INTEGRON MEDIATED ANTIBIOTIC RESISTANCE GENES IN SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* AND THEIR TRANSFER

by

SUPAKANA NAGACHINTA

(Under the Direction of JINRU CHEN)

ABSTRACT

Approximately 7.9 and 0.6% of the Shiga toxin-producing *Escherichia coli* (STEC) isolates in our laboratory collection were positive for class 1 and 2 integron, respectively. These positive isolates were resistant to multiple antibiotics. However, only some of the antibiotic resistance traits were mediated by the integrons. A single gene cassette of *aadA1* was identified on the class 1 integron and three gene cassettes, *dfrA1, sat1,* and *aadA1*, were found on the class 2 integron. The class 1 integron mediated antibiotic resistance genes were transferable to *E. coli* MG1655 during conjugation in nutrient broth. The transconjugants expressed the antibiotic resistance traits encoded by integron mediated, as well as non-integron mediated antibiotic resistance genes. Transfer of class 1 integron occurred in samples usually found in farm environments such as bovine feces and storm water. Environmental temperature and sample pH influenced the transfer frequencies of the integron mediated antibiotic resistance genes.

INDEX WORDS: Shiga toxin-producing *E. coli*, antibiotic resistance, integron, and conjugation

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DEDICATION

I would like to dedicate this thesis to my loving family for all their support.

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CHAPTER 1

INTRODUCTION

Shiga toxin producing *Escherichia coli* (STEC) is an important group of foodborne pathogens and is responsible for causing serious clinical conditions such as diarrhea, hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (136). *E. coli* O157:H7 is the serotype most commonly associated with foodborne diseases and outbreaks. Several other serotypes including O26, O103, O111, O121, and O145 were also involved (103). Cattle are major reservoirs of STEC which persist in farm environments for a length of time. The contamination rate of STEC in retail meats corresponds to levels of STEC contamination in farm areas (16). The highest rate of fecal excretion of STEC cells by cattle occurs in the warmer months (87) which corresponds to the higher incidence of STEC infections from May to October (157).

Strains of STEC have recently been found to resist to the antibiotics commonly used in human and veterinary medicine (46). The most commonly resisted antibiotics include streptomycin, sulfisoxazole and tetracycline (224). Antibiotic use in food animals is believed to select for resistance to antibiotics in commensal and pathogenic microorganisms. Bacteria in response to selective pressure acquired antibiotic resistance through genetic mutation and horizontal transfer of antibiotic resistance genes. The later mechanism has been recognized as a successful tool of resistance acquisition among bacteria. Genetic elements including plasmid, transposons, and integron/gene cassettes are the most important elements facilitating dissemination of antibiotic resistance genes

(179). An integron is a site-specific recombination system that recognizes and captures antibiotic resistance gene cassette (85). Transfer of integron and antibiotic resistance gene cassette is usually mediated by plasmid and transposons (85). Bacteria with integron therefore serve as reservoir of antibiotic resistance genes.

The objectives of this study were to:

1. Determine the prevalence of integrons in a group of STEC strains and the role of integrons in dissemination of antibiotic resistance genes from STEC to a non-pathogenic *E. coli* strain under laboratory conditions.
2. Determine whether the transfer of integron-mediated antibiotic resistance genes from STEC to generic *E. coli* can take place in samples commonly found in farm environments such as storm water and bovine feces and whether environmental temperature and sample pH influence the transfer frequency of the antibiotic resistance genes.

CHAPTER 2

LITERATURE REVIEW

ESCHERICHIA COLI AND SHIGA TOXIN PRODUCING *E. COLI*

Escherichia coli

Escherichia coli are gram negative, non-spore forming facultative anaerobic rods in the family of *Enterobacteriaceae*. They are one of many species of bacteria living in the lower intestine of mammals. Physiologically, *E. coli* can grow in the presence or absence of oxygen. Under anaerobic condition, they utilize substrate and gain energy via fermentation producing mixed acids and gas as end products (100). They can also grow by means of anaerobic respiratory using nitrate or fumarate as final electron acceptors (98). This allows growth of *E. coli* in the colon.

E. coli may be commensal or pathogenic. One way of classifying the pathogenic strains of *E. coli* is the use of virotypes. The virotypes of *E. coli* are defined based on their pathogenic features including enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC), cytolethal distending toxin (CDT)-producing *E. coli*, cell-detaching *E. coli* (CDEC) or cytotoxin nacrotizing factor (CNF) producing *E. coli* (116, 136). Because some strains can have the characteristics related with more than one virotypes, the Kauffman serotyping approach instead is widely used for its convenience to identify strains involved in foodborne outbreaks or diseases. This method classifies strains of *E. coli* based on

bacterial surface antigens O (somatic), H (flagella) and K (capsular) (136, 169). Specificity of O antigens comes from the immunogenicity of polysaccharide repeating units. Some *E. coli* strains though lack of these repeating side chain and become autoagglutinable (designated as OR for rough). H antigens are based on different types of flagellin present on flagella structure. Some strains are nonmotile and designated as NM or H^-. There are 174 O antigens and 53 H antigens in the international serotyping scheme. The *E. coli* isolates serotype is defined by combination of O and H antigens (172).

Shiga toxin-producing *E. coli*

General characteristics

Shiga toxin-producing *E. coli* (STEC) has been associated with outbreaks of gastrointestinal diseases (33, 147). Strains of STEC produce at least one of the potent cytotoxins called Shiga toxins (Stx1 and Stx2). The name Shiga toxin is used because their resemblance to the cytotoxin produced by *Shigella dysenteriae* serotype 1 (141). The strains of virotypes EHEC denote a subset of STEC strains that cause hemorrhagic colitis and hemolytic uremic syndrome (HUS), cause attaching and effacing (A/E) lesion, express Stx, and possess c.a. 60-MDa plasmid (116, 136). STEC strains expressing somatic (O) antigen 157 and flagella (H) antigen 7 have been strongly associated with HUS worldwide (185), however strains of non-O157 have appeared to be more common cause of HUS in Australia, Germany and Austria (68, 77).

Physiological characteristics

Similar to other *E. coli*, cells of STEC can be grown easily in the laboratory on general or selective media at 37°C. Culture of STEC is usually grown aerobically in

laboratory condition although the organisms are facultative anaerobes. For *E. coli* O157:H7, the maximum and minimum growth temperatures were reported at 44-45.5°C and 8-10°C, respectively (64, 137, 144). The minimum growth temperature, for non-O157:H7 *E. coli* isolates was 4.9-11.2°C and maximum growth temperature was 47.3-48.0°C (166). STEC strains are acid resistant. They can grow at pH 4.6 and some survive at extreme acidic condition as low as pH 2.5 (133). The optimum growth of STEC is in the pH range of 6-7 but decline rapidly at the lower pH (35). In alkaline condition, STEC cells could grow up to pH of 9.0 (79). In food, type of acid (e.g. organic vs. inorganic) and acid concentration both influence the growth of STEC. Abdul-Raouf et al. (1993) reported that in beef slurries, the relative inhibitory activity of organic acids on *E. coli* O157:H7 was acetic > lactic ≥ citric (1). *E. coli* O157:H7 grows vigorously in 2.5% NaCl, slowly in 6.5% NaCl, and does not grow in 8.5% NaCl. The minimum water activity required for STEC strains is about 0.95, similar to other *E. coli* cells.

Biochemical characteristics

STEC strains belong to a wide range of serotypes. Strains of non-O157 do not differ significantly in their biochemical characteristics from the typical *E. coli*. They are lactose-fermentive, oxidase-negative, and indole-positive. However, for strains of *E. coli* O157:H7, some biochemical characteristics are used for their isolation. *E. coli* O157:H7 cells are unable to ferment sorbitol within 24 h of incubation and produce colorless colonies on sorbitol-MacConkey (SMAC) agar media (62, 148). The sensitivity of SMAC is improved by supplementing with cefixime and tellutile to inhibit *Proteus* spp, and other strains of *E. coli* (50, 223). Tellurite-cefixime-SMAC agar is effectively in isolating *E. coli* O157:H7 from clinical samples. Another characteristic of *E. coli*

O157:H7 is its inability to produce beta-glucuronidase which hydrolyzes 4-methyl-umbelliferyl-D-glucuronidase (MUG) (199). Hydrolysis of MUG by most non-O157:H7 strains produces a fluorescent compound thus this substrate has been incorporated into some agar media (e.g. Rainbow Agar O157) to differentiate among *E. coli* O157:H7 (136). Nearly all *E. coli* O157:H7 and a significant portion of non-O157 STEC cells produce EHEC hemolysin (hly) forming small turbid zones of hemolysis on washed sheep and O^+ human red blood cells agar plates as a result of their distinct enterohemolytic characteristics.

Sub-typing of STEC

In epidemiological studies, outbreak investigation often requires sub- typing data to identify outbreak-related strains (10). Typing methods differ in their speed, cost, difficulty of the technique and ability to discriminate between strains. These methods fall into two broad categories: phenotypic and genotypic typing methods.

Phenotypic methods

Phenotyping differentiates strains based on the properties expressed by the bacteria. Biotyping identifies bacteria based on their biochemical profiles. The method is relatively low cost and available through commercial kits (API-20E system). However, the method has poor discriminatory power and low reproducibility (95). Antimicrobial susceptibility testing determines the pattern of resistance to selected antibiotic expressed by the bacteria. However, because some mobile genetic elements (plasmid and transposons) carrying resistance genes can be transferable between cells strains could be falsely classified (95). Phage typing differentiates strains by their patterns of susceptibility to a selected group of bacteriophage (154). Phage typing for *E. coli*

O157:H7 has been extended to 62 different bacteriophage and is used in Canada, the United States, Japan, Australia, and England (106). Serotyping is one of the classic strain typing techniques used over the years. The method characterizes the strains based on antigens present on the cell's surface. Karmali et al. (2003) classified STEC into 5 seropathotypes, A to E (103). *E. coli* O157:H7 and O157:NM (non-motile; mutation of strains without a H-antigen) are in seropathotype A and are the most virulent. Seropathotype B consists of strains that are associated with outbreaks and HUS but at lower frequency than serotype O157:H7. The seropathotype B includes serotypes O26:H11, O103:H2, O111:NM, O121:H19 and O145:NM. Seropathotype C is composed of strains that are associated with sporadic HUS but not outbreaks including O91:H21 and O113:H21. Seropathotype D and E comprise of serotypes that have been implicated in sporadic cases of diarrhea and serotype that caused disease only in animal, respectively. Seropathotype is a useful method to study bacterial pathogenesis and their important virulent features as it provides comparison between the more virulent and the less virulent groups.

Genotypic or molecular methods

Genotyping utilizes the genetic variations on bacterial genome to identify the differences between STEC strains. The methods subtype STEC strains based on the results of DNA restriction analysis, DNA amplification using polymerase chain reaction (PCR), and plasmid profiling (197).

- *Restriction analysis*. The typing techniques fall in this category are divided into two methods. The first method uses restriction enzymes known as frequent cutters to generate many pieces of DNA fragments followed by DNA separation using the

conventional agarose gel electrophoresis. The restriction fragments within a size range of 25 to 0.5 kb give discernible banding pattern. The second method uses infrequently cutting restriction enzymes to generate fewer but larger (1 kb up to 1,000 kb) fragment bands. These fragment bands are separated on gel run in special form of electrophoresis (29, 176). Restriction fragment-length polymorphism (RFLP) referred to variations in the number and size of the fragments detected by hybridization. RFLP analysis using probe is a modification of restriction enzyme analysis and conventional electrophoresis. The resulting restriction fragments are separated according to length by gel electrophoresis as mentioned above. The resulting banding patterns are sometimes too complex to analyze yet can be enhanced by southern blotting using suitable probes of DNA or RNA. Ribotyping is a common typing technique which uses the same chromosomal DNA preparations and a ribosomal RNA probe. For STEC isolates, the restricted genomic DNA can be probed with λ DNA or *stx* probe. Levine et al. (1987) developed DNA probe prepared from 3.4-kilobase segment of the EHEC plasmid (117). Samdpour et al. (1993) reported RFLP typing using λ DNA probe had more discriminatory power than probing with rRNA (ribotyping) or *stx* fragments (170). Pulsed-field gel electrophoresis (PFGE) generates distinct pattern fragment bands approximately 10 to 30 restriction fragments ranging from 10 to 800 kb by using restriction enzyme that has relatively few recognition sites (197). PFGE uses a specially designed chamber with three sets of electrodes that cause DNA to migrate through gel in back-and-forth movement resulting in the higher level of fragment resolution. Due to its accuracy and high reproducibility between laboratories PFGE has become a standard method

for subtyping STEC in public health laboratories (107). "Pulsenet" is national network of public health laboratories performing PFGE fingerprints of foodborne causing bacteria allowing rapid comparison between patterns.

- *Typing methods using PCR.* The hallmark of PCR is the ability to amplify copies of a particular DNA segment from minute quantities of sample DNA. The technique has been modified for use in different typing tools (197, 211). Random amplification of polymorphic DNA (RAPD) analysis employs a single short primer that is not targeted to amplify any specific sequence. Rather, the primers hybridize at multiple random locations on DNA resulting in PCR products of different sizes visualized on agarose gel (197). Recently developed PCR-based fingerprints employ DNA primers corresponding to naturally occurring interspersed repetitive elements in bacteria. In repetitive extragenic palindromic-PCR (REP-PCR), amplification of the DNA between adjacent repetitive extragenic elements is used to obtain strain specific DNA fingerprints which can be easily analyzed with pattern recognition computer software (14, 61). Similarly, amplification of DNA between the enterobacterial repetitive intergenic consensus (ERIC) sequences is employed to obtain DNA fingerprint in enterobacterial repetitive intergenic consensus –PCR (ERIC-PCR). Brocchi et al (2006) reported REP-PCR, although possessing discriminatory power; did not separate the 30 commensal and 49 pathogenic strains of the tested *E. coli* (32). On the contrary, clonal clusters generated by REP and EPIC-PCR tested on 50 *E. coli* strains in the study by Carvalho de Moura et al (2001) corresponded to strains with different degrees of pathogenicity (40). Genomic events such as DNA polymerase slippage and recombination cause a variation in the number of repeated units in short

sequence repeat (SSR) motifs. A class of SSRs, named variable number of tandem repeats (VNTRs) has proven to be a suitable target for genomic polymorphisms typing in bacteria (26). VNTR-based methods rely on direct PCR amplification of the repeat loci thus variation of bacteria at the repeat loci resulted in different sizes of PCR products. Noller et al.(2004) recently developed a multilocus variable-number tandem repeat (TR) analysis (MLVA) assay for *E. coli* O157:H7 (139). The assays were adapted to multicolored capillary electrophoresis (CE) which resulted in system for rapid genotyping of *E. coli* O157:H7 (139).

- *Plasmid typing*. Plasmid profile of bacterial strains can be produced when intact plasmids are separated by gel electrophoresis. As shown in previous literature, plasmid profiling results of *E. coli* O157:H7 have shown no individual plasmid or combination of plasmids was significantly linked to the nature of an infection (143, 158). Ratnam et al. (1988) analyzed plasmid profiles of a total of 174 *E. coli* strains collected from cases of HUS and non-bloody diarrhea across Canada and the U.S. (158). They found three basic plasmid profiles of *E. coli* O157:H7. However, none of the characteristics could be related with geographical distribution of strains or the nature of infection. Ostroff et al (1989) showed a considerable diverse of plasmid content among 93 *E. coli* O157:H7 isolates collected from a single geographical region over a discrete period of time (143). The problem with plasmid typing can arise when the organism acquires or loses a plasmid which makes this method an unreliable marker for typing.

