERT:	013 - VET
ORGANISMS TO BE TESTED IN THE PLATE:	E. Coli, Shigella, Salmonella spp.
MISCELLANEOUS:	NARMS - INVENTORY

a)

SENSITITRE CUSTOM PLATE FORMAT

0409081420

Plate Code: **CMV3AGPF**

	1	2	3	4	5	6	7	8	9	10	11	12
A	TGC 0.015	TGC 0.03	TGC 0.06	TGC 0.12	TGC 0.25	TGC 0.5	ERY 0.25	ERY 0.5	ERY 1	ERY 2	ERY 4	ERY 8
B	TET 1	TET 2	TET 4	TET 8	TET 16	TET 32	CIP 0.12	CIP 0.25	CIP 0.5	CIP 1	CIP 2	CIP 4
C	CHL 2	CHL 4	CHL 8	CHL 16	CHL 32	PEN 0.25	PEN 0.5	PEN 1	PEN 2	PEN 4	PEN 8	PEN 16
D	DAP 0.25	DAP 0.5	DAP 1	DAP 2	DAP 4	DAP 8	DAP 16	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4
E	STR 512	STR 1024	STR 2048	NIT 2	NIT 4	NIT 8	NIT 16	NIT 32	NIT 64	VAN 8	VAN 16	VAN 32
F	TYLT 0.25	TYLT 0.5	TYLT 1	TYLT 2	TYLT 4	TYLT 8	TYLT 16	TYLT 32	GEN 128	GEN 256	GEN 512	GEN 1024
G	SYN 0.5	SYN 1	SYN 2	SYN 4	SYN 8	SYN 16	SYN 32	LIN 1	LIN 2	LIN 4	LIN 8	NEG
H	LZD 0.5	LZD 1	LZD 2	LZD 4	LZD 8	KAN 128	KAN 256	KAN 512	KAN 1024	POS	POS	POS

ANTIMICROBICS

TGC	Tigecycline
TET	Tetracycline
CHL	Chloramphenicol
DAP	Daptomycin
STR	Streptomycin
TYLT	Tylosin tartrate
SYN	Quinupristin / dalfopristin
LZD	Linezolid
NIT	Nitrofurantoin
PEN	Penicillin
KAN	Kanamycin
ERY	Erythromycin
CIP	Ciprofloxacin
VAN	Vancomycin
LIN	Lincomycin
GEN	Gentamicin
POS	Positive Control
NEG	Negative Control

PLATE TYPE:	MIC
REQUIRED FOR (system):	SWIN
READ METHOD:	ARIS/AUTOREAD/VIZION/SENSITOUCH/MANUAL
PRODUCT LABELLING - ORGANISM:	Susceptibility testing of veterinary isolates
SUBSTRATE IN WELLS:	GRAM POSITIVE
RECONSTITUTION VOLUME:	50µl
INOCULUM CONCENTRATION:	1 x 10^5 cfu/ml
PRODUCT LABELLING - USE:	FOR VETERINARY USE ONLY
LABEL COLOUR:	BLUE
SUPPLY FORMAT PADS:	N/A
PLATE EXPIRY:	24 Months
PACK INSERT:	013 - VET
ORGANISMS TO BE TESTED IN THE PLATE:	GRAM POSITIVE
MISCELLANEOUS:	

b)

Appendix 7. Antimicrobial Sensitivity Testing by Disc Diffusion Assay

Screening of ESBL producing *E. coli* phenotype

Follow the instructions according to Clinical and Laboratory Standard Institute guidelines (82)

1. Make 0.5 McFarland standard of the bacterial suspension as explained in appendix 5.
2. Dip a cotton swab into the inoculum tube and spread onto the Mueller Hinton Agar surface by rotating the plate four times in order to have uniform distribution of the inoculum.
3. Place antimicrobial discs on the agar surface one at a time using a sterile forcep.
4. Sterilize the forcep by dipping in 70% alcohol and igniting in Bunsen burner before placing each new disc.
5. Place 8 antimicrobial discs on a 150 mm plate to maintain at least 24 mm centre to centre between the discs.
6. Incubate at 36^0C for 18 hours.
7. Record the inhibition zone diameter in mm and interpret the result as sensitive, intermediate, or resistant to that particular drug according to CLSI guidelines (82).
8. *Use E. coli* ATCC 25922 as control.

