獸醫學博士學位論文

Molecular diagnostic and epidemiological investigations of Shiga toxin-producing *Escherichia coli* and *Campylobacter jejuni* in dairy cattle.

젖소에서 Shiga toxin-producing *E. coli* 와
C. jejuni 의 분자진단 및 분자역학 연구

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동 희 진

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By

Hee-Jin Dong

Supervisor: Professor Seongbeom Cho, D.V. M., PhD.

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Program in Zoonotic Animal Diseases,

College of Veterinary Medicine

Graduate School of Seoul National University

Abstract

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Hee-Jin Dong

(Supervisor: Seongbeom Cho, D.V.M., Ph.D.)

Program in Zoonotic Animal Disease
College of Veterinary Medicine
Graduate School of Seoul National University

Foodborne illness is a significant public health concern, contributing to 25 to 30% of gastroenteritis. Among bacterial causes, Shiga toxin-producing *Escherichia coli* (STEC) is an important enteric pathogens that causes bloody diarrhea, hemorrhagic colitis, or hemolytic uremic syndrome which occasionally leads to death at high rate.

E.coli O157, the most notorious of the STEC strain, and hundreds of STEC non-O157 strains are associated with both endemic and epidemic infection. *Campylobacter* is also recognized as the leading cause of diarrheal disease especially in developed countries. In order to minimize human infections, rapid detection and characterization, especially for the virulence potentials of STEC and *Campylobacter jejuni* are needed. Cattle are a natural reservoir of STEC and have recently been recognized as a major source of *C. jejuni* contamination. In this study, molecular diagnostic and epidemiological investigations were performed for the STEC and *C. jejuni* strains isolated from cattle farm samples, a primary source of infection. First, loop-mediated isothermal amplification (LAMP) assay, the newest and the most advanced method for amplification of nucleic acid, was developed for rapid and sensitive detection of STEC and *C. jejuni*. Next, the prevalence and virulence potentials of STEC in cattle were investigated. Finally, the influence of microbiota on shedding STEC or *C. jejuni* was investigated.

The first study as discussed about the development of real-time LAMP assay for detection of *C. jejuni* targeting *hipO*. The developed LAMP assay was specific (100% inclusivity and exclusivity for 84 *C. jejuni* and 41 non-*C. jejuni* strains, respectively), sensitive (detection limit of 100 fg/μl), and quantifiable ($R^2 = 0.9133$). When applied the LAMP assay on all *C. jejuni* strains (n = 51) isolated from cattle farm during

2012 to 2013, the *hipO* gene was successfully amplified within 30 min (mean = 10.8 min) demonstrating the accuracy and rapidity. In addition, the LAMP assay was applied to the enriched broth cultures of the naturally-contaminated cattle farms samples. In the comparison between LAMP and PCR, the higher sensitivity of the LAMP assay (84.4% vs. 35.5%) was observed in boiled DNA samples, indicating less susceptibility of LAMP assay to the existence of inhibitors in sample material. The detection of this organism in cattle and their environment is important for the control of *C. jejuni* transmission and the prevention of Campylobacteriosis. However, isolation of *C. jejuni* using culture method is difficult due to its high oxygen sensitivity and difficulty in reading typical phenotypes on media. The use of LAMP assay together with culture method would enhance identifying and screening of *C. jejuni* in cattle farm samples, and play an important role in the prevention of *C. jejuni* contamination in the food chain, thereby reducing the future risk of human Campylobacteriosis.

The second study was also focused on the development of LAMP for detection of STEC targeting *stx* genes. Whilst LAMP assay was accepted as a novel nucleic acid amplification method, the application of LAMP assay to several genes was limited due to formation of cauliflower-like amplified product. In this study, multiplex LAMP assay was developed targeting *stx1* and *stx2* genes of STEC. The mLAMP

was able to distinguish two target genes based on different T_m values (85.03 ± 0.54°C for *stx1* and 87.47 ± 0.35°C for *stx2*). The mLAMP was highly specific (100% inclusivity and exclusivity), sensitive (with a detection limit as low as 10 fg/μL), and quantifiable (R^2 = 0.9313). In addition, the mLAMP assay was able to type shiga toxin types of a total of 12 (12/253; 4.7%) and 17 (17/253; 6.7%) STEC O157, and 11 (11/236; 4.7%) STEC non-O157 strains, which were isolated from cattle farm samples by conventional selective culture, immunomagnetic separation, and PCR-based culture methods, respectively. Due to lack of characteristic phenotype of STEC non-O157, it is hard to isolate STEC non-O157 by culture method, thus a molecular based detection method is often accompanied. Application of mLAMP assay would be helpful not only to detect STEC but also to identify its shiga toxin type simultaneously. Furthermore, the high detection rate of specific genes from enriched broth samples indicates the potential utility of this assay as a screening method for detecting STEC in cattle farm samples.

The third study was discussed about the prevalence and the virulence potentials of STEC in cattle farm samples. In total, 63 STEC were isolated from 496 cattle farm samples, and temperature and rainfall affected STEC prevalence ($p < 0.001$). The O157 serogroup was most prevalent, followed by O108, O8, O84, O15, and O119. In the stx variant test, high prevalence of *stx2a* and *stx2c* (known to be associated

with high STEC virulence) were observed, and *stx2g*, a bovine STEC variant, was detected in STEC O15 and O109. Additionally, *stx1c* was detected in eae-positive STEC, suggesting genetic dynamics among the virulence genes in the STEC isolates. STEC non-O157 strains were resistant to tetracycline (7.9%), ampicillin (6.4%), and cefotaxime (1.6%), while STEC O157 was susceptible to all tested antimicrobials, except cefotaxime. The antimicrobial resistance genes, bla_{TEM} (17.5%), *tetB* (6.3%), and *tetC* (4.8%), were only detected in STEC non-O157, whereas *tetE* (54.0%) was detected in STEC O157. *AmpC* was detected in all STEC isolates. Clustering was performed based on the virulence gene profiles, which grouped STEC O84, O108, O111, and O157 together as potentially pathogenic STEC strains. Finally, PFGE suggested the presence of a prototype STEC that continues to evolve by genetic mutation and causes within- and between-farm transmission within the Gyeonggi province.

The final study was dealt with the bovine gut microbiota in relation to the presence of the foodborne zoonotic pathogens, STEC and *C. jejuni*. Recent studies have focused on the shedding lebel of fooborne pathogens. The host shedding pathogens at high levels called high-shedder or super-shedder, which is thought to be related to the high prevalence or incidence of the pathogens. In this study, the fecal microbiota of dairy cattle (n = 24) was investigated using next-generation sequencing to identify

the microbial impact on shedding STEC or *C. jejuni*. The core microbiota (9 phyla, 13 classes, 18 orders, 47 families, 148 genera, and 261 species) was identified, which covered 80.0~100.0% of the fecal microbial community. The presence of STEC had a minor effect on alpha-diversity and relative abundance (RA) of taxa, identifying 2 and 3 genera that were significantly higher and lower, respectively, in STEC shedding cattle. On the other hand, a high diversity index was observed in *C. jejuni*-positive samples. In addition, *C. jejuni*-positive cattle had a higher RA of *Bacteroidetes* ($p = 0.035$) and a lower RA of *Firmicutes* ($p = 0.035$) compared to *C. jejuni*-negative cattle. In genus level, the RA of 6 and 3 genera were significantly higher and lower, respectively, in cattle shedding *C. jejuni*. While diverse microbial communities were observed between cattle shedding foodborne pathogens and non-shedding cattle, these differences had a minor influence on the overall microbial community.

Rapid, and sensitive detection of STEC and *C. jejuni* at cattle farm is especially important in terms of the initial stage of the food chain, which prevent further contamination along the food production line. With the use of developed LAMP assay in this study, effective control and prevention would be possible. In addition, considerable numbers of STEC non-O157 were isolated from cattle farms, and the virulence and antimicrobial resistance features were different between

the STEC O157 and non-O157 strains. STEC from cattle with virulence or antimicrobial resistance genes might represent a threat to public health and therefore, continual surveillance of both STEC O157 and non-O157 would be beneficial for controlling and preventing STEC-related illness. Finally, diverse microbial community was observed in cattle groups shedding STEC or *C. jejuni*, implying the interaction between indigenous bacteria and foodborne pathoges. While several factors are known to be associated with bacterial colonization, the underlying microbial factors have not been clarified. Microbiota is thought to be one of the important animal factor causing super-shedding, because microbiota is closely reltated to survival and colonization of STEC and *C. jejuni* as a niche for bacterial pathogens. These finding would provide fundamental information on bacterial ecology in cattle feces, and would be useful to develop strategies for controlling bacterial shedding.

Keywords: Foodborne zoonotic pathogens, STEC, *C. jejuni*, LAMP, gut microbiota, cattle

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A/E	Attaching and effacing
AM	Ampicillin
AMC	Amoxicillin-clavulanic acid
AN	Amikacin
ATCC	American type culture collection
ATM	Aztreonam
C	Chloramphenicol
CAZ	Ceftazidime
CDT	Cytolethal distending toxin
CFU	Colony forming units
CPD	Cefpodoxime
CRO	Ceftriaxone
CTX	Cefotaxime
DAEC	Diffusely adherent *E. coli*
dNTP	Deoxynucleoside triphosphate
DI	Diversity index
EAEC	Enteroaggregative *E. coli*

EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
GM	Gentamicin
HC	Hemorrhagic colitis
HUS	Hemolytic uremic syndrome
IMP	Imipenem
IMS	Immunomagnetic saperation
LAMP	Loop-mediated isothermal amplification
LEE	Locus of enterocyte effacement
mLAMP	Multiplex Loop-mediated isothermal amplification
NA	Nalidixic acid
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
RA	Relative abundance
STEC	Shiga toxin-producing *E. coli*

STX	Trimethoprim-sulfamethoxazole
TE	Tetracycline
UPGMA	Unweighted pair group method with arithmetic mean analysis
VBNC	Viable but not culturable
VTEC	Verotoxin-producing *E. coli*

General introduction

Foodborne illness is one of the most significant public health concern worldwide. The large number of episodes are reporting, and the number of episodes are increasing these days. In the United States, more than 9 million of foodborne illness cases were estimated each year, and the number of foodborne illness cases are also increasing. Most important concern is that many of foodborne pathogens are evolving or newly recognized. The relationship between climate changing and occurrence of waterborne disease was reported. Because waterborne and foodborne diseases are commonly share their transmission route and foodborne diseases are commonly linked to contamination of water, the changing in occurrence of foodborne disease are expected. In addition, the increase of direct and indirect contacts of human bodies including material resources facilitates outbreak and expansion of a new virulent strain of bacteria. For instance, in 2011, starting from Germany, *E.coli* has spread the entire Europe within a few months causing more than 4,000 cases of infection, including more than 50 cases of death. The causative agent was found to be *E.coli* O104:H4 which increased capabilities of survivability and virulence by acquiring both aggregative factor and antimicrobial resistance factor. It was the representative case of outbreak which was occurred by a new virulence strain

of bacteria, highlighting needs of a molecular epidemiological research, particularly based upon the antimicrobial resistance, routines of transmission, and evolution of its own virulence. In addition both a faster detection and investigation of virulence potential is necessary to develop strategies for disease control and prevention.

An effective detection technique could give financial benefits along with physical advantages. The LAMP is the newest nucleic acid amplification technique, which can amplify specific target gene rapidly with high sensitivity. Due to auto-cycling displacement of the target, LAMP assay is able to amplify target gene at a single temperature, which enable to react within 60 min by saving time required for denaturation, annealing, and extension. In addition, recognition of 6 to 8 distinct regions on the target sequence using 4 to 6 primers ensures high specificity and sensitivity of the LAMP assay. Several studies had revealed the high sensitivity of LAMP assay compared to that of the PCR or real-time PCR. Owing to its high sensitivity, LAMP assay can be applied for detection and screening of specific pathogens.

Investigation of the prevalence and characterization of STEC in cattle farm is crucial because cattle, a major reservoir of STEC, could be a primary source of infection. The surveillance of STEC in cattle farm enable to react rapidly in an outbreak setting. Many of studies were conducted on STEC in cattle, and most works were concentrated in STEC O157 serotypes. Recently, the importance of STEC non-

O157 is being magnified as an etiological agent of foodborne pathogen causing serious illness to human with high prevalence. *C. jejuni* is leading cause of foodborne diarrheal disease, especially in developed countries. *C. jejuni* infection is often under diagnosed due to difficulty in culture and isolation of the pathogen. In addition, whilst the poultry is known to be a major source for *C. jejuni* contamination, consumption of raw milk and direct contact with cattle are also considered to be significant risk factors, contributing considerable portion of human infection.

Investigation of risk factors for shedding STEC or *C. jejuni* is important to develop strategies to reduce infection in human. Recent studies have focused on the shedding level of foodborne pathogens. The host shedding pathogens at high levels called high-shedder or super-shedder, which is thought to be related to the prevalence or incidence of the pathogens. Compared to that of the *C. jejuni*, numerous studies are ongoing to understand epidemiology and risk factors for STEC super-shedders in cattle. Previous studies identified that STEC colonizes at the terminal rectum of cattle, and this attribute is closely related to the shedding level of STEC. The presence of high-shedder increases the prevalence of STEC in environment by within- and between- farm transmissions, and accordingly, increases the incidence of human disease risk. Microbiota is thought to be one of the cattle factors because it is a niche for STEC, and closely related to STEC survival in terminal rectum.

While few studies were reported the link between microbiota and cattle, most of them were focused on beef cattle.

The studies on prevalence, virulence potentials, and microbial impact of STEC and *C. jejuni* would provide fundamental information on bacterial ecology in cattle feces and the more effective and comprehensive knowledge to the epidemiology of infection.

Literature review

I. Foodborne illness

A. General introduction

Ensuring food safety is a common priority to maintain public health worldwide. Since the end of 20th century, incidence of foodborne illness, outbreaks, and emergence of a new virulent strain of foodborne pathogens has been dramatically increased (1). Foodborne illness is one of the most significant public health concern worldwide. In the United States, more than 9 million of foodborne illness cases were estimated each year, and the number of foodborne illness cases are also increasing. In addition, the increase of direct and indirect contacts of human bodies including material resources facilitates outbreak and expansion of etiological agents leading to increase concern of global foodborne-related health risk (1).

Foodborne illness refers any illness caused by ingestion of food. Foodborne illness is comprehensive problem, which is hard to identify etiological agents due to its large and complexity of transmission route, progression, clinical symptoms, etc. Moreover, many of foodborne illness cases are under-reported, limiting epidemiological data. Therefore, to control and prevent foodborne illness, accumulating information on the epidemiology and risk of foodborne illness is important (2). While physical agents

can enter into food that cause injuries or choking, most of foodborne illness caused by microbiological or chemical agents which results in infection, intoxications, metabolic food disorders, allergies, or idiosyncratic diseases (2). The metabolic food disorders, allergies, or idiosyncratic diseases are often considered as an individual adverse effect to foods, but infections and intoxications can be occurred in everyone in the population causing gastrointestinal disorder including diarrhea, vomiting, abdominal pain etc., or other extraintestinal disease (Table 1). There are many risks to cause foodborne illness in the food supply chain, namely from farm to table, which categorized into three major groups, physical, chemical, and biological hazards (3).

Table 1. Sites of action for foodborne toxicants. Adapted from Mortimore et al., 2001

Tissue site	**Toxin affecting the site**
Blood components	Nitrite
Brain	Domoic acid
Gastrointestinal tract	Staphylococcal enterotoxins, Trichothecene mycotoxins
Heart	Erucic acid (rapeseed oil)
Kidney	Ochratoxin
Liver	Aflatocins, Ethanol
Lung	Paraquat
Nervous system	Saxitoxin, Botulinum toxins
Skelectal system	Lead (Pb)
Skin	Histamine, Trichothecene mycotoxins

B. Physical hazards

Physical hazards includes a variety of physical foreign materials that can enter a food product. They consist of glass, metal, stones, wood, plastic, pests, or intrinsic materials. While some of foreign materials just gives an unpleasant feeling to the customer, many of them has a potential to bring about a health risk to the consumer. People can be injured by consuming sharp or hard items, or may be choked by blocking the airways. Some materials can also act as a transporter of microbiological agents into the food product (4).

C. Chemical hazards

Chemical hazards may be the most complicated category within food industry. There is limited numbers of toxicological expertise, and dependent of level of risk on the consumer of the final customer or uncertainty of long-term effect of chemicals makes it hard to understand and set up the hazards in this field. Some chemicals, e.g. melamine, was added in food as a protein replacer, now become economic adulteration due to its toxic effect. However, it is still in use in animal food industry. Another examples is salt and fat. High levels of these ingredients in food has shown to be associated with development of chronic disease like coronary heart disease, these materials not thought to be a chemical hazards due to the variety of human diets and its positive effect on overall health conditions. Still, there are many

limitations in expertized knowledge and experience. While major chemical hazards are listed below, there are many other chemical hazards that can carelessly, unnecessarily, or accidently enter at any stage of food processing and cause acute or chronic disease (2, 4).

Allergens and Food intolerances

Approximately 2% and 7% of adults and children, respectively, are thought to be sensitive to specific food products causing mild to severe adverse response. The common food that cause allergies or food intolerances are milk, eggs, wheat, soy, peanuts, tree nuts, fish, and shellfish, which contributes 90% of food allergies. Dependent on the amount of exposure, individual sensitivity, a severe anaphylactic shock can be seen. To control and prevent this, accurate labelling is needed.

Cleaning chemicals

Cleaning residues may be commonly added in food during processing equipment or may be accidently contaminated during cleaning other materials.

Mycotoxins

Mycotoxin is a toxin produced my fungi and can cause both short term and long term toxic effects depending on the exposure time and level. Some categorizes

mycotoxin as a biological hazards because they produced by microbial agents. There consists aflatoxins, patulin, deoxynivalenol, and fumonisins. Once the mycotoxins are generated, it is very hard to remove due to its high survivability during further food processing including heating processes. Therefore prevention of entry, especially in the early stages of food processing is key to reduce foodborne illness.

Nitrates, nitrites, and nitrosamines

Nitrogen is one of the constituent of air and has been used for preservation of food products. However, high level of hitries, nitrates, and N-nitroso compounds in food has a toxic effect including carcinogenic effect, and now regulated to be used as adequate amount to the products.

Pesticides

Many pesticides including algicides, animal repellents, avicides, food storage protectors, fungicides, herbicides, insecticides, masonry biocides, rodenticides, wood preservatives, etc. are used broadly in variety of plant and animal agriculture, industry, or shipping. The use of these pesticides are needed to protect product, increase product yield, or preserve for storage, but it is important to check the pesticide is accepted to be used in food as well as on the residue limits are acceptable.

Polychlorinated biphenyls (PCBs)

PCBs had been used widely in many industries, but has been limited in use due to its toxic effect and high stability to environment. PCBs are known to be accumulated in tissue, especially tissues with high lipid contents.

Toxic Heavy metals

Heavy metal can be found in the earth naturally, but might cause illness when it becomes concentrated. Metal can enter to the food chain through various sources including environmental pollution, soil, equipment, water, etc. The metals significantly concerning are aluminum, antimony, arsenic, bismuth, cadmium, chromium, cobalt, copper, lead, mercury, nickel, selenium, silver, tin, and zinc.

Veterinary residues

Domestic animals are often treated with hormones, antimicrobial agents, or growth regulators, which may remain in the animal-originated food product. Carry-over of antimicrobial agents are of significant public health concern, which may cause treatment failure and, more critically, death.

D. Biological hazards

While microbiological and macrobiological agents can be a biological hazards, but normally microbiological agents commonly considered as microbiological hazards due to its high risk to human illness. Microbiological agents can cause disease to human directly by infection or indirectly by toxin produced by microorganisms (Table 2) (5).

Infection occurs by presence of viable microorganisms, which ingested with food, at the site of inflammation. The microorganisms multiply in the gastrointestinal track or rarely in some other organ to generate infectious disease. These microorganism include bacteria, viruses, protozoa, and parasites. On the other hand, intoxications are involved in nonviable agents. While synthetic toxicants classified as a chemical agent, natural toxicants classified as biological agents because they originate from microbial sources (3, 4).

Table 2. Estimated annual number of episodes of domestically acquired foodborne illnesses caused by 31 pathogens, United States. Adapted from Scallen *et al*., 2011.