STEC reservoirs

Cattle are major reservoir of both *E. coli* O157 and non-O157. In addition, sheep, goat, deer, and birds were identified as natural reservoirs of STEC (49, 87, 88). Calves more frequently carry *E. coli* O157:H7 than do adult cattle. The organisms persist in manure, feeds, water troughs, and other places on farms (88). Fecal excretion of STEC cells by cattle occurs at highest rate in warmer months (87) which correlates with the start of seasonal peak of human STEC infection. Studies have shown that STEC cells can remain viable in soil for more than 60 days at 25°C and 100 days at 4°C (97). STEC cells contaminated surface and groundwaters through surface runoff, contaminated soil, and animal feces (2). Survival of STEC cells in bovine feces at different temperatures (5, 15, and 25°C) was studied by Fukushima et al. (1999). The survival period of three different serotypes including STEC O26, O111 and O157 ranged from 1 to 18 weeks. The length of STEC survival depended on level of inoculation and storage temperature. In samples inoculated with 10^1 CFU/g, the organisms survived at 5 and 25°C for 1 to 4 weeks and at 15°C for 1 to 8 weeks. With higher inoculations (10^3 and 10^5 CFU/g), the organisms survived at 25°C for 3 to 12 weeks, at 15°C for 1 to 18 weeks and at 5°C for 2 to 14 weeks. Their results showed that STEC pathogens can survive in feces for a long time (75). Wang and Doyle (1998) reported survival of *E. coli* O157:H7 in environmental water at 8, 15 and 25°C. The organisms can survive up to 90 days with the greatest survival at 8°C (208).

Contamination of STEC in food

A variety of food has been identified as source of STEC infections. Foods originated from bovine have been the most frequently implicated source of STEC

infections, particularly ground beef. The hides and feces of animals at slaughtering are major sources of STEC in processing plants (67). STEC cells contaminate meat products by transfer of the organisms from fecal material onto meat surfaces. The total STEC prevalence on carcasses was 71.9% pre-evisceration and 10.1% post-processing during the summer months (13). STEC cells were found in 12.1% of beef (n=91), 17.1% of lamb (n=37), 4.0% of pork (n=35) but none of chicken samples collected from markets in Bunedin, New Zealand over a 13-month period (33). Doyle and Schoeni (1987) recovered *E. coli* O157:H7 from 6 (3.7%) of 164 beef, 4 (1.51%) of 264 pork samples, 4 (1.5%) of 263 poultry samples, and 4 (2.0%) of 205 lamb samples obtained from retail outlets from Madison, WI., and Calgary, Alberta, Canada (63). STEC has been found in dairy products such as unpasteurized cheese, milk and mayonnaise (209, 212). They were isolated from 5 of 131 (3.8%) bulk tank milk samples collected from dairy herds in eastern South Dakota and western Minnesota (94). A similar STEC incidence rates of 3.9% for raw milk (n=127) and 2.1% for certified raw milk (n=146) from 5 different regions in Germany were reported by Klie et al. (1997) (108). Vegetables and fruits can also be contaminated with STEC during cultivation, harvesting, handling, processing and distribution. The presence of STEC in farm areas could lead to contamination of irrigation water which used for washing of the produce or contamination of soil in which produce is grown. STEC contamination in these two resources could cause contamination of fresh produce. Prevention of food contamination should be emphasized especially for foods that are consumed raw. STEC outbreaks associated with spinach, lettuce and alfalfa sprouts were traced to contamination at farm level (45). Consumption of unpasteurized apple juice or cider has been implicated in *E. coli* O157:H7 infections.

Apple drops which are commonly used for making cider can be contaminated with animal manure on the ground or during the processing (44).

Disease transmission

In most countries, *E. coli* O157:H7 is the serotype associated with most cases of disease and outbreak (12). Several other serogroups are also implicated, including O26, O103, O111, O121, and O145 (103). The most common cause of human infection with STEC is through consumption of contaminated food or water. Other causes include personal contact with infected patients or direct contact with animals (34, 90, 217). Most foodborne outbreaks involved consumption of contaminated food derived from cattle such as ground beef and raw milk (153, 207). Spectrum of vehicles implicated STEC infection is expanding. Outbreaks have been linked to consumption of mayonnaise(208), unpasteurized apple juice and fermented hard salami (19, 42). Outbreaks related to contamination of fruits and vegetables such as lettuce, apple cider, unpasteurized apple juice, and alfalfa sprouts(43, 44). Outbreaks have also been linked to drinking and swimming water (5, 104). Transmissions *via* direct contact with animals and infected person are related to occupational exposure among nurses and microbiologists (81). Outbreaks of STEC infections have occurred in day-care facilities and school camps (17, 39, 150).

Pathogenesis

STEC are shown to be able to survive in acidic foods and cause diseases (15). This acid tolerance facilitates survival of STEC in the low pH environment in human gastrointestinal tracts. Most STEC strains are capable of causing HUS, and are *eae*-positive (70). The *eae*-positive STEC strains possess a pathogenicity island called the

locus of enterocyte effacement which encodes a type III secretion apparatus, a translocated intimin receptor (Tir), intimin and *E. coli* secretion proteins (Esp), responsible for the formation of attaching and effacing (A/E) lesion (136). Massive loss of microvilli caused by the A/E lesion leads to malabsorption and diarrhea. Inflammatory cytokines and circulating of bacterial lipopolysaccharide increase the process of diarrhea.

Stxs produced by STEC is essential for the development of bloody diarrhea and hemorrhagic colitis (136, 159). The toxins produced in the intestines enter the blood system and damage epithelial cells of the kidneys resulting in post diarrheal HUS (131). Two major groups in the Stx family are Stx1 and Stx2. The structure of Stx1 is highly conserved and identical to Stx from *S. dysenteriae* serotype 1, but the Stx2 consists of several antigenic variants (74, 196). Stx molecules are heterohexamer A_1B_5 structure. The A-subunit is non-covalently bounded with a pentamer of B-subunits and functions by cleaving a specific adenine base from the 28S rRNA. As a result, cell protein synthesis is inhibited leading to cell apoptosis (190). The B-subunits bind the toxin to a specific glycolipid receptor, globotriaosylceramide or Gb_3 which is present in high concentration on the human renal endothelial cells (30, 151). Stx damages and causes lesions on endothelial cells. Bloody diarrhea is a result from lesions in small blood vessels of the colon while HUS is resulted from renal glomerular lesions that caused by damaged renal endothelial cells.

The *ehxA* encoding the EHEC hemolysin (enterohemolysin) is located on a 60-MDa plasmid (20, 173). The role of enterohemolysin is unclear but lysis of erythrocytes could serve as a source of iron for the bacteria (111). Two other phage encoded hemolysins, called Ehly1 and Ehly2, have been reported in many STEC strains (21, 192)

but the role of these hemolysins in STEC pathogenesis is unknown (reviewed in Nataro and Kaper, 1998)

Among STEC isolates, the 60-MDa plasmid is present in *E. coli* O157:H7 and non-O157 STEC strains including O26:H11 (117). Beutin et al. (1994) reported 90% prevalence of this plasmid from 2,165 STEC isolates from human patients in Germany (20). The plasmid, designated as pO157, is highly conserved among *E. coli* O157:H7 and varies in size from 93.6 to 104 kb (174, 175). The role of the plasmid was suggested in adherence of STEC cells, however there is no consisting evidence in findings (reviewed in Nataro and Kaper, 1998). *In vitro* study, Karch et al (1987) reported plasmid was required for fimbriae expression and adhesion to epithelial cells (102). In contrary, several *in vivo* studies suggested that the presence of plasmid had no effect to the intestinal hispathology (204)

Other putative virulence factors produced by STEC may also play roles in pathogenesis. Cytholethal distending toxin (CDT), a toxin interferes with cell cycling, may cause vascular endothelial cell death. The toxin was detected in a small number of *eae*-negative STEC and recently was reported in O157:H^- (22, 23). The genes encoding for CDT were either chromosomal and homologous to *cdt*-V (serotypes O73:H18, O91:H21, and O113:H21) or plasmid-borne and homologous to *cdt*-III (serotype O153:H18) (22). CDT-V was shown to cause cell cycle arrest and irreversible damage to human endothelial cells suggesting its role in process of infection by STEC isolates (23). Enteroaggregative *E. coli* heat-stable enterotoxin (EAST), first described in EAEC, is also found in among STEC strains. The gene encoding for EAST is the *astA* gene was reported in *E. coli* O157:H7 which possibly account for some of the watery diarrhea

(171). Seriwatana et al. (1988) reported total of 9% of heat-labile (LT) toxin II-producing *E. coli* isolates (3 of 34) from cows and buffalo hybridized with DNA probes for genes coding for verocytotoxin 2 (VT2), and 18% (6 of 34) hybridized with a DNA probe coding for EHEC adhesin fimbriae (181). ETEC strains cause diarrhea through the action of the enterotoxins LT and ST. LT-II is primarily found in animal isolates yet has not been showed to be associated with disease (136).

The infectious dose for some STEC strains (O111:H^- and O157:H7) are estimated to be 1 to 100 organisms (147, 203). The infection dose of *E. coli* O157:H7 responsible for the large outbreak in the U.S. in 1993 has been estimated to be less than 700 organisms (203).

ANTIBIOTIC USE IN FOOD ANIMALS

Historical background

The introduction of penicillin and sulfa drugs into clinical use in the 1930s and 1940s, respectively (52) advanced the treatment of infectious diseases. Clinical success of these drugs and the need for treatment of bacterial infection in war-related wounds during WWII led to discovery of different antibiotics including streptomycin, chloramphenicol, tetracycline, erythromycin, rifamycin, vancomycin and cephalosporin between 1940 and 1960 (review in Yoneyama et al. 2006) (221). Striking impact of antibiotics led to optimism for curing of infectious diseases. However, bacteria developed resistances soon after the use of every new drugs causing re-emergence of previously experienced infections that appeared to be more virulent (52).

The term antibiotic refers to low-molecular-weight microbial metabolites that at low concentrations inhibit the growth of other microorganisms (110). Antibiotic possesses a defined chemical structure having a relative mass of at most a few thousand.

Therefore, this definition does not include enzymes such as lysozyme that also have antibacterial properties. The specifier "at low concentration" excluded compounds such as ethanol and fermentative products of some microorganisms that also display antibacterial activity but only at high concentrations. With the term "low concentration", the value generally mean well below 1 mg/ml (110). The term "antibiotic" denotes chemical substances produced by a microorganism, and semi-synthetic modification of natural products such as β-lactams antibiotics (124). This definition distinguished antibiotics from antimicrobial agents that are totally synthesized. Three classes of chemically synthesized antimicrobials used for antibacterial purpose include the sulfa drugs, the quinolones, and oxazolidinone (124). However, according to literatures, it should also be mentioned that the requirement that antibiotic be "microbial metabolite" is no longer strictly applied (110). Antibiotics block the growth of sensitive microorganisms by inhibiting the action of a molecule essential for cell multiplication. This means that the antibiotic molecule must bind to a specific site on the target molecule, forming a molecular complex which is no longer able to accomplish its original function (110). The specific mechanism by which inhibition takes place is the mode of action of the antibiotic. According to the process of which they interfere, antibiotics are divided into the following groups: 1) inhibitors of cell wall synthesis, 2). inhibitors of the replication or transcription of genetic material, 3). inhibitors of protein synthesis, 4) inhibitors of cell membrane functions and 5). antimetabolites (110).

The use of antibiotics in food animals was introduced shortly after WWII. Moore et al. (1946) suggested addition of antibiotics in animal feeds or water because they appeared to promote faster growth in chicken (134). Since then, research continued to

report benefits of sub-therapeutic doses (defined by the U.S. FDA as < 200 g per ton of feed) of antibiotics experimented in different animals such as swine and calves (58, 96, 120, 140). The mechanism of how antibiotics improve growth rate and feed efficiency has never been well-understood. Several theories have however, been proposed, including 1). biochemical effects that involve nitrogen excretion, efficiency in phosphorylation in cells, and protein synthesis, 2). effects of antibiotics on the generation of essential vitamins and cofactors by intestinal microflora, and 3). reduction in subclinical population of pathogenic organisms (57, 96).

Antibiotic uses in farms

In animal husbandry, the use of antibiotics can be divided into 4 common practices (177). Firstly, therapeutic treatment which intends to control existing bacterial infection by treating infected animals. Secondly, metaphylaxis treatment which aims to treat infected animals as well as to medicate other animals for prevention purpose. Thirdly, prophylaxis treatment which is applied during high risk period of infection as a preventive measure but not treating infected animal (such as in calves after lactation to prevent mastitis). And lastly, use of antibiotics agents for growth promotion purpose.

Tetracycline, penicillin, erythromycin and other important drugs used in human medicine are used extensively in food animal production. A study released by the Union of Concerned Scientists in 2001 suggested an estimated of 24.6 million pounds of antimicrobial use in animals. The major portion accounted for usage in poultry production with a dramatic 307% increase since the 1980s. Their estimates suggested that nontherapeutic use in livestock accounted for 70% of total antimicrobial use (129). The figures derived in their study were based on extrapolations and indirect methods.

Accurate estimates antimicrobial use data for animals are still needed to determine which drugs are used for what purpose and in what quantity.

In the following examples of antibiotics used in different types of animal production are listed.

Poultry: Production of broiler chickens has become highly integrated. This integration leads to a need of standardized practices including drug treatment procedures to minimize infection among the flock. Antimicrobials are usually administrated through water and feed to an entire chicken flock because individual treatment is not practical. For example, antibiotics such as ionophores and sulfonamides which are used to control coccidiosis (a parasitic disease cuased by coccidia protozoa) are contained in broiler rations. Bacitracin, bambermycin, chlortetracycline, penicillin, virginiamycin, arsenical compounds are approved for growth promotion and feed efficiency in broilers, turkeys, and egg layers (140). New antibiotics e.g. fluoroquinolones are used to treat *E. coli* infections because older drugs such as the tetracyclines are ineffective (due to emergence of microbial resistance (126, 155). Hatching eggs may also be dipped in gentamicin to reduce contamination of mycoplasma and bacteria (126).

*Cattle***:** Calves after weaning are fed on high-grain diets in order to achieve rapid fattening before slaughter. Antibiotics are administrated on feedlots for many reasons including deterring liver abscesses due to possible damage on the stomach lining causing bacteria to enter bloodstream and grow in the liver, promoting weigh gain, and preventing and controlling of respiratory infectious diseases. Example of drugs administrated into feed and water for prophylaxis and growth promotion include monensin, lasalocid, chlortetracycline, chlortetracycline-sulfamethazzine, oxytetracycline

and tetracycline. Antibiotics such as cephalosporins, penicillins, macrolides and fluoroquinolones are used in feedlots for individual animal therapy (126).

Dairy calves are often treated individually or in groups to prevent pneumonia and diarrhea; a major cause of high mortality in calves (215). Antibiotics such as tetracyclines, penicillins, and sulfonamides are administered orally or by injection once calves are housed in groups after weaning. Lactating cows receive few antimicrobials in feed. However, some antibiotics such as penicillins, cephalosporins, erythromycin, and oxytetracycline are administered through intramammary infusion to treat mastitis (126).

Swine: Antibiotics are mainly administered in feed for growth promotion and prophylaxis purposes. Antibiotics are given to pigs after weaning (in starter rations) when they are most vulnerable to infectious diseases (60). Antibiotics such as gentamicin, apramicin and neomycin are used to treat bacterial diarrhea caused by *E. coli* and *Clostridium perfringens*. Pneumonia is controlled by antibiotics such as sulfonamides, tetracyclines, ceftiofur, and tiamulin. Macrolides and lincomycin are used to control swine dysentery *(Serpulina hyodysenteriae*) (66). Antibiotic treatments are typically removed to avoid residue at the finishing stages of production (126).