Screening of AmpC *E. coli* phenotype

1. Make 0.5 McFarland standard of the bacterial suspension as explained in appendix 5 and spread the inoculum on MHA plate as in described above.
2. Place Mast D69C: DiscA, DiscB and DiscC (Mast Group Ltd, UK) on the agar surface with a sterile forcep and incubate for 18 hours at 36^0C.

3. Record the difference in inhibition zone diameter between discC and discA; discC and discB.
4. Interpret according to manufacture's guidelines: Zc-Za, Zc-Zb= ≥5 mm, AmpC positive, ≤3 mm, AmpC negative
5. Use *E. coli* ATCC 25922 as control.

Screening of *mec*A mediated methicillin resistant *Staphylococcus* spp.

1. Use cefoxitin disc (30µg) screen for *mec*A mediated methicillin resistant *Staphylococcus* spp.
2. Follow the procedures as described above.
3. Incubate the plate at 35^0C for 18 hours.
4. Record the inhibition zone diameter and interpret the result as follows according to CLSI guidelines.

   Stahpylococcus aureus: ≤ 21 mm: *mec*A positive; ≥22 mm: *mec*A negative

   Coagulase Negative Staphylococci: ≤ 24 mm: *mec*A positive; ≥25 mm: *mec*A negative
5. Use *Staphylococcus aureus* ATCC 29213, and ATCC 25923 as controls.

Appendix 8. Antimicrobial Discs and Their Amount Used in This Study

Antimicrobial Discs	Amount (µg)	Manufacturer
Ampicillin	10	Oxoid Company, ON, Canada
Amoxycillin	10	Oxoid Company, ON, Canada
Amoxycillin-Clavulanic Acid	20/10	Oxoid Company, ON, Canada
Cefoxitin	30	Oxoid Company, ON, Canada
Ceftriaxone	30	Oxoid Company, ON, Canada
Ceftiofur	30	Becton, Dickinson and Company, MD, USA
Cefotaxime	30	Oxoid Company, ON, Canada
Ceftazidime	30	Oxoid Company, ON, Canada
Cefotaxime-Clavulanic acid	30/10	Becton, Dickinson and Company, MD, USA
Ceftazidime-Clavulanic acid	30/10	Becton, Dickinson and Company, MD, USA
Imipenem	10	Oxoid Company, ON, Canada
Mast D69C		Mast Group Ltd, UK

Appendix 9. Polymerase Chain Reaction

Genomic DNA isolation

1. Take one mL overnight broth culture of bacterial isolate in a two mL microcentrifuge tube.
2. Centrifuge at highest speed (14,800 rpm) for two minutes.
3. Remove the supernatant carefully and mix the bacterial pellet with 100 µL of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems Inc. ON, Canada)
4. Vortex the sample for approximately 30 seconds.
5. Place the microcentrifuge tube on a heat block and boil at 100^0C for 10 minutes.
6. Allow the sample to cool for two minutes and centrifuge at highest speed (14,800 rpm) for two minutes.
7. Collect the supernatant containing DNA and store at 4^0C for PCR experiments.

Plasmid DNA Isolation

1. Suspend a single colony of *E. coli* from overnight culture in a three mL LB broth and incubate at 36^0C for 8 hours at 250 rpm in an orbital shaker.
2. Transfer five µL of the broth culture to three mL LB broth and incubate at 36^0C for 16 hours at 250 rpm in an orbital shaker.
3. Use Qiagen Plasmid Mini kit (Qiagen Inc Toronto, ON) to extract plasmid DNA from the broth culture.
4. Centrifuge the overnight broth culture at 6000xg for 15 minutes at 4^0C and discard the supernatant carefully.