Pathogen	Laboratory confirmed	Multipliers: Under-reporting	Multipliers: Under-diagnosis	Travel related, %	Foodborne, %†	Domestically acquired foodborne, mean (90% credible interval)
Bacteria						
Bacillus cereus, foodborne	85‡	25.5	29.3	<1	100	63,400 (15,719–147,354)
Brucella spp.	120§	1.1	15.2	16	50	839 (533–1,262)
Campylobacter spp.	43,696¶	1.0	30.3	20	80	845,024 (337,031–1,611,083)
Clostridium botulinum, foodborne	25§	1.1	2.0	<1	100	55 (34–91)
Clostridium perfringens, foodborne	1,295‡	25.5	29.3	<1	100	965,958 (192,316–2,483,309)
STEC O157	3,704¶	1.0	26.1	4	68	63,153 (17,587–149,631)
STEC non-O157	1,579¶	1.0	106.8	18	82	112,752 (11,467–287,321)
ETEC, foodborne	53‡	25.5	29.3	55	100	17,894 (24–46,212)
Diarrheagenic *E. coli* other than STEC and ETEC	53	25.5	29.3	<1	30	11,982 (16–30,913)
Listeria monocytogenes	808¶	1.0	2.1	3	99	1,591 (557–3,161)
Mycobacterium bovis	195¶	1.0	1.1	70	95	60 (46–74)
Salmonella spp., nontyphoidal	41,930¶	1.0	29.3	11	94	1,027,561 (644,786–1,679,667)
S. enterica serotype Typhi	433¶	1.0	13.3	67	96	1,821 (87–5,522)
Shigella spp.	14,864¶	1.0	33.3	15	31	131,254 (24,511–374,789)
Staphylococcus aureus, foodborne	323‡	25.5	29.3	<1	100	241,148 (72,341–529,417)
Streptococcus spp. group A, foodborne	15‡	25.5	29.3	<1	100	11,217 (15–77,875)
Vibrio cholerae, toxigenic	8§	1.1	33.1	70	100	84 (19–213)
V. vulnificus	111§	1.1	1.7	2	47	96 (60–139)
V. parahaemolyticus	287§	1.1	142.4	10	86	34,664 (18,260–58,027)
Vibrio spp., other	220§	1.1	142.7	11	57	17,564 (10,848–26,475)
Yersinia enterocolitica	950¶	1.0	122.8	7	90	97,656 (30,388–172,734)
Subtotal						3,645,773 (2,321,468–5,581,290)
Parasites						
Cryptosporidium spp.	7,594¶	1.0	98.6	9	8	57,616 (12,060–166,771)
Cyclospora cayetanensis	239¶	1.0	83.1	42	99	11,407 (137–37,673)
Giardia intestinalis	20,305§	1.3	46.3	8	7	76,840 (51,148–109,739)
Toxoplasma gondii		1.0	0.0	<1	50	86,686 (64,861–111,912)
Trichinella spp.	13§	1.3	9.8	4	100	156 (42–341)
Subtotal						232,705 (161,923–369,893)
Viruses						
Astrovirus	NA	NA	NA	0	<1	15,433 (5,569–26,643)
Hepatitis A virus	3,576§	1.1	9.1	41	7	1,566 (702–3,024)
Norovirus	NA	NA	NA	<1	26	5,461,731 (3,227,078–8,309,480)
Rotavirus	NA	NA	NA	0	<1	15,433 (5,569–26,643)
Sapovirus	NA	NA	NA	0	<1	15,433 (5,569–26,643)
Subtotal						5,509,597 (3,273,623–8,355,568)
Total						9,388,075 (6,641,440–12,745,709)

*All estimates based on US population in 2006. Modal or mean value shown unless otherwise stated; see online Technical Appendix 3 (www.cdc.gov/EID/content/17/1/7-Techapp3.pdf) for the parameters of these distributions. STEC, Shiga toxin–producing *Escherichia coli*; ETEC, enterotoxigenic *E. coli*; NA, not applicable. An expanded version of this table is available online (www.cdc.gov/EID/content/17/1/7-T2.htm).
†Percentage foodborne among domestically acquired illnesses.
‡Passive surveillance data on outbreak-associated illnesses from the Foodborne Disease Outbreak Surveillance System. Estimates based on the number of foodborne illnesses ascertained in surveillance and therefore assumed to reflect only foodborne transmission.
§Passive surveillance data from Cholera and Other *Vibrio* Illness Surveillance or the National Notifiable Disease Surveillance System.
¶Active surveillance data from Foodborne Diseases Active Surveillance Network, adjusted for geographic coverage; data from the National Tuberculosis Surveillance System for *M. bovis*.

Bacteria

Several bacteria including *Salmonella, Campylobacter,* Pathogenic *E. coli, Listeria*, etc. are known to cause infections. Food intoxication occurs by consumption of food contaminated with toxin produced by bacteria, which includes *Staphylococcus aureus, Clostridium botulinum*. In some case, toxin is released in the intestine by bacteria (e.g. *Clostridium perfringens*), cause illness.

Gram-negative foodborne pathogens rarely fetal to healthy people, but may be fatal to young, old, or immunocompromised people. Gram-negative foodborne pathogens include *Campylobacter* species, *Salmonella enterica, Escherichia coli* (STEC), *Vibrio* species*, Shigella* species*,* and *Yersinia enterocoliticai.* These bacteria commonly found in intestine of human, animal, and environment, therefore infection can occur by consumption of raw agricultural product like raw milk, undercooked meat or fish, and raw shellfish. Cross-contamination from raw material during food processing, improper processing, poor sanitation or hygiene may result in the contamination of food and causing human illness.

Gram-positive bacteria generally show a rapid onset and short-lasting, which include *Bacillus cereus,* some of *Clostridium* species, *Listeria monocytogenes*, and *Staphylococcus aureus.*

Viruses

The incidence of viral gastroenteritis is drastically increasing, and hepatitis A and norovirus are involved in many of outbreaks. Shellfish is the common source of contamination due to its filter-feeding processing. Relatively less information is available regarding viral foodborne illness due to difficulty on detecting viral pathogens.

Parasites and Protozoa

Parasites, flatworms, tapeworms, and flukes, can enter into human via consumption of raw or undercooked meat from infected pig, cattle, wild animal, or fish. Several pathogenic parasites are known including *Clonorchis sinensis* (fish), *Taenia saginata* (beef), and *Trichinella spiralis* (pork). Infection from these parasites effectively prevented by heating, freezing, drying, etc.

Protozoa *including Cyclospora cayetanensis*, *Cryptosporidium parvum*, *Giardia intestinalis,* and *Toxoplasma gondii,* can enter into human by consumption of infected meat, raw milk, contaminated water, or direct contact with infected animal. Infections can be prevented by heating, freezing, or drying.

Prions

Prions are composed of protein material, but are transmissible and has an infectivity to cause brain cell damage leading to formation of spongiform encephalopathies. Several animal and human disease including Bovine Spongiform Encephalopathy (BSE), scrapie, and creutzfeldt-Jacob disease (CJD) and its variant (vCJD) are known to be caused by prions. Of them, the most well-known prion disease that has been threaten food safety is BSE, which closely associated with developmemt of vCJD, by consumption of beef or beef-product from infected cattle.

Emerging pathogens

Emerging pathogens refers for the organisms that has not been recognized as an etiology of human disease, or has been previously recognized but occurred in a new geographical areas, populations, or a new vehicles. Many of food pathogens, including *E. coli* O157:H7, *E. coli* O104:H4, *Campylobacter, Cronobacter, Listeria monocytogenes* etc. have emerged in the past few decades, causing serious human foodborne illness. In addition, the emergence of a new food pathogens continues to increase.

II. Bacterial Foodborne pathogens

A. Shiga toxin-producing *Escherichia coli* (STEC)

General introduction

Escherichia coli is a large and diverse group of bacteria that lives in the intestinal tract of human and animal. They are mostly harmless and play important role as a normal gut flora. However, some groups of *E. coli* are pathogenic causing not only intestinal, but also extra intestinal illness including urinary tract infections (UTIs), sepsis, or meningitis (6, 7). The clinical symptoms of *E. coli* infection varies from mild gastrointestinal problems to the life-threatening disease. The pathogenic *E. coli* categorizes into 6 major pathotypes, Shiag toxin-producing *E. coli* (STEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely adherent *E. coli* (DAEC) (Fig. 1) (6).

STEC is the most serious of these pathotypes, which is characterized by producing of shiga toxins, STX type 1 and 2. STEC was first discovered in 1977 by Konowalchuk *et al*., reporting a strains of *E. coli* that producing cytotoxin active on vero cells (a African Green Monkey kidney cell) (8). The strain was called a verotoxin-producing *E. coli* (VTEC) following its attribute to produce a verotoxin.

In 1982, two outbreaks occurred in the United States by *E. coli* O157:H7 infection, and the infection was characterized with unusual gastrointestinal illness showing bloody diarrhea (9). The *E. coli* O157:H7 strain had a high level of cytotoxin that were active on HeLa and Vero cell as well as neutralized by anti-Shiga toxin. Because the toxin was highly similar in structure and biological activity to shiga toxin generated by *Shigella dysenteriae*, the toxin was called a shiga-like toxin (SLT) (10). Consequently, these two toxin, verotoxin and SLT, were found to be the identical, and the term Verotoxin-producing *E. coli* and Shiga-like toxin producing *E. coli* were used interchangeably. Finally, the toxin was designated as shiga toxin, thus the strains of *E. coli* producing these toxin was proposed to be named as STEC (11).

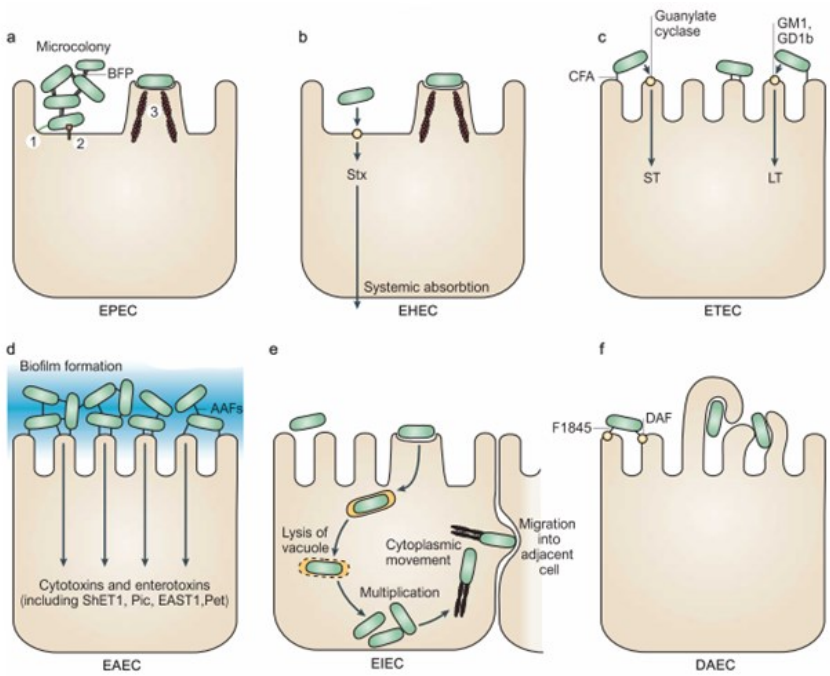


Figure 1. Pathogenic schema of diarrheagenic *E. coli.* Adapted from Kaper *et al*., 2004.

Importance of STEC as a foodborne pathogen

STEC, also called enterohemorrhagic *E. coli* (EHEC) or verocytotoxic-producing *E. coli* (VTEC), is characterized by producing of shiga toxins, STX type 1 and 2. The STEC is one of the most commonly reported pathotype in association with foodborne outbreak. The STEC infection often cause severe illness including hemorrhagic colitis, thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome (HUS), which may result in death at high rate (12-14). Due to its high

incidence and high rate of progress in severe disease, STEC is considered as the most notorious pathotype among pathogenic *E. coli.*

More than 400 serotypes of *E. coli* are able to produce shiga toxins (15). Of these, a serotype O157:H7 is the most notorious serotypes, which led to further categorize STEC serotypes into STEC O157 and non-O157. Since its first recognition in 1982, STEC O157 has become a single most common serotype. Because of its low infectious dose and high rate of progression in severe disease of STEC O157:H7, it has subjected of many studies and regulatory framework (16). STEC O157 is specially associated with the progression to HUS, showing high mortality rate of 2 to 10%. Of the 176,000 illness, 2,400 hospitalizations, and 20 deaths caused by STEC annually in the United States, serotype O157 is implicated in roughly 35% of illnesses, 89% of hospitalizations, and 100% of deaths (17).

However, it is obvious that STEC non-O157 become a significant cause of human illness. While STEC non-O157 tend to cause watery diarrhea more often than bloody diarrhea, severe illness including HUS caused by STEC non-O157 is also reported in high portion in some geographic regions (18, 19). In Denmark, STEC non-O157 contributed 41% of all STEC strains isolated during 2003 to 2005 with O103, O146, and O26 being the most common serotypes (20). In Australia, STEC non-O157 was also responsible for 42% of all STEC strains isolated during 2000 to 2010, with O111 and O26 being the most common serotypes (21). US Department of Agriculture

(USDA) has announced the STEC serogroups of O26, O45, O103, O111, O121, and O145 as a "Big 6 STEC non-O157 strains", showing higher incidence with severe illness in human during 1983 to 2002. The "top 6" serotypes were accounted for about 75% of all STEC non-O157 illness (16, 22).

In the Republic of Korea, STEC is one of the causative agents of bacterial foodborne illness estimating approximately 100 cases of illness and 3-4 cases of outbreak occurred annually (23). In addition, the outbreak cases are continuously increase in every year, causing social and economic problems (23). Although STEC O157 is the most common serotype rating 20.4% of all STEC infection, numerous STEC non-O157 strains were isolated from diarrheic patients. Of STEC non-O157, O103, O26, O91, and O8 were the top four most frequently identified serotype in the Republic of Korea (24).

Virulence potentials of STEC

-Shiga toxins

Shiga toxins are the principal virulence factor for STEC as its name implicated. Two types of Shiga toxin, *Stx1* and *Stx2*, vary antigenically, and each has several antigenic variants (10, 25). Although *Stx1* is much more homogeneous, there exists many variants in *Stx2*. (26). The numerous STEC isolates produce more than two

forms of *Stx2* variants differing by a few amino acid, but the pathogenesis of different forms of toxins are not understood (27, 28). There are several evidence that STEC harboring *Stx2* is more associated with severe illness in human than STEC harboring *Stx1* only or both *Stx1* and *Stx2* (29, 30). In addition, *Stx2* has shown to be 1,000 times more toxic to the human renal microvascular endothelial cells, the target cell of Shiga toxin for development of HUS (31, 32). While the majority of STEC O157 harbors *Stx2* only, many of STEC non-O157 harbors various Stx type, which coincided with the fact that higher HUS rate in STEC O157 infection (26).

-Adhesion

Attaching and effacing (A/E) lesions: A/E lesions are the most important characteristics of STEC O157, some other STEC non-O157, and EPEC (33). A/E lesions are characterized by cytoskelectal changes, effacement of intestinal epithelial microvilli, formation of pedestal-like structure, and protein translocation by Type III secretion system (34). The genes required for formation of A/E lesions are encoded in pathogenicity island (PAI) called locus of enterocyte effacement (LEE) (35). The genes in LEE can be divided into several groups; i) secreted proteins necessary for signal transduction and A/E activity (*EspA, EspB, EspD*), ii) type III secretion apparatus, and iii) *eae* (encoding intimin) and *Tir* (encoding Tir protein for translocated intimin receptor) (33, 36)

-Attaching and effacing Activity: Whilst there are many factors that associated with STEC adhesion, most research focused on intimin as a potential adhesion factor. In EPEC, intimin is responsible for both initial and intimate attachment, and reduced virulence was observed in intimin-deficient EPEC mutant (37-39). STEC intimin is also responsible for attachment to HEp-2 cells, and the reduced virulence was observed in intimin-deficient STEC O157 mutant as well (40, 41). However, the intimin of EPEC and STEC is different in receptor binding specificities, suggesting the different tissue tropisms (41). Different antigenic structures also observed among STEC strains, showing less than 60% homology with the intimin of STEC O157, which alter the adhesive properties as well as the ability to cause disease (42).

While almost all STEC O157 express intimin, intimin in STEC non-O157 reported less frequently which coincide with the severe illness reported in STEC O157 (43). In particular, eae-positive strains were isolated higher in human than in animal, supporting its role in pathogenesis, which supports the fact that reduced virulence was observed in intimin-negative STEC non-O157.

-Acid resistance

To cause gastrointestinal disease, STEC had to pass through the acidic environment, stomach. To overcome acidic environment, three major acid resistance

systems operates; i) an acid-induced oxidative system, ii) an acid-dependent arginine-dependent system, and iii) a glutamate-dependent system (44-46). Although acid tolerance does not ensure the enhanced virulence of STEC (47), it has benefit to enhance the survival of STEC in acidic environment including food vehicles, or water (48). In addition, the acid-resistant STEC may be involved in the infection of STEC with low dose (26, 49).

-Enterohemolysin

Enterohemolysin (Ehx) was first discovered by Beutin et al. in 1989 (50). Ehx is characterized by formation of small zones of hemolysis on blood agar after 18-24h of incubation, which is differed from α -hemolysin of *E. coli* by formation of larger zones after 4h incubation (51). Whilst the exact function of Ehx has not been elucidated yet, the presence of highly conserved *ehx* operon in STEC strains suggests significant role in STEC survival (52). Enhanced production of Ehx under the conditions with low or absented oxygen in vitro suggests that the toxin may be actively produced in large intestine of human and animal (53). Ehx is commonly produced by STEC serogroup of O157, O26, and O111, as well as in eae-positive STEC (26, 54). In addition, Ehx incidence was higher in STEC causing HUS than that of the diarrhea, indicating that Ehx may be associated with the development of HUS (55).

-Extracellular serine protease

EspP is a plasmid-encoded, extracellular serine protease, which pathogenesis is largely unknown (56). It has proteolyrtic activity including human coagulation factor V. Coagulation factor V is involved in the blood clotting cascade, which indicates that cleavage of factor V may induce a prolonged bleeding. Therefore, degradation of factor V by STEC could induce bloody diarrhea (51). However, absence of Esp also causes both HC and HUS, indicating EspP might be not essential to develop such diseases in humans (26). Unlike from other extracellular proteins like EspA or EspD (a plasmid-encoded, extracellular serine protease), EspP is neither involved in formation of A/E legion nor encoded in the LEE (33, 56)

-Catalase/Peroxidase

The gene *katP* encodes a bifunctional catalase-peroxidase. Although the role of this enzyme is not clear, it has suggested that the protein may help STEC escaping from host defense as well as assist recovery of STEC from heat stress (57). The presence of KatP depends on the serotypes of STEC, reporting its present in 66% of STEC O157, 38% of STEC non-O157, and none of sorbitol-fermenting STEC O157 (58, 59).

Epidemiology of STEC

-STEC infection

STEC infection first implicated as a foodborne pathogen in 1982 through 2 outbreaks occurred in the Oregon and Michigan states in the United States. At least 47 people were infected by STEC O157:H7 and the illness was characterized with bloody diarrhea with severe crampy abdominal pain, and little or no fever (9). Before then, STEC O157:H7 was rare serotype of *E. coli*, only reported in 1975 for sporadic case of HC. The etiological agent of the 1982 outbreak was the consumption of undercooked beef patty in fast food restaurant, which led to arouse the significant of STEC as a foodborne pathogen (9).

Since then, several sporadic and large outbreak were reported worldwide, and become a significant public health concern. The low infectious dose is one of the most important factor for STEC infection. Infectious dose for STEC infection estimated less than 100 organisms (60), and one study estimated between 20 to 700 organisms (61). The clinical symptoms of STEC infection varies that ranged from mild, self-limiting gastrointestinal problem to severe, life-threatening complications, including HUS or HC (13, 14, 28). While bloody diarrhea and HC with severe abdominal pain is frequently observed in STEC infected patients, 1 to >20% of patients may develop to HUS and which bring about a mortality rate of 2 to 10% (13,

26). In particular EHEC, a subset of STEC, is known to be more associated with HC and HUS (7).

-STEC outbreaks

STEC O157, the most notorious of the STEC strain, and hundreds of STEC non-O157 strains are associated with both endemic and epidemic infection worldwide. Ruminant, especially cattle, is a major reservoir of STEC, and consequently, many of outbreaks were associated with contamination with cattle manure, diary product, or direct contact with animals (62, 63). Besides bovine sources, raw vegetables including lettuce (64, 65), radish sprouts (66), and spinach (67), processed foods including salami (68), sausage (69) etc. were involved with large outbreaks.

In North America, 17 outbreaks caused by STEC O157:H7 occurs annually resulting over 75,000 human infections (70, 71). From 1982 to 2002, a total of 350 STEC O157 outbreaks were reported in the United States. Of them, 183 (52%), 50 (14%), 31 (9%), and 11 (3%) were foodborne, person-to-person, waterborne, and animal contact, respectively (71). Of 183 foodborne outbreak, 93 (51%) were associated with cattle (ground beef, other beef, and dairy products) (71). STEC O157 outbreaks were also reported in other countries such as Canada (72), Japan (66), Cameroon (73), and the Republic of Congo (74).

STEC non-O157 outbreak was first reported in 1990, and a total of 46 outbreaks were reported up to 2010 in the United States (75). The number of STEC non-O157 outbreaks are increased after 1998, and 27 (59%) outbreaks occurred during 2007 – 2010. The most frequently reported serotype were serogroup O111 (14 outbreaks) followed by O26 (11 outbreaks), O45 (4 outbreaks), O103 (2 outbreaks), O121 (2 outbreaks), O145 (2 outbreaks), O104 (1 outbreak), O165 (1 outbreak), undetermined (1 outbreak). The STEC non-O157 caused 1,727 illness, 144 hospitalizations, and 1 death, and serogroup O111 was responsible for the death (75). In Europe, while STEC O157 is accounted for the most infections, STEC non-O157 infection cases had increased as well as the rate of development to HUS which were increase as many as 50% in Germany (76), Italy (77, 78), France (79), United Kingdom (29), and Norway (69).

While few examples of STEC O157 and non-O157 outbreaks were described above, many more outbreaks are continuously occurring globally. Moreover, the increase of direct and indirect contacts of human bodies including material resources facilitates outbreak and expansion of a new virulent strain of bacteria. In 2011, starting from Germany, *E.coli* has spread the entire Europe within a few months causing more than 4,000 cases of infection, including more than 50 cases of death. The causative agent was *E.coli* O104:H4 which increased capabilities of

survivability and virulence by acquiring both aggregative factor and antimicrobial resistance factor (80, 81).