Aquatic food animal: Antibiotics administrated in aquaculture feed are subject to FDA approval. The antibiotics must be mixed in feed at concentrations that are specified in FDA medicated-feed regulations (140). No antibiotics are approved for growth promotion in the U.S. for salmonids and catfish (126). Ormetoprim-salfadimethoxine and oxytetracycline are approved for treatment of bacterial infections such as hemorrhagic, septicemia, and psedomonase disease in salmonids, catfish, and pacific

salmon (140). Antibiotics maybe administrated *via* feed to the entire group or treated individually (126).

Consequences of antibiotics used in food animals

Studies suggest antibiotics used in food animals select antibiotic resistant commensal bacteria, such as enterococci and non-pathogenic *E. coli*, and zoonotic enteropathogens such as *Salmonella*, *Campylobacter, Yersinia* and pathogenic *E. coli* (119, 121) (69, 118). However, other studies indicate that antibiotic resistances were easier to acquire in some bacterial species and did not show a link to antibiotics use in food animals (59, 210). Nevertheless, antibiotics used in food animals provided a selective pressure forcing bacteria to develop resistance mechanisms and evidently contributed to dissemination of antibiotic resistance among bacteria.

Because most of antibiotics used in food animals belong to the classes of antibiotics which are also used in human medicine, emergence of antibiotic resistant bacteria has become a special concern in food safety (182). Antibiotic resistant strains of zoonotic enteropathogens as well as commensals are likely to be transmitted *via* food chain to human. Holmberg et al. (1984) reported *Salmonella* infection linked to consumption of hamburgers contaminated with *S. enterica* serovar Newport resistant to ampicillin, carbenicillin, and tetracycline. The causative pathogens were tracked to the origin beef cattle in South Dakota that fed with subtherapeutic doses of chlorotetracycline as growth promoter. Antibiotic therapy taken during colonization with antibiotic resistant bacteria may cause progression into clinical disease. The selective effects provided by antibiotics administrated in drug therapy offer a specific advantage for certain resistance pathogens to outgrowth microflora (91). Rosenthal et al. (1969) reported penicillin

treatment on a mild infection of *Salmonella* serovar Typhimurium had developed into a severe infection by the ampicillin resistant serovar Typhimurium strain (165).

Increased level of bacterial resistances makes antibiotic therapy less efficacious. Treatment failure due to increased resistance of *Salmonella* to fluoroquinolones such as nalidixic acid and ciprofloxacin has been reported since the 1990s (135). The drugs have been proven to be very effective for treatment of a number of illnesses including typhoid fever and invasive *Salmonella* Virchow infections (146). Infections with antibiotic resistant bacterial strains become more difficult to treat due to limited choice of treatment after diagnosis, and increased virulence of the antibiotic resistance strains (132).

Antibiotic resistant bacteria of animal origin can cause serious human illness and failure in treatment of drug treatment failure in both veterinary and human medicine. More prudent use of antibiotics is needed for controlling of the emergence and spread of antibiotic resistant pathogens

MOBILE ELEMENTS FACILITATING HORIZONTAL TRANSFER OF ANTIBIOTIC RESISTANCE

Sensitive bacterial cells gain resistance to antimicrobial agents through genetic mutations and gene transfer. There are mainly three ways of gene transfer between bacterial cells including transformation, transduction and conjugation (198). Transformation is mediated by chromosomally encoded proteins which develop a regulated physiological state of competence allowing the cells to uptake extracellular DNA. After the uptake, the transferred DNA must integrate into bacterial genome or, in case of plasmid; it must reconstitute a replication-proficient form to persist in the new host cells (198). Natural transformation plays limited role in the transfer of resistance

genes (18). Woegerbauer et al. (2002) reported transformation of plasmid-borne antibiotic resistance genes in *E. coli* wild-type strains was possible by transformation under naturally occurring conditions in aquatic environments containing concentrations of calcium but was suppressed in nitrogen-rich fluid like urine (218). Antibiotic resistance genes can be packaged into bacteriophage and spread *via* transduction. The amount of DNA that can be packaged into a phage head is limited, and the process requires the presence of phage receptors on the surface of host cells. Phage transduction of antibiotic resistance genes are widely demonstrated among bacteria (27, 28, 123, 216). Transfer of Shiga toxin and chloramphenicol resistance genes in *E. coli via* phage transduction was reported under natural condition in house fly gut (152). Conjugation refers to self-transfer of replicating genetic elements including conjugative plasmids, and chromosomally integrated conjugative elements or conjugative transposons. These elements encode proteins that facilitate their transfer both intra-and intercellular. Conjugation is a major antibiotic resistance gene transfer mechanism under *in vivo* condition (178).

Among mobile genetic elements, plasmid, transposons, and integron/gene cassettes are the most important in horizontal transfer of antibiotic resistance genes (177). These elements possess different genetic components therefore are unique in their property and mobility.

Plasmids

Plasmids are extrachromosomal DNA that replicate and control their own copy number. Besides their replication system, plasmids contain genes that encode for accessory traits such as resistance to antimicrobials, metabolic properties, virulence

properties and fertility functions (191). Resistance plasmids can sometimes carry one or more resistance gene(s) in addition to the genes coding for other functions. Large plasmids carry *tra* gene complex (>15 kb in gram-positive bacteria and >30 kb in gram-negative bacteria), encoding for conjugal transfer apparatus can mediate their transfer between cells (178). The conjugation process involves establishment of mating pair, signaling of DNA transfer and transfer of DNA (41, 122). The mating pair apparatus called pilus mediates cell-cell contact and generates a junction and pore through which DNA could be transferred to the recipient cell (41). Relaxase nicks DNA to give a single-stranded form that is suitable for the transfer. Coupling proteins, belonging to the TraG-like family of ATPases, facilitate the DNA transfer through a specialized pore and are thought to "pump" DNA into the recipient cells (80). Conjugative plasmids also act as vectors for nonconjugative transposons and integrons to transfer between bacteria cells. Plasmid NR1 isolated from *Shigella flexneri* is an example of multiple drug resistant plasmid that carries *tra* gene complex as well as transposon Tn*2670* nested with Tn*21* that carries integron-mediated antibiotic resistance genes (183). Small nonconjugative plasmids, however, can utilize the transfer apparatus of the co-reside conjugative plasmid if they have an *ori*T or possibly *mob* (mobilization) genes (178). Conjugative plasmids carrying transposons or integron with one or more antibiotic resistance genes have been widely reported in gram-positive and gram-negative bacterial pathogens (11, 128, 168, 213). Transfers of antibiotic resistance genes in *vivo* condition are believed to mostly occur *via* conjugation and mobilization (178).

The efficiency of plasmid transfer between bacterial strains depends on the plasmid, the donor strain, and the recipient strain (71, 162, 188, 189) as well as other

factors such as temperature, pH, nutrients and mating time conditions. The optimal condition depends on the identity of the plasmid, the characteristics of donors and recipient cells, or various combinations of these and other factors (189).

Various conjugation studies in nutrient broth and environmental samples including wastewater and soil found that low temperatures have a negative effect on plasmid transfer while detected maximum rate of transfer between the range of 20 to 30°C (6, 76, 161, 189). These studies also found maximum transfer frequencies at pH value close to neutral, while acid pH values affect the conjugation negatively (109, 161, 189). Several studies demonstrated that conjugation frequencies increase upon addition of a carbon source. Trevors and Oddie (1986) found that plasmid transfer in sterile stream water occurs only when it is adjusted with diluted nutrient broth (201). Fernandez et al (1992) observed gradual decrease in transfer frequencies as the concentration of total organic carbon in the mating medium descended from 11,510 to less than 1 mg of carbon liter^{-1} (71).

Transposons:

Transposons (Tn) are DNA elements that can transpose from one place to another in bacteria DNA *via* the function mediated by non-homologous recombinase enzyme called transposase. Transposons do not have replication system and must integrate themselves into chromosomal DNA or plasmids. The size of transposons varies from <1 kb to 60 kb (177). Insertion sequence (IS) is the smallest transposon which contains only the transposase gene which allows integration and excision of the element. Composite transposons such as Tn*9* (7), Tn*10* (47, 112), , andTn*5706* (105) contain one or more antimicrobial resistance genes and insertion sequence at the end of the sequence (105)

(47, 112). Complex transposons such as Tn*2610* have inverted repeats that are situated between the antibiotic resistance part and the transposase genes or at each end of the sequence (219). Non-conjugative transposons can integrate themselves as part of a conjugative element for intracellular transfer; however, some transposons carrying *tra* gene complex are conjugative transposons such as Tn*916* family predominant among gram-positive bacteria (160, 168).

Integrons

Integron has been recognized as an effective means of antibiotic resistance gene dissemination. Integron itself is not mobile, but relies on transferable elements such as transposons and conjugative plasmids. Integrons are found as part of these genetic elements as well as on the bacterial chromosome (73).

Structure and classification

An integron is composed of integrase gene, promoter gene, and gene cassette which normally contains genes encodes for resistance to antibiotics or disinfectants. This structure allows integron to capture additional antibiotic resistance genes and to express them under the promoter that resides in the integron. The integrase gene excises and integrates antibiotic resistance gene cassette into or out of the integron at the specific receptor site (*att*). At least three classes of antibiotic-resistance-encoding integrons have been described, and each has its own integrase (85). The majority of integrons described belongs to class 1. In addition to integrase, promoter and the gene cassettes, class 1 integron structures often carry *qacEΔ1* and *sul1*, which are the genes conferring resistance to ethidium bromide/quaternary ammonium compounds (qac) and sulfonamides, respectively. The genes are found at the conserved region located

downstream of the cassettes (also called the 3'conserved segment or 3'CS) however, not all members of class 1 integrons possess *sul1* gene (86).

Class 1 integrons are found in Tn*21* and Tn*21*-related transposons. These transposons generally are located on conjugative plasmid which generally enhances the spreading of gene cassette (72, 73). A gene cassette consists of one coding sequence (usually antibiotic resistance gene) and may contain a variable number of non-translated nucleotides. At the 3' end of the coding sequence, a so-called 59-base element (59-be recombination site) is located which interacts with the *att* specific receptor site on the integrons (86). Class 1 integron gene cassettes encoding resistance against antibiotics cover a wide range of antibiotic classes including *β*-lactam, aminoglycosides, trimethoprim, chloramphenicol, rifampin, quaternary compounds and erythromycin (72, 85).

Class 2 integron is included in the Tn*7* family of transposons containing three integrated gene cassettes (*dfr1-sat-aadA1*) adjacent to a defective integrase gene (*intl2*) located at the 5'CS. The integrase gene of class 2 integron contains an internal stop codon thus there is a limited variation of gene cassettes found among class 2 integrons. The gene cassette *dfr1-sat-aadA1* encodes resistance to trimethoprim, streptothricin and streptomycin, respectively. Tn*7* are transferred to bacterial cells on a conjugative plasmid, however it was shown that the transposons preferentially inserts into a unique site of the bacterial chromosomes from which it is transferred by transposition onto a conjugative plasmid (89). The location of class 2 integron has been indicated on conjugative plasmid and bacterial chromosomes (164).

Class 3 integron has been far less reported than class 1 and class 2 integrons. Gene cassette associated with the third class of integron are gene cassettes of bla_{IMP} genes that confers resistance to broad-spectrum β-lactams including carbapenems (56).

Fluit and Schmitz (2004) reviewed the epidemiology of these three classes of integrons among bacteria. Class 1 integrons have been reported in many gram-negative genera including *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Burkholderia*, *Campylobacter*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Psedomonas*, *Salmonella*, *Serratia*, *Shigella* and *Vibrio*. Class 2 integrons have been found in *Acineobacter*, *Shigella* and *Salmonella*. Class 3 integrons have been described in *Psedomonas aeruginosa*, *Serratia marcescens*, *Alcaligenes xylosoxidans*, *Psedomonas putida* and *Klebsiella pneumoniae* (73).

By using integrons, bacteria can stockpile different exogenous genes to increase their antibiotic resistances. Integrons are not restricted to only gram-negative bacteria, class 1 integrons have also been found in gram-positive bacteria including corynebacteria, aerococci, and staphylococci (125).

Antibiotic resistance gene cassettes

B-lactam resistance: Different resistance genes encoding for β-lactamase have been identified in integron gene cassettes. Most of these genes encode serine β-lactamases belonging to class A (carbenicillin hydrolyzing enzyme) or class D (oxacillin hydrolyzing enzyme) (85). Gene cassette encoding for class A (bla_{P1}, bla_{P2} and bla_{P3}) and class D (bla_{oxa}) β-lactamases are often found in *Pseudomonas* but are also found in *Enterobacteriaceae* (129). Gene encoding for a class B metallo β-lactamase known as

bla$_{IMP1}$ (carbapenems and broad spectrum β-lactam hydrolyzing enzyme) also has been found in class 1 and 3 of gram-negative rods (179).

Aminoglycosides resistance: Variants of *aadA* gene encoding aminoglycoside (3') adenylytransferases, AAD(3'), or streptomycin-spectinomycin modifying enzymes are widely distributed among class 1 integron of gram-negative bacteria. The *aadA1* gene cassette has been found within the transposon Tn*21* and Tn*7* and in antibiotic-resistant strains such as *Salmonella, E. coli, Staphylococcus* and *Klebsiella pneumoniae* (85, 130, 163, 184). Several *aac* genes inactivate various types of aminoglycosides e.g. kanamycin, neomycin, gentamicin, tobramycin, amikacin, and isepamicin. The variants of *aac* such as *aac3*, and *aac6* are widely distributed in resistant strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* (51, 65, 85, 220, 225)

Chloramphenicol resistance: A group of *catB* genes encoding for a new group of chloramphenicol acetyletransferases (CATB) are mostly found among gene cassettes (36, 145). This novel group of CATB enzymes is distinct from the better known chloramphenicol acetyletransferases A (CATA), in both structure and in that they can only acetylate chloramphenicol and not 1-acetylechloramphenicol (145). Several *cmlA* genes are cassette associated encoding inner membrane proteins that confer for resistance *via* efflux pump. The *cmlA* cassettes were found in Tn*1696* of *P. aeruginosa* (25), and on the plasmid of a *Salmonella* Typhimurium strain (38).

Resistance to antiseptics-disinfectants and resistance to sulfonamides (the 3'CS of class 1 integrons): As it has been mentioned previously, the 3'conserved segment of class 1 integron contains a *qacEΔ1* and *sul1* genes that confer resistance to ethidium bromide/the quaternary ammonium compounds (qac) and sulfonamide drugs,

respectively. However, class 1 integrons lacking of *sul1* gene on the 3'CS have been reported (83). The *qacE*Δ*1* is a truncated version of the *qacE* cassette that has lost its 59-be recombination site (149, 156) and encodes for inner membrane protein that exports the quaternary ammonium compounds (149). Hall and Collis (1998) suggested that the presence of *sul1* gene may also have originally been part of a functional cassette (85).