5. Re-suspend the bacterial pellet in 300 µL of re-suspension buffer P1 containing RNase and vortex vigorously.
6. Add 300 µL precipitation buffer P2 and mix well by inverting 4-5 times; incubate at room temperature for 5 minutes.
7. Add 300 µL of pre-chilled neutralization buffer P3 and mixed well by inverting 4-5 times and incubate on ice for 5 minutes.
8. Centrifuge at highest speed (14,800 rpm) for 10 minutes.
9. Place the Qiagen® Tip-20 QIArack and equilibrate by applying one mL buffer QBT.
10. Apply the supernatant from step eight to Qiagen® Tip-20 and allow passing through the resin by gravity flow.
11. Wash the Qiagen® Tip-20 twice with two mL wash buffer QC.
12. Add 800 µl of elution buffer QF in the Qiagen® Tip-20 and collect the eluate in a two mL microcentrifuge tube.
13. Precipitate eluted DNA by adding 560 µL of molecular biology grade 100% isopropanol and centrifuge at 14,800 rpm for 30 minutes.
14. Remove the supernatant carefully and wash the pellet DNA one ml 70% ethanol.
15. Centrifuge at 10,000 rpm for 10 minutes.
16. Remove the supernatant carefully and allow the pellet air dry for five minutes.
17. Re-dissolve the plasmid DNA pellet in 40 µL of TE buffer and store at 4^0C.

PCR reaction set up

Reaction kit used: Taq polymerase PCR kit (Qiagen Inc, Toronto ON)

1. Calculate the amount of reagents required for 22 µL (including 10% error rate) for each reaction volume in the master mix using following formula.

   C1xV1=C2xV2

where, C1=initial concentration, V1=initial volume, C2=final concentration, V2= final volume.

The mastermix composed of following components for seven PCR reactions.

Table: Composition of PCR mastermix calculated for PCR reactions.

Components	Final concentration	Final volume
10x PCR buffer	1x	15.4µl
dNTP mix	200 µM of each dNTP	3.1µl
Primer-Forward	0.4 µM	6.1 µl
Primer-Reverse	0.4 µM	6.1 µl
Taq polymerase	0.5 units/reaction	0.5 µl
*Template	≅ 50 ng	1 µl
Nuclease Free Water	Variable	

Note: *Plasmid DNA ≅ 10 ng

3. Aliquot the mastermix equally into seven PCR tubes.
4. In each tube, add one µL of template DNA. Do not add template in No Template Control tube.
5. Add nuclease free water in each tube to make final volume of 22 µL.
6. Run the PCR on a Veriti® 96 well thermal cycler (Applied Biosystems Inc, ON).

Note:

1. Optimize PCR reactions for annealing temperature of the primer and concentration of the template.
2. Dilute the DNA template to 1:10, 1:20, 1:50 and 1:100 in nuclease free water.
3. Take the template dilution that showed a clear visible band on gel electrophoresis as standard template concentration for PCR.
4. Take the temperature at which PCR product show clear visible band on gel electrophoresis as annealing temperature of the primers.

Table: PCR parameters for amplifying class1 and class2 integrons

Initial denaturation	94^0C	3 min
3 steps cycling		
Denaturation	94^0C	1 min
Annealing	56^0C	1 min
Extension	72^0C	5 min
No of cycles - 30		
Final Extension		10 min

Table: PCR parameters for amplifying *ampC* promoter in *E. coli*

Initial denaturation	94^0C	1.5 min
3 steps cycling		
Denaturation	94^0C	1.5 min
Annealing	57^0C	30 sec
Extension	72^0C	1 min
No of cycles - 30		
Final Extension		10 min

Table: PCR parameters for amplifying *bla*CMY-2 and *bla*CTX-M-1 group in *E. coli*

Initial denaturation	94^0C	3 min
3 steps cycling		
Denaturation	94^0C	1 min
Annealing	58^0C	1 min
Extension	72^0C	1 min
No of cycles - 30		
Final Extension		10 min

Appendix 10. Agarose Gel Electrophoresis

Preparation for making Agarose Gel

Calculate the amount of agarose powder to make 0.7% to 1.2% agarose gel.