-Prevalence of STEC in cattle

Ruminants are the principal reservoir of STEC, reporting 435 serotypes of STEC from cattle isolates. Indeed, among more than 470 serotypes isolated from human, most of them were also recovered from cattle isolates. Due to absence of stx receptor, globotriaosylceramide (Gb3), in the intestinal tract of cattle, they do not cause disease and act as an asymptomatic carrier (82). As a major reservoir of STEC, cattle were subjected for many prevalence studies, mainly for STEC O157:H7. The prevalence of STEC in cattle varies dramatically depending on sampling or isolation methodologies as well as factors associated with STEC shedding.

Many of studies were focused on the prevalence of STEC O157:H7 due to its significant in causing human disease. In beef cattle, the prevalence of STEC O157 reported 9.2% to 11.4% in USA (83, 84), 8.9% in Japan (85). On the other hand, the prevalence of STEC O157 in dairy cattle was reported as 1.2 to 69.0% in Japan (86, 87). In Korea, the prevalence of STEC O157 was investigated in beef and dairy cattle, reporting 1.7% and 6.7% in beef and dairy cattle, respectively (88), which is coincided with recent study reporting the higher prevalence in beef (21%) than dairy

cattle (13%) (89). The difference prevalence between beef and dairy cattle seemed to be the difference management or hygiene rather than breed of cattle (85, 89).

Some of studies compared the prevalence of STEC by serotypes, mainly STEC O157 and non-O157. In USA, the prevalence of STEC O157 and non-O157 in beef cattle ranged from 0.3 to 19.7% and 4.6 to 55.9% in feedlot, and 0.7 to 27.3% and 4.7 to 44.8% on pasture, respectively (90), while the prevalence of STEC O157 and non-O157 in dairy cattle was 0.2 to 48.8% and 0.4 to 74.0%, respectively (91). In EU, the prevalence of STEC and STEC O157 in cattle ranged from 2.2 to 6.8 % and 0.5 to 2.9%, respectively (92). In Swiss, 57.6% of STEC was detected by PCR, among them STEC O157 accounted for 8.0% (93).

-Factors influencing excretion of STEC

Seasonality: Seasonal variations in prevalence of STEC were reported. Numerous studies implicates the STEC is highly excreted in warmer season, especially during a late summer to early autumn, while the prevalence of STEC shows decrease in cold, winter season (89, 94-96). In meta-analysis, fecal prevalence of STEC O157 was significantly higher during summer in both beef and dairy cattle (7.87% vs. 4.21% in beef cattle, 2.27% vs. 0.36% in dairy cattle) (97). However, some of studies reported the opposite results or no seasonal difference in prevalence of STEC (84, 98-100).

Age of cattle: The prevalence of STEC was also investigated in cattle with different age groups, which is likely associated with weaning and age. Several studies resulted similarly that STEC excretion was more dominated in post-weaning calves, especially in calves aged 4 to 12 months than pre-weaned calves (62, 101-103). Although some hypothesized that the colostrum might prevent from STEC infection, they found no correlation between them (104, 105).

Diet: Many studies implicates the correlation between diet and STEC shedding. The difference in diet may bring about the difference in gastrointestinal environment, including pH and VFA concentrations, and this may be linked to the environmental conditions for STEC growth. One of the generally accepted relation is the positive association of barley grain and *E. coli* O157 shedding. Several observational and experimental studies found the higher concentration of STEC O157 in cattle fed barley grain than those fed corn-based diet. Cattle fed grain or molasses had a higher risk than cattle fed barley silage (102). In addition, cattle fed brewers grains had a higher risk of *E. coli* O157 shedding (OR: 2.87) (106). In calves, feeding hay had a significantly higher risk than calves not fed hay on STEC O157 shedding (102)

Lactation: In dairy cattle, STEC shedding was significantly higher during first lactation (Odd ratio; OR: 1.8), especially less than 30 days in milking (OR:3.9), suggesting that negative energy balance together with the stress during lactation may be associated with the STEC prevalence (89).

Phage type: Several virulence factors were compared between cattle shedding high level of STEC and low-level of STEC. While no association were identified between virulence genes and levels of STEC shedding, phage type (PT) was found to be related to the STEC shedding level. PT 21/28 was significantly higher in high-level shedder, whereas PT32 was significantly higher in low-level shedder (107).

B. *Campylobacter jejuni*

General introduction

Campylobacter was believed to be first discovered by Theodore Escherich in 1886, reporting non-culturable spiral shaped bacteria (108). However, due to its fastidious growth condition, *Campylobacter* was able to be isolated in 1913 from aborted bovine fetuses (109). After that, the *C. jejuni* and *C. coli* were isolated from feces of diarrheic cattle and pig, respectively, but named as *Vibrio jejuni* and *V. coli* (110, 111). The Genus *Campylobacter* got its name in 1963, which characterized

with small, slender spirally curved rod shaped gram negative bacterium that grow under the microaerophilic conodition (112). Under unfavorable condition, *C. jejuni* can adopt a viable but not culturable (VBNC) form, while retaining its infectivity (113, 114). The VBNC forms might be able to convert culturable form after passing through the intestinal tract of chicken (115, 116).

Until now, approximately 20 Campylobacter species were identified (117). *Campylobacter* was first recognized as etiologic agent of animal disease, but *Campylobacter* has become interested as a cause of human disease, reporting high incidence in diarrheic human (116, 118)

Importance of *C. jejuni* as a foodborne pathogen

Camplyobacter is one of the most commonly reported pathogen of bacterial gastroenteritis in the United State and in the United Kingdom. *C. jejuni,* which accounts for approximately 90% of *Campylobacter* infections, is a major cause of global foodborne illness (119, 120). The FOODNET, the foodborne diseases active surveillance network in the USA, estimated that the incidence per 100,000 populations for *Campylobacter* spp. was 14.3 in 2012, which was the second highest incidence among foodborne pathogens, and a 14% increase compared to the incidence reported between 2006 and 2008 (119). In Europe, the Campylobacteriosis has been the most prevalent zoonotic pathogens reporting more than 200,000

confirmed cases in 2009, and the incidence for *Campylobacter* spp. was much higher than USA scoring 45.2 cases per 100,000 populations (121, 122).

Until now, a few studies have been reported regarding *campylobacter* infection in the Republic of Korea. The first recognized *C. jejuni* outbreak was occurred in 2009 rating an attack rate of 11.6% (123), and the number of sporadic and outbreak cases cause by *Campylobacter* infection has increasing year by year, highlighting the need of continual monitoring of *C. jejuni* in various sources (Fig. 2) (124). Although Campylobacteriosis is usually self-limiting, in rare cases severe and long-lasting illness can occur, and may develop to more serious illness including Guillain-Barre´ syndrome and reactive arthritis (125).

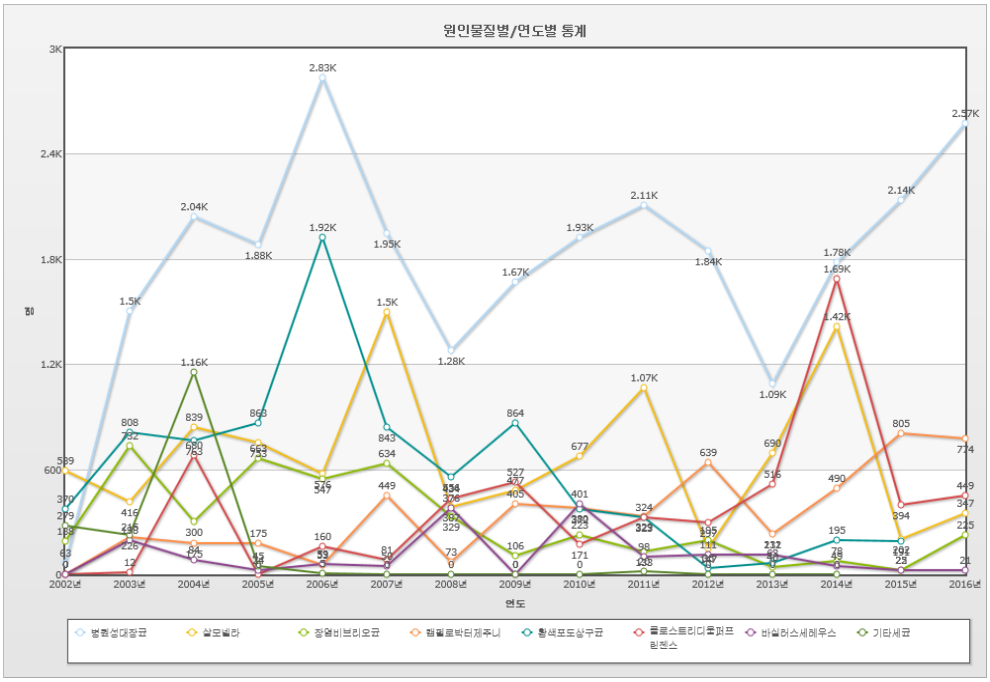


Figure 2. Foodborne illness cases caused by bacterial pathogens in Korea from 2002 to 2016. Adapted from KCDC (last updated at 2016.12) (http://www.foodsafetykorea.go.kr/portal/healthyfoodlife/foodPoisoningStat.do?menu_no=519&menu_grp=MENU_GRP02).

Virulence potentials of *C. jejuni*

The pathogenesis of *Campylobacter* is not fully understood. Few virulence factors including flagella, cytolethal distending toxin, and resistance to gastric acid and bile salts were known to be associated with bacterial virulence.

-Genetic variation and natural transformation

C. jejuni is a genetically diverse bacterium showing extensive genetic variation among stains as well as high rate of genetic exchange between strains (126). In addition, *C. jejuni* is able to acquire DNA not only from other bacterium via horizontal transfer but also from environment, which ensuring genome plasticity to increase survival of the bacterium (127, 128). High frequency of variation was reported especially in regions encoding capsult, lipooligosacchraide (LOS), and flagellum, which involved in surface-structure modification or biosysthesis (126).

-Lipooligosaccharide (LOS) and capsule

LOS and capsule structure of *C. jejuni* is highly variable, which are responsible for the avoidance of immune response (129). The similar molecular structure of LOS is thought to be bring about the autoimmune disease including Guillain-barre syndrome or Miller-Fisher syndrome in human with Campylobacteriosis (125). In addition, the *C. jejuni* capsule is also responsible for serum resistance, and adhesion and invasion of epithelial cell (130).

-Flagella

Flagella and Flagella motility is an essential not only for colonization of the small intestine but also for virulence, secretion, and host-cell invasion (126). The

regulation of flagella motility is important for bacterial survival, especially in the various ecological conditions in the gastrointestinal tract (131).

-Protein Glycosylation

There are 2 protein glycosylation system, O-linked and N-linked glycosylation (132). O-linked glycosylation is important for assembling flagella filament. Defects of it bring about loss of motility, reduced virulence, and decreased adhesion and invasion to host cell (133). N-linked glycosylation modifies arginine residues on other proteins. While the role of N-linked glycan is not elucidated, it is suggested that might be involved in fundamental role, considering their highly conserved N-linked glycan in all *C. jejuni* (134). It is assumed that N-linked glycan might be responsible for evading the host immune response by changing the immunoreactivity of some glycosylated proteins (132).

-Cytolethal distending toxin (CDT)

CDT is produced by diverse group of gram negative bacteria. The toxin composed of three subunits, CdtA, CdtB, and CdtC, and causes cell to arrest at G1/S or G2/M phase of the cell cycle, leading to cell death (135, 136). While the role of CdtA and CdtC is not clear, one or both are thought to be required for CdtB delivery and binding to host cells (137). Once entering to host cell, CdtB localized in nucleus,

breaking double stranded DNA by DNase I activity (135). In addition, CDT is thought to be associated with long-term, asymptomatic infection by avoiding host immune-response (138, 139).

Epidemiology of *C. jejuni*

-*C. jejuni* infection

Campylobacter infection commonly causes acute gastroenteritis which is characterized with abdominal pain, fever, and diarrhea (140) . Interestingly, there are two disease manifestations observed in *campylobacter* infection according to the socio-economic status. While bloody diarrhea is frequently observed in developed countries, watery diarrhea is predominant in developing countries (141). The incidence per 100,000 populations for *Campylobacter* spp. was 14.3 and 45.2 in the United States and the United Kingdom, respectively (119, 121, 122). The information regarding the incidence of Campylobacteriosis in developing countries is limited due to lack of national surveillance system, and many of Campylobacteriosis is undiagnosed or unreported.

-*C. jejuni* outbreaks

Sporadic cases of Campylobacteriosis arise from a variety of transmission routes, such as waterborne transmission or animal contact and, more commonly, foodborne transmission, which accounted for 86% of cases in the United States between 1997 and 2008 (142). Given that *C. jejuni* can infect poultry, cattle, and swine without causing disease (143), humans are prone to *C. jejuni* infection by consumption of contaminated meat or dairy products prepared from asymptomatic animals.

Although sporadic cases is commonly reported in human Campylobacteriosis, outbreaks of Campylobacteriosis could be more common than previously expected. Recently, the number of outbreaks were reported worldwide including United Kingdom (144, 145), Greece (146), USA (147), Canada (148), and Norway (149), and the implicated food were found to be the contaminated drinking water, raw milk, and chicken products.

-Prevalence of *C. jejuni* in cattle

Although poultry are known to be a major source for *C. jejuni* contamination, recent studies have revealed that cattle are also one of the major sources for human Campylobacteriosis. The prevalence of *C. jejuni* was reported at 19.6 to 34.1% in the United States (150, 151), 35.9% in the United Kingdom (152), and 20.2% in Spain (153) in cattle farms, and at 47% in Denmark (154) in a slaughterhouse. In

addition, consumption of undercooked meat, tripe, or raw milk and direct contact with cattle are also considered to be significant risk factors (155, 156). Similarly, molecular epidemiological studies have indicated that cattle-associated *C. jejuni* isolates contribute to a considerable portion of human infection together with poultry-associated *C. jejuni* isolates (157-159).

III. Detection of STEC and *C. jejuni*

A. Isolation of STEC by standard culture method

E. coli is a gram negative, fermentative, and rod-shaped bacterium that inhabits the intestinal tract of human and warm-blooded animals. It can grow under aerobic and anaerobic condition, preferentially at 37°C. *E. coli* can be isolated directly from stool sample by plating on selective agar. MacConkey agar is a commonly used selective agar appearing red or pink on media for lactose-fermenting *E. coli* (160).

STEC O157 is traditionally detected by a selective culture method based on the inability to ferment sorbitol (28), appearing white colonies on sorbitol MacConkey agar (161). In addition, there are some commercial product to aid isolation of STEC O157. CHROMagar^{TM} O157 is used for the selective isolation and differentiation of *E. coli* O157, which appears mauve color on media whereas other bacterium appears blue, colorless or inhibited their growth (162). Moreover, IMS step can be added for increasing detection rate. IMS is a method that can efficiently isolate cells out of body fluid or cultured cells using STEC O157 antibody-coated magnetic beads. Higher sensitivity of IMS techniques were reported compared to that of the direct culture method (163-165). The identified colonies then further tested for its serotype,

O157 and H7. These antigens could be identified by antigen-antibody agglutination assay, which commercial kits are also available (166).

There are several challenges for isolation of STEC non-O157 due to the lack of phenotypic characterization. Although a few selective culture media were developed including rhamnose-MacConkey agar for STEC O26, and tellurite-containing media for STEC O26, O111, and O145, the use of these media is limited for specific serotypes (167-169). In addition, some of commercially available chromogenic agar, CHROMagar STEC and modified rainbow agar, can be used for isolation of STEC non-O157, further analysis for serotyping is required (170). Several immunological-based methods are used to identify *E. coli* O antigens for detection and identification of STEC non-O157. Antigen-antibody agglutination test are generally used, but it is time consuming and labor-intensive (171). Some of commercially available latex agglutination kits, IMS product, flow cytometry, and enzyme-linked immunosorbent assays (ELISA) have been adapted for identifying serogroup, but it is limited to only a few serotypes, especially for the "top 6 STEC non-O157" serogroup (172, 173).

To confirm STEC, Shiga toxin must be detected. Traditionally, cell cytotoxicity assay was performed to detect Shiga toxin, but this method is time-consuming and labor-intensive technique (18, 28). Besides cell cytotoxicity assay, several commercially available kits including enzyme immunoassay (EIAs) kit, Shiga toxin colony immunoblot are also in use (174-176).

B. Isolation of *C. jejuni* by standard culture method

C. jejuni generally requires microaerophilic (2–10% O_2), capnophilic (3–5% CO_2), and thermophilic (30–43°C) growth conditions. Because *campylobacter* species are highly sensitive to oxygen, several selective media were developed to reduce the level of oxygen including blood, ferrous iron, or pyruvate. Several selective media were developed and those selective media could be categorized into two major groups based on the use of animal blood or not. Skirrow medium, Blaser medium, and Preston medium utilize blood as supplement (177-179), but these media have several limitations including relatively expensive cost, variable quality of blood, and vulnerability to contamination (180). On the other hand, there are several blood-free selective media including Karmali medium, cefoperazone amphotericin teicoplanin (CAT) medium, Cephazolin charcoal deoxycholate (CCD), and modified cefoperazone charcoal deoxycholate (mCCDA) agar (180-183). For confirmation of *C. jejuni*, morphological profiling and biochemical test are required. To differentiate *C. jejuni* from *C. coli*, hippurate hydrolysis test is generally used (184).

C. Application of molecular-based methods

Due to lack of phenotypic characterization of STEC non-O157, standard culture methods for isolation STEC non-O157 is often accompanied with molecular-based method to detect shiga toxin gene. Fast, sensitive, and specific nucleic acid-based amplification (NAAT) methods have recently been adopted to supplement the conventional culture method. The standard manuals established by the United States Department of Agriculture (USDA) and the United States Food and Drug Administration (FDA) recommend PCR and real-time PCR (qPCR) as screening methods for detection of STEC by targeting various genes, including *stx1* and *stx2* (185, 186).

Although conventional culture is the current gold standard for the detection of *C. jejuni*, the isolation and identification of this organism are problematic due to its fastidious growth condition requirements and the morphologic similarity to other *Campylobacter* spp. Indeed, the performance of conventional culture methods has been shown to be compromised for *C. jejuni* at low doses or in a state of VBNC (113, 187). Moreover, characterized phenotype on selective media is sometimes difficult to read due to expression of atypical phenotype. These limitations may be overcomed by applying NAAT, such as PCR and real-time PCR. Several studies employed PCR and real-time PCR techniques for detection of *C. jejuni* targeting *hipO* gene (188,

189). The uniqueness and high within-species conservation DNA sequence of the *C. jejuni*-specific *hipO* gene, which encodes hippurate hydrolase, make it suitable for the identification of *C. jejuni,* as well as for its differentiation from other *Campylobacter* spp. (188-190).

D. LAMP assay for detection foodborne zoonotic pathogen

Loop-mediated isothermal amplification (LAMP)

Recently, a novel NAAT named LAMP assay has developed as a rapid, simple, and accurate technique (191). The use of three primer sets, namely, an inner primer set (FIP and BIP), an outer primer set (F3 and B3), and a loop primer set (LF and LB), results in highly specific, sensitive, and rapid reactions, which can amplify low titers of DNA templates (191, 192). The LAMP assay recognizes 6–8 distinct regions of the DNA template, thereby providing high specificity and sensitivity (191, 192). In addition, a special DNA polymerase, *Bst* polymerase or *Gsp* polymerase, reacts under isothermal conditions at 60°C to 65°C within 1 h (191, 193). The amplified product can be detected by simple inspection of insoluble magnesium pyrophosphate, gel electrophoresis, or measuring the turbidity of the product (191, 194, 195).

Multiplex Loop-mediated isothermal amplification (mLAMP)

Despite the many advantages of the LAMP assay, multiplex LAMP (mLAMP) approaches are limited due to difficulty of differentiating more than two specific genes. Visual observation of LAMP products is indirect and cannot be used to distinguish multiple target genes. Gel electrophoresis after restriction enzyme digestion has been attempted (196-198), but is laborious and time-consuming. Moreover, the high sensitivity of the LAMP assay raises the concern of carry-over contamination during post-amplification analysis (199). Probe-based detection is also not applicable due to the various types of cauliflower-like structures of LAMP products (199).

Application of LAMP assay for detection STEC or *C. jejuni*

Several groups have used LAMP assays for detection of STEC strains targeting *stx1*, *stx2* (194, 195, 200), *eae* (194), *rfbE* (201), and serogroup-specific genes *wzx* and *wzy* (202), and have shown that the LAMP assay is more sensitive than PCR or qPCR.

Only a few studies are published for employing LAMP assay to detect *C. jejuni.* The previous studies targeted cj0414 gene, *C. coli*, and *C. fetus* for detection of *Campylobacter* spp. Using LAMP, revealing high sensitivity and specificity of LAMP assay compared to that of the culture method (203-206).

IV. Influence of enteric pathogens to Microbial community in intestine

A. Fecal shedding of STEC in cattle

During past decades, numerous researcher has focused on the level of shedding STEC in cattle, found out the heterogeneity of STEC carriage and shedding level (107, 207). While the most cattle shed STEC in at concentration of less than 10^2 CFU per gram of feces, some cattle shed high level of STEC at concentration of up to more than 10^7 CFU per gram of feces (107, 207-209). Chase-topping et al. analyzed the distribution of *E. coli* O157 in cattle (107). The mixture-distribution analysis revealed that there are two subpopulation among STEC shedding cattle fitting with normal-normal distribution. The threshold for a super-shedder was defined as cattle shedding more than 10^4 CFU/g in their feces based on the point of overlap of the two-component normal distributions (107, 210). In addition, some cattle shed STEC O157:H7 not only in high concentration, but also for longer duration, which named as a super-spreader (210-212).