Other antibiotic resistances: Several cassette associated *dfrA* genes encoding trimethoprim-resistant dihydrofolate reductases (DHFR) have been identified. The *dfrA1, dfrA5, dfrA7, dfrA12* and *dfrA14* genes have all been found in clinical and non-clinical isolates from various countries and *dfrA1* is most often associated with Tn*7* (3, 92, 186). The cassette-associated *sat* gene determines for streptothricin acetyltransferase been found in class 1 and 2 integrons (Tn7 and relatives) (187, 195, 200). The presence of a cassette-associated erythromycin resistance *ereA* genes encoding for erythromycin esterase have been identified in *E. coli* class 1 and class 2 integron (24, 48). Rifampin ADP-ribosylating transferase encoding by cassette-associated *arr2* gene has been identified within class 1 integron in *Enterobacteriaceae*, and *Pseudomonas* species (11, 202).

Mode of antibiotic resistance gene transferred mediated by integron

Integron encoded site-specific recombinase enzyme, integrase (IntI) facilitates the insertion of a free circular gene cassette. This insertion occurs between the *att* site of the integron and a 59-be site of the gene cassette (53). The gene cassette is correctly placed in an orientation having the 59-be portion downstream while the expression of the antibiotic resistance genes relies on the upstream promoter (P_{ant}). Further insertions are possible through the same process as evident by integrons carrying multiple gene

cassettes (65). This suggests a potential increase of integrons containing different types of antibiotic resistance genes. The integron encoded integrase can catalyse recombination events that lead to the deletion of cassettes and the formation of free circular cassette (55). This process allows relocation of gene cassette yet recombination between sites of existing integrons in the same cell can also occur (86).

Gene cassettes in an integron are expressed by a common promoter called P_1 (P_{ant}) located upstream of the gene cassettes. However, in some of class 1 integrons, a second promoter, P_2 is present and co-responsible for expression of the resistance genes. Four variants of P_{ant} promoter of class 1 integrons have been sequenced. The strength of P_{ant} promoters relative to the derepressed *E. coli tac* promoter revealed that each variant of the promoters had a different strength. Different versions of P_{ant} promoters lead to differences of up to 20-fold in the level of resistance conferred by the same gene (54, 115).

All of the gene cassettes within an integron are cotranscribed from upstream P_{ant} (or P_{ant} and P_2). The expression of the antibiotic resistance genes vary depending on the versions of the promoters and the location of the gene cassette on the integron. When more than one cassette present in an integron, this event results in low level of the full length transcript of each gene cassette (54). Lower levels of mRNA molecules were detected when the same gene was located further downstream and lower level of resistance was expressed as a consequence (54).

Integron gene cassettes associated antibiotic resistance among *Enterobacteriaceae*

Integron has been associated with antibiotic resistance among *Enterobacteriaceae*. Sallen et al. reported 59% of 49 clinical isolates of six different

species of *Enterobacteriaceae* from one location in France were positive for integrons (167). Similar results were reported in a study by Jones et al (1997). More than half of the 135 clinical isolates of seven species of *Enterobacteriaceae* from the Netherlands carried integrons (99).

Class 1 and class 2 integrons have been described in *Salmonella* (37, 73). The multidrug resistance region (MDR) of *Salmonella enterica* serovar Typhimurium DT104 harboring resistance genes for ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su) and tetracycline (T) has been identified within the same chromosomal locus called *Salmonella* genomic island1 (SG1) (37). Within this region, two class 1 integrons were identified carrying gene cassette of *aadA2* gene and the ampicillin resistance- *pse1* gene, respectively. The region between the integrons contains the *floR* gene conferring resistance to florfenicol-chloramphenicol and the *tetR* and *tetA* conferring resistance to tetracyclines (31, 37).

AadA1 containing integrons were found in serovars Agona, Enteritidis, Hadar, Panama, Poona, Typhimurium, Virchow and Worthington of *Salmonella*. *DfrA1-aadA1* containing integron were present in serovars Brandenburg, Ohio, Panama, and Virchow of *Salmonella*. *AadB-catB, dfrA14-aadA1*, *oxa1-aadA1a* and, *sat1-aadA1a* containing integrons were found in *S*. Typhimurium (84). A class 2 integrons carrying *dfrA1-sat1-aadA1* gene cassettes were identified in serovars of Typhimurium, Panama, Grumpensis and Worthington (164, 206).

A class 1 integron with resistance gene *dfrA1, oxa1* and *aadA2* has been described in *Shigella sonnei* (127, 138). Ahmed et al. (2006) described integron gene cassette found among *Shigella* spp. isolated from human since 2000-2004 in Hiroshima

prefecture, Japan (4). They found classical type of class 2 integron carrying *dfrA1-sat1-aadA1* in *S. sonnei,* and *S. flexineri*. In addition, class 2 integron containing only *dfrA1-sat1* was also found in strains of *S. sonnei*.

Class 1 integrons with gene cassettes of *aadA2, aac4*, *dfr1,* and *dfr9* were identified in *Campylobacter jejuni* and *Campylobacter coli* (78, 113, 142). No class 2 integron has been reported among *Campylobacter* spp. In *E. coli*, the genes commonly found in class 1 integrons were those of aminoglycoside (*aadA)* and trimethoprim *(dfr)* resistances such as *aadA1*, *aadA2*, *blaP1-aadA2*, *dfrA12-aadA2* and *dfrA17-aadA5*. Yu et al. (2003) showed that integron has accumulated gene cassettes over time as they screened class 1 integrons in *E. coli* isolates collected in Korea during the last two decades. Single-gene cassettes were mainly found during the 1980s, while multigene cassettes predominated from the 1990s on (222).

STEC isolates have increased the resistance to multiple antibiotics commonly used in human and veterinary medicine including ampicillin, kanamycin, sulfisoxazole, streptomycin, tetracyclines and ticarcillin (214). The most common antibiotic resistance pattern was to streptomycin-sulfisoxazole-tetracycline (187, 224). Integron associated antibiotic resistances in STEC has been studied by several groups of researchers (187, 224). The study by Zhao et al. in 2001 reported class 1 integrons were identified among STEC serotype O157:H7, O111:H11, O111:H8, O111:NM, O103:H2, O45:H2, O26:H11 and O5:NM. The integron gene cassettes found in these isolates are of *aadA* genes and *dfrA* genes. In 2005, the same group of researchers identified class 1 integrons with variants of the *aadA* gene and *dfrA* gene, as well as a novel gene cassette of *sat* gene.

Similar gene cassettes were observed among STEC class 1 integrons reported in recent studies (187, 205, 214).

Class 2 integrons were identified among clinical isolates of *E. coli* including STEC serotypes O145 and O111 from human and cattle (82, 205). The class 2 integron often carried *dfA1- sat1 - aadA1* gene cassette. Only limited variation of class 2 integron was found due to mutation at codon position 179 resulted in stop codon as mentioned earlier (73).

Conjugal transfer of integron harbored plasmid in *Enterobacteriaceae*

As mentioned earlier, integrons rely on conjugative plasmid or transposons for their mobility. Zhao et al. (2001) reported the transfer of integron from *E. coli* O157:H7 and O111: NM to another strain of *E. coli* O157:H7 and to several strains of *Hafnia alvei* (224). Kang et al. (2005) reported transferable class 1 integrons carrying different set of gene cassettes including *dfrA1-aadA2*, *dfrA12-orfF-aadA2,* and *dfrA17-aadA5* gene cassette between *E. coli* isolates. A non-transferable class 1 integrons was however, observed on *E. coli* chromosome (101). A plasmid-borne (>70 kb) class 1 integron with a bla_{oxa} gene cassette in *S.* Typhimurium was transferred to a susceptible strain of *E. coli* K802N. The *Salmonella* donor and its transconjugants were also resistant to amoxicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline. These resistances were all encoded by genes on the same plasmid carrying a bla_{oxa} integron gene cassette (9). Transfer of integron mediated antibiotic resistance genes often associated with co-transfer of non-integron mediated antibiotic resistance genes located on the same conjugative plasmid. An example of *S. sonnei* plasmid carrying class 1 integron with

dfrA12-orfF-aadA2 gene also encodes for resistance to tetracycline, kanamycin and ampicillin (180).

Class 1 integrons in STEC strains were transferable *via* conjugative plasmid to *E. coli* recipient strain *in vitro* (187, 224). The antibiotic resistance genes that are most frequently co-transferred with the integron mediated antibiotic resistances in STEC are the genes that encode the resistance to tetracycline (101, 187, 193). These suggest a genetic linkage between the two types of genes or possibility of co transferring of different plasmids that contain these two genes.

Similar to class 1 integrons, genetic locations of class 2 integrons were identified on both chromosomal DNA and conjugative plasmid (4, 164, 180). Rodriguez et al. (2007) reported that strains of *Salmonella enterica* serovar Virchow carried two copies of a class 2 integron: one copy on a intact Tn7 transposon located on the chromosome, and a second copy of the integron on a truncated Tn*7* transposon located on a conjugative plasmids (164). The plasmid mediated integron was transferable to a strain of *E. coli* K12. The *dfrA1-sat-aadA1* gene cassette of a class 2 integron located on the Tn7 in a strain of *S. sonnei* isolates was nontransferable and later found to be located on chromosomal DNA (180). Class 2 integron in strains *E. coli* isolated in the Netherlands in 2004 with the gene cassette *dfrA1-sat-aadA1* were demonstrated to be transferable (206).

The mating/incubating times used by different research groups vary, but are typical between 16 and 24 h (93, 101, 114). The transfer of genetic materials between the donor and recipient cells depend on bacterial strains and experimental conditions, but could take place within 3-4 min (8). The cells of transconjugants grow during the

extended incubation period resulting in misleadingly higher conjugation efficiencies. With an overnight mating/incubation period, the conjugation efficiencies of plasmid carrying integron among *Enterobacteriaceae* were reported at 10^{-2} to 10^{-4} in nutrient broth (114, 194). Transfer frequencies of integron from *E. coli, K.oxytoca,* and *E. cloacae* to *E. coli* K 12 strain in LB broth were reported at 1 to 3 x 10^{-2} transconjugants per recipient cell; from *C. freundii* at 4 to 8 x 10^{-3} transconjugants per recipient cell and from *K. pneumoniae* at 10^{-2} to 10^{-4} transconjugants per recipient cells (114).

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CHAPTER 3

INTEGRON MEDIATED ANTIBIOTIC RESISTANCE IN SHITGA TOXIN-PRODUCING *ESCHERICHIA COLI* [1]

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ABSTRACT

The aim of this study was to investigate the prevalence of integrons among a group of Shiga toxin-producing *Escherichia coli* (STEC), and the abilities of the integrons in dissemination of antibiotic resistance genes from STEC to *E. coli* K-12 MG1655. A total of 177 STEC isolates from our laboratory collection were analyzed for antimicrobial susceptibility and the presence of integrons. Class 1 integrons were detected in 14 STEC isolates and a class 2 integron was identified in 1 STEC isolate. The class 1 integron positive STEC isolates resisted streptomycin (MICs >128 μg/ml) and sulfisoxazole (MICs >1,024 μg/ml), while the class 2 integron positive isolate resisted streptomycin (MIC =128 μg/ml), trimethoprim (MIC >256 μg/ml), and streptothricin (MIC >32 μg/ml). Results of restriction digestion and nucleotide sequencing revealed that the cassette regions of the class 1 integrons amplified from the 14 STEC isolates had a uniform size of approximately 1.1 kb and contained a nucleotide sequence identical to that of *aadA1*. The class 2 integron cassette region amplified from the STEC isolate was at a size of 2.0 kb and carried nucleotide sequences homologous to those of*, dfrA1, sat1,* and *aadA1*. Conjugation between the integron positive isolates and MG1655 suggested that horizontal transfers of class 1 integrons through conjugative plasmids are responsible for the dissemination of antibiotic resistance genes from STEC to MG1655. Antibiotic resistance traits not mediated by integrons such as resistance to tetracycline and oxytetracycline were co-transferred with the integron mediated antibiotic resistance genes.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is an important group of foodborne pathogens, that cause a broad range of manifestations such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (25). STEC strains have more than 200 serotypes, only a few serotypes are however, responsible for the majority of recorded outbreaks and sporadic cases of STEC-associated diseases in humans (8, 21, 24, 40). In the United States, approximately 73,000 cases of food-related illnesses were estimated to have attributed to serotype O157:H7 and more than 36,000 to STEC with non-O157:H7 serotypes each year (22). STEC cells colonize in the gastrointestinal tracts of animals. Fecal contamination of foods of animal origin is one of the major causes of STEC infection to humans. Treatment with antibiotics in the early stage of STEC infection has been suggested to prevent disease progression (36). However, antibiotic treatment to STEC infection is considered controversial. The mechanisms of how antibiotics increase the risk of disease progression remain unknown, but they could involve bacterial lysis which liberates Shiga toxins (15, 41).

Antibiotics are used for disease prevention and growth promotion in animal production in addition to therapeutic use in veterinary and human medicine. The non-therapeutic use of antibiotics is speculated to play a role in the increase in the dissemination of antibiotic resistances in pathogenic bacteria. It is believed that bacteria develop resistance in response to the presence of antibiotic pressures. Ratnam et al. reported in 1988 that strains of STEC including those of O157:H7 were susceptible to many antibiotics such as ampicillin, cephalothin, tetracycline, kanamycin, trimethoprim, sulfisoxazole, and nalidixic acids (29). Recent studies have however, shown that

antibiotic resistance in STEC is on the rise (5, 44). Strains of STEC have recently been found to resist many of the antibiotics commonly used in human and veterinary medicine (6, 23, 34). The most commonly resisted antibiotics include streptomycin, sulfisoxazole, and tetracycline (5, 44).

Horizontal transfer of antibiotic resistance genes is believed to be one of the mechanisms contributing to the increased resistance of STEC to various antibiotics. Self-transmissible DNA elements such as plasmid, transposons, and bacteriophages all facilitate the acquisition and dissemination of antibiotic resistance genes. In addition, a genetic element known as integron has been shown to play a role in the acquisition and dissemination of antibiotic resistance genes among bacteria (14, 43). The integron is a site-specific recombination system that recognizes and captures mobile gene cassettes which normally encode for antibiotic resistance. An integron encoded site-specific recombinase enzyme, integrase, mediates the insertion of the gene cassettes into integrons at the *attI* recombination site (11). The insertion places gene cassettes in the correct orientation and the expression of the antibiotic resistance genes relies on the upstream promoter (P_{ant}) in the integron (11). Three classes of antibiotic-resistance-encoding integrons and over 60 distinct gene cassettes have been identified (8, 10, 30). These integrons are distinguished by their respective integrase genes (42). Class 1 integrons are most commonly found in clinical isolates of gram-negative bacteria (10). Relatively little is known about integrons in STEC strains. The objective of this study was to determine the prevalence of integrons in a group of STEC strains and the role of integrons in dissemination of antibiotic resistance from STEC to a nonpathogenic *E. coli* strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 177 STEC isolates from our laboratory collection were inoculated on tryptic soy agar (TSA; Becton, Dickinson and Co., Sparks, MD) and grown at 37°C for 16 h. A nalidixic acid resistant derivative of *E. coli* K-12 MG1655 strain CA32 was obtained from Dr. Wondwossen A. Gebreyes at North Carolina State University, Raleigh, NC, USA. All cultures were examined for purity on MacConkey (MAC) and Sorbitol MacConkey agar (SMAC) (Becton, Dickinson and Co., Sparks, MD) under the same growth conditions described above. A single colony from MAC and SMAC plate was streaked on TSA and incubated under the same growth conditions. The resulting cultures from TSA were used in later experiments.