1. Weigh the required amount of Gel Pilot® LE Agarose powder (Qiagen Inc, Toronto ON) and boil in 1xTAE buffer in a microwave.
2. Allow the solution to cool for approximately 65^0C.
3. Add two µL ethidium bromide (0.5µg/ml) per 100 mL gel and swirl well.
4. Pour the agarose solution into the gel-casting tray with appropriate combs.
5. Mix 1.25 µL GelPilot® DNA Loading Dye 5x (Qiagen Inc, Toronto, ON) to five µL of PCR product.
6. Once the gel gets solidified, take the comb out and transfer the gel into the gel tank.
7. Pour 1xTAE running buffer gel so that the gel is immersed into the buffer by 3-5 mm.
8. Pipette each PCR product mixed with loading dye into each of the wells. Use appropriate DNA ladder into the first well.
9. Plug in the cords. Make sure the blue cord is in negative end and red cord is in positive end.
10. Make sure that the wells lie towards the negative end of the gel.

Table. Agarose %, voltage, time period applied and DNA ladder used in the study.

Target	Agarose %	Voltage	Time	DNA ladder
Integrons (Class 1and Class2)	0.7	60 v	2 hours	Mid range ladder (Qiagen Inc Toronto, ON)
*amp*C promoter	1%	100 v	30 min	100 bp ladder (Qiagen Inc Toronto, ON)
*bla*CMY-2	1%	100 v	30 min	Mid range ladder (Qiagen Inc Toronto, ON)
*bla*CTX-M-1 group	1%	100 v	30 min	Mid range ladder (Qiagen Inc Toronto, ON)
Plasmid profiling	0.7 %	60 v	2 hours	1kb plus ladder (Qiagen Inc Toronto, ON)

Note: bp, base pair

Appendix 11. Purification of PCR Amplicons

QIAquick® PCR purification kit (Qiagen Inc, Toronto, ON) was used to purify the PCR amplicons

Procedure

1. Add five volumes of binding buffer PB to one volume of PCR product.
2. If the colour of the mixture is violet or orange, add 10 µL of 3M sodium acetate to make the solution yellow so as to maintain the required pH of ≤7.0
3. Apply the solution to t QIAquick® Column and centrifuge at 14,800 rpm for one minute.
4. Discard the flow through and wash the column with 750 µL wash buffer PE.
5. Centrifuge at 14,800 rpm for one minute.
6. Discard the flow through and centrifuge again to remove residual wash buffer.
7. Elute the purified PCR product in the column with 40 µL TE buffer in a 1.5 mL collection tube by centrifuging at 14,800 for one minute.

Appendix 12. Plasmid Gel extraction

QIAEX® II gel extraction kit was used to extract plasmid from agarose gel.

Procedure

1. Weigh an empty 2.0 mL microcentrifuge tube.
2. Place the agarose gel on a UV trans-illuminator and cut the visible bands with a scalpel.
3. Place the gel slice in the microcentrifuge tube and calculate the weight of the gel slice by subtracting the weight of the empty tube from the weight of the tube with the gel.
4. Per volume (or gram) of gel slice, add buffer QX1 as follows. This buffer solubilizes agarose gel and helps bind DNA to QIAEX II silica particles.

DNA fragments	<100bp	6 volumes
	100bp-4kb	3 volumes
	<4kb	3 volumes of buffer and 2 volumes of water

5. Re-suspend the binding solution QIAEX II by vortexing for 30 seconds and pipette 10 µL in the gel slice tube.
6. Incubate the tube in water-bath at 50^0C for 10 min. In every two minutes, vortex the tube well so as to keep in suspension.
7. Centrifuge at 13,000 rpm for one min and remove the supernatant carefully.
8. Wash the pellet with 500 µL high salt wash buffer QX1; vortex well and centrifuge as described above to remove the residual agarose.
9. Remove the supernatant carefully.
10. Add 500 µL wash buffer PE to the pellet, vortex well and centrifuge as above.
11. Remove the supernatant carefully. This buffer containing ethanol removes the salt

contaminants in the tube.

12. Air dry the pellet until it becomes white.
13. Elute the DNA by adding 20 µL of TE buffer (pH 8.5), vortex well and incubate as follows.