The epidemiology of super-shedding is significant to control and prevent STEC. The presence of super-shedder results in within-farm transmission as well as between-farm transmission, which increase the prevalence of STEC in environment

(210, 213). Some study reported that less than 10% of super-shedder are responsible for more than 90% of the STEC shedding (107, 214). In addition, increased prevalence of STEC may increase the risk to spread STEC in environment, which influence on the human exposure of STEC, as well as the disease (210). What make super-shedder is not yet elucidated. Several risk factors might be related to the host, pathogen, or environment. A study reported that *E. coli* O157 with specific phage type is associated with high- or low-shedder (107), and another study suggests the relations between specific genotypes and the PT of *E. coli* O157 (215). Animal factors are also important for shedding STEC. Several studies implicated that STEC colonized in the mucosal epithelium at the terminal rectum are strongly associated with STEC shedding in both concentration and duration (212, 216, 217).

B. Microbial community analysis

The use of high-throughput sequencing-based molecular techniques, namely, NGS, provides more extensive information regarding the microbial ecosystem and is not limited to culturable bacteria, enabling multilateral approaches for understanding the microbial community in diseased and healthy states of the host. Several studies have utilized NGS techniques to characterize the microbiota of various organs such as the oral cavity, esophagus, stomach, intestine, or vagina in

humans, revealing not only bacterial species that are difficult to culture and the core microbiota, but also the relationship between microbiota and health status (218, 219).

Modern medicine considered gut microbiota as a third organ, recognizing its function and importance in human disease and health. Moreover, recent studies revealed the microbial diversity is the key to establish the interaction of microbiota and human. Gut microbiota is especially crucial for cattle due to its high dependency on microbiome for digestion of food, and numerous studies investigated the microbiota in reticulorumen to understand contribution of microbes to digestion. Investigation on the fecal microbiota is also one of the fascinated fields of study to understand microbial diversity and its application in controlling and preventing disease.

C. Indigenous microbes and STEC or *C. jejuni* shedding

Research on the individual factors underlying the bacterial shedding of STEC or *Campylobacter* in cattle has been limited. Microbiota comprise an important individual factor, playing a critical role in animal health, physiology, productivity, and bacterial shedding (210, 220). Indigenous microbes may inhibit or promote the colonization of pathogens by competing for nutrition or by using the byproducts of indigenous bacteria (221, 222). It is reported that organic acids and volatile fatty

acids and the presence of butyrate-producing bacteria might inhibit STEC shedding, and several bacterial species might promote or inhibit STEC shedding (223, 224). An increase in generic *E. coli* might inhibit *Campylobacter* colonization in mice (225). However, these studies involved culture-based techniques, limiting the understanding of microbial ecology in cattle.

D. Microbial community of cattle shedding STEC or *C. jejuni*

Fecal microbial communities were analyzed in beef and dairy cattle, revealed that they shared many of the bacterial groups, whereas the RA of the groups were profoundly differed. In particular, the phyla *Firmicutes* and *Bacteriodetes* were predominated in both beef and dairy cattle, which is coincided with previous report in mammalian samples, indicating their vital role in the mammalian gut (219, 226, 227). However, while the average RAs of *Firmicutes* and *Bacteroidetes* were 77.3~81.3% and 14.4~18.7%, respectively (228, 229), a lower concentration of *Firmicutes* (52.6~62.8%) and higher concentration of *Bacteroidetes* (29.5~42.1%) were reported in beef cattle (227, 230).

The microbial diversity in relation to STEC or *C. jejuni* shedding has been reported but not in dairy cattle (227, 230-232). Bacterial shift has been reported in *Campylobacter*-infected humans and mice (140, 225), and differences in bacterial

communities have been reported in *Campylobacter*-shedding chickens (233). Moreover, while STEC infection may be asymptomatic in cattle, the presence of STEC might influence the composition of intestinal microbiota in beef cattle (223, 230). Limited information on this aspect is available for dairy cattle, the management of which differs from that of beef cattle. This necessitates the characterization of microbial populations in relation to foodborne pathogen shedding in dairy cattle. Furthermore, the influence of *Campylobacter* infection on cattle microbiota has not been investigated.

Chapter I.

Development of a loop-mediated isothermal amplification assay for rapid, sensitive detection of *Campylobacter jejuni* in cattle farm samples

Abstract

Campylobacter jejuni is a leading cause of bacterial foodborne disease worldwide. The detection of this organism in cattle and their environment is important for the control of *C. jejuni* transmission and the prevention of Campylobacteriosis. Here, the development of a rapid and sensitive method for the detection of *C. jejuni* in naturally contaminated cattle farm samples was deveolped based on real-time loop-mediated isothermal amplification (LAMP) assay of the *hipO* gene. The LAMP assay was specific (100% inclusivity and exclusivity for 84 *C. jejuni* and 41 non-*C. jejuni* strains, respectively), sensitive (detection limit of 100 fg/μl), and quantifiable (R^2 = 0.9133). The sensitivity of the LAMP assay was then

evaluated for its application to the naturally-contaminated cattle farms samples. *C. jejuni* strains were isolated from 51/246 (20.7%) of cattle farm samples and the presence of the *hipO* gene was tested using the LAMP assay. Amplification of the *hipO* gene by LAMP within 30 min (mean = 10.8 min) in all *C. jejuni* isolates (n = 51) demonstrated its rapidity and accuracy. Next, template DNA was prepared from a total of 186 enrichment broth cultures of cattle farms samples either by boiling or using a commercial kit, and the sensitivity of detection of *C. jejuni* was compared between the LAMP and PCR assay. In DNA samples prepared by boiling, the higher sensitivity of the LAMP assay (84.4%) compared with the PCR assay (35.5%), indicates that it is less susceptible to the existence of inhibitors in sample material. In DNA samples prepared using a commercial kit, both the LAMP and PCR assays showed 100% sensitivity. This LAMP assay, which is the first of its kind for the identification and screening of *C. jejuni* in cattle farm samples, would play an important role in the prevention of *C. jejuni* contamination in the food chain, thereby reducing the future risk of human Campylobacteriosis.

Keywords: *Campylobacter jejuni* (*C. jejuni*), cattle feces, cattle farm, *hipO*, loop-mediated isothermal amplification

1.1. Introduction

Foodborne illness is a significant public health concern and an important cause of morbidity and mortality worldwide. *C. jejuni,* which accounts for approximately 90% of *Campylobacter* infections, is a major cause of global foodborne illness (119, 120). The Foodborne Diseases Active Surveillance Network in the United States estimated that the incidence per 100,000 populations for *Campylobacter* spp. was 14.3 in 2012, which was the second highest incidence among foodborne pathogens, and a 14% increase compared to the incidence reported between 2006 and 2008 (119). Sporadic cases of Campylobacteriosis arise from a variety of transmission routes, such as waterborne transmission or animal contact and, more commonly, foodborne transmission, which accounted for 86% of cases in the United States between 1997 and 2008 (142). Given that *C. jejuni* can infect poultry, cattle, and swine without causing disease (143), humans are prone to *C. jejuni* infection by consumption of contaminated meat or dairy products prepared from asymptomatic animals (142, 157). Although poultry are known to be a major source for *C. jejuni* contamination, recent studies have revealed that cattle are also one of the major sources for human Campylobacteriosis. The prevalence of *C. jejuni* was reported at 19.6 to 34.1% in the United States (150, 151) , 35.9% in the United Kingdom (152), and 20.2% in Spain (153) in cattle farms, and at 47% in Denmark (154) in a slaughterhouse. In addition, consumption of undercooked meat, tripe, or raw milk and direct contact

with cattle are also considered to be significant risk factors (155, 156). Similarly, molecular epidemiological studies have indicated that cattle-associated *C. jejuni* isolates contribute to a considerable portion of human infection together with poultry-associated *C. jejuni* isolates (157-159). While *C. jejuni* can be introduced at any point in the "farm to table" food production process (234), intervention for the control and prevention of Campylobacteriosis is most effective at the original source of contamination, namely, infected farm cattle.

C. jejuni generally requires microaerophilic (2–10% O_2), capnophilic (3–5% CO_2), and thermophilic (30–43°C) growth conditions. Under unfavorable condition, *C. jejuni* can adopt a VBNC form, while retaining its infectivity (113, 114). Although conventional culture is the current gold standard for the detection of *C. jejuni*, the isolation and identification of this organism are problematic due to its fastidious growth condition requirements and the morphologic similarity to other *Campylobacter* spp. Indeed, the performance of conventional culture methods has been shown to be compromised for *C. jejuni* at low doses or in a state of VBNC (113, 187). These limitations may be overcome by applying nucleic acid-based amplification methods, such as PCR and real-time PCR. Several studies employed PCR and real-time PCR techniques for detection of *C. jejuni* targeting *hipO* gene (188, 189). The uniqueness and high within-species conservation DNA sequence of the *C. jejuni*-specific *hipO* gene, which encodes hippurate hydrolase, make it suitable

for the identification of *C. jejuni,* as well as for its differentiation from other *Campylobacter* spp. (188-190).

LAMP is a recently developed nucleic acid amplification-based assay that utilizes polymerases such as *Bst* or *Gsp*, which are active under isothermal conditions between 60°C and 65°C, and provides rapid, accurate, and simple detection of target genes (191). Moreover, the use in this assay of three primer sets, namely, an inner primer set (FIP and BIP), an outer primer set (F3 and B3), and a loop primer set (LF and LB), results in highly specific, sensitive, and rapid reactions (191, 192). Traditionally, the amplification products can be visualized using magnesium pyrophosphate- or gel electrophoresis-based techniques (191). Recently, direct detection in closed test tubes reported in a recently developed real-time LAMP assay (235, 236) minimizes post-amplification contamination of samples. To date, a limited number of LAMP studies for *C. jejuni* have been conducted. Yamazaki *et al*. described the use of the LAMP assay for detection of *C. jejuni* in human stool (204) and in naturally contaminated chicken meat (206) through targeting the *cj0414* gene; however, detection of the *hipO* gene of *C. jejuni* from cattle farm samples has not yet been described.

Here, the development of a real-time LAMP assay was described targeting the *hipO* gene for the rapid, sensitive, and simple detection of *C. jejuni* in cattle farm samples. The performance of the LAMP assay with respect to *C. jejuni* isolates and standard enrichment broth culture from cattle feces and farm environment samples

was compared with that of a PCR assay. Finally, the potential influence of inhibitors present in sample material on the performance of the LAMP assay and PCR assays was evaluated.

1.2. METERIALS AND METHODS

Bacterial strains.

C. jejuni (ATCC 33560) was used for optimization of the LAMP assay and as a positive control for the LAMP, PCR, and standard culture assays. A total of 84 *C. jejuni* (ATCC 33560, ATCC 33291, NCTC 11168, and 81 isolates) and 41 non-*C. jejuni* gram-negative and -positive bacteria (Table 1.1.) were used for the inclusivity and exclusivity tests.

Design of LAMP primer sets targeting the *hipO* gene.

Six *hipO* gene DNA sequences (Genbank accession Nos.: NC017279, NC017281, CP000814, CP001900, CP000025, and CP000538) were aligned using the CLUSTALW program (Lasergene® 10.1.1, USA) to generate a consensus *hipO* sequence. The LAMP Designer program (Optigene Ltd., UK) was used to design the inner, outer, and loop primers based on this consensus sequence (Table 1.2.).

Table 1.1. Bacterial strains used for the inclusivity and exclusivity tests

Organism	**No. of strains**
Bacterial strains used for the inclusivity test	
Campylobacter jejuni reference strains (ATCC 33560, ATCC 33291, NCTC 11168)	3
Campylobacter jejuni isolates (49 cattle feces 2 soil from cattle feedlots, 27 duck carcasses, 1 chicken meat, 2 human stools)	81
Bacterial strains used for the exclusivity test	
Campylobacter coli (ATCC 33559, duck carcasses, chicken meat)	8
Escherichia coli (ATCC 43888, ATCC 43889, ATCC 43890, ATCC 43894, ATCC 19853, ATCC 35150, ATCC 51434, NCCP 15661, NCCP 15659, NCCP 15663, NCCP 15660, NCCP 13935, NCCP 14540)	13
Salmonella enterica serovar (Typhimurium; ATCC 43971, Enteritidis; ATCC 13076, Hadar, Montevideo, Schwarzengrund, Senftenberg, Lagos)	7
Shigella flexneri (ATCC 29903)	1
Shigella sonnei (ATCC 25931)	1
Bacillus cereus (ATCC 11778)	1
Staphylococcus aureus (ATCC 33586)	1
Listeria monocytogenes (ATCC 15313)	1
Listeria innocua (ATCC 33090)	1
Listeria ivanovii (ATCC 19119)	1
Listeria seeligeri (ATCC 35967)	1
Listeria welshimeri (ATCC 35897)	1
Listeria grayi (ATCC 25401)	1
Clostridium perfringens (NCCP 10347)	1
Enterobacter cloacae (wild type)	1
Serratia spp. (wild type)	1
Total	125

Table 1.2. Primer sets used for the PCR and LAMP assays

Assay	Primer name	Nucleotide sequences (5′ to 3′)	Amplicon size	Reference
LAMP	**FIP_*hipO***	CTGCTGAAGAGGGTTTGGGTGCATATTGTGCCATCCAA		
	BIP_*hipO*	GCTAAATACTTTGCAGCAAGCAGCTTTGCCTTTACAAGAATGC		
	LF_*hipO*	GGTGCTAAGGCAATGATAGAAG		This study
	LB_*hipO*	CATCATGACCGCAAGCATG		
	F3_*hipO*	GAAGAAGCCATCATCGCA		
	L3_*hipO*	AATAGGACTTCGTGCAGATATG		
PCR	**F_*16s rRNA***	GGATGACACTTTTCGGAGC	816 bp	(237)
	R_*16s rRNA*	CATTGTAGCACGTGTGTC		
	F_*cj0414*	CAAATAAAGTTAGAGGTAGAATGT	161 bp	(237)
	R_*cj0414*	CCATAAGCACTAGCTAGCTGAT		
PCR	**F_*hipO***	ACTGCAAAATTAGTGGCG	383 bp	(189)
	R_*hipO*	CGCTACCAAAAGGCATAT		

DNA extraction.

Genomic DNA was extracted from gram-negative bacteria and *C. jejuni* isolates using a boiling method. Briefly, 1 ml of bacterial cell suspension was boiled for 10 min, chilled on ice for 3 min, and centrifuged for 3 min. The supernatant was used as a template for the LAMP and PCR assays. Genomic DNA was extracted from gram-positive bacteria, using InstaGene™ Matrix (Bio-Rad, USA) according to the manufacturer's instructions. For the optimization and detection limit tests of the LAMP assay, template DNA of *C. jejuni* (ATCC 33560) was prepared using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions.

Optimization of the LAMP assay.

The LAMP assay was performed using a Genie® II instrument (Optigene Ltd., UK) in a total volume of 25 μl, containing 1× Buffer, 1 M betaine, 4 mM $MgSO_4$, 0.5 mM each dNTP, 0.8 μM of each FIP and BIP primer, 0.4 μM of each LF and LB primer, 0.2 μM of each F3 and B3 primer, 1× Evagreen (Solgent, Seoul, Korea), 8 U *Gsp* polymerase (Optigene Ltd., UK), and 4 μl template DNA. Nuclease-free distilled water (Invitrogen, USA) was used as a negative control. The reaction conditions were optimized using the block gradient function of the Genie® II system (OptiGene Ltd., UK), with a constant temperature ranging from 60°C to 67°C for 30 min. On the basis of the amplification ratio, the optimal reaction temperature was

determined and the LAMP reactions were carried out at 65°C for 30–60 min, with annealing curve analysis starting from 98°C to 78°C. LAMP results were analyzed on the basis of the T_p value (amplification time; min) and the T_m value (the temperature at which the double stranded LAMP product separates to single strands).

To determine the detection limit of the LAMP assay, serially diluted DNA templates (100 ng/µl–1 fg/µl) of *C. jejuni* (ATCC 33560) were tested in triplicate and the results were compared with those of the PCR assay (described below). To evaluate the quantifiability of the LAMP assay, a standard curve was generated by plotting the T_p value against the DNA concentration in log scale. The linearity and the correlation coefficient of determination (R^2) were calculated using Microsoft Excel software (Seattle, USA).

PCR assay.

The PCR assay was performed on a MyCycler thermal cycler (Bio-Rad, CA) using the previously designed primers (Table 1.2.). The reaction mixture contained 1× Emerald Master Mix (Takara Biotechnology, Japan), 0.5 µM each primer, and 1 µl template. Nuclease-free distilled water (Invitrogen, USA) was added to a final volume of 20 µl. The *C. jejuni* (ATCC 33560) strain and nuclease-free distilled water (Invitrogen, USA) were used as positive and negative controls, respectively. The reaction conditions were as follows: a single pre-denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and

72°C for 45 s (extension); and a final extension step at 72°C for 5 min. For identification of *C. jejuni* isolates, multiplex PCR assays targeting the *Campylobacter*-specific *16s rRNA* and *C. jejuni*-specific *cj0414* genes were conducted as described previously (237).

Cattle farm samples.

A total of 246 cattle farm samples (232 cattle feces, 11 soil samples from cattle feedlots, one raw milk sample, one forage sample, and one water sample) were collected between August 2012 and May 2013 from 15 enrolled cattle farms located in Gyeonggi-do, Korea. *C. jejuni* was isolated using a conventional culture method as described previously (238) . Briefly, approximately 1 g of each sample was enriched in 9 ml of Bolton broth (Oxoid, UK) containing 5% Laked Horse Blood and antibiotic supplement (Oxoid, UK) at 42°C overnight under microaerobic conditions. One loop of enrichment broth was then streaked onto *Campylobacter* blood-free selective agar containing antibiotic supplement (mCCDA; Oxoid, UK) and incubated at 42°C for 48 h under microaerobic conditions. Up to four presumed colonies were confirmed using the PCR assay as described previously.

Evaluations of the LAMP assay in application to cattle farm samples.

To evaluate the assay sensitivity for the *C. jejuni* isolates from cattle farm samples, the presence of the *hipO* gene in all *C. jejuni* isolates was tested using the LAMP and PCR methods as described previously. Moreover, the assay sensitivity and specificity in enrichment broth cultures of naturally-contaminated cattle farm samples were evaluated using the LAMP and PCR assays. The standard culture results were considered a gold standard for sensitivity (No. of test positive/No. of culture positive) and specificity (No. of test negative/No. of culture negative) analysis. In addition, to evaluate assay sensitivity regarding influences of potential inhibitors, DNA from enrichment broth culture was extracted with two different methods: boiling method and using the DNeasy Blood and Tissue Kit (Qiagen, Germany) as described in the "DNA extraction" section. The results of LAMP and PCR assay were compared.

1.3. RESULTS

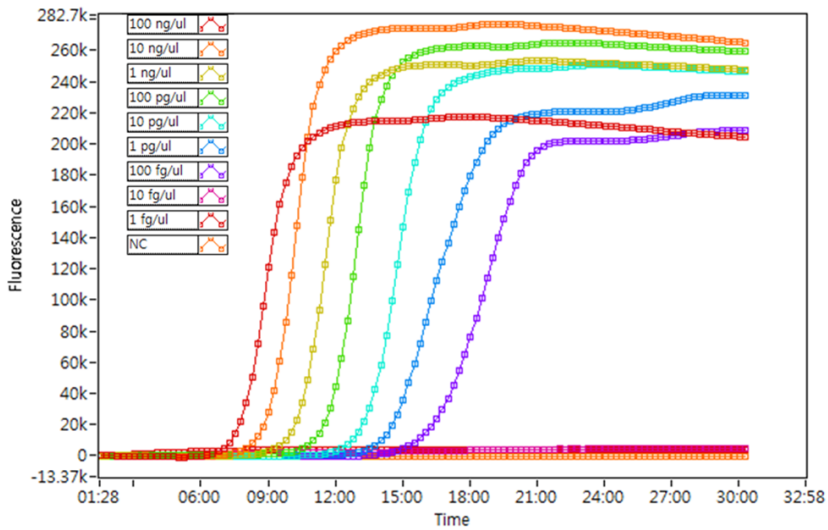
Inclusivity and exclusivity of the LAMP assay

To evaluate the inclusivity and exclusivity of the LAMP assay, 125 bacterial strains were tested. The *hipO* gene was successfully amplified from 84 *C. jejuni* strains (3 reference and 81 wild-type strains; T_p = 9.67 min and T_m = 84.29 ± 0.68°C), but not from 41 non-target strains, demonstrating 100% inclusivity and exclusivity (Table 1.1.).

Detection limit of the LAMP assay

The detection limit of the LAMP assay was found to be 100 fg/µl in three independent tests. Figure 1.1.A shows a general amplification curve generated by the LAMP instrument. The quantification equation for the LAMP assay was determined to be y = -1.3988x + 19.355, with a coefficient of determination (R^2) = 0.9133 (Fig 1.1.B). For the PCR assay, the detection limit was found to be 1 pg/µl in three independent tests.