Detection of integrase gene and integron gene cassettes. The conserved regions of integrase genes, *intI1, intI2,* and *intI3* were amplified using degenerate primers hep35-36 as described by White et al (42, 43). Cassette regions of the class 1 and class 2 integron were amplified using primers hep58-59 and hep74-51, respectively following the method of White et al (42, 43). The primers were synthesized by Invitrogen Life Technologies (Carlsbad, CA), and their nucleotide sequences are shown in Table 3.1. The DNA template was prepared by boiling lysis of 1 ml overnight-grown STEC culture in Luria-Bertani (LB) broth (DIFCO Laboratories, Detroit, MI) at 37°C for 16 h with shaking. The cell cultures were centrifuged for 3 min at 16,000 × *g* (Eppendorf Centrifuge 5415 C, Westbury, NY). The pellets were washed twice with, and re-suspended in 100 µl of sterile distilled water before being heated in boiling water for 10 min. Total cellular DNA was collected from the supernatant fluid after centrifugation at

16,000 x *g* for 5 min and used as the DNA template. PCR amplifications were performed in a 50 µl of PCR mix containing 1 U of *Taq* DNA polymerase (Promega, Madison, WI), 0.1 mM of each deoxynucleoside triphosphate (Roche Diagnostics, Indianapolis, IN), 1.5 mM of $MgCl_2$ (Promega, Madison, WI) , 5 µl of DNA template, and 32 µl of dH_2O. DNA thermal cycler PE 480 was used for PCR amplification (Perkins Elmer, Waltham, MA). The thermal cycling reaction parameters were 30 cycles at 94°C for 2 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min before the samples were maintained at 4°C. Amplified products were electrophoresed on 0.8% agarose gels (GIBCO BRL, Gaithersburg, MD) which were stained with 0.5 µg/ml of ethidium bromide (Fisher Biotech, Pittsburgh, PA). The agarose gels were de-stained in distilled water before being visualized under shortwave UV light, and photographed with a gel documentation system (Bio-Rad, Hercules, CA.).

Nucleotide sequence analysis of integrase genes and integron gene cassettes. The PCR products amplified from the conserved regions of the integrase genes were digested with either *Hinf*I or *Rsa*I (Promega, Madison, WI) in order to determine the class of the integron carried by each STEC isolate (43). The PCR products amplified from the class 1 integron cassette regions were digested with restriction enzyme *Bcl*I, *Bgl*I, *Dra*III, *Hae*II, and *Sty*I, respectively in order to identify possible nucleotide sequence variations on the gene cassettes. The *Bcl*I, *Bgl*I, and *Sty*I were purchased from Fisher BioReagents, (Fairlawn, NJ), while the *Dra*III and *Hae*II were purchased from New England Biolabs (Boston, MA). The digestion reactions were performed in a 25 µl of reaction mix containing 5 µl of each PCR product, 2.5 µl of 10 x restriction enzyme buffer, 0.5 µl of acetylated bovine serum albumin, 2 µl of restriction enzyme, and 10 µl of dH_2O.

Additionally, PCR products amplified from the class 1 and class 2 integron cassette regions in STEC 7-13 and 7-38, respectively were sequenced, using the ABI 3100 sequencer (Applied Biosystems, Foster City, CA). Prior to DNA sequencing analysis, the PCR products were purified using the micro-column YM-100 (Millipore Co., Bedford, MA) according to the manufacturer's instruction. Briefly, approximately 250 µl of each PCR product were placed into the sample reservoir of the filtrate vial and centrifuged at 500 x *g* for 10 min. Following centrifugation, the sample reservoir was removed from the filtrate vial and placed upside down into a new vial for recovery of the purified PCR product after centrifugation at 1,000 x *g* for 3 min.

Antimicrobial susceptibility of STEC strains. Antibiotic resistance profiles of the integron-positive STEC isolates and the MG1655 strain were determined by the standard disc diffusion assay according to the guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS) (currently the Clinical and Laboratory Standards Institute) (27). A single colony of each STEC culture from a TSA plate was transferred into tryptic soy broth (TSB) (Becton, Dickinson and Co., Sparks, MD) and incubated at 37°C for 12 h. The cell populations of the resulting cultures were adjusted with TSB to an optical density of 0.1 at 600 nm (Novaspec II Visible Spectrophotometer, Pharmacia Biotech, Cambridge, UK). Each adjusted STEC culture was inoculated onto a Mueller-Hinton agar (Becton, Dickinson and Co., Sparks, MD) plate with a sterile cotton swab. The plate was allowed to dry for 5 min before five different antibiotic discs (Sensi-Disc Antimicrobial Susceptibility Test Discs, BBL, Becton Dickinson) were placed onto each plate using sterile forceps. The plates were incubated at 37°C for 16 h. A total of twelve different antibiotics were tested which

included ampicillin (AM), cephalothin (CF), chloramphenicol (C), gentamicin (GM), kanamycin (K), nalidixic acid (NA), neomycin (N), novobiocin (NB), oxytetracycline (T), streptomycin (S), sulfisoxazole (G), and tetracycline (TE). The diameter of the zone of growth inhibition around each disc was measured to the nearest whole mm, and each tested STEC strain was determined as resistant, intermediate, or susceptible to the antibiotics tested according to the guidelines provided by the supplier of the antibiotic discs.

Minimum inhibitory concentration (MIC). The MICs of seven different antibiotics towards the cells of the STEC isolates and the MG1655 strain, respectively were determined by a twofold macrodilution method (26). The antibiotics included ampicillin, chloramphenicol, kanamycin, oxytetracycline, sulfisoxazole, tetracycline (Sigma Chemical Company, St. Louis, MO), and streptomycin (MP Biochemicals, Solon, Ohio) because these antibiotics were commonly resisted by the integron positive STEC isolates tested in this study. The STEC isolate positive for the class 2 integron was also tested for trimethoprim and streptothricin (MP Biochemicals, Solon, Ohio) resistance in addition to the antibiotics mentioned above. All tested antibiotics were weighed, dissolved, diluted in the appropriate diluents, and added to TSB to make a dilution series of 0.25-512 μg/ml except for sulfisoxazole (0.25-1,024 μg/ml), trimethoprim (0.25-256 μg/ml), and streptothricin (0.25 -32 μg/ml). A single colony of each STEC isolate was inoculated in TSB and incubated at 37°C for 12 h. The cell population of the culture was adjusted to obtain 10^5 CFU/ml before inoculation into TSB supplemented with an appropriate concentration of each tested antibiotic. The inoculated broth cultures were incubated at 37°C for 16 h without shaking. The MIC of each antibiotic was determined

as the lowest concentration of the antibiotic that completely inhibited the growth of STEC cells.

Conjugation. Six selected class 1 integron positive STEC isolates and one class 2 integron positive STEC isolate were used as donor strains, and the Nal^r derivate of *E. coli* MG1655 strain CA32 was used as the recipient strain in the conjugation experiment. Both the donor and recipient strains were purified on SMAC prior to each conjugation experiment. A single colony of each culture was inoculated in TSB and grown at 37°C for 12 h. The optimal density of each culture was adjusted to 0.08-0.10 at a wavelength of 600 nm (approximately 10^8 CFU/ml). The donor and recipient cells were subsequently mixed at a ratio of 1:10 in a sterile microcentrifuge tube and mated for 90 min at 37°C without shaking. Subsequently, 100 µl of the donor and recipient mixture were inoculated onto TSA plates and incubated at 37°C for 16 h. The antibiotic resistant transconjugants were selected on SMAC agar plates supplemented with nalidixic acid (100 µg/ml) and one of the following antibiotics, dependent upon the donor strain used, including ampicillin (100 µg/ml), chloramphenicol (50 µg/ml), kanamycin (50 µg/ml), oxytetracycline (30 µg/ml), streptomycin (100 µg/ml), streptothricin (30 µg/ml), tetracycline (30 µg/ml) or trimethoprim (50 µg/ml). Conjugation efficiencies were determined by mating without the overnight incubating period followed by plating the donor-recipient mixture on SMAC plates supplemented with nalidixic acid (100 µg/ml) and one of the following antibiotics: streptomycin (100 µg/ml), tetracycline (30 µg/ml) or oxytetracycline (30 µg/ml). The populations of the recipient cells per ml of the culture were determined by plating appropriate dilutions of *E. coli* MG1655 on SMAC plates.

Conjugation efficiencies were calculated as the ratio of the number of transconjugants to the number of recipients.

Characterization of antibiotic resistant transconjugants. To confirm the transfer of integron-related antibiotic resistance genes, each type of obtained transconjugants was examined for 1). antibiotic susceptibility, 2). plasmid profile, and 3) presence of integrons and integron-related antibiotic resistance genes. The experiment was designed based on the assumption that integron associated antibiotic resistance genes are located either on STEC chromosomes or transmissible plasmids. If the transfer of integron-associated antibiotic resistance genes is mediated by a conjugative plasmid, the susceptible recipient cells will become resistant by acquiring the antibiotic resistance genes on the plasmid through conjugation. Consequently, the plasmid can be detected in the transconjugant cells, and the integrons and integron-related antibiotic resistance genes will be detected on the conjugative plasmid.

The antibiotic susceptibility of the transconjugants was determined using the disc diffusion method described above. Integron gene cassettes were detected by PCR using the plasmid DNA of the transconjugant cells as templates. Respective donor strains were included in each experiment as controls. The plasmid profiles of the donors and the transconjugant cells were analyzed and compared. Plasmid DNA was extracted using the High Pure Plasmid Isolation kit (Roche Diagnostics, Indianapolis, IN). Specifically, a single colony of each isolate was inoculated into 10 ml of LB broth and incubated at 37°C for 16 h. The cell pellet was obtained from centrifugation at 3,200 x *g* for 30 min (GS-6R centrifuge, Beckman, Fullerton, CA). The pellet was re-suspended in 300 µl of suspension buffer (50 mM Tris-HCl ,10 mM EDTA, pH 8.0 and 1 mg/ml RNase), and the

resulting suspension was transferred to a sterile microcentrifuge tube for centrifugation at 16,000 × *g* using an Eppendorf Centrifuge (Model 5415 C)). The re-suspended pellet was mixed well, and 300 µl of lysis buffer (0.2 M NaOH and 1% SDS) were subsequently added. The mixture was incubated at room temperature for 5 min. After the incubation, 400 µl of chilled binding buffer (4 M guanidine hydrochloride and 0.5 M potassium acetate pH 4.2) were added. The tube was incubated on ice for 5 min and centrifuged at 16,000 x *g* for 10 min. The supernatant obtained from centrifugation was transferred into a High Pure plasmid filter tube and washed with 700 µl of washing buffer (20 mM NaCl, and 2 mM Tris-HCl, pH 7.5 in ethanol solution). After washing, the filter tube was transferred into a new sterile microcentrifuge tube into which 100 µl of elution buffer (10 mM Tris-HCl, pH 8.5) were added for the recovering of plasmid DNA. The extracted plasmid DNA was analyzed by agarose gel electrophoresis for the presence of conjugative plasmids.

RESULTS

Presence of integron and integron-associated genes. Of the 177 STEC isolates tested, 15 (8.5%) tested positive for the 491 bp *intI* PCR product. The PCR products amplified from these 15 STEC isolates were digested with *Rsa*I and *Hinf*I, respectively. The analysis revealed that 14 (7.9%) STEC isolates carried the *intI1*, and 1 isolate (0.6%) carried the *intI2* (Table 3.2). The serotypes of the class 1 integron positive STEC isolates included O18:H7, O26:H$^-$, O26:H11, O55:H$^-$, O55:H6, O103:H2, O111:H$^-$, O111:H8, and O157:H7. The serotype of the class 2 integron positive STEC isolate was O55:H7.

To identify the structures of the integrons, the cassette regions on the class 1 and class 2 integron, respectively were amplified by using total cellular DNA of the STEC

isolates as templates. Results of the PCR amplification revealed that the class 1 integron cassette regions in the 14 STEC strains had a uniform size of approximately 1.1 kb (Fig. 3.1). Additionally, a PCR product of approximately 200 bp was amplified from 4 STEC isolates using the primer pair hep58-59 (Fig. 3.1). These four isolates however, tested negative for the *intI1*. Class 2 integron was detected in only 1 STEC isolate and the size of the cassette region was approximately 2.0 kb (Fig. 3.1).

Analysis of integron gene cassette. To identify possible nucleotide sequence variations, the 1.1 kb PCR products amplified from the class 1 integron cassette region of the 14 STEC isolates, respectively was digested with *Bcl*I, *Bgl*I, *Dra*III, *Hae*II, and *Sty*I, respectively. The results showed that all 14 PCR products had the same restriction patterns (Fig. 3.2) indicating that the class 1 integron shared similar structures. Nucleotide sequencing revealed that the class 1 integron contained a single gene cassette for aminoglycoside adenylyltransferase (*aadA*) (GenBank accession number ABI188267) which encoded resistance to streptomycin and spectinomycin. The class 2 integron contained three gene cassettes for dihydrofolate reductase (*dfrA1*), streptothricin acetyltransferase (*sat1*), and aminoglycoside adenylyltransferase (*aadA1*) (GenBank accession number ABI188272) which encoded resistance to trimethoprim, streptothricin, and streptomycin/spectinomycin, respectively. The nucleotide sequences of the class 1 and class 2 integron gene cassettes shared approximately 99 and 98% homologous, respectively (Data not shown) with their respective sequences previously deposited in the GenBank (accession number ABI188267 and ABI188272).

Nucleotide sequence analysis also revealed that the ~200 bp PCR product amplified from STEC 7-62 was flanked by an approximately 400 bp sequence at the 5'

end (position 220-633), which shared 89% homology with a short segment (position 775-1173) of the *aadA1* (GenBank accession number EF417896.1) on *E. coli* class 1 integron, and an approximately 200 bp sequence at the 3' end (position 6-195), which shared 96% homology with a small region (position 5046054 – 5046269) of a hypothetical protein (GenBank accession number BA000007) of *E. coli* O157:H7 Sakai strain.

Antimicrobial susceptibility and minimal inhibitory concentration. The antimicrobial susceptibility of the integron-positive isolates is shown in Table 3.3. All of the integron positive isolates examined in this study were resistant to at least three different antibiotics. STEC isolates positive for the class 1 integron demonstrated the resistances conferred by the genes located on the class 1 integron, i.e. resistance to sulfisoxazole (14/14) and streptomycin (14/14). In addition to these, they were also resistant to tetracycline (11/14), oxytetracycline (11/14), novobiocin (11/14), cephalothin (5/14), and/or chloramphenicol (4/14). Three separate STEC isolates among the 14 class 1 integron positive STEC isolates examined in the study were resistant to ampicillin, kanamycin or neomycin. All examined isolates were susceptible to gentamicin. The majority of class 1 integron positive isolates (10/14) shared the same antibiotic resistance pattern having resistance to sulfisoxazole, streptomycin, as well as novobiocin, tetracycline and oxytetracycline. The 4 STEC isolates, from which the ~200 bp PCR products were amplified, were resistant to novobiocin. Two of them were also resistant to cephalothin. All four of them were however, susceptible for all other antibiotics tested in the study including streptomycin and sulfisoxazole. The STEC isolate positive for the class 2 integron was resistant to trimethoprim, streptomycin and streptothricin, which

were encoded by the class 2 integron gene cassettes, as well as neomycin and novobiocin. The recipient strain MG1655 was resistant to only novobiocin and nalidixic acid.

The MICs of the antibiotics against selected integron positive STEC isolates and *E. coli* MG1655 are shown in Table 3.4. The STEC isolates positive for the class 1 integron were all resistant to streptomycin and sulfisoxazole, and the MICs of streptomycin and sulfisoxazole were 128-512 and >1,024 μg/ml, respectively. The MICs of streptomycin, trimethoprim, and streptothricin against the class 2 integron positive STEC isolate were 128, >256, and >32 μg/ml, respectively. Resistance to tetracycline and oxytetracycline were not controlled by integrons but are common in class 1 integron positive STEC isolates examined in this study. Their MICs were also determined and documented in Table 3.4.