DNA fragments	≤ 4kb	5 min at room temperature
	4-10kb	5 min at 50^0C
	>10kb	10 min at 50

14. Centrifuge the tube and collect supernatant in a 1.5 mL microcentrifuge tube.
15. Step 12, 13 and 14 can be repeated to increase the yield. Combine the eluates.

Appendix 13. Nucleotide Sequencing

Prepare the PCR product for sequencing according to the instructions given by University of Calgary DNA sequencing facility as follows:

DNA template required: 50-100 ng per kb PCR product

Primer required: 3.2 pmole

Final volume: 12 µL

1. Take two separate PCR tubes for each forward and reverse primer for a PCR amplicon.
2. Add PCR template in each tube. Add forward primer in one tube and reverse primer in another tube.
3. Add nuclease free water to make final volume of 12 µL and send to the University of Calgary DNA sequencing facility for sequencing.

Appendix 14. Procedure for Minimum Biofilm Eradication Concentration Assay

Preparation of Universal Neutralizer

Chemicals required: L-Histidine - 1 gm, L-Cysteine -1 gm, Reduced glutathione - 2.0 gm

1. Mix and make upto 20 mL in double distilled water.
2. Pass through a syringe with a 0.20 µm filter to sterilize.

Preparation of inoculum

1. From the cryogenic bead stock at -80^{0}C, prepare a first culture on TSA and incubate at 36^{0}C for 24 hours.
2. From the first sub-culture, prepare a second sub-culture on TSA and incubate at 36^{0}C for 24 hours.
3. Aseptically, pick an isolated colony and inoculate on 100 mL TSB
4. Place the culture in a humidified incubator (approx. 95%) on an orbital shaker at 110 rpm and incubate at 36^{0}C for 24 hours.
5. Pipette out 100 µL and transfer to 100 mL TSB and adjust the inoculum to an approximate cell density of 10^5 CFU/mL.
6. For inoculum check, pipette out 100 µL of the diluted organism, serially dilute (10^0-10^{-7}) and spot plate on TSA.
7. Pipette out 150 µL of the culture and transfer it into the wells of NuncTM 96-Well Polysterene MicroWell^{TM} plate.
8. Place the lid of MBEC^{TM} device over the 96 well plate containing the organism.

9. Place the plate in a humidified incubator (approx. 95%) on an orbital shaker at 110 rpm and incubate at 36^0C for 24 hours.

Growth Control and Sterility Control

1. Following incubation, break the pegs corresponding to sterility control wells and growth control wells.
2. Place each peg into 200 µL neutralizer solution.
3. Sonicate the pegs using VWR B2500A-MT Ultrasonic cleaner (VWR International. Edmonton, AB) at 42 Hz. for 30 minutes.
4. Serially dilute to 10^0-10^{-7} and spot plate on TSA.

Preparation of Antimicrobial Susceptibility Plate

1. Open the Sensititre® AVIAN1F panel (Trek Diagnostic Systems, OH. USA) one per organism of interest.
2. Reconstitute the antimicrobials in the well by adding 50 µL Cation Adjusted Mueller Hinton Broth (CAHMB) according to manufacturer's recommendation.
3. Prepare a working stock solution of antibiotics in a growth medium: CAHMB
4. Pipette out 200 µL of the working solution in the appropriate wells of the susceptibility plate.
5. Add sterile CAHMB to the growth control and sterility control wells.

Determination of MIC and MBEC

1. Prepare a rinse plate by pipetting 200 µL of PBS on a 96 well plate.
2. Place the biofilm grown peg lids over the wells for 1-2 minutes to release the loosely attached planktonic cells.
3. Then, place the lid over the susceptibility plate and incubate at 36^0C for 24 hours.
4. Following incubation, remove the peg lids from the susceptibility plate and place it on the rinse plate for 1 min.
5. Check visually the turbidity on the susceptibility plate. The wells showing the turbidity indicates bacterial growth while the clear wells indicated growth inhibition. This is the minimum inhibitory concentration (MIC).
6. Discard the susceptibility plate after recording MIC.
7. For MBEC, remove the peg lid from the rinse plate and place over the recovery plate that contains 200 µL recovery medium (CAHMB) with neutralizer.
8. Sonicate the pegs using pegs using VWR B2500A-MT Ultrasonic cleaner (VWR International. Edmonton, AB) at 42 Hz. for 30 minutes.
9. Then incubate the recovery plate at 36^0C for 24 hours.
10. Next, Check visually the turbidity on the wells. Clear wells indicate biofilm eradication. This is the Minimum Biofilm Eradication Concentration.