A



B

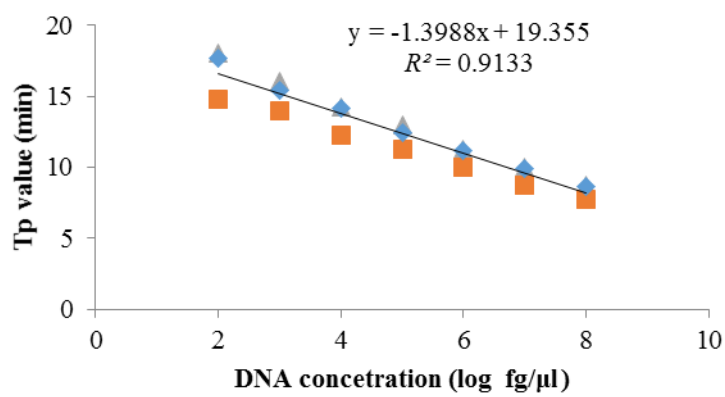


Figure. 1.1. Real-time LAMP analysis and standard curve generation in the detection limit test targeting the *hipO* gene of *C. jejuni*

A. The X-axis corresponds to time (min), while the Y-axis shows fluorescence (K) in the amplification graph. The amplification curve was generated from 100 ng/µl to 100 fg/µl of template DNA of *C. jejuni* (ATCC 33560)
B. A standard curve generated from triplicate data of the detection limit test. The X-axis shows DNA concentration (log fg/µl), while the Y-axis shows time (min). DNA concentrations 1 to 8 correspond to template DNA from 100 fg/µl to 100 ng/µl in log scale. The quantification equation and R^2 value are shown on the graph.

LAMP application in cattle farm samples

C. jejuni strains were isolated from 51/246 (20.7%) of cattle farm samples by using the conventional culture method, and the presence of the *hipO* gene was tested using the LAMP and PCR assays. Both LAMP and PCR detected *hipO* gene from all 51 *C. jejuni* isolates, showing 100% sensitivity. The mean T_p and T_m values of 51 isolates were 10.8 min (range: 5.52–19.28 min) and 84.30 ± 0.74°C. No peak was detected for the negative control.

The LAMP assay was then applied to the enrichment broth culture. DNA was extracted from 45 *C. jejuni* culture positive and 141 *C. jejuni*–negative enrichment broth cultures using two method, boiling and commercial kit. In DNA extracted using the commercial kit, the LAMP and PCR methods both amplified the *hipO* gene from all *C. jejuni* culture-positive samples with 100% sensitivity (45 of 45), compared with the gold standard culture method. For 141 *C. jejuni* culture–negative enrichment broth samples, LAMP detected *hipO* gene from 12 (8.5%; 91.5% specificity) samples, whereas PCR did not detect *hipO* gene from all sample (100% specificity). In DNA extracted by boiling, the LAMP assay detected *hipO* gene from 38 *C. jejuni*-culture positive broth culture (84.4% sensitivity, 38 of 45) and 25 *C. jejuni* culture–negative broth culture (82.3% specificity, 25 of 141). PCR detected *hipO* gene from 16 *C. jejuni*-culture positive broth culture (35.5% sensitivity, 16 of 45), while none of *hipO* gene was detected from *C. jejuni* culture–negative broth culture (100% specificity, 0 of 141) (Table 1.3.).

Table 1.3. Evaluation of the LAMP and PCR assays for the detection of the *hipO* gene in enrichment broth cultures prepared using two different DNA extraction methods.

No. of enrichment broth samples		DNA extracted by boiling		DNA extracted using a commercial kit[a]	
		PCR results	LAMP results	PCR results	LAMP results
Culture-positive samples (n = 45)	No. of positives [b] (%)	16 (35.5%)	38 (84.4%)	45 (100%)	45 (100.0%)
	No. of negatives (%)	29 (64.4%)	7 (15.5%)	0 (0.0%)	0 (0.0%)
Culture-negative samples (n = 141)	No. of positives (%)	0 (0.0%)	12 (8.5%)	0 (0.0%)	25 (17.7%)
	No. of negatives [c] (%)	141 (100.0%)	129 (91.5%)	141 (100.0%)	116 (82.3%)

[a] DNeasy Blood and Tissue Kit (Qiagen; Germany) was used according to the manufacturer's instructions

[b] Sensitivity of the LAMP assay in enrichment broth culture

[c] Specificity of the LAMP assay in enrichment broth culture

1.4. DISCUSSION

In this study, a novel real-time LAMP assay targeting the *hipO* gene was developed, which allows for rapid and simple in-tube detection of *C. jejuni* with high specificity and sensitivity. The LAMP assay enabled in-tube detection by monitoring the fluorescence of LAMP reactions and its unique T_m value. The detection limit of the LAMP assay (100 fg/μl) was 10 times more sensitive than that of the PCR assay. Previously reported detection limits for LAMP assays targeting *cj0414* gene were 5.6×10^3 CFU/g in spiked human stool (239) and 7.9 CFU/test tube in samples of chicken meat (206). Based on calculations from preliminary data (not shown), the detection limit of the LAMP assay (100 fg/μl) corresponds to 2.5×10^2 CFU/ml (1.0 CFU/test tube), indicating that it is comparable to or more sensitive than these previous studies. To evaluate the quantitative capability of the LAMP assay, the quantification equation was calculated (y = -1.3988x + 19.355, coefficient of determination (R^2) = 0.9133). This R^2 value was reported within the range of 0.904 to 0.997 in previous LAMP studies (194, 240), indicating that the LAMP assay showed adequate quantification capability.

Next, the suitability of the LAMP assay for the detection of *C. jejuni* in cattle feces and farm environmental samples was evaluated. Despite the significance of cattle farms as an initial contamination source for *C. jejuni*, there have been no reports to date of the use of LAMP assays for the detection of *C. jejuni* in cattle farm

samples. The sensitivities of both methods for detection of the *hipO* gene in all 51 tested isolates (LAMP, PCR = 100%), are comparable to values of 100% in the analysis of human stool (239) and 98.5% in analysis of chicken meat samples (206) targeting *cj0414* gene of *C. jejuni*. The mean T_p values of 51 isolates were 10.8 min (range: 5.52–19.28 min), indicating that the LAMP assay identified the *C. jejuni* isolates within 30 min. Considering that the conventional PCR method takes more than 3 h including the post-amplification process, this results indicate that the LAMP assay provides faster and accurate identification of the *C. jejuni* isolates. Moreover, the real-time LAMP assay enables direct in-tube detection, preventing possible post-contamination with aerosolized LAMP product.

Next, the performance of the LAMP assay as a screening tool for early diagnosis of the presence of the *hipO* gene in DNA extracted from 45 *C. jejuni* culture positive and 141 *C. jejuni*–negative enrichment broth cultures was evaluated (Table 1.3.). In DNA extracted using the commercial kit, both the LAMP and PCR methods showed 100% sensitivity, compared with the gold standard culture method. Considering that the duration of the standard culture method is at least 4 days after the enrichment step, the LAMP assay represents a highly sensitive screening method for enrichment broth that is less labor- and time-intensive. In DNA extracted by boiling, the LAMP assay had a higher sensitivity than that of the PCR assay (84.4% vs. 35.5%), indicating that the LAMP assay may be less susceptible than the PCR assay to potential inhibitors in samples, such as cattle feces or soil. This results indicate that

although the LAMP assay is more sensitive when commercial kit–extracted DNA is used as the starting material, the use of DNA extracted by boiling offers savings in speed, cost, and labor without appreciable loss of sensitivity. Further studies may be needed to investigate the effectiveness of other DNA extraction methods in the detection of *C. jejuni* using the LAMP assay.

The PCR assay did not detect the *hipO* gene in any of the 141 *C. jejuni* culture–negative enrichment broth samples (100% specificity), irrespective of the DNA extraction method used. By contrast, the LAMP assay detected the *hipO* gene from 12 (91.5% specificity) and 25 (82.3% specificity) *C. jejuni* culture–negative enrichment broth samples when DNA was extracted by both boiling and the commercial kit, respectively. It is possible that some samples included *C. jejuni* cells that were either dead or in an injured or viable but not culturable state that prevented their recovery using the standard culture method. Moreover, it is also possible that the amounts of *C. jejuni* in these enrichment cultures were below the previously reported detection limit of the culture method of 10^3 organisms per gram in animal feces (187), but were nevertheless detectable with the LAMP assay. Compared with the PCR assay, LAMP yielded a higher detection rate in culture-negative samples, possibly because the detection limit of LAMP is 10 times lower in pure culture and is less susceptible to potential inhibitors in fecal and soil samples. The identification by the LAMP assay of *C. jejuni* in nominally *C. jejuni*–negative enrichment broth indicates its usefulness as a screening tool for samples from which *C. jejuni* might

not be isolated using the standard culture method. Given that ingestion of as low as 500 cells of *C. jejuni* is sufficient to cause disease in humans (120), sensitive detection of this organism at the initial stages of the food chain is critical to prevent further contamination along the food production line. Further studies will be needed to evaluate the efficacy of our LAMP assay as a screening tool for the detection of *C. jejuni* in other food production lines such as slaughterhouses, processing plants, or retail mark

1.5. Conclusion

The real-time LAMP assay targeting the *hipO* gene was highly sensitive, specific, and quantifiable for the detection of *C. jejuni*. Moreover, the LAMP assay showed high sensitivity in enrichment broth culture, suggesting its application to cattle farm samples as an effective screening tool. The use of this rapid, sensitive, and simple LAMP assay in cattle farm samples represents a starting point for the control of *C. jejuni* contamination, and will potentially ultimately reduce *campylobacter* infection in humans.

Chapter II.

Development of a multiplex loop-mediated isothermal amplification assay to detect Shiga toxin-producing *Escherichia coli* in Cattle

Abstract

A multiplex loop-mediated isothermal amplification assay (mLAMP) assay was developed for simultaneous detection of the *stx1* and *stx2* genes and applied for detection of Shiga toxin-producing *Escherichia coli* (STEC) in cattle farm samples. Two target genes were distinguished based on T_m values of 85.03 ± 0.54°C for *stx1* and 87.47 ± 0.35°C for *stx2*. The mLAMP assay was specific (100% inclusivity and exclusivity), sensitive (with a detection limit as low as 10 fg/μL), and quantifiable (R^2 = 0.9313). The efficacy and sensitivity of the mLAMP assay were evaluated for their applicability to cattle farm samples. A total of 12 (12/253; 4.7%) and 17 (17/253; 6.7%) STEC O157, and 11 (11/236; 4.7%) STEC non-O157 strains were isolated

from cattle farm samples by conventional selective culture, immunomagnetic separation, and PCR-based culture methods, respectively. The coinciding multiplex PCR and mLAMP results for the types of Shiga toxin revealed the value of the mLAMP assay in terms of accuracy and rapidity for characterizing shiga toxin genes. Furthermore, the high detection rate of specific genes from enrichment broth samples indicates the potential utility of this assay as a screening method for detecting STEC in cattle farm samples.

Keywords: cattle farm, *E. coli* O157, LAMP, shiga toxin, *stx*

2.1. Introduction

STEC is a food-borne zoonotic pathogen that frequently causes human illness ranging from mild gastrointestinal problems to serious fatal complications (12, 13). It is estimated that approximately 176,000 cases of foodborne illnesses caused by STEC occur annually in the United States (17). Among the 2,400 hospitalizations and 20 deaths caused annually by STEC, serotype O157 is implicated in roughly 35% of illnesses, 89% of hospitalizations, and 100% of deaths (17). Because cattle are known to be a natural reservoir of STEC, asymptomatically infected cattle can transmit STEC to humans (62, 63). Therefore, investigating the prevalence of STEC in cattle and their environment is important for control and prevention of STEC transmission to humans.

STEC harbors essential virulence genes *stx1* and/or *stx2* (28). STEC O157 is traditionally detected by a selective culture method based on the inability to ferment sorbitol (28); however, detection of STEC non-O157 is difficult due to the lack of phenotypic characterization. Fast, sensitive, and specific nucleic acid-based amplification methods have recently been adopted to supplement the conventional culture method. The standard manuals established by the United States Department of Agriculture (USDA) and the United States Food and Drug Administration (FDA) recommend PCR and real-time PCR (qPCR) as screening methods for detection of STEC by targeting various genes, including *stx1* and *stx2* (185, 186).

The LAMP assay is a promising novel nucleic acid amplification method in terms of its accuracy, simplicity, and rapidity (191). The use of 4–6 specially designed primers (FIP, BIP, F3, B3, LF, and LB) results in high efficiency of the assay, which can amplify low titers of DNA templates (192). The LAMP assay recognizes 6–8 distinct regions of the DNA template, thereby providing high specificity and sensitivity (191, 192). In addition, a special DNA polymerase, *Bst* polymerase or *Gsp* polymerase, reacts under isothermal conditions at 60°C to 65°C within 1 h (191, 193). The amplified product can be detected by simple inspection of insoluble magnesium pyrophosphate, gel electrophoresis, or measuring the turbidity of the product (191, 194, 195).

Several groups have used LAMP assays targeting *stx1*, *stx2* (194, 195, 200), *eae* (194), *rfbE* (201), and serogroup-specific genes *wzx* and *wzy* (202) to detect STEC, and have shown that the LAMP assay is more sensitive than PCR or qPCR.

Despite the many advantages of the LAMP assay, mLAMP approaches are limited due to difficulty of differentiating more than two specific genes. Visual observation of LAMP products is indirect and cannot be used to distinguish multiple target genes. Gel electrophoresis after restriction enzyme digestion has been attempted (196-198), but is laborious and time-consuming. Moreover, the high sensitivity of the LAMP assay raises the concern of carry-over contamination during post-amplification analysis (199). Probe-based detection is also not applicable due to the various types of cauliflower-like structures of LAMP products (199).

In this study, a real-time mLAMP assay was developed to detect *stx1* and *stx2* simultaneously based on different annealing temperatures determined by annealing curve analysis. To evaluate the applicability of the mLAMP assay to cattle farm samples, assay efficacy and sensitivity using cattle feces and environmental samples was assessed.

2.2. Materials and Methods

Bacterial strains

Bacterial strains used for inclusivity and exclusivity testing are listed in Table 2.1. Three STEC strains, ATCC 43984 (harboring both *stx1* and *stx2* genes), ATCC 43890 (*stx1*), and ATCC 43889 (*stx2*), were tested to evaluate the mLAMP assay. STEC ATCC 43894 was also tested to optimize the mLAMP assay and determine the detection limit.

Sample collection

A total of 253 fecal and environmental samples were collected from 15 cattle farms located in Gyeonggi-do, South Korea, from August 2012 to May 2013. Cow fecal samples were collected directly by rectal retrieval, and at least one environmental sample from each farm was collected. All samples were transported to the laboratory at 4°C for microbiological tests and analyzed immediately upon arrival.

Table 2.1. Bacterial strains used for inclusivity and exclusivity test

Species	Number of strains	Shiga toxin type	mLAMP assay	
			stx1	*stx2*
Bacterial strains used for the inclusivity test				
Escherichia coli O157:H7 (ATCC 43890)	1	*Stx1*	+	−
Escherichia coli O157:H7 (ATCC 43889, 00-2, 00-10, 00-12, 00-16)	5	*Stx2*	−	+
Escherichia coli O157:H7 (ATCC 43894, ATCC 35150, 00-11, 00-13, 00-14, 00-15)	6	*Stx1,2*	+	+
Escherichia coli O91:H21 (ATCC 51434)	1	*Stx2*	−	+
Escherichia coli O111 (NCCP 13935, NCCP 14540)	2	*Stx1*	+	−
Escherichia coli O84 (wild type)	1	*Stx1*	+	−
Escherichia coli O108 (wild type)	1	*Stx1*	+	−
Escherichia coli O185 (wild type)	1	*Stx2*	−	+
Escherichia coli O119 (wild type)	1	*Stx2*	−	+
Bacterial strains used for the exclusivity test				
Escherichia coli (ATCC 43888, ATCC 19853, NCCP 15661, NCCP 15659, NCCP 15663, NCCP 15660)	6	None	−	−
Salmonella enterica serovar (Typhimurium; ATCC 43971, Enteritidis; ATCC 13076, Hadar, Montevideo, Schwarzengrund, Senftenberg, Lagos)	7	None	−	−
Shigella flexneri (ATCC 29903)	1	None	−	−
Shigella sonnei (ATCC 25931)	1	None	−	−
Campylobacter jejuni (ATCC 33560, ATCC 33291)	2	None	−	−
Campylobacter coli (ATCC 33559)	1	None	−	−
Bacillus cereus (ATCC 11778)	1	None	−	−
Staphylococcus aureus (ATCC 33586)	1	None	−	−
Listeria monocytogenes (ATCC 15313)	1	None	−	−
Listeria innocua (ATCC 33090)	1	None	−	−
Listeria ivanovii (ATCC 19119)	1	None	−	−
Listeria seeligeri (ATCC 35967)	1	None	−	−
Listeria welshimeri (ATCC 35897)	1	None	−	−
Listeria grayi (ATCC 25401)	1	None	−	−
Clostridium perfringens (NCCP 10347)	1	None	−	−
Enterobacter cloacae (wild type)	1	None	−	−
Seratia spp. (wild type)	1	None	−	−
Total	48			

Isolation of STEC O157 strains

STEC O157 was isolated by conventional selective culture (conventional) and IMS methods. For the conventional method, approximately 1 g of each sample was homogenized in 9 mL modified EC broth (mEC; Becton, Dickinson and Company, USA) supplemented with novobiocin (20 mg/L, Oxoid, UK) and incubated overnight at 37°C. Following incubation, one loop of mEC broth culture was streaked onto sorbitol MacConkey agar (Becton, Dickinson and Company, USA) supplemented with potassium tellurite (T-SMAC; 2.5 mg/L, Sigma-Aldrich, Canada) and incubated at 37°C overnight. A maximum of six typical colonies were subcultured onto MacConkey agar (MAC; Becton, Dickinson and Company) and CHROMagar O157 (CHROM; CHROMagar Microbiology, France) and then incubated overnight at 37°C. Typical colonies, pink in MAC and mauve in CHROM, were selected for testing using the *E. coli* O157 latex test kit (Oxoid).

For the IMS method, Dynabeads MAX anti-*E. coli* O157 (Dynal; Invitrogen, USA) was used according to the manufacturer's instructions. The suspension of immunomagnetic beads was spread onto T-SMAC and incubated at 37°C overnight. Up to four typical colonies were selected and identified by applying the same criteria as described in the conventional method. If the four colonies yielded no STEC O157 strain, up to four additional colonies were tested.

Isolation of STEC non-O157 strains

STEC non-O157 strains were isolated from STEC O157-negative samples by PCR-based culture assay as previously described, with minor modification (241). Briefly, one loop of enriched mEC broth culture was streaked onto T-SMAC and incubated overnight at 37°C. DNA was extracted from randomly swiped areas of heavy bacterial growth and tested for the presence of shiga toxin genes by PCR. Plates possessing shiga toxin genes were subcultured onto MAC and incubated overnight at 37°C. The presumptive colonies were tested for the presence of shiga toxin genes by PCR. Colonies possessing shiga toxin genes were assessed by conventional agglutination tests to determine the serotype of the isolates using commercial antiserum (Joongkyeom, Korea).

DNA extraction

DNA of gram-negative bacteria and enrichment broth culture were extracted using boiling method as described in the "Materials and Methods" section in the Chapter I. Instagene matrix (BioRad Laboratories, USA) was used to extract DNA from gram-positive bacteria, and the genomic DNA of three STEC strains (ATCC 43890, 43889, and 43894) was extracted with blood and tissue kits (Qiagen, Germany) according to the manufacturer's instructions.

Primer design and the LAMP assay

To design primer sets targeting shiga toxins genes, the genomic sequences of *stx* genes from various serotypes of STEC were collected from GenBank. For the *stx1* gene, the genomic sequences of STEC O26, O79, O103, O111, and O157 (GenBank Accession No. AP010953, FR875154, FE94195, AP010958, AP010960, and M19473) were collected and the genomic sequences of STEC O26, O157, and O178 (GenBank Accession No. FR850034, AB048240, X07865, FR850037) were collected for the *stx2* gene. The consensus sequence of each gene was generated by multiple alignment and comparison in ClustalW (Lasergene 10.1.1, USA). Each primer set was designed from the consensus sequence using LAMP designer (Optigene, UK; Table 2.2.).

The mLAMP assay was evaluated using two primer sets targeting *stx1* and *stx2*. To differentiate between targets, 100 ng/μL of DNA from three STEC strains (ATCC 43984, ATCC 43890, and ATCC 43889) were tested in triplicate. The mLAMP assay was performed on a Genie® II instrument (Optigene) in a total reaction volume of 25 μL containing 1× Buffer, 1 M Betaine, 4 mM $MgSO_4$, 0.8 mM each dNTP, 0.8 μM each of FIPs and BIPs, 0.4 μM each of LFs and LBs, 0.2 μM each of F3s and B3s, 1× EvaGreen (SolGent, Korea), 8 U *Gsp* polymerase (Optigene), and 4 μL template DNA. Distilled water (Invitrogen, USA) was used as a negative control. The LAMP reactions were carried out at 64°C for 30 to 60 min, with annealing curve analysis was conducted from 98°C to 80°C.

To determine the detection limit of the mLAMP assay, 10-fold serially diluted DNA templates with concentrations ranging from 100 ng/μL to 1 fg/μL were tested in triplicate. A standard curve was generated by plotting T_p values versus concentration of template DNA ranging from 100 ng/μL to 1 fg/μL on a log scale for each set, and the correlation coefficient of determination (R^2) was calculated. The detection limit was evaluated and compared to mPCR.