Conjugal transfer of integrons. Results of the conjugation experiments showed that the class 1 integrons in 5 out of the 6 selected STEC donor strains, 6-20, 6-22, 7-13, 7-57, and 7-63 were transferred to MG1655. The conjugation efficiencies of streptomycin resistance gene ranged from 3.33×10^{-10} to 2.00×10^{-9} (Table 3.5). The conjugation efficiencies for tetracycline and oxytetracycline resistance genes were 3.33×10^{-10} to 8.67×10^{-5}, and 3.33×10^{-10} to 3.23×10^{-4}, respectively (Table 3.5). The class 1 integron in STEC 6-35 was either not transferable or had a conjugation efficiency lower than 3.33×10^{-10}. No transconjugant was obtained from the conjugation experiment involving the class 2 integron positive isolate and MG1655.

Analysis of transconjugants. Among the transconjugants obtained from the conjugation experiments involving 6-20, 6-22, 7-13, 7-57, and 7-63, respectively, integron-mediated resistances to streptomycin and sulfisoxazole were detected. Non-

integron-mediated antibiotic resistance genes, i.e. genes for tetracycline and oxytetracycline resistance were co-transferred from 6-20, 6-22, 7-13, and 7-63 with the class 1 integron mediated antibiotic resistance genes. The transconjugants recovered by using streptomycin as a selectable pressure were found to be resistant to tetracycline and oxytetracycline. Similarly, transconjugants collected by using tetracycline and oxytetracycline as selectable makers were resistant to streptomycin. The MICs of streptomycin against the transconjugants selected by using streptomycin were 128-256 μg/ml, whereas the MICs of streptomycin against the transconjugants selected by using tetracycline and oxytetracycline were 128 μg/ml. These values were slightly lower than the MICs of the antibiotics against their respective donors (128-512 μg/ml). It was noticed that transconjugants derived from STEC 7-57 and 7-63 selected by using streptomycin had lower resistance to streptomycin (256 μg/ml) than their respective donors (512 μg/ml). The transconjugants derived from STEC 7-57 and MG1655 shared the same antibiotic resistance pattern with STEC 7-57 donor having resistance to streptomycin and sulfisoxazole, but not tetracycline and oxytetracycline. The plasmid DNA from 6-20, 6-22, 7-13, 7-57, and 7-63 as well as 6-35 tested positive for the class 1 integron. The plasmid DNA from the respective transconjugants of 6-20, 6-22, 7-13, 7-57, and 7-63 also tested positive for the class 1 integron. However, the class 2 integron and integrase genes were not detected from the plasmid DNA of the isolate positive for the class 2 integron.

DISCUSSION

Integrons and antibiotic resistances found in STEC isolates. The results of the present study showed that class 1 integrons were more common than class 2 integrons

among the examined STEC isolates. These results were in agreement with previous studies involving *E. coli* isolates from human and animal origins (13, 33). Among the 16 multiple-drug resistant *E. coli* isolates selected from the 515 tested strains in the study of Saenz et al. class 1 integrons were detected in 11 isolates, class 2 integrons were detected in 3 isolates, and 1 isolate tested positive for both types of integrons. Kang et al. detected class 1 integrons in 175 *E. coli* isolates, class 2 integrons in 9 isolates, and both integrons in 5 isolates out of the 664 *E. coli* isolates examined in their study. Results of the antibiotic susceptibility test in the present study agree with those reported in previous literature. In addition to integron mediated streptomycin and sulfisoxazole resistance, tetracycline and oxytetracycline resistance were also observed (37, 44).

Analysis of gene cassettes on integrons. Although class 1 integrons with more complicated gene cassettes have been reported previously (14, 37, 44), the present study identified only the class 1 integrons with the gene cassettes carrying a single antibiotic resistance gene, *aadA1*. Class 1 integrons with gene cassettes similar to those identified in the present study have been observed previously in *E. coli* isolated from human and animal origins as well as clinical isolates of *Vibrio cholera* O139 and *Salmonella* (3, 14, 16, 37, 44). The prevalence of the *aadA1* gene cassette among the class 1 integrons shown by the present and previous studies pointed to a common origin of the gene cassettes. Martinez-Freijo et al. studied the distinct structures of class 1 integrons among species of *Enterobacteriaceae,* including those with the 1.1 kb inserted regions containing only *aadA*1 (19). The study revealed that transfer of such integrons is a more frequent event compared to the occurrence of gene transfer mediated by other

mechanisms, which could explain why the class 1 integrons with the 1.1 kb *aadA*1 gene cassettes are commonly found among *E. coli* strains (18, 19).

According to previous literature, class 2 integrons were found to be associated with six different resistance gene cassettes including *sat1*, *ere*(A), *aadA1, dfrA1, dfrA14*, and *sat2* (4, 20, 28). The gene cassettes of class 2 integron found in this study were the same as those embedded in Tn*7* transposons in *Salmonella,* and *E. coli* (13, 32). The limited diversity of class 2 integron gene cassettes is most likely due to the fact that the gene of the integrase contains a nonsense mutation codon at position 179 and thus yields a non-functional protein (12).

Transfer of integrons and antibiotic resistance genes. The sequencing data of the present study suggested that the class 1 integron were responsible for the resistance to only streptomycin and sulfisoxazole. The results of the conjugation experiments have shown however, that multiple resistances including resistance to ampicillin, chloramphenicol, kanamycin, tetracycline, and oxytetracycline were transferred to the recipient cells. Plasmid profile showed that the donor and their respective transconjugants cells might carry one or more conjugative plasmid(s). Transconjugants might become resistant to tetracycline, oxytetracycline as well as other antibiotics through acquisition of the resistance genes on the plasmid which carried the integron, or on a different plasmid that was co-transferred by conjugation with the integron-bearing plasmid. Sunde and Norstrom reported *tetA*, the gene encoding for tetracycline resistance and class 1 integrons in *E. coli* are often located on the same conjugative plasmid (38). Agerso and Sandvang reported that class 1 integron and *tetA* gene of *Alcaligenes faecalis*

were detected on a plasmid of approximately 36 kb in both donor and transconjugant cells by using DNA hybridization (1).

The conjugation efficiencies observed in this study were much lower than those reported by previous studies (17, 39). Sunde and Sorum reported the conjugation transfer of class 1 integron transmissible plasmid in *E. coli* at 4×10^{-4} to 6×10^{-2} (39). Similar rates of conjugation transfer were reported by Leverstein et al. (17). who studied the transfer of integron-mediated resistance genes among *Enterobacteriaceae*. These differences were most likely due to the conjugation procedure used in this study, which included a 90 min mating period at 37°C while in the study of Leverstein et al. a longer mating/incubating time of 24 h was used. The longer incubation could lead to an increase in growth of transconjugants cells and therefore, result in misleading conjugation efficiencies. Antibiotic resistant transconjugants were not recovered from the conjugation experiment involving STEC 6-35. Class 1 integron was however, detected on its plasmid. This suggested that the plasmid carried by 6-35 was either not transferable or had a lower than detectable conjugation efficiency ($<3.33 \times 10^{-10}$). Increasing the volumes of the conjugation mixture or the populations of the donor and recipient may be able to slightly improve the detection limit but will not be able to prove whether the plasmid was indeed transferable.

The conjugation experiment involving the class 2 integron positive STEC isolate did not yield any transconjugants. A previous study reported that a class 2 integron carried by transposon Tn*7* integrated into a unique site of bacterial chromosomes or conjugative plasmid (12). Class 2 integrons have been detected from the chromosome of *Vibrio cholerae*, *Salmonella enterica* serovar Virchow and *Shigella sonnei* (2, 31, 35).

The class 2 integron in *S. sonnei* was reportedly non transferable, and antibiotic resistant transconjugants were therefore, not detected after conjugation (35). The class 2 integron was not detected on the plasmid DNA of the STEC isolate positive for the class 2 integron in the present study and no transconjugants were recovered, suggesting that the integron may not be plasmid-borne.

In summary, the results of this study showed that a certain percentage of the STEC isolates examined in the present study was resistant to the selected antibiotics. The commonly resisted antibiotics include streptomycin, sulfisoxazole, as well as tetracycline and oxytetracycline. The presence of class 1 integrons in the STEC isolates may offer an explanation for their common resistance to sulfisoxazole and streptomycin. These integrons are responsible for the horizontal transfer of the antibiotic resistance genes from STEC to *E. coli* MG1655 under laboratory conditions. Conjugative plasmid plays a crucial role in the dissemination of both integron and non integron mediated antibiotic resistance genes. Intensive use of antibiotics at sub-therapeutic levels during food animal production may have provided the pressure to select the antibiotic resistance bacteria in an agricultural environment.

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TABLE 3.1. Primer sequences, target genes and sizes of amplified PCR products

Primers	Sequences	Target genes	Sizes of PCR products	References
hep 35 hep 36	TGCGGGTYAARGATBTKGATTT CARCACATGCGTRTARAT B=C or G or T, K=G or T, R=A or G and Y=C or T	The conserved regions of integron-encoded integrase genes *intI1, intI2,* and *intI3*	491 bp	(43)
hep 58 hep 59	TCATGGCTTGTTATGACTGT GTAGGGCTTATTATGCACGC	Class 1 integron gene cassette	1.1 kb	(43)
hep 51 hep 74	GATGCCATCGCAAGTACGAG CGGGATCCCGGACGGCATGCACGATTTGTA	Class 2 integron gene cassette	2 kb	(43)

TABLE 3.2. Classification of integrons found in the STEC isolates

Class of integron	Serotype	Product size resulted from enzyme restriction analysis	
		RsaI	*HinfI*
Class 1 integron	O18:H7, O26:H-, O26:H11, O55:H-, O55:H6, O103:H2, O111:H-, O111:H8, and O157:H7	491 bp	491 bp
Class 2 integron	O55:H7	334,157 bp	300,191 bp

TABLE 3.3. Antibiotic resistance profiles of the integron positive STEC strains [a] and the MG1655 *E. coli* K-12 recipient strain.

Antibiotic agents	5-1	6-9	6-11	6-20	6-22	6-25	6-35	7-13	7-34	7-35	7-57	7-60	7-63	7-125	7-38 [b]	Recipient strain MG1655
AM	S[c]	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S
CF	S	I[c]	S	S	S	R	R	S	R	R	S	S	S	S	S	S
C	S	S	S	S	S	S	R	R	R	R	S	S	S	S	S	S
GM	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
K	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
NA	S	I	S	S	S	S	S	S	R	S	S	S	S	S	S	R
N	S	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S
NB	R[c]	R	S	R	R	R	R	R	S	S	R	R	R	R	R	R
T	R	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S
S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S
G	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S
TE	R	R	R	R	R	R	R	R	S	S	S	R	R	R	S	S

[a] Antibiotic agents include ampicillin (AM), cephalothin (CF), chloramphenicol (C), gentamicin (GM), kanamycin (K), nalidixic Acid (NA), neomycin (N), novobiocin (NB), oxytetracycline (T), streptomycin (S), sulfisoxazole (G), and tetracycline (TE). [b]Class 2 integron positive isolate STEC 7-38 also was resistant to trimethoprim and streptothricin. [c] Sensitive (S), Intermediate resistant (I), and Resistant (R).

TABLE 3.4. Minimal inhibitory concentrations (μg/ml) of selected antibiotics against the integron positive STEC strains, and the MG1655 *E. coli* K-12 recipient strain.

MICs of antibiotic agents (μg/ml)	Resistant breakpoint	6-20	6-22	6-35	7-13	7-57	7-63	7-38[a]	Recipient strain MG1665
Ampicillin	≥32	2	2	4	>128	4	4	2	4
Chloramphenicol	≥32	8	8	256	512	8	8	8	8
Kanamycin	≥64	16	16	16	16	>512	16	16	8
Streptomycin	≥64	256	256	256	128	512	512	128	16
Oxytetracycline	≥16	>512	>512	>512	256	2	>512	2	2
Tetracycline	≥16	256	256	256	64	2	128	2	2
Sulfisoxazole	≥512	>1024	>1024	>1024	>1024	>1024	>1024	256	256

[a] The class 2 integron positive isolate was resistant to trimethoprim (>256 μg/ml) and streptothricin (>32 μg/ml)). Resistant breakpoints described by the National Clinical Laboratory Standards (NCCLS) (27) except for streptomycin and oxytetracycline. Isolates were considered resistant to oxytetracycline if MIC ≥ 16 μg/ml (similar to tetracycline resistant breakpoint described by NCCLS). Streptomycin resistant breakpoint (≥ 64 μg/ml) described by the US Food and Drug Administration, US Department of Agriculture, and Centers for Disease Control standard (7)

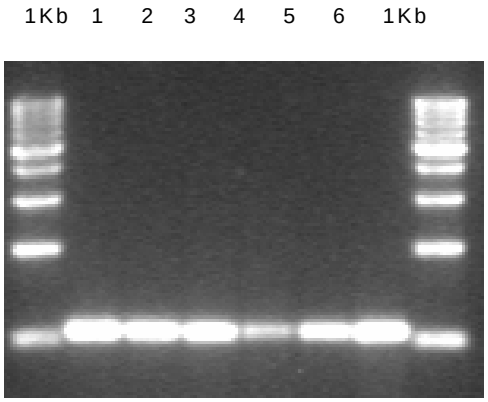
TABLE 3.5. Conjugation efficiencies of selected antibiotic resistance genes transferred from integron positive STEC strain to the MG1655 *E. coli* K-12 recipient strain.

Selectable pressure/ STEC isolates	Streptomycin–nalidixic acid	Tetracycline-nalidixic acid	Oxytetracycline-nalidixic acid
6-20	2×10^{-9}	1.67×10^{-5}	1.83×10^{-4}
6-22	1×10^{-9}	8.67×10^{-5}	3.23×10^{-4}
6-35	$<3.33 \times 10^{-10}$	$<3.33 \times 10^{-10}$	$<3.33 \times 10^{-10}$
7-13	3.33×10^{-10}	3.33×10^{-10}	3.33×10^{-10}
7-57	3.33×10^{-10}	n/a	n/a
7-63	3.33×10^{-10}	3.33×10^{-10}	2×10^{-9}

n/a: Not applicable

FIG. 3.1. PCR products amplified with total cellular DNA of STEC using primers targeting the specific regions of class 1 and class 2 integron gene cassettes. Fig. 3.1a shows the 1.1 kb product amplified from the class 1 integron gene cassette in STEC 6-20, 6-22, 6-35, 7-13, 7-57, and 7-63, respectively. Fig. 3.1b shows the 1.1 kb (lane 1 and 2) and the ~200 bp (lane 3 and 4) PCR products amplified from the class 1 integron gene cassette, as well as the 2 kb PCR product (lane 5) amplified from the class 2 integron gene cassette. Lane 6 shows the result of amplification using the total cellular DNA of MG1655 as target DNA. Lane L is the low DNA mass ladder.

a.



b.