Disinfectant Assay

Preparation of Disinfectant susceptibility Plate

1. Prepare a 10x stock solution of disinfectants in sterile hard water.
2. Take out a microtitre plate for one organism of interest per disinfectant to be tested.

3. Place 200 µL of the 10X disinfectant solution on the wells of 2nd and 3rd column. These serve as a 10 X concentration test. Exclude the wells of row H.
4. Place 160 µL of 0.9% neutralizer solution on the wells of 4 and 5 and add 40 µL of 10X stock solution. This serves as 2X concentration test. Exclude the wells of row H.
5. Place 180 µL of 0.9% neutralizer solution on the wells of 6 and 7 and add 20 µL of 10X stock solution. This serves as recommended concentration test. Exclude the wells of row H.
6. Place 198 µL of 0.9% neutralizer solution on the wells of 8 and 9 and add 2 µL of 10X stock solution. This serves as 1/10th concentration test. Exclude the wells of row H.
7. Place 200 µL of TSB on the well H1 and H2. This serves as the device sterility control.
8. Place 200 µL sterile neutralizer on the wells of H4, H5, H6 and H7. This serve as neutralizer toxicity control.
9. Place 100µL of sterile neutralizer on the wells or column 1 (excluding the well of row H) and add 100 µL disinfectant stock solution. This serve as neutralizer function test.
10. Place 200 µL TSB on well H8 and H9. This serves as biofilm growth control.

Determination of MBEC

1. Place the overnight grown biofilm on peglids over the disinfectant susceptibility plate as described above.
2. Incubate the plate at room temperature (21^0C) for 10 minutes.
3. Then, transfer the pegs to a neutralizer plate, then to a recovery plate and sonicate for 30 minutes to release biofilm bacteria from the pegs.
4. Incubate the pegs at 36^0C for 24 hours in humidified incubator on an orbital shaker.

5. Visually check for the turbidity in the wells indicating +/- growth.

Sample Preparation for Scanning Electron Microscopy

1. Break the pegs from the lid of MBEC^{TM} device with the help of pliers.
2. Rinse the pegs once with 0.9% Phosphate Buffered Saline to release loosely attached planktonic cells.
3. Place the biofilm pegs in an empty vials.
4. Fix the biofilm on the pegs by adding 2.5% gluteraldehyde in 0.1M cacodylate buffer pH 7.5.
5. Completely cover the pegs with the fixative agent.
6. Close the lid of the vial and incubate at 4^0C for 20 hours.
7. Decant the fixative with a pipette rinse the pegs with double distilled water.
8. Dehydrate the pegs with 70% alcohol and allow the pegs to air-dry for 72 hours before mounting on the microscope.

Table: Antibiotics their concentration used fro MBEC Assay

Antibiotic	**Range (µg/mL)**
Enrofloxacin	0.12 - 2
Gentamycin	0.5 - 8.0
Ceftiofur	0.25 - 4
Neomycin	2 - 32
Erythromycin	0.12 - 4
Oxytetracycline	0.25 - 8
Tetracycline	0.25 - 8
Amoxicillin	0.25 - 16
Spectinomycin	8 - 64
Sulfadimethoxine	32 - 256
Trimethoprim-Sulfamethoxazole	0.5/9.25 - 2/38
Florfenicol	1 - 8
Sulfathiazole	32 - 256
Penicillin	0.06 - 8
Streptomycin	8 - 024
Novobiocin	0.5 - 4
Tylosin tartarate	2.5 - 20
Clindamycin	0.5 - 4

Appendix 15. Preparation of 50x TAE Buffer Solution.