Table 2.2. Primer sets used for the PCR and LAMP assay

Assay	Primer name	Nucleotide sequences (5' to 3')	Ampli-con size	Refer-ence
***Stx1*-PCR**	m*STX1*_F	CAGTTAATGTGGTGGCGAAGG	348bp	(186)
	m*STX1*_R	CACCAGACAATGTAACCGCTG		
***Stx2*-PCR**	m*STX2*_F	ATCCTATTCCCGGGAGTTTACG	584bp	(186)
	m*STX2*_R	GCGTCATCGTATACACAGGAGC		
+93uid A-PCR	mUIDA_F	GCGAAAACTGTGGAATTGGG	252bp	(186)
	mUIDA_R	TGATGCTCCATCACTTCCTG		
γ-eaeA -PCR	mEAE_F	ATTACCATCCACACAGACGGT	397bp	(186)
	mEAE_R	ACAGCGTGGTTGGATCAACCT		
***Stx1*-LAMP**	*Stx1*_FIP	GCGATTTATCTGCATCCCCGTATGTCTGGTGAC AGTAGCTAT		This study
	*Stx1*_BIP	GGAACCTCACTGACGCAGTCCTTCAGCTGTCA CAGTAACA		
	*Stx1*_LF	ACTGATCCCTGCAACACG		
	*Stx1*_LB	TGTGGCAAGAGCGATGTT		
	*Stx1*_F3	ACAACAGCGGTTACATTGT		
	*Stx1*_B3	GATCATCCAGTGTTGTACGAA		
***Stx2*-LAMP**	*Stx2*_FIP	GGCGTCATCGTATACACAGGAGCGCTTCAGGC AGATACAG		This study
	*Stx2*_BIP	AGACGTGGACCTCACTCTGAAACTCTGACACC ATCCTCTC		
	*Stx2*_LF	CAGACAGTGCCTGACGAA		
	*Stx2*_LB	GGCGAATCAGCAATGTGC		
	*Stx2*_F3	GCATCCAGAGCAGTTCTG		
	*Stx2*_B3	CAGTATAACGGCCACAGTC		

PCR assay

PCR assay

Multiplex PCR (mPCR) targeting *stx1* and *stx2* was performed to determine the detection limit using serially diluted template DNA of STEC ATCC 43894 as for the mLAMP assay, and results of both assays were compared. mPCR was also used to identify STEC in STEC-positive enrichment broth cultures. For genotypic characterization of STEC isolates from cattle farm samples, *stx1*, *stx2*, *eae,* and *uidA* were tested, as described, on BAM (Table 2.2.) (186).

The reaction mixture included 1× Emerald Master Mix (Takara Bio, Japan), 0.5 μM each primer, and 1 μL template. Distilled water was added to a final volume of 20 μL. The optimal reaction conditions were defined as follows: pre-denaturation at 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 sec, 57˚C for 30 sec, and 72°C for 45 sec, and then final extension at 72˚C for 5 min. mPCR was performed on a MyCycler thermal cycler (BioRad Laboratories) and the products were electrophoresed in 1.5% agarose gels

mLAMP assay application in cattle farm samples

The mLAMP assay was used to verify the types of Shiga toxin in all STEC isolates relative to the mPCR assay. To evaluate assay sensitivity, the presence of *stx1* and/or *stx2* in enrichment broth cultures was assessed by mLAMP and mPCR.

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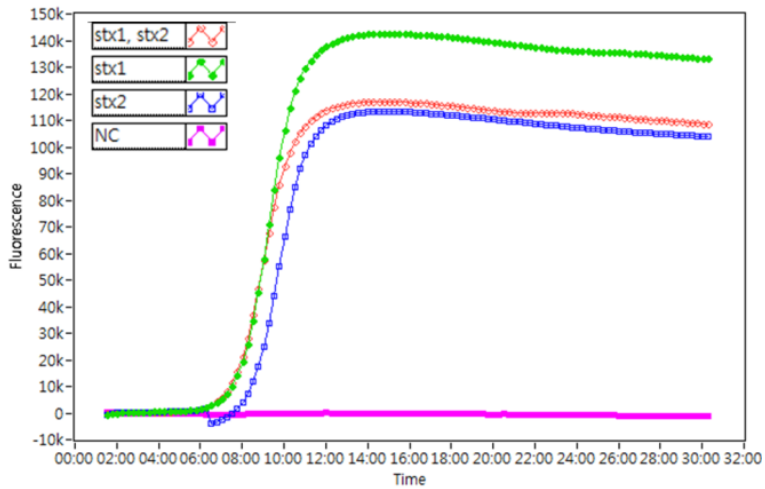
2.3. Results

mLAMP evaluation

To evaluate the utility of the mLAMP assay, three STEC strains with different Shiga toxin types were tested. The results are shown with the T_p value, which represents the time required to detect the amount of fluorescence required to produce a positive peak, and the T_m value (annealing temperature) determined by annealing curve analysis. For well 1 (ATCC 43894; *stx1* and *stx2* genes), the mean T_p value was 9:06 (min:sec) and T_m values (mean) were observed at 84.55 ± 0.32°C and 87.22 ± 0.21°C. For well 2 (ATCC 43890; *stx1*), the mean T_p and T_m values were 8:57 and 85.59 ± 0.02°C, respectively. For well 3 (ATCC 43889; *stx2*), the mean T_p and T_m values were 9:23 and 87.71 ± 0.17°C, respectively. There was no product amplification in the negative control (Fig. 2.1.).

A



B

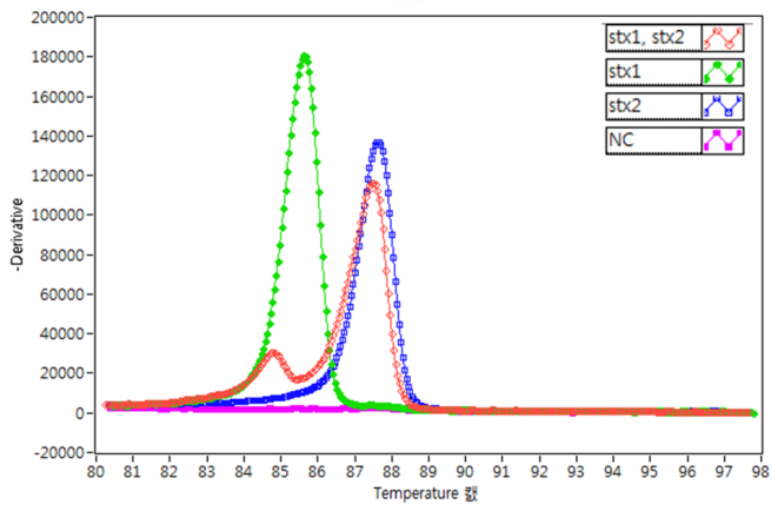


Figure. 2.1. The mLAMP assay used to detect *stx1* and/or *stx2*.

A. The amplification curve shows the time in minutes on the X-axis and fluorescence on the Y-axis

B. The annealing curve shows temperature on the X-axis and fluorescence on the Y-axis. Well 1, STEC 43894 (*stx1* and *stx2*), red; Well 2, STEC 43890 (*stx1*), green; Well 3, STEC 43889 (*stx2*), blue; Well 4, NC (negative control), purple.

Inclusivity and exclusivity of the mLAMP assay

To evaluate the inclusivity and exclusivity of the mLAMP assay, 48 bacterial strains were tested. mLAMP amplified and distinguished the shiga toxin genes in all 19 STEC strains according to their Shiga toxin type. The mean T_p value was 10:12 (8:24 to 15:46) and the mean T_m value was 85.32 ± 0.60°C for *stx1* and 87.77 ± 0.38°C for *stx2*, demonstrating 100% sensitivity in the mLAMP assay. T_m values were not generated for 29 gram-negative and gram-positive strains of non-Shiga toxin-producing bacteria, demonstrating 100% specificity.

Detection limit of the mLAMP assay

The detection limit of the mLAMP assay was found to be 10 fg/µL in three independent experiments. At 1 fg/µL, *stx2* was detected in all three experiments, but *stx1* was detected in only one of three experiments. Figure 2.2. shows a general amplification and annealing curve generated by the LAMP instrument. The DNA templates were amplified with mean T_p values ranging from 9:06 to 22:16 for concentrations ranging from 100 ng/µL to 10 fg/µL. The mean T_m values were 84.63 ± 0.30°C for *stx1* and 87.24 ± 0.25°C for *stx2*. The quantification equation for the mLAMP assay was determined to be $y = -1.8145\times + 23.671$, with a correlation coefficient (R^2) of 0.9313. For the mPCR assay, the detection limit was 1 pg/µL in three independent experiments.

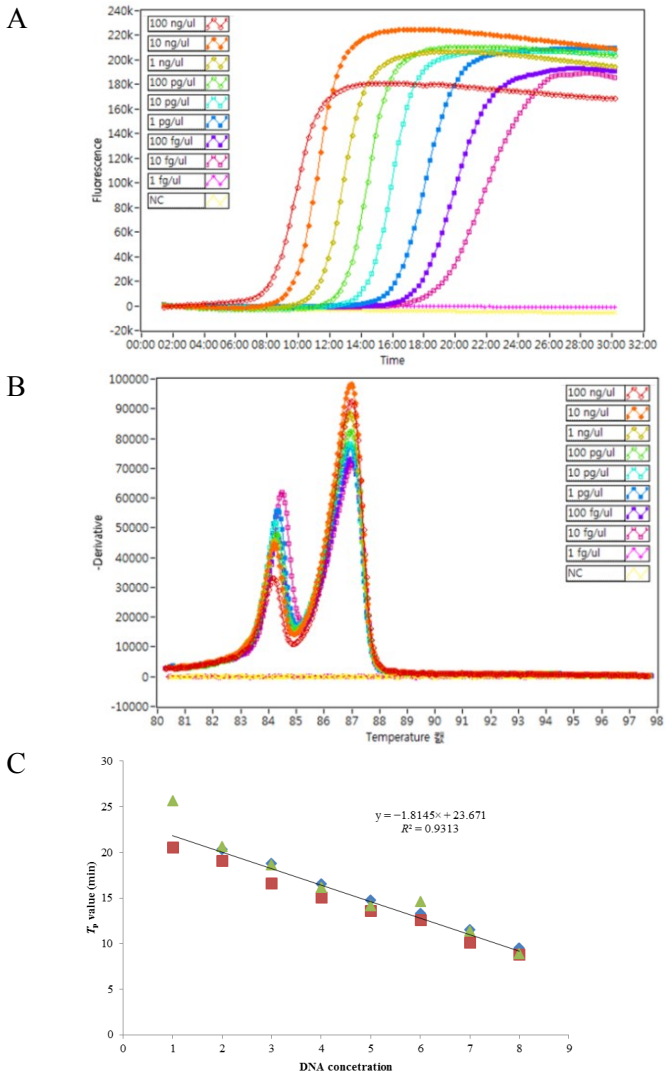


Figure. 2.2. Detection limit and standard curve of the mLAMP assay.

A. Ten-fold serial dilutions of STEC 43894 template DNA from 100 ng/µL to 1 fg/µL tested in triplicate. The amplification curve shows the amplification of *stx1* and/or *stx2*
B. Specific genes differentiated by annealing curve analysis
C. The standard curve shows the amount of template DNA on the X-axis and T_p values (min:sec) on the Y-axis. Samples 1 to 8 correspond to STEC (ATCC 43894) template DNA from 10 fg/µL to 100 ng/µL in log scale

mLAMP assay application in cattle farm samples

A total of 253 cattle farm samples (consisting of 237 cattle feces, 13 ground samples, one raw milk, one water, and one forage sample) were tested for STEC. The conventional culture method yielded 12 (4.7%) STEC O157 strains from cattle fecal samples. The IMS method yielded 17 (6.7%) STEC O157 strains from cattle feces and ground samples. Eleven (4.7%) STEC non-O157 strains were isolated from a total of 236 STEC O157-negative cattle fecal samples by the PCR-based culture method. As shown in Table 2.3., the STEC non-O157 isolates belonged to four different serotypes, O84 (2 isolates), O108 (5 isolates), O185 (1 isolate), and O119 (3 isolates).

Among 28 STEC isolates, seven (25.0%) and 13 (46.4%) harbored *stx1* and *stx2*, respectively, while eight (28.6%) strains harbored both *stx1* and *stx2* (Table 2.3.). All 17 STEC O157 isolates had both *eae* and *uid* genes, while STEC non-O157 did not harbor these genes. The Shiga toxin types of 28 STEC isolates were tested by mPCR and mLAMP. The mLAMP determinations were identical to those of the mPCR assay (Table 2.3.). The T_p values of 28 isolates ranged from 8:21 to 12:03 (mean T_p = 10:03), and the mean T_m values were 85.76 ± 0.42°C for *stx1* and 87.73 ± 0.29°C for *stx2*.

Enrichment broth cultures that were positive for STEC were used to evaluate the sensitivity of the mLAMP assay relative to mPCR. Among the 12 enrichment broth cultures that were positive for STEC O157 by the conventional culture method, shiga

toxin genes were detected in three (25.0%) by mPCR and in 12 (100.0%) by mLAMP. For the 17 enrichment broth cultures that were positive for STEC O157 by the IMS method, shiga toxin genes were detected in four (23.5%) and 15 (88.2%) samples by mPCR and mLAMP, respectively. For the ten enrichment broth cultures that were positive for STEC non-O157, mLAMP detected shiga toxin genes in nine (90.0%) samples, while none were detected by mPCR assay (Table 2.4.).

Table 2.3. Characteristics of STEC isolated from cattle feces and environmental samples in Gyeonggi-do, Korea

Farm	Isolates	Isolation method[a]			Sample source	Serotype		Shiga toxin type[d]	
		Conventio -nal ulture (SMAC)	IMS (O157)	PCR based culture[b]					
STEC O157									
Farm S	0806-3	+	+	UT	Feces	O157			*stx1, stx2*
	0806-5	+	+	UT	Feces	O157			*stx1, stx2*
	0806-20	−	+	UT	Ground	O157			*stx1, stx2*
Farm 4	0823-2	+	+	UT	Feces	O157			*stx1, stx2*
	0823-4	+	+	UT	Feces	O157			*stx1, stx2*
	0823-5	−	+	UT	Feces	O157			*stx1, stx2*
	0823-8	+	+	UT	Feces	O157			*stx1, stx2*
Farm 7	0827-1	+	+	UT	Feces	O157		*stx2*	
	0827-2	+	+	UT	Feces	O157		*stx2*	
	0827-3	+	+	UT	Feces	O157		*stx2*	
	0827-5	+	+	UT	Feces	O157		*stx2*	
	0827-6	+	+	UT	Feces	O157		*stx2*	
	0827-7	+	+	UT	Feces	O157		*stx2*	
	0827-8	−	+	UT	Feces	O157		*stx2*	
	0827-9	+	+	UT	Feces	O157		*stx2*	
	0827-10	−	+	UT	Ground	O157		*stx2*	
Farm 13	0905-7	−	+	UT	Feces	O157			*stx1, stx2*
STEC non-O157									
Farm 11	0904-9	−	−	+	Feces	O 84	*stx1*		
Farm 12	0904-16	−	−	+	Feces	O185		*stx2*	
Farm S	1015-9	−	−	+	Feces	O 84	*stx1*		
	0527-1	−	−	+	Feces	O108	*stx1*		
	0527-4	−	−	+	Feces	O108	*stx1*		
	0527-8	−	−	+	Feces	O119		*stx2*	
	0527-15-1[c]	−	−	+	Feces	O108	*stx1*		
	0527-15-2[c]	−	−	+	Feces	O119		*stx2*	
	0527-19	−	−	+	Feces	O108	*stx1*		
	0527-23	−	−	+	Feces	O108	*stx1*		
	0527-24	−	−	+	Feces	O119		*stx2*	
Total*		12/253 (4.7%)	17/253 (6.7%)	11/236 (4.7%)	Ground: 2/28 (7.1%) Feces: 26/28 (92.9%)		7/28 (25.0%)	13/28 (46.4%)	8/28 (28.6%)

[a] Twenty-eight STEC strains were isolated from a total of 253 cattle farm samples from 15 enrolled cattle farms by three different methods. For isolation of O157STEC, a conventional culture method based on SMAC agar media was used; while immunomagnetic beads coated with anti-O157 were used for the IMS method.

[b] A PCR-based culture method was used for isolation of STEC non-O157 strains. From a total of 253 samples, 17 STEC-O157 positive samples were excluded from the PCR assay (UT, untested).

[c] Two strains with different serotypic and genotypic characterizations were isolated from the same sample.

[d] Shiga toxin types were determined by mPCR and the mLAMP assay, which were concordant.

Table 2.4. Comparison of *stx1* and/or *stx2* detection results obtained by mPCR and by mLAMP

Isolation method	Number of positive tests from enrichment broth (%)[a]		Number of positive STEC from enrichment broth
	PCR	LAMP	
STEC O157			
Conventional culture (SMAC)	3 (25.0)	12 (100.0)	12
IMS (O157)	4 (23.5)	15 (88.2)	17
STEC non-O157			
PCR based culture	0 (0.0)	9 (90.0)	10[b]

[a] In enrichment broth culture, detection of *stx1* and/or *stx2* was considered positive.
[b] The number of STEC positive broth cultures is ten because two strains (0527-15-1 and 0527-15-2) were isolated from one sample.

2.4. Discussion

A real-time mLAMP assay was developed for simultaneous detection of the STEC *stx1* and *stx2* genes. This assay is highly specific, sensitive, rapid, and quantifiable. When applied to cattle farm samples, the mLAMP assay provided determinations of shiag toxin types within 30 min and provided a much high detection rate from enrichment broth culture.

The mLAMP assay was designed for simultaneous detection of two different types of shiga toxin genes. As shown in Figure 2.1., two sets of primers successfully amplified two target genes, which could be distinguished by their different T_m values. The mean T_m values for *stx1* and *stx2* in all tested samples were 85.03 ± 0.54°C and 87.47 ± 0.35°C, respectively, and were thus easily distinguished. Similarly, in a recent study of sex determination in the plant *Carica papaya*, two target genes were also differentiated by annealing curve analysis (193).

The limit of detection in the mLAMP assay was 10 fg/μL, which was 100 times more sensitive than mPCR. Kouguchi (199) reported a detection limit of 100 CFU/mL in STEC pure culture. In this study, the detection limit was measured at the DNA concentration level rather than the CFU level, so the results cannot be compared directly. However, in preliminary tests, 6.2 ± 1.74 × 10^8 CFU/mL corresponded to 140.5 ± 54.7 ng/μL (data not shown). Thus, 10 fg/μL corresponds

to 10 CFU/mL, making this assay 10 times more sensitive than the one reported by Kouguchi (199).

In this study, the mean T_p values ranged from 9:06 to 22:16 for the concentration range of 100 ng/μL to 10 fg/μL, which was much faster than previously reported for a LAMP assay conducted using a turbidimeter, in which 21.4 ~ 45.2 min was required to detect the *stx2* gene from a 10 ~ 10^5 CFU/reaction (194). These results are consistent with those of other studies designed to detect *Vibrio parahaemolyticus* or ammonia-oxidizing bacteria in which the detection time for the fluorescent-based LAMP assay was faster than that of the turbidity-based LAMP assay (240, 242). In previous mLAMP studies based on the post-amplification analysis of two target genes, detection limits were 10 times more sensitive than those of PCR for detecting *Salmonella* or *Shigella* spp. and 10^3 to 10^5 times more sensitive for differentiating *Babesia* spp. (196, 198).

The quantitative capability of the LAMP assay was reported previously (194, 202, 240). In the present study, a standard curve was generated based on triplicate T_p values (detection time) versus the concentration of template DNA. The standard curve had a linear relationship with R^2 values of 0.9313 within the range of 100 ng/μL and 10 fg/μL, indicating that the mLAMP assay can estimate unknown STEC contamination levels in samples. Indeed, this result is unique in terms of its wide coverage of concentrations. In another study, the R^2 values fell within a relatively

narrow range of concentrations (10^2 to 10^5 CFU/mL), possibly due to the delayed detection time observed at lower concentrations (194, 240).

Until now, only a few LAMP methods for detecting STEC have been used to analyze samples from beef, beef trimmings, lettuce, spinach, and human stool (194, 202, 243). However, no LAMP assay has been used to screen for STEC on cattle farms. As a primary source of human STEC infection, cattle and their environment are important to public health and need to be monitored. This is the first report of use of a real-time mLAMP assay for detection of STEC targeting shiga toxin genes that was applied to cattle farm samples.

To evaluate the efficacy of the mLAMP assay, all STEC strains were examined for the presence of *stx1* and/or *stx2*. The Shiga toxin types of each isolate were identical when tested by mPCR and mLAMP, indicating high accuracy of the mLAMP assay. Moreover, the mLAMP assay detected shiga toxin genes in 11 STEC non-O157 strains, indicating that the mLAMP assay can be used to detect various serotypes of STEC. In addition, the mLAMP process was completed within 30 min (mean T_p value = 10:03), while a PCR assay generally takes 3 h, including the post-amplification processes. Overall, these findings indicate that the mLAMP assay could be useful for rapid and accurate characterization of various STEC serotypes.

The sensitivity of the mLAMP assay was evaluated in enrichment broth cultures that were found to be positive for STEC by conventional culture, IMS, and PCR-based culture methods. As shown in Table 2.4., the mLAMP assay detected shiga

toxin genes in all 12 broth cultures that were positive for STEC O157 by the conventional culture method, which is regarded as the gold standard (185, 186). In addition, the mLAMP assay was able to detect shiga toxin genes in nine (90.0%) enrichment broth cultures that were positive for STEC non-O157 strains. These results indicate that the detection rate of the mLAMP assay is comparable to the culture-based detection method for STEC strains. Considering that the culture method is labor intensive and takes at least three days to complete, the application of the mLAMP assay to screening enrichment broth cultures would provide a detection rate similar to culture-based methods, but with less time and labor.

The STEC O157 strains were isolated by conventional culture and IMS methods. While 12 strains were isolated by the conventional method, five more strains were isolated by the IMS method. Considering IMS is known to be the most sensitive culture method (244), the contamination of these five samples with STEC O157 seems to be at a low level that may not be detected by conventional culture. The mLAMP assay detected shiga toxin genes in enrichment cultures of three of these five samples, demonstrating high sensitivity. Indeed, the assay could detect even levels of contamination below those that could be detected using the conventional culture method.