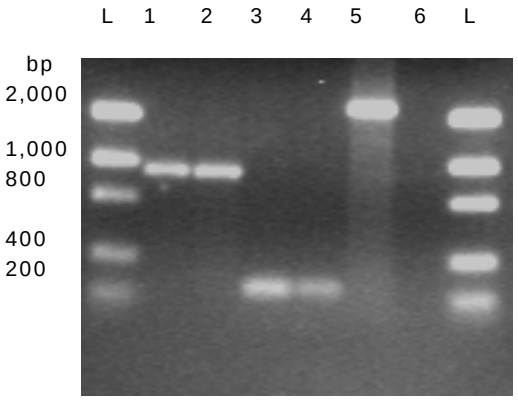
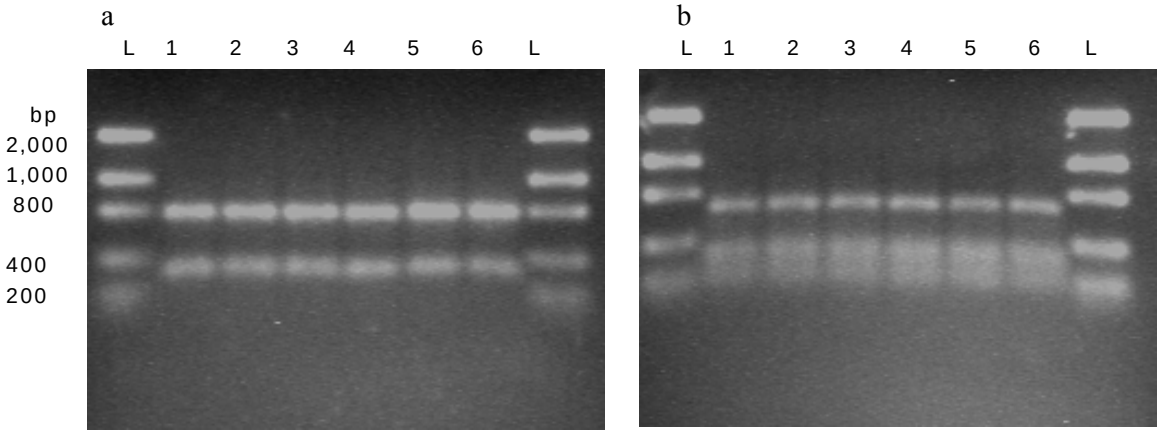


FIG. 3.2. Restriction digestion analysis of class 1 integron gene cassettes. PCR products amplified from the class 1 integron gene cassette were analyzed for possible nucleotide sequence variations using *Bcl*I, *Bgl*I, *Dra*III, *Hae*II and *Sty*I. Fig. 3.2a shows restriction pattern generated by *Bcl*I. Fig. 3.2b shows restriction pattern generated by *Dra*III.



CHAPTER 4

TRANSFER OF CLASS 1 INTEGRON MEDIATED ANTIBIOTIC RESISTANCE GENES FROM SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* TO A SUSCEPTIBLE *E. COLI* K-12 STRAIN IN STORM WATER AND BOVINE FECES [1]

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ABSTRACT

Transfer of class 1 integron mediated antibiotic resistance genes has been demonstrated under laboratory conditions. However, knowledge on the transfer of the genes in an agricultural environment is lacking. The present study sought to determine if integron mediated streptomycin and sulfisoxazole resistance genes could be transferred from Shiga toxin producing *Escherichia coli* (STEC) 6-20 (O157:H7) and 7-63 (O111:H8) to a susceptible strain of *E coli* K12 MG1655 in bovine feces (pH 5.5, 6.0 or 6.5) and storm water (pH 5, 6, 7 or 8) at 4, 15, or 28^{o}C, which are average seasonal temperatures for winter, spring-fall, and summer, respectively in Griffin, GA area. The results indicated that at 28°C, the integron mediated antibiotic resistance genes were transferred from both the STEC donors in bovine feces. Higher conjugation efficiencies were however, observed in the conjugation experiments involving STEC 6-20. In storm water, the resistance genes were only transferred from STEC 6-20. Greater numbers of transconjugants were recovered in the conjugation experiments in bovine feces with a pH value of 6.5, and in storm water with a pH value of 7. Antibiotic susceptibility tests confirmed the transfer of integron mediated streptomycin and sulfisoxazole resistances as well as the transfer of non-integron mediated oxytetracycline and tetracycline resistances in the transconjugant cells. These results suggest that the antibiotic resistance genes in STEC could serve as a source of antibiotic resistance genes disseminated *via* conjugation to susceptible cells of other *E. coli* strains in an agricultural environment.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) has been associated with foodborne outbreaks of infections worldwide. The diseases caused by STEC range from mild diarrhea to very severe conditions such as hemolytic-uremic syndrome (HUS). The organisms have been traced to farm animals, primarily in cattle (3, 9). They have been isolated from manure, water troughs, feedlots, and other places on cattle farms (9). The occurrence of STEC on animal carcasses and retail meats corresponds to the level of STEC contamination at the farm level (1). Human STEC infections associated with the consumption of contaminated fresh produce and water have been linked to the organisms originating from such farm areas (5, 12).

Antibiotic use in an agricultural environment is believed to play a key role in dissemination of antibiotic resistance genes among bacteria (16, 17, 22). Cells of bacteria acquire antibiotic resistance through genetic mutation and horizontal transfer of antibiotic resistance genes in response to the selective pressures. The increased prevalence of antibiotic resistance among pathogens such as STEC has raised concerns because some of the antibiotics are used in treatment of infectious diseases in both humans and animals (15, 26). Overuse of antibiotics in animal husbandry is, therefore suggested to create a threat to human and veterinary medicine.

Antibiotic resistance was primarily observed among clinical isolates of STEC, including resistance to streptomycin, sulfonamides, and tetracycline (28). The genes for resistance to the antibiotics are transmissible through conjugative plasmids to homologous and heterologous bacterial species under laboratory conditions (21, 26, 30). The transfer of antibiotic resistance genes is often related to integrons which are capable

of capturing and inserting antibiotic resistance genes into its structure (8). The transfer of integron-mediated antibiotic resistance genes has often been studied in controlled laboratory conditions, however, knowledge on the transfer of such genes under simulated agricultural conditions is lacking.

The goal of the present study was to determine whether the transfer of integron-mediated antibiotic resistance genes from STEC to generic *E. coli* could take place in samples commonly found in the farm environment, such as storm water and bovine feces, and whether environmental temperature and sample pH could influence the transfer frequency of the antibiotic resistance genes. It has been reported that the average seasonal temperatures for winter, spring-fall, and summer in the Griffin, GA area are 4, 15 and 28^{o}C, respectively (http://www.weather.com). The average pH levels of natural water range from 5 in acid lakes to 8 in estuaries (24, 29), while the fecal materials of cattle have average pH levels ranging from 5.5 to 6.5 (10, 11).

MATERIALS AND METHODS

Bacterial strains and environmental samples. STEC isolates 6-20 (O157:H7) and 7-63 (O111:H8), both from our laboratory collection were used as donor strains in the study. These isolates carry class 1 integrons which are responsible for their resistance to streptomycin and sulfisoxazole. They are also resistant to novobiocin, tetracycline, and oxytetracycline, which are likely mediated by an antibiotic resistance plasmid (Nagachinta and Chen, Unpublished). The recipient strain is a nalidixic acid resistant derivative of *E. coli* K-12 MG1655 CA32, provided by Dr. Wondwossen A. Gebreyes at North Carolina State University, Raleigh, NC, USA. The cultures were grown on MacConkey (MAC) or Sorbitol MacConkey agar (SMAC) (Becton, Dickinson and Co.,

Sparks, MD) at 37°C for 16 h. A single colony of each culture was inoculated on tryptic soy agar (TSA; Becton, Dickinson and Co., Sparks, MD) and incubated under the same conditions described above.

Storm water was collected from storm water discharge in Griffin, GA during the month of February (pH at collection = 6.4). The collected storm water was dispensed into glass bottles at a volume of 40 ml each before being autoclaved at 121°C for 15 min. Bovine feces were collected from a single cattle farm in Griffin, GA during the month of April (pH at collection = 6.5). The sample was distributed in glass beakers, weighing 40 g each and autoclaved at 121°C for 30 min. The sterility of the storm water and bovine feces was confirmed by plating the autoclaved samples on TSA plates for bacterial growth.

Preparation of donor and recipient cells. Single colonies of the donor and recipient cells on TSA plates were transferred into tryptic soy broth (TSB), and the inoculated broth was incubated at 37°C for 16 h. The resulting cultures (10^8 CFU/ml) were then centrifuged at 3,000 *g* for 15 min. The pellet of each culture was re-suspended in sterile saline solution (0.85% NaCl) in order to remove the nutrient residues from the culture media.

Conjugation in stormwater. Storm water (40 ml) was inoculated with donor and recipient cells prepared as described above. The inoculation ratio of the donor to recipient cells was 0.4: 4 ml (10^8 CFU/ml). The inoculated storm water samples were held for two weeks in the incubation chambers with an ambient temperature of 4, 15, or 28°C, which represent the average ambient temperature of winter, fall-spring, and summer in the Griffin, GA area, respectively. These average temperatures were

calculated based on average high and low monthly temperatures of the area provided by http://weather.msn.com/monthly_averages.aspx?&wealocations=wc%3aUSGA0251&setunit=C. Storm water inoculated with only the donor (0.4 ml) or the recipient cells (4 ml) both at 10^8 CFU/ml, as well as uninoculated storm water were included in the conjugation experiments as negative controls. The numbers of transconjugant cells were determined every two days during the experiment period.

Subsequently, the effect of storm water pH on the efficiency of the conjugation was investigated. Response surface methodology was used to design the experiments. The pH of the storm water was adjusted, with either HCl or NaOH, to 5, 6, 7, or 8, which represent the pH levels of natural water resources such as lake and estuary waters (24, 29). The storm water samples were held at 28^{o}C for one week. Samples were taken every two days and the numbers of antibiotic resistant transconjugant cells were determined.

Conjugation in bovine feces. A similar design was used in the conjugation experiment of bovine feces. The prepared donor and recipient cells (10^8 CFU/ml) were inoculated into 40 g of sterile fecal material using the same ratio and volumes of the donor to the recipient cells as described in the storm water experiment. Uninoculated fecal samples and samples inoculated with only the donor and recipient cells were included in the study as negative controls. All samples were held for two weeks at the temperatures specified above. Similar to the conjugation experiment of storm water, the numbers of transconjugant cells in bovine feces were enumerated every two days during the incubation period.

To study the influence of pH on the conjugation efficiency in bovine feces, the pH of the fecal material were adjusted with either HCl or NaOH to obtain a level of 5.5, 6.0, or 6.5, which represent the pH range of bovine feces (10, 11). The donor and recipient cells were inoculated into the fecal material at the ratio specified above. The inoculated samples were mixed well and kept at 28°C for a period of one week. The numbers of antibiotic resistant transconjugants were determined every two days.

Estimation of conjugation efficiency. To determine the numbers of transconjugants derived from the conjugation experiment conducted on storm water, 1 ml of each inoculated storm water sample was placed into a disposable, conical polypropylene centrifuge tube and centrifuged at 3,000 *g* for 15 min (GS-6R centrifuge, Beckman, Fullerton, CA). The cell pellets were re-suspended in 100 µl of sterile saline solution before being plated onto SMAC agar supplemented with nalidixic acid (100 µg/ml) and one of the following antibiotics: streptomycin (100 µg/ml), tetracycline (30 µg/ml), and oxytetracycline (30 µg/ml), respectively. The populations of the recipient cells per ml of culture were determined by plating appropriate dilutions of the *E. coli* MG1655 culture onto SMAC agar plates supplemented with only nalidixic acid (100 µg/ml).

To determine the number of transconjugants cells in bovine feces, 1 g of the fecal material was suspended in 1 ml of sterile saline solution in the conical tube described above, and centrifuged at 500 *g* for 10 min in order to remove the solid substances in the fecal material. The supernatant was then collected, and re-centrifuged at 3,000 *g* for 15 min to sediment the *E. coli* cells. The cell pellet was re-suspended in 100 µl of sterile saline solution and inoculated on the selective agar plates described above. When

necessary, the supernatants were serially diluted and appropriate dilutions were inoculated on the selective agar plates.

The absence of antibiotic resistant transconjugant cells in the negative control storm water and bovine fecal samples were confirmed by plating the samples on the selective plates described above. Conjugation efficiency was calculated as the ratio of the number of transconjugant cells to the number of recipient cells per ml of storm water or per g of bovine feces.

Analysis of transconjugants. It has been shown in a previous study conducted in our laboratory, that resistance to tetracycline and oxytetracycline, which are mediated by an antibiotic resistance plasmid, were co-transferred with resistance to streptomycin, an antibiotic resistance trait mediated by a class 1 integron. The antibiotic resistant transconjugant cells derived from each conjugation experiment in the present study were therefore, tested for their resistance to the antibiotics that were not used as the selective markers in the conjugation experiment. The presence of the class 1 integrons in the transconjugant cells were confirmed by determination of their susceptibility to streptomycin and sulfisoxazole. Standard disc diffusion assay was performed according to the guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS) (currently the Clinical and Laboratory Standards Institute) (19). Selected transconjugant cells derived from each selective plate were inoculated onto a TSA plate for a single colony and incubated at 37°C for 12 h. A single colony of each culture was transferred into tryptic soy broth (TSB) (Becton, Dickinson and Co., Sparks, MD) and incubated under the same conditions. The cell populations in the resulting cultures were adjusted with TSB to achieve an optical density of 0.1 at 600 nm (Novaspec II Visible

Spectrophotometer, Pharmacia Biotech, Cambridge, UK). The adjusted cell cultures were inoculated onto Mueller-Hinton agar (Becton, Dickinson and Co., Sparks, MD) plates, respectively with sterile cotton swabs and allowed to dry 5 min before the application of antibiotic discs (Sensi-Disc Antimicrobial Susceptibility Test Discs, BBL, Becton Dickinson) onto the plates using sterile forceps. The plates were incubated at 37°C for 16 h. The diameters of the zone of growth inhibition around each disc were measured to the nearest whole mm, and each tested cell culture was determined as resistant, intermediate, or susceptible to the antibiotics tested according to the guidelines provided by the supplier of the antibiotic discs.

RESULTS

Influence of temperature. Among the three temperatures used in the study, antibiotic resistant transconjugants were only recovered from the storm water and bovine feces held at 28°C (Table 4.1). The antibiotic resistance genes were transferred at higher frequencies from STEC 6-20 compared to 7-63 at this particular temperature. In bovine feces, the antibiotic resistance genes from STEC 6-20 were transferred at an approximately 100 times higher frequency than were the genes from STEC 7-63 (Table 4.1). In storm water, however, only the antibiotic resistance genes from 6-20 were transferred.

In storm water held at 28°C, the average of transfer frequencies showed that transconjugants of STEC 6-20 selected from the oxytetracycline agar plates (10^{-6}) were 100 times higher than the numbers of transconjugants recovered from tetracycline agar plates (10^{-8}) (Table 4.1). In bovine feces kept at the same temperature, however, similar

the average of transfer frequencies suggested that numbers of transconjugants were recovered from both types of selective agar plates for each donor strain (Table 4.1).

In general, the populations of STEC 6-20 transconjugants in bovine feces and storm water increased over time during the sampling period, except for the transconjugants recovered from storm water and grown on tetracycline agar plates which were detected only at day 7 (Table 4.1). The populations of STEC 7-63 transconjugants decreased over time during the sampling period (Table 4.1).

Influence of pH. Because the transconjugants were not recovered from the conjugation mixtures held at lower temperatures in the previous experiments, the influence of pH on conjugation efficiency was determined only at 28^{o}C.

Integron mediated antibiotic resistance genes in STEC 6-20 were transferred at higher frequencies in storm water with a pH of 7 (Fig 4.1a and 4.1b). The conjugation efficiencies decreased as the pH of the storm water increased or decreased from this value. Relatively lower conjugation efficiencies from STEC 6-20 were observed at a pH of 5 (Fig 4.1a and 4.1b). No transconjugant was obtained from the conjugation experiment involving STEC 7-63 in storm water.