Reagents required: Tris-base, Glacial Acetic Acid, 0.5M EDTA solution (pH 8.0)

Procedure to make 1000 ml stock solution

1. Dissolve 242 gm of tris-base powder in 750 mL of de-ionized water using magnetic stirrer.
2. Add 100 mL EDTA (0.5M) and 57.1 mL glacial acetic acid to the mixture and dissolve well by magnetic stirrer.
3. Adjust the pH of the solution to 8.0 using pH meter.
4. Add de-ionized water to make the final volume of 1000 mL.
5. Autoclave at 121^0C and 17 psi for 40 minutes and store at room temperature.

Preparation of working stock solution (1X TAE Buffer)

1. To make 1000 ml, use the following formula:

   C1xV1=C2xV2

   where, C1=initial concentration, V1=initial volume, C2=final concentration, V2= final volume.

Mix 20 mL of 50X TAE buffer was mixed with 980 mL of de-ionized water to make 1x TAE working solution.

Appendix 16. Preparation of 0.5M EDTA Solution

Reagents required: Di-sodium EDTA dehydrate (EDTA.Na_2.$2H_20$, mol wt-372.24), Sodium hydroxide pellet, deionized water.

Procedure to make 1000 ml solution:

1. Mix 186.12 gm of Di-sodium EDTA dehydrate with 800 ml of de-ionized water.
2. Add 20 gm of sodium hydroxide pellets to the mixture and dissolve well using magnetic stirrer.
3. Adjust the pH of the solution to 8.5 by adding sodium hydroxide pellet if needed.
4. Add de-ionized water to make final volume of 1000 mL and autoclave at 121^0C and 17 psi for 40 minutes.

Appendix 17. Preparation of Tris EDTA (TE) Buffer

Reagents required: 1M Tris (hydroxymethyl) aminomethane, 0.5M EDTA

Procedure

1. First, dissolve 60.57 gm Tris in 500 mL double distilled water.
2. Use magnetic stirrer to dissolve quickly.
3. Adjust the pH to 8.5 using HCL

To make 500 mL of TE stock solution.

1. Add 1 mL 0.5M EDTA solution to 5 ml Tris solution.
2. Add 496 mL distilled water to make final volume of 500 mL
3. Autoclave at 121^0C and 17 psi for 40 min.

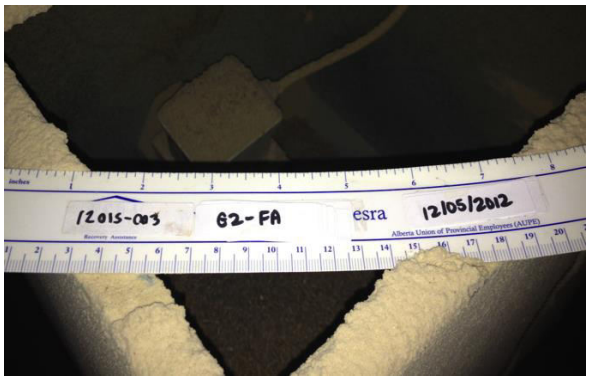
Appendix 18. Pictures of samples taken from poultry farms and processing plants of Alberta.

a)

b)

c)

d)



e)

f)

Photographs depicting the equipment and materials sampled in the broiler farms. The rulers give size reference, and indicate the date of sample collection.

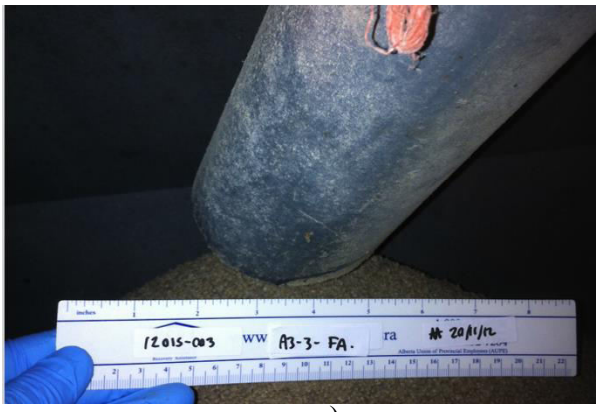
a, b) drinking nipple; c, d) feed pan; e, f) feed auger

a)

b)

c)

d)