The mLAMP assay may not detect shiga toxin genes in the presence of low amounts of STEC in enrichment broth cultures (under our detection limit, 10 fg/μL), but still showed greater sensitivity than the conventional culture method. In addition,

the mLAMP assay was much more sensitive than the mPCR assay in enrichment broth culture, which was expected considering the high sensitivity of the LAMP assay over the PCR assay in pure culture. Conversely, it is possible that the mLAMP assay may be less sensitive than the mPCR assay to inhibitors in the sample matrix. Other studies have compared methods such as PCR, qPCR, or LAMP for detection of target genes from food or clinical samples and shown that the LAMP assay is less sensitive to inhibitors than the PCR or qPCR assay (194, 198, 243)

2.5. Conclusion

The mLAMP assay simultaneously amplifies the *stx1* and *stx2* genes, enabling detection of STEC, and enables identification of Shiga toxin types more rapidly and accurately than current methods. Moreover, the high detection rate of specific genes from enrichment broth cultures indicates the potential utility of this assay as a primary screening tool for detecting STEC in cattle farm samples.

Chapter III.

Prevalence, virulence potentials, and pulsed–field gel electrophoresis profiling of Shiga toxin-producing *Escherichia coli* strains from cattle

Abstract

As a primary source of Shiga-toxin-producing *Escherichia coli* (STEC) infection, cattle are often targeted to develop strategies for reducing STEC contamination. Monitoring the virulence potentials of STEC isolates from cattle is important for tracing contamination sources, managing outbreaks or sporadic cases, and reducing the risks for human infection. This study aimed to investigate the prevalence of STEC in cattle farm samples in South Korea and to assess their virulence potentials. In total, 63 STEC were isolated from 496 cattle farm samples, and temperature and rainfall affected STEC prevalence ($p < 0.001$). The O157 serogroup was most prevalent, followed by O108, O8, O84, O15, and O119. In the *stx* variant test, high prevalence of *stx2a* and *stx2c* (known to be associated with high STEC virulence)

were observed, and *stx2g*, a bovine STEC variant, was detected in STEC O15 and O109. Additionally, *stx1c* was detected in *eae*-positive STEC, suggesting genetic dynamics among the virulence genes in the STEC isolates. STEC non-O157 strains were resistant to tetracycline (7.9%), ampicillin (6.4%), and cefotaxime (1.6%), while STEC O157 was susceptible to all tested antimicrobials, except cefotaxime. The antimicrobial resistance genes, bla_{TEM} (17.5%), *tetB* (6.3%), and *tetC* (4.8%), were only detected in STEC non-O157, whereas *tetE* (54.0%) was detected in STEC O157. *AmpC* was detected in all STEC isolates. Clustering was performed based on the virulence gene profiles, which grouped STEC O84, O108, O111, and O157 together as potentially pathogenic STEC strains. Finally, PFGE suggested the presence of a prototype STEC that continues to evolve by genetic mutation and causes within- and between-farm transmission within the Gyeonggi province. Considerable numbers of STEC non-O157 were isolated from cattle farms, and the virulence and antimicrobial resistance features were different between the STEC O157 and non-O157 strains. STEC from cattle with virulence or antimicrobial resistance genes might represent a threat to public health and therefore, continual surveillance of both STEC O157 and non-O157 would be beneficial for controlling and preventing STEC-related illness.

Keywords: Shiga toxin-producing *Escherichia coli*, cattle, *stx* variant, antimicrobial resistance, virulence gene, PFGE

3.1. Introduction

Since the identification of STEC O157:H7 as a foodborne zoonotic disease in 1982 (9), human infections by STEC have been reported worldwide. While numerous studies have focused on STEC O157:H7, the most well-known and notorious serotype, >400 serotypes of STEC non-O157 have been implicated as etiological agents of several outbreaks and in sporadic cases of STEC infection (15). Recently, STEC non-O157 infection cases have increased globally, highlighting the significance of investigating STEC non-O157 (67, 69, 75). Among the STEC non-O157 serotypes, O26, O45, O103, O111, O121, and O145, were reported as the six major STEC non-O157 linked to human diseases (16, 22). Scallen *et al*. reported that ~63,000 and 112,000 cases of foodborne illness caused by STEC O157 and non-O157, respectively, occur in the United States annually (5). The progression of STEC infection varies, causing symptoms ranging from mild gastrointestinal symptoms to severe HC or HUS (13, 26, 28). Predicting the risk of STEC is especially important for public health because STEC infection might develop into a life-threatening disease, and is often associated with large and multinational outbreaks (7, 26, 81).

Although the pathogenicity of STEC is not fully understood, several virulence factors have been identified (26), including Shiga toxins, intimin, and the 60-mDa plasmids (enterohemolysin or serine protease) (26). Shiga toxins are the principal virulence factors of STEC, and two major types of Shiga toxins are known, Stx1 and

2 (25). While the DNA sequence of *stx1* is highly conserved and only a few *stx1* variants have been reported (including *stx1c* and *stx1d*), the *stx2* sequence shows 84%–99% similarity among the *stx*2 variants (26, 27). Because the variants are related to the properties of Shiga toxin, subtyping of the *stx* variants is important for predicting the virulence potential of STEC in human infection (245). Among Shiga toxin and its variants, Stx2 is most associated with severe disease (29, 30). Stx2 is a 1,000 times more toxic than Stx1 to renal microvascular endothelial cells, and Stx2 and Stx2c are more commonly reported in HUS patients (31, 32, 246). Intimin, one of the proteins encoded by *eae* in the locus of enterocyte effacement, which is responsible for the formation of attaching and effacing (A/E) legions (26, 33, 37). Several other factors also contribute to the virulence of STEC. EhxA (EHEC-enterohemolysin) disrupts the cytoplasmic membranes of mammalian cells (26, 247). EspP (a serine protease) potentiates STEC colonization in the human gut (56, 58), and KatP (catalase peroxidase) (57), SubAB (subtilase), and Saa (STEC autoagglutinating adhesin) are associated with the virulence of STEC (25, 248, 249).

Cattle are a primary source of STEC infection and are often targeted to develop strategies for reducing contamination. Therefore, monitoring the virulence potentials of STEC isolates from cattle is important for tracing the sources of contamination, managing outbreaks or sporadic cases, and reducing the risks for human infection. This study investigated the prevalence of STEC O157 and non-O157 in cattle farm samples in South Korea and assessed the virulence potentials of STEC isolates from

these samples by characterizing *stx* variants, antimicrobial resistance, and virulence genes. Finally, genetic analysis was performed to analyze the genetic dynamics of STEC strains isolated over a four-year period.

3.2. Materials and Methods

Sample collection

Samples were collected from 15 cattle farms located in the Gyeonggi province in Korea during 2012–2015. Each farm was visited one to nine times during the sampling period (median = 1, average = 1.9), and cattle farm samples, including feces, ground soil, and water, were collected. Fecal samples were collected by direct rectal retrieval using disposable gloves. Environmental samples in the farm were collected using sterilized spatulas. Each sample collected had a mass of at least 5 g (or a volume of at least 5 mL for liquid samples). A total of 469 samples (419 fecal, 47 ground soil, one water, one raw milk, and one forage sample) were collected and transported immediately to the laboratory for STEC isolation.

Isolation of STEC strains

STEC was isolated using the standard selective culture, IMS, and PCR-based culture method as described in the "Materials and Methods" section in Chapter II.

Antimicrobial-susceptibility test

A standard disk-diffusion test was performed to determine antimicrobial susceptibility for the following 14 antimicrobial drugs: AM (10 μg), C (30 μg), IMP (10 μg), TE (30 μg), AN (30 μg), AMC (20/10 μg), CAZ (30 μg), GM (10 μg), NA

(30 µg), STX (1.25/23.75 µg), CRO (30 µg), ATM (30 µg), CTX (30 µg), and CPD (10 µg). For quality control, *E. coli* ATCC 25922 was used as the reference strain. Antimicrobial susceptibility was interpreted as guided by the Clinical Laboratory Standard Institute (CLSI).

Detection of stx variants, virulence genes, and antimicrobial resistance genes

The presence of virulence and antimicrobial resistance genes was determined by PCR using a MyCycler thermal cycler (Bio-Rad Laboratories, USA). For DNA preparation, a single colony of each isolate was suspended in 1 mL of normal saline and centrifuged for 3 min at 6,000 × *g*. The pellets were re-suspended with 200 µL of sterile water and boiled for 10 min. The suspension was centrifuged for 3 min at 6,000 × *g*, and the supernatant was used as the DNA template. PCR was conducted as described previously to detect Shiga toxin genes (*stx1, stx1c, stx1d, stx2, stx2a, stx2c, stx2d, stx2e, stx2f,* and *stx2g*), virulence genes (*eae*, *tir*, *espB*, *espD, ehxA, katP, espP, iha, subA, stcE,* and *saa*), and antimicrobial resistance genes (*ampC, tetA, tetB, tetC, tetD, tetE, tetG, cat, cml,* bla_{OXA}, bla_{CMY}, bla_{TEM}, and *qnr*). The primer sequences and reaction conditions for each gene are summarized in Table 3.1.

Table 3.1. Primer sequences and the PCR conditions used in this study

Target gene	Nucleotide sequences (5' - 3')	PCR conditions				Ampli-con size (bp)	Refer-ences
		Denatu-ration	Anneal-ing	Exten-sion	cycle		
stx1	CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG	94°C, 60 s	56°C, 60 s	72°C, 60 s	25	348	(186)
stx2	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	94°C, 60 s	56°C, 60 s	72°C, 60 s	25	584	(186)
stx1c	TTTTCACATGTTACCTTTCCT CATAGAAGGAAACTCATTAGG	94°C, 60 s	56°C, 60 s	72°C, 60 s	30	498	(250)
stx1d	CTTTTCAGTTAATGCGATTGCT AACCCCATGATATCGACTGC	94°C, 60 s	56°C, 60 s	72°C, 60 s	30	192	(250)
stx2a	GCGATACTG**R**G**B**ACTGTGGCC CCG**K**CAACCTTCACTGTAAATGTG	94°C, 50 s	65°C, 40 s	72°C, 30 s	25	349	(251)
stx2c	GCGGTTTTATTTGCATTAGT AGTACTCTTTTCCGGCCACT	94°C, 60 s	56°C, 60 s	72°C, 60 s	30	124	(250)
stx2d	GGTAAAATTGAGTTCTCTAAGTAT CAGCAAATCCTGAACCTGACG	94°C, 60 s	56°C, 60 s	72°C, 60 s	30	175	(250)
stx2e	ATGAAGAAGATGTTTATAGCG TCAGTTAAACTTCACCTGGGC	94°C, 60 s	56°C, 60 s	72°C, 60 s	30	267	(250)
stx2f	AGATTGGGCGTCATTCACTGGTTG TACTTTAATGGCCGCCCTGTCTCC	94°C, 60 s	56°C, 60 s	72°C, 60 s	30	428	(250)
stx2g	GTTATATTTCTGTGGATATC GAATAACCGCTACAGTA	94°C, 60 s	56°C, 60 s	72°C, 60 s	30	573	(250)
eae	ATTACTGAGATTAAGGCTGAT ATTTATTTGCAGCCCCCCAT	94°C, 20 s	58°C, 20 s	72°C, 90 s	35	682	(15)
tir	CATTACCTTCACAAACCGAC CCCCGTTAATCCTCCCAT	94°C, 40 s	57°C, 60 s	72°C, 75 s	30	1,550	(252)
espB	GCCGTTTTTGAGAGCCAGAAT ATCATCCTGCGCTCTGCGAAC	94°C, 40 s	63°C, 45 s	72°C, 60 s	30	633	(252)
etpD	CGTCAGGAGGATGTTCAG CGACTGCACCTGTTCCTGATTA	94°C, 30 s	54°C, 60 s	72°C, 90 s	30	1,062	(253)
ehxA	GTTTATTCTGGGGCAGGCTC CTTCACGTCACCATACATAT	94°C, 60 s	56°C, 60 s	72°C, 60 s	25	166	(186)
KatP	CTTCCTGTTCTGATTCTTCTGG AACTTATTTCTCGCATCATCC	94°C, 30 s	58°C, 60 s	72°C, 150 s	30	2,125	(57)
espP	AAACAGCAGGCACTTGAACG GGAGTCGTCAGTCAGTAGAT	94°C, 30 s	58°C, 60 s	72°C, 150 s	30	1,830	(56)
iha	CTGGCGGAGGCTCTGAGATCA TCCTTAAGCTCCCGCGGCTGA	94°C, 60 s	57°C, 60 s	72°C, 120 s	30	827	(254)
subA	CGGCTTATCATCCTGTCAGC TATAGCTGTTGCTTCTGACG ATGGACATGCCTGTGGCAAC	94°C, 45 s	57°C, 60 s	74°C, 60 s	30	233	(255)

Table 3.1. Primer sequences and the PCR conditions used in this study (cont')

Target gene	Nucleotide sequences (5' - 3')	PCR conditions				Ampli-con size (bp)	Refer-ences
		Denatu-ration	Anneal-ing	Exten-sion	cycle		
stcE	GGCTCCGGAGGTGGGGGAAT GAAGCCGGTGGAGGAACGGC	94°C, 30 s	60ºC, 60 s	72ºC, 15 s	30	399	(256)
saa	CGTGATGAACAGGCTATTGC ATGGACATGCCTGTGGCAAC	94°C, 60 s	56ºC, 60 s	72ºC, 60 s	30	119	(257)
tetA	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAG	95°C, 60 s	58ºC, 60 s	72ºC, 60 s	30	210	(258)
tetB	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	95°C, 60 s	56ºC, 60 s	72ºC, 60 s	30	659	(258)
tetC	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	95°C, 60 s	58ºC, 60 s	72ºC, 60 s	30	418	(258)
tetD	AAACCATTACGGCATTCTGC GACCGGATACACCATCCATC	95°C, 60 s	60ºC, 60 s	72ºC, 60 s	30	787	(258)
tetE	AAACCACATCCTCCATACGC AAATAGGCCACAACCGTCAG	95°C, 60 s	58ºC, 60 s	72ºC, 60 s	30	278	(258)
tetG	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC	95°C, 60 s	60ºC, 60 s	72ºC, 60 s	30	468	(258)
ampC	CCCCGCTTATAGAGCAACAA TCAATGGTCGACTTCACACC	94°C, 60 s	61ºC, 120 s	72ºC, 180 s	35	634	(259)
catA1	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	95°C, 60 s	57ºC, 70 s	72ºC, 120 s	32	547	(62)
cmlA	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	95°C, 60 s	57ºC, 70 s	72ºC, 120 s	32	698	(62)
floR	TATCTCCCTGTCGTTCCAG AGAACTCGCCGATCAATG	94ºC, 30 s	52ºC, 30 s	72ºC, 30 s	30	399	(260)
bla_{CMY}	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	94°C, 60 s	49ºC, 90 s	72ºC, 60 s	35	462	(261)
bla_{OXA}	TATCTACAGCAGCGCCAGTG CGCATCAAATGCCATAAGTG	94°C, 60 s	62ºC, 120 s	72ºC, 60 s	31	199	(259)
bla_{TEM}	TACGATACGGGAGGGCTTAC TTCCTGTTTTTGCTCACCCA	94°C, 60 s	62ºC, 60 s	72ºC, 60 s	30	717	(262)

Virulence gene profiling

A phylogenetic dendrogram of the virulence profiles was constructed by using the UPGMA for binary data using BioNumerics, version 6.6 (Applied Maths NV, Belgium).

Pulsed-field gel electrophoresis

PFGE was performed following the CDC PulseNet protocol using CHEF MAPPER (Bio-Rad, Hercules, CA, USA). Briefly, STEC colonies were suspended in cell suspension buffer (100 mM Tris: 100 mM EDTA, pH 8.0) and then adjusted to the 4.0 McFarland scale (McF). The adjusted cell suspension (400 µL) was mixed gently with 20 µL of proteinase K (20 mg/mL) and 400 µL of 1% SeaKem Gold melted agarose gel to build a plug. The plug was soaked in a proteinase K-containing cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% sarcosyl) for 2 h to lyse the cells, after which it was washed twice with sterile water for 15 min and then four times with TE buffer (10 mM Tris: 1 mM EDTA, pH 8.0) for 15 min. The plug was then digested with 50 U of *Xba*I restriction enzyme for 2 h. PFGE was performed with a pulse time of 2.16–54.17 s for STEC O157 and 6.76–35.38 s for STEC non-O157; *S.* Braenderup ATCC BAA664 was used as a size ladder marker. A Dice similarity coefficient with a UPGMA dendrogram was generated based on 1.5% tolerance windows and 1.5% optimization, using BioNumerics, version 6.6 (Applied Maths NV, Belgium).

Statistical analysis

To identify factors potentially associated with prevalence, farm-management factors, environmental factors, and animal information were collected, if present (Table 3.2.). Farm-management factors (farm size, ground soil hygiene, and diet), and animal information (age and breed) were obtained from the veterinarian in charge of each farm. Environmental factors included average temperature on sampling date, humidity, and rainfall within three days prior to sampling, which were obtained from the data provided by the Meteorological Administration (http://www.kma.go.kr/weather/observation/past_table.jsp). The association between STEC prevalence and farm management and environmental factors was analyzed by using the chi-squared test, and the association between STEC prevalence and animal factors was analyzed by Fisher's exact test. The statistical analysis was performed using SPSS, version 22.0 (SPSS IBM, New York, NY, USA), and the variables were considered to be significantly associated when the *p*-value was <0.05.

Table 3.2. Data summary of 15 cattle farms and their STEC prevalence

Farm	Farm size	Hygiene of sawdust[a]	Diet	Loca-tion[b]	No of visit	Tempe-rature [c,d] (℃)	Humi-dity	Rain[c,d]	Sampling date (YYYYMM)	No. of samples collected	No. of STEC isolates	Farm prevalence	ID (breed, serotype)[d] of STEC isolates
1	250	High	TMR[e]	GP	1	22.5	93.4	Yes	201208	8	0	0.00	
2	60	Mid	TMR	GP	1	22.5	93.4	Yes	201208	10	0	0.00	
3	60	Low	TMR	GP	1	22.5	93.4	Yes	201208	8	0	0.00	
4	50	Low	Hay	CP	2	22.4	80.1	Yes	201208	9	4	44.44	0823-2 (D, O157), 0823-4 (D, O157), 0823-5 (D, O157), 0823-8 (D, O157)
						21.4	78.6	Yes	201309	6	1	16.67	0909-5 (D, O157)
5	30	Low	TMR	GP	1	22.4	80.1	Yes	201208	7	0	0.00	
6	40	High	TMR	GP	1	23.1	80.9	Yes	201208	9	0	0.00	
7	160	High	TMR	GP	2	27.7	69	Yes	201208	10	9	90.00	0827-1 (D, O157), 0827-2 (D, O157), 0827-3 (D, O157), 0827-5 (D, O157), 0827-6 (D, O157), 0827-7 (D, O157), 0827-8 (D, O157), 0827-9 (D, O157), 0827-10 (G, O157)
						20	98.9	Yes	201309	8	1	12.50	0911-3 (D, NT)
8	150	High	TMR	GP	1	27.7	69	Yes	201208	10	0	0.00	
9	50	High	TMR	GP	1	27.8	63.8	Yes	201208	10	0	0.00	
10	70	Low	TMR	CP	2	27.8	63.8	Yes	201208	10	0	0.00	
						21.4	78.6	Yes	201309	4	1	25.00	0909-9 (G, O157)
11	30	Mid	TMR	GP	2	21.8	94.3	Yes	201209	9	1	11.11	0904-9 (G, O84)
						21.4	78.6	Yes	201309	7	5	71.43	0909-11 (D, O157), 0909-14 (D, O157), 0909-15 (D, O157), 0909-16 (D, O157), 0909-17 (G, O157)
12	50	Low	TMR	GP	2	21.8	94.3	Yes	201209	10	1	10.00	0904-16 (D, O185)
						20	98.9	Yes	201309	9	2	22.22	0911-3 (D, O169), 0911-11 (D, O157)
13	80	High	TMR	GP	2	21.8	76.9	Yes	201209	11	1	9.09	0905-7 (D, O157)
						20	98.9	Yes	201309	8	0	0.00	
14	60	High	TMR	GP	1	21.8	76.9	Yes	201209	12	0	0.00	

Table 3.2. Data summary of 15 cattle farms and their STEC prevalence (cont')

Farm	Farm size	Hygiene of sawdust[a]	Diet	Location[b]	No of visit	Temperature [c] (℃)	Humidity	Rain[c]	Sampling date (YYYYMM)	No. of samples collected	No. of STEC isolates	Farm prevalence	ID (breed, serotype)[d] of STEC isolates
15	150	High	Hay	YP	9	30.5	62.9	No	201208	24	3	12.50	0806-3 (D, O157), 0806-5 (D, O157), 0806-20 (G, O157)
						11.0	63.6	No	201210	28	1	3.57	1015-16 (G, O84)
						-4.2	75	No	201301	32	0	0.00	
						19.7	81.3	Yes	201305	36	8	22.22	0527-1 (D,O108), 0527-4 (D,O108), 0527-8 (D,O119), 0527-15-1 (D, O108), 0527-15-2 (D,O119), 0527-19 (D,O108), 0527-23 (D,O108), 0527-24 (D,O119)
						20.7	92.1	Yes	201408	34	13	38.24	0814-4 (D, O157), 0814-5 (D, O157), 0814-7 (D, O157), 0814-8 (D, O157), 0814-11 (B, O157), 0814-13 (D, O15), 0814-16 (D, O8), 0814-20 (D, O157), 0814-22 (D, O157), 0814-25 (D, O15), 0814-31 (D, O157), 0814-32 (D, O55), 0814-34 (D, O157)
						16.8	54.6	No	201410	40	4	10.00	1013-6 (C, O111), 1013-12 (B, O8), 1013-19 (G, O157), 1013-21 (D, O84)
						0.1	90.8	No	201412	27	3	11.11	1215-7 (D, O15), 1215-8 (D, O109), 1215-24 (B, O109)
						25.4	65.6	Yes	201507	40	5	12.50	0709-6 (D, O8), 0709-7 (D, O8), 0709-29 (B, NT), 0709-32 (C, O111), 0709-35 (C, O84)
						19.2	60.1	No	201509	33	0	0.00	

[a] Hygiene level of sawdust: Low; very wet, dirty, and slippery, Mid; normal, High; very dry.