In bovine feces, integron-mediated antibiotic resistance genes were transferred from both the STEC donors to the recipients. Similar numbers of antibiotic resistant transconjugants were received from the selective agar plates supplemented with oxytetracycline and tetracycline, respectively (Fig. 4.2a, 4.2b, 4.3a, and 4.3b). Greater rates of antibiotic resistance gene transfer were found in bovine feces with a pH of 6.5 (Fig 4.2 and 4.3). The conjugation efficiencies declined at the lower fecal pH. The

antibiotic resistance genes were transferred from both the donor strains at relatively lower efficiencies in bovine feces with a pH of 5.5.

Overall, the numbers of transconjugants derived from both the STEC donors increased over time before day 5 and dropped slightly after this sampling point (Fig. 4.1, 4.2 and 4.3).

Analysis of transconjugants. The antibiotic susceptibility test showed that the obtained transconjugants were resistant to streptomycin and sulfisoxazole, indicating the transfer of class 1 integron mediated antibiotic resistance genes. The transconjugants also resisted tetracycline and oxytetracycline, suggesting that the genes encoding for resistance to tetracycline and oxytetracycline were co-transferred with the integron mediated antibiotic resistance genes.

DISCUSSION

Conjugation in storm water and bovine feces. Transfer of integron-mediated antibiotic resistance genes from both the STEC donors occurred at higher efficiencies when the conjugations took place in bovine feces. Bovine feces is semi-solid and contains waste materials from animal digestive tracts, which provides supports for bacterial cells to be in contact during the conjugation process, a critical step for the transfer of genes associated with plasmids. Storm water, nevertheless contains much less solid waste and therefore, lacks many of the conditions that favor bacterial conjugation.

Bovine feces contain relatively higher amounts of organic materials compared to storm water, and may therefore, have more utilizable nutrients to bacterial cells. Previous research has shown that the presence of nutrients in conjugation mix resulted in a greater efficiency of gene transfer. Gotz and Smalla (1997) reported the transfer rates of an IncQ

plasmid increased more than 10-fold when conjugation took place in soil supplemented with pig manure compared to non-manured soil (7). It has been shown that F pili expression is essential in forming the mating bridge between Gram-negative bacterial cells at the early stage of the conjugation (4). Curtiss et al. (1969) reported the expression of F pili was influenced by a growth media (4). The cells of *E. coli* expressed a greater amount of F pili in minimal growth media supplemented with 0.5% casamino acids and glucose (4). It is worthy to note though that an increase in the number of transconjugants may partially be caused by the growth of transconjugant cells after the transfer of antibiotic resistance genes has actually occurred, which may not necessarily be the result of increased conjugation efficiency.

Influences of temperature. According to the results of the present study, the incubation temperatures of 4 and 15°C did not favor the transfer of integron-mediated antibiotic resistance genes in bovine feces and storm water. Previous studies have shown that a temperature higher than 15°C was required for conjugal transfer of R-plasmids between *E. coli* cells (23, 25). Singleton and Anson (1981) found that the transfer of an R-plasmid between *E. coli* cells occurred from 17 to 37°C after studying the transfer of the plasmid at six different temperatures, i.e. 15, 17, 20, 22, 27 and 37^{o}C. No transconjugants were however, obtained when the conjugation experiment was conducted at 15°C. In the present study, the transfer of integron-mediated antibiotic resistance genes from both the donor strains took place at 28°C, an average summer temperature in Griffin, GA. The influence of temperature on the efficiency of conjugal transfer may be related to the expression of F pili at various temperatures (20). The expression of F pili in *E. coli* has been shown to be regulated by temperature (20). Novotny and Lavin

(1971) reported that optimal expression of F pili occurred at temperatures between 37 and 42°C, decreased at temperatures below 37°C, and diminished at any temperature below 25°C (20).

Influences of pH. Transfer of antibiotic resistance genes occurred in bovine feces and storm water with a near neutral pH, except for the conjugation experiment involving STEC7-38 in storm water. Previous studies observed optimum conjugation efficiencies in nutrients broth and in soil samples with a pH near 7 (24). Krasovsky and Stotzky (1987) found that although the donor cells were able to survive at a pH as low as 5.7 in soil samples, conjugation only occurred when pH was near neutrality. Sub-optimal pH inhibited the growth of bacteria cells, and lowered the transfer frequencies of DNA (14). DNA could undergo DNA depurination under acidic conditions. DNA replication in recipient cells after conjugation could be suppressed by the sub-optimal pH. As what happened at 28^{o}C, the higher numbers of antibiotic resistant transconjugants in samples with a neutral pH may partially be influenced by the higher growth rate of the transconjugant cells.

Antibiotic resistant STEC in an agricultural environment. Cattle are known as the primary reservoir for both O157 and non-O157 STEC (2). The occurrence of STEC in an agricultural environment can lead to the contamination of both water and food. STEC infections have been linked to contaminated water supplies (5, 12). The organisms have shown to persist for as long as 4 to 8 weeks at 25°C in bovine feces (6), and up to 90 days in river water (27). Studies have shown that fecal excretion of *E. coli* O157:H7 in cattle occurred in the highest rates during the spring and late summer months

(3, 18). This time period of high excretion generally reflects the start of a seasonal peak (spring and fall) of *E. coli* O157:H7 infections (13).

Transfer of antibiotic resistance genes among bacteria is a threat to both human and veterinary medicine. In this study, the transfer of integron mediated-antibiotic resistance genes in STEC isolates was demonstrated in bovine feces and storm water with a natural pH at a temperature representing the average temperature of the summer months in the Griffin, GA area. The conjugation efficiencies of antibiotic resistance genes varied between the two STEC donors. The precise genetic basis for this variation is currently unknown. This study provided an evidence of antibiotic resistance genes transfer between pathogenic strain and nonpathogenic strain of *E. coli* in agricultural environments.

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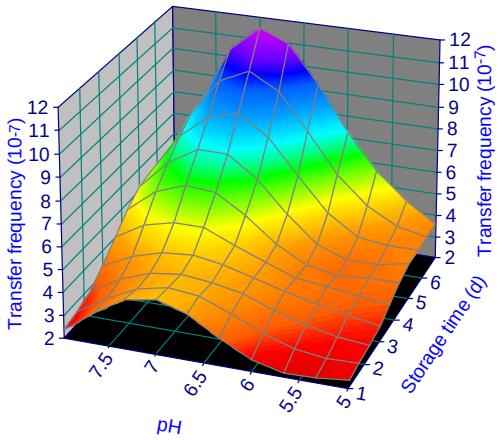
TABLE 4.1. Frequencies of antibiotic resistance gene transfer from STEC isolate 6-20 and 7-63 to the MG1655 *E. coli* K12 recipient strain in storm water and bovine feces held at 28°C.

Mating pair/ Selective antibiotics	Transfer frequencies at different sampling time (d)							
	1	3	5	7	9	11	13	Average
Stormwater 6-20 – oxy	8.93×10^{-7}	1.86×10^{-6}	7.50×10^{-7}	1.04×10^{-6}	8.93×10^{-7}	9.64×10^{-7}	1.04×10^{-6}	1.06×10^{-6}
6-20 – tet	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	2.50×10^{-7}	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	$< 4.43 \times 10^{-8}$
Bovine feces 6-20 - oxy	3.75×10^{-6}	7.65×10^{-6}	7.10×10^{-6}	1.26×10^{-5}	7.25×10^{-6}	1.12×10^{-5}	1.71×10^{-5}	9.52×10^{-6}
6-20 - tet	1.59×10^{-6}	1.32×10^{-6}	1.22×10^{-6}	3.04×10^{-6}	8.12×10^{-6}	2.29×10^{-5}	2.96×10^{-5}	9.68×10^{-6}
7-63 - oxy	1.01×10^{-7}	1.01×10^{-7}	2.90×10^{-8}	1.45×10^{-8}	4.35×10^{-8}	$< 1 \times 10^{-8}$	1.45×10^{-8}	$< 4.45 \times 10^{-8}$
7-63 - tet	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	$< 1 \times 10^{-8}$	4.35×10^{-8}	4.35×10^{-8}	2.90×10^{-8}	$< 2.23 \times 10^{-8}$

Detection limit: 1×10^{-8}

FIG.4.1. Transfer frequencies of antibiotic resistance genes from STEC 6-20 to MG1655 in stormwater with a pH level of 5, 6, 7, and 8, respectively at 28°C. Fig.4.1a shows the transconjugants selected on SMAC supplemented with oxytetracycline. Fig.4.1b shows the transconjugants selected on SMAC supplemented with tetracycline.

a.



b.

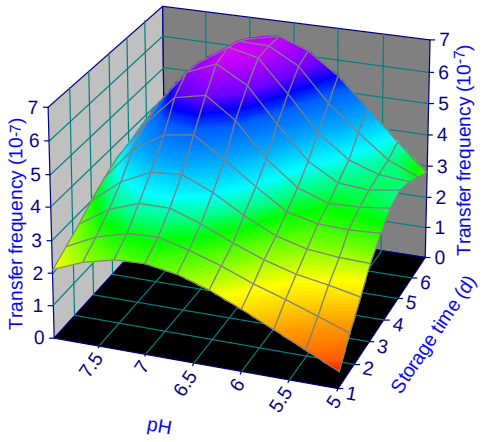
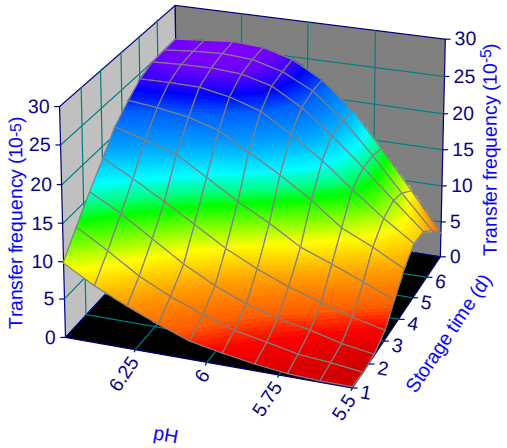


FIG.4.2. Transfer frequencies of antibiotic resistance genes from STEC 6-20 to MG1655 in bovine feces with a pH level of 5.5, 6.0 and 6.5, respectively at 28°C. Fig. 4.2a shows the transconjugants selected on SMAC supplemented with oxytetracycline. Fig. 4.2b shows the transconjugants selected on SMAC supplemented with tetracycline.

a.



b.

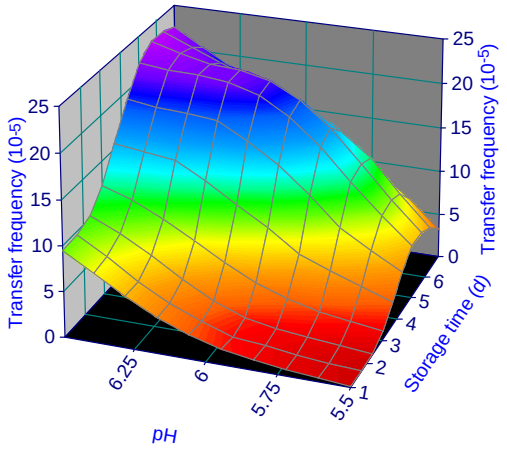
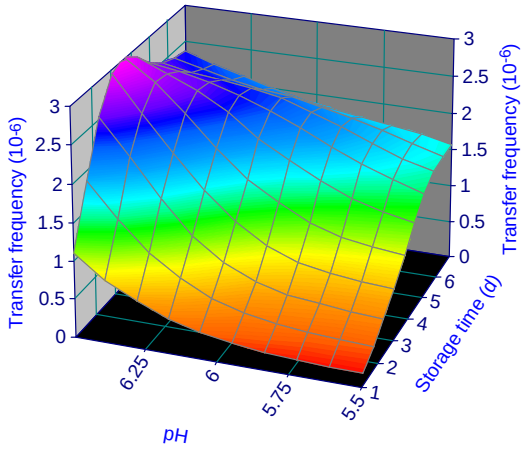
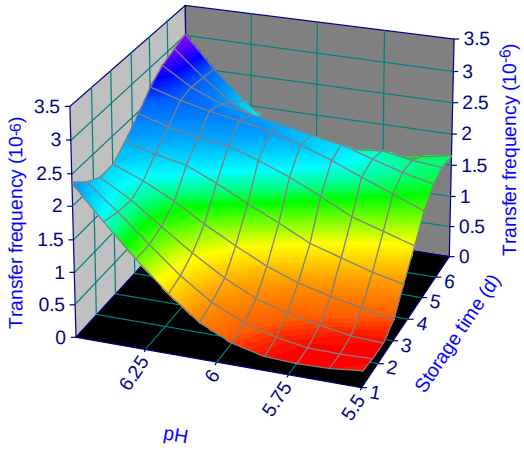


FIG.4.3. Transfer frequencies of antibiotic resistance genes from STEC 7-63 to MG1655 in bovine feces with a pH level of 5.5, 6.0 or 6.5, respectively at 28°C. Fig. 4.3a shows the transconjugants selected on SMAC supplemented with oxytetracycline. Fig. 4.3b shows transconjugants selected on SMAC supplemented with tetracycline.

a.



b.



CHAPTER 5

CONCLUSIONS

Results from the studies described in Chapter 3 and 4 can be concluded as in following:

1. Integrons were identified in 15 (8.5%) of the 177 STEC isolates from our laboratory collection. Fourteen (7.9%) and one (0.6%) isolate(s) were positive for class 1 and class 2 integron, respectively. The positive isolates are resistant to multiple antibiotics. The common resisted antibiotics include streptomycin, sulfisoxazole, as well as tetracycline and oxytetracycline. The class 1 integron of the 14 positive isolates had a uniform size of approximately 1.1 kb and carried a gene cassette of *aadA1* (streptomycin-spectinomycin resistance) gene. The presence of class 1 integron explained the common resistance to streptomycin and sulfisoxazole among the integron positive isolates. The class 2 integron was at a size of 2.0 kb and carried three gene cassettes of *aadA1*, *sat1* (streptothricin resistance) and *dfrA1* (trimethoprim resistance) gene, respectively. The antibiotic resistance genes mediated by integron are only responsible for part of the antibiotic resistance traits expressed by STEC isolates. Horizontal transfer of class 1 integrons from STEC to susceptible strain of K12 MG1655 was mediated by conjugative plasmids. Transconjugants acquired resistances mediated by integron, as well as those that are not mediated by integron. This part of the study determined the prevalence of integrons among the STEC isolates and the transfer

of the antibiotic resistance genes to a susceptive *E. coli* strain under laboratory condition.

2. Two class 1 integron positive isolates (STEC 6-20 and 7-63) were used as donors in this part of the study to determine the transfer of class 1 integron mediated antibiotic resistance genes in storm water and bovine feces. The integron mediated antibiotic resistance genes were transferred from both the STEC donors in bovine feces with natural pH (pH 5.5., 6.0, and 6.5) at 28°C, a temperature representing the average temperature of the summer months in Griffin, GA area. Higher conjugation efficiencies were observed in the conjugation experiments involving STEC 6-20. In storm water, the resistance genes were only transferred from STEC 6-20. Greater numbers of transconjugants were recovered from bovine feces with a pH value of 6.5, and storm water with a pH value of 7. Antibiotic susceptibility tests confirmed the transfer of integron mediated streptomycin and sulfisoxazole resistance as well as non-integron mediated tetracycline and oxytetracycline resistance. The part of the study showed that antibiotic resistance genes in STEC could be transferred to susceptible *E. coli* strains in an agricultural environment.