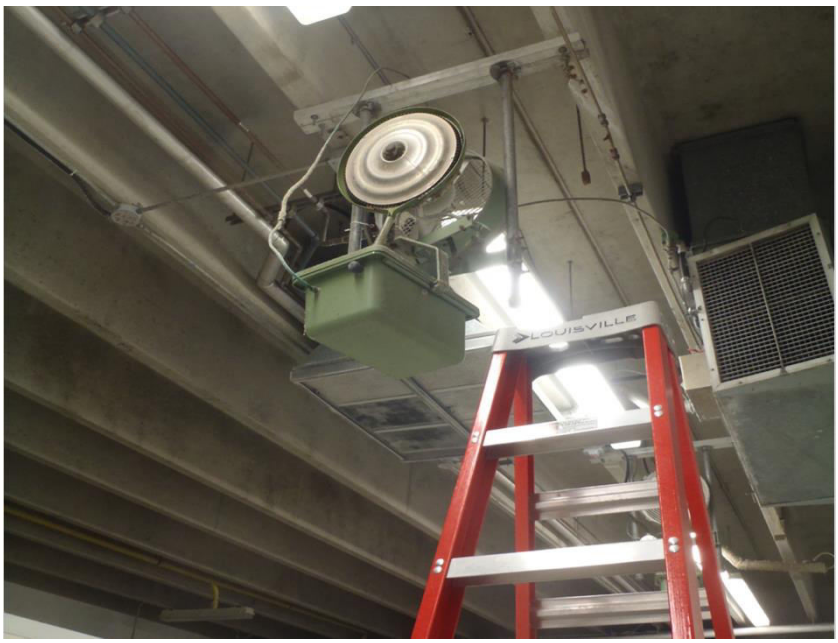


e)

f)

Photographs depicting the equipment and material type sampled in the layer farms. The rulers give size reference, and indicate date of sample collection.

a, b) drinking nipple; c, d) feed pan; d, e) feed auger.



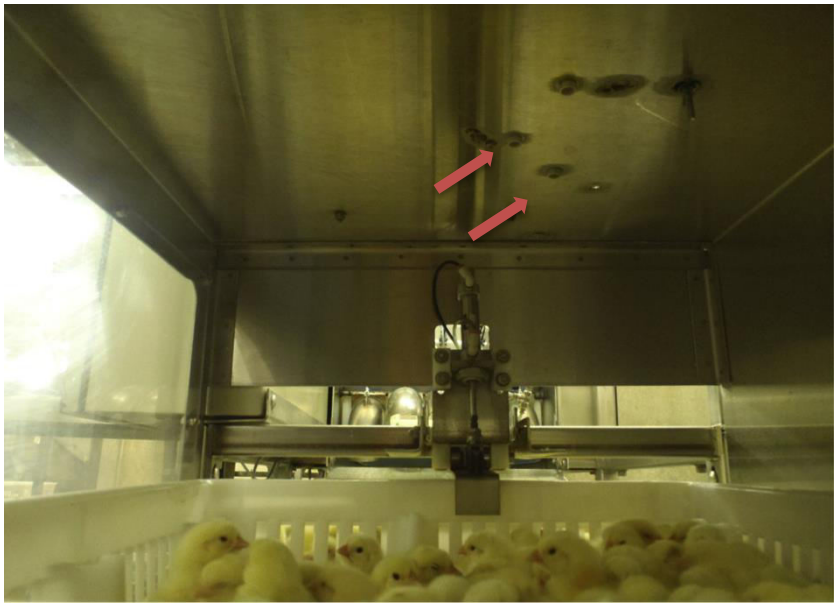
a)

b)

Photographs depicting the equipment and material type sampled in the hatchery.

a) humidifier blade; b) humidifier reservoir water.

a)



b)

Photographs depicting the equipment and materials sampled in the hatchery.

a) bronchitis vaccine tube inside the flask; b) spray nozzles in day-of-age spray cabinet vaccinator (shown in red arrows)

a)

b)

c)

d)

e)

f)

Photographs of depicting the equipment and materials sampled in the poultry processing plants. Rulers give size reference and indicate sample collection date.

a) picker fingers; b) eviscerator; c) stunning bath; d) scald tank; e) chilled tank; f) saw guard.