[b] GP: Gapyeong; CP: Chungpyeong; YP: Yangpyeong

[c] Temperature: average temperature of sampling day, rain: raining within 3 days prior to sampling date considered yes. Prevalence of STEC were significantly higher at the temperature above 20℃ and when rained within 3 days prior to sampling date ($p < 0.001$, Chi square test, two tailed).

[d] D:dairy cattle, G:Ground soil; B:beef cattle; C: calf

[e] TMR: Total mixed ration.

3.3. Results

STEC prevalence in cattle farms

Of 496 samples collected from 29 visits to 15 farms, 63 STEC were isolated from 17 visits to seven farms (Table 3.2.). Most STEC were isolated from feces (54 from adult cow and three from calves), and six STEC were isolated from ground soil. No STEC were isolated from feed, water, or milk samples. The farm prevalence of STEC varied from 0.0%–90.0%, but no farm management factors were found to be associated with STEC prevalence. STEC prevalence was significantly higher when the average temperature was above 20 °C ($p < 0.001$, two tailed chi-squared test, odds ratio [OR] = 2.3), and when rain was reported within three days prior to sampling ($p < 0.001$, two tailed chi-squared test, OR = 3.5). Moreover, while the STEC prevalence was higher in calves (3/19; 15.8%) than in adults (54/405; 13.3%), the difference was not significant. By breed, STEC prevalence was significantly higher in beef cattle (4/9; 44.4%) than in dairy cattle (50/396; 12.63%; $p < 0.05$, two-tailed Fisher's exact test, OR = 5.5).

Serogroup

Of the 63 STEC isolates, 61 were serogrouped. Thirty-five (55.6%) and 26 (41.3%) isolates were found to be STEC O157 and non-O157, respectively, whereas two isolates were non-typeable (NT). Among STEC non-O157, the most common

serogroup was O108 (five isolates, 7.5%), followed by O8 and O84 (four isolates each, 6.0%), O15 and O119 (three isolates each, 4.5%), O109 and O111 (two isolates each, 3.0%), and O55, O169, and O185 (one isolate each, 1.5%)

Prevalence of Shiga toxin and its variant genes

The Shiga toxin gene types of the 63 STEC isolates were determined by PCR. STEC harboring both *stx1* and *stx2* (28/63, 41.8%) were the most common strains, followed by STEC with either s*tx1* (13/63, 19.4%) or *stx2* (22/63, 32.8%; Table 3.3.). STEC harboring *stx1* were only detected in STEC non-O157 (O55, O84, O108, and O111), while STEC O157 harbored *stx2* only or both *stx1* and *stx2* with a similar distribution. Of the 35 STEC harboring *stx1*, 34 (97.1%) carried the *stx1c* variant, while no *stx1d* variants were detected. All STEC harboring *stx2* (n = 50) carried at least one *stx2* variant, *stx2a* (45; 90.0%)*, stx2c* (34; 68.0%), and *stx2g* (5; 10.0%). *Stx2d, stx2e*, and *stx2f* were not detected. When the distribution of the *stx* variants was analyzed based on serotypes, *stx2a* was observed in O8, O119, O157, O169, and O185; *stx2c* in O157 (33/35, 94.3%), and O185 (1/1, 100.0%); and *stx2g* in O15 (3/3, 100.0%) and O109 (2/2, 100.0%).

Table 3.3. Shiga toxin genotypes of STEC isolates

Shiga toxin gene type	Serotype	No. of isolates	*stx* variants			
			stx1c	*stx2a*	*stx2c*	*stx2g*
stx1 only	O55	1	1	-	-	-
	O84	4	4	-	-	-
	O108	5	5	-	-	-
	O111	2	2	-	-	-
	NT[a]	1	1	-	-	-
	Subtotal	13	13	-	-	-
stx2 only	O15	3	-	0	0	3
	O109	2	-	0	0	2
	O119	3	-	3	0	0
	O157	18	-	18	18	0
	O169	1	-	1	0	0
	O185	1	-	1	1	0
	Subtotal	28	-	23	19	5
Both *stx1* and *stx2*	O8	4	3	4	0	0
	O157	17	17	17	15	0
	NT	1	1	1	0	0
	Subtotal	22	21	22	15	0
Total		63	34	45	34	5

[a] NT: non-typeable for STEC serogroup

Antimicrobial resistance of the STEC isolates

Disc diffusion tests were conducted using 14 antimicrobial agents. While resistance was observed for TE (five isolates, 17.9%), AMP (four isolates, 14.3%), and CTX (one isolate, 3.6%) in 28 STEC non-O157 strains, resistance was not observed in 35 STEC O157 strains. Four and five STEC O157 and non-O157 STEC, respectively, showed intermediate resistance to CTX. Of them, only one STEC non-O157 isolate exhibited multi-drug resistance to both on TE and AMP (Table 3.4.).

All of the 63 STEC isolates carried more than one antimicrobial resistance genes. Of 13 tested antimicrobial resistance genes, genes *tetA, tetD, tetG, cat, cml,* bla_{OXA}, bla_{CMY}, and *qnr* were not detected. All STEC isolates carried *ampC* gene (63/63; 100.0%), and 11 isolates carried bla_{TEM}, (11/63; 17.5%) gene. Of tetracycline resistance gene, *tetE* (34/63; 54.0%) was the most prevalent, followed by *tetB* (4/63; 6.3%) and *tetC* (3/63; 4.8%; Table 3.4.).

Table 3.4. Phenotypic and genotypic antimicrobial resistance profiles of STEC isolates

Antimicrobial class	Phenotypic profiles			Genotypic profiles		
	antimicrobial [a]	No. of STEC isolates [b] (%)		Antimicrobial resistance gene [c]	No. of STEC isolates (%)	
		STEC O157	STEC non-O157 [b]		STEC O157	STEC non-O157
B-lactams	Ampicillin	0 (0.0)	4 (14.3)	*ampC*	35 (100.0)	28 (100.0)
	Cefotaxime [d]	0 (0.0)	1 (3.6)	Bla_{TEM}	0 (0.0)	11 (36.3)
Tetracycline	Tetracycline	0 (0.0)	5 (17.9)	*tetB*	0 (0.0)	4 (14.3)
				tetC	0 (0.0)	3 (10.7)
				tetE	34 (97.1)	0 (0.0)

[a] All STEC showed susceptibility on chloramphenicol, imipenem, amikacin, amoxicillin-clavulanic acid, ceftazidime, gentamicin, nalidixic acid, trimethoprim-sulfamethoxazole, ceftriaxone, aztreonam, and cefpodoxime on standard disk-diffusion test

[b] Antimicrobial resistance profiles were tested for 35 and 28 STEC O157 and non-O157 strains, respectively

[c] Antimicrobial resistance genes of *tetA, tetD, tetG, cat, cml,* bla_{OXA}, bla_{CMY}, and *qnr* were not detected from all STEC isolates

Virulence gene profiles

The prevalence of virulence genes was investigated. The prevalence of each virulence gene was as follows: *eae* (77.8%), *tir* (76.2%), *espB* (74.6%), *espD* (65.1%), *ehxA* (92.1%), *katP* (55.6%), *espP* (84.1%), *iha* (66.7%), *subA* (7.9%), *stcE* (65.1%), and *saa* (9.5%).

When clustering analysis was conducted based on the profiles of the virulence genes, including Shiga toxin and its variant genes, six clusters were generated (based on 50% similarity; Fig. 3.1.). STEC strains in clusters 1, 2, and 6 were composed of a single serotype each, O119, O185, and O55, respectively. The strains showed 100% prevalence of *eae*, *ehxA, stx2,* and *stx2c* in cluster 1; *espP, stx2, stx2a*, and *stx2c* in Cluster 2; and *tir*, *stx1*, and *stx1c* in cluster 3. Strains in cluster 3 were characterized as having *stx2g* variants, and belonged to the O15 and O109 serogroups. Cluster 4 was composed of STEC O84, O108, O111, and O157 strains with a high prevalence of *eae*, *tir*, *espB*, *espD*, *ehxA, katP, espP, iha, stcE, stx2, stx2a,* and *stx2c*. Strains in cluster 5 were characterized as having *subA* and *saa* (Table 3.5.).

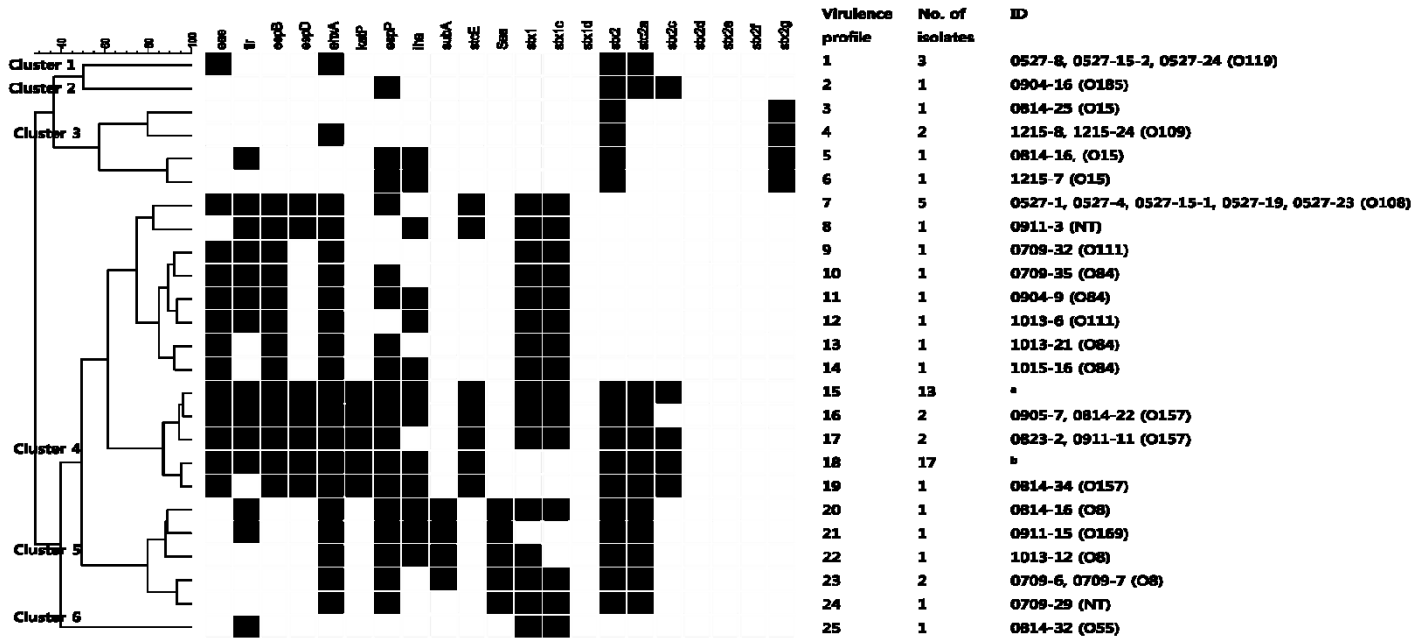


Figure 3.1. UPGMA dendrogram of STEC O157 and non-O157 based on the profiles of virulence genes. Six clusters were generated with the UPGMA method based on 50% similarity.

[a] 0806-3, 0806-5, 0806-20, 0823-4, 0823-5, 0823-8, 0909-5, 0909-9, 0909-11, 0909-14, 0909-15, 0909-16, 0909-17 (O157)

[b] 0827-1, 0827-2, 0827-3, 0827-5, 0827-6, 0827-7, 0827-8, 0827-9, 0827-10, 0814-4, 0814-5, 0814-7, 0814-8, 0814-11, 0814-20, 0814-31, 1013-19 (O157)

Table 3.5. Prevalence of virulence genes in each cluster

Cluster	No. of isolates	Serotype (No. of isolates in each serotype)	Prevalence of virulence genes (%)										
			eae	*tir*	*esp*B	*esp*D	*ehx*A	*kat*P	*esp*P	*iha*	*sub*A	*stc*E	*Saa*
1	3	O119 (3)	100.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
2	1	O185 (1)	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
3	5	O15 (3), O109 (2)	0.0	20.0	0.0	0.0	40.0	0.0	40.0	40.0	0.0	0.0	0.0
4	47	O84 (4), O108 (5), O111 (2), O157 (35), NT (1)	97.9	93.6	100.0	87.2	100.0	74.5	93.6	78.7	0.0	87.2	0.0
5	6	O8 (4), O169 (1), NT (1)	0.0	33.3	0.0	0.0	100.0	0.0	100.0	50.0	83.3	0.0	100.0
6	1	O55 (1)	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

NT; non-typeable

Genetic relatedness of the STEC isolates

The PFGE patterns of *Xba*I-digested STEC O157 and non-O157 were analyzed to determine how the clonal relatedness of STEC isolates changed with temporo-spatial variation. The DNA fingerprints of 35 STEC O157 strains showed high similarity, generating 16 different PFGE profiles with 15–20 discernible fragments (mean: 17.4, median: 17). The 16 PFGE profiles clustered into four groups, based on 90% similarity; each group was composed of one to 23 isolates (Fig. 3.2.A).

PFGE was performed for STEC strains belonging to 10 different serogroups, other than O157 and two strains that were non-typeable for the O serotype. Diverse PFGE patterns of *Xba*I-digested STEC non-O157 were observed, resulting in 20 different PFGE profiles with 14–23 discernible fragments (mean: 18.2, median: 19), except for one isolate (0911-15), which was untypeable by PFGE. A UPGMA dendrogram, generated using Dice coefficient analysis, clustered STEC non-O157 into 12 different groups, based on 80% similarity. Each group was composed of one to five strains, and STEC strains belonging to the same serotype were grouped together regardless of differences in the sampling period or geographical location of each farm (Fig. 3.2.B).

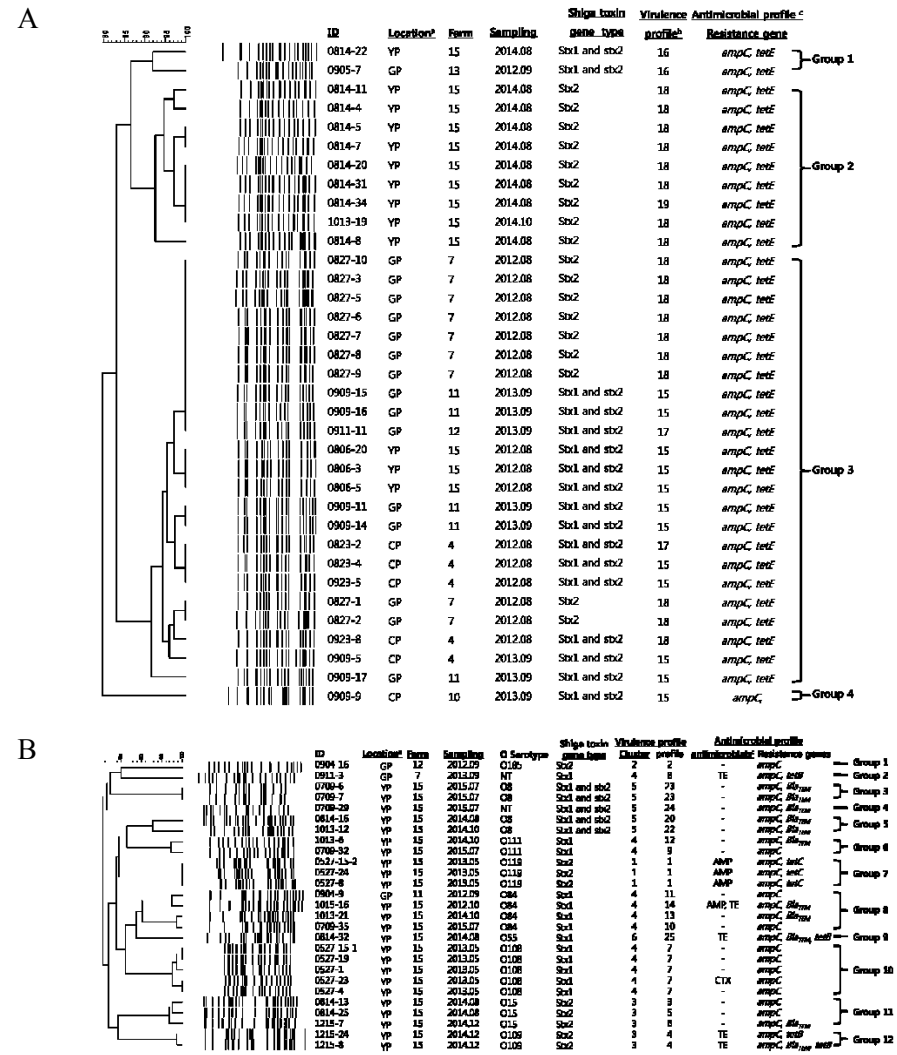


Figure 3.2. Phylogenetic analysis of STEC isolates from cattle. The UPGMA method was used with a 1.5% optimization and 1.5% tolerance window using Bionumerics software.

[a] CP: Cheongpyeong, GP: Gapyeong, YP: Yangpyeong. [b] All STEC O157 strains were belonged to cluster 4. [c] AMP: ampicillin, CTX: cefotaxime, TE: tetracycline

A. STEC O157 strains were clustered into four groups (based on 90% similarity).
B. STEC non-O157 strains clustered into 12 groups (based on 80% similarity), STEC strains with the same serotype clustered together, except for STEC O8. The STEC O169 strains were not typeable by PFGE.

3.4. Discussion

The prevalence of STEC in 15 different cattle farms, virulence-gene profiles, antimicrobial resistance, and genetic relatedness of STEC isolates were analyzed to investigate the virulence potentials of STEC in cattle farm.

During the sampling period, 63 STEC were isolated from 469 cattle farm samples collected from 15 cattle farm in Gyeonggi province in South Korea. Numerous studies are ongoing to identify the factors associated with STEC prevalence. In this study, high temperature and rain were found to be associated with STEC prevalence. Similarly, a previous study reported higher STEC prevalence in hot seasons than in cold seasons (89, 94, 97). In addition, rainfall has been considered an important transmission factor for STEC. The pathogens may be transported via sediments to vast geographical regions as far away as 32 km, resulting in an increased prevalence in the environment (263, 264). Many published reports have shown that STEC O157 prevalence is higher in calves, especially in post-weaned calves, than in adult cattle (101-103). However, no obvious link between age and STEC non-O157 prevalence has been reported, and some investigators even observed a higher prevalence of STEC non-O157 in adult groups (102, 265, 266). In this study, the adult group showed a higher prevalence for STEC O157 (calves: 0/19, 0.0% vs. adults: 31/405, 7.7%) and a lower prevalence for STEC non-O157 (calves: 3/19, 15.8% vs. adult: 24/405, 5.9%). This discrepancy with respect to previous data may be due to the

collection of a relatively small number of calf feces samples, compared to the number of samples collected from adults. Thus, further studies may be needed to investigate the effect of age on STEC non-O157 prevalence. Here, beef cattle showed higher STEC prevalence than dairy cattle. Although only a few beef cattle were included in this study, the results are consistent with data from previous studies (89, 97).

While many studies have focused on the O157 serotype, the significance of STEC non-O157 in human infection has become clear recently (22, 75). In this study, 11 different serotypes of STEC were identified and >40% of the STEC were non-O157, highlighting the need for active surveillance of STEC non-O157 and understanding their virulence potential in humans. Of the identified serotypes, O8, O15, O55, O84, O109, O111, O119, and O157 have been reported frequently in dairy cattle worldwide (265). Among them, several serogroups have also been reported frequently in human clinical cases. The O111 serogroup is the second most common serogroup in human infections, and is the most common cause of HUS. Moreover, it accounts for half the STEC non-O157 outbreaks. The O15, O84, and O119 serogroups also frequently cause human illness (22, 265). In addition, human-pathogenic STEC O8, O15, and O109 serotypes have been detected in food samples, highlighting the possible transmission of STEC via the food chain (267).

The genetic variation of Shiga toxin causes changes in its amino acid composition, which may directly influence the virulence of STEC, resulting in a

change in the toxin receptor tropism or toxicity of Shiga toxin (28). In this study, high prevalence of *stx1c, stxs2*, and *stx2c* was detected. The *stx1c* variants are associated with ovine-originated STEC strains (250, 268, 269), but the high prevalence of *stx1c* in buffaloes, cattle, and goats was reported to account for 80% of the *stx1* variants, indicating a wide distribution of *stx1c* variants in STEC of bovine origin (270). *stx1c* variants have been found as *stx1c* only or in combination with *stx1, stx2*, or *stx2d.* However, in this study, combinations involving *stx1, stx1c, stx2, stx2a,* and *stx2c* (16 isolates); *stx1, stx1c, stx2*, and *stx2a* (six isolates); and *stx1* and *stx1c* (13 isolates) were newly found. In addition, *stx1c*-producing STEC is considered a subset of *eae*-negative STEC, and is responsible for asymptomatic or mild disease (250, 269, 271). However, in this study, 29 *stx1c*-producing STEC harbored *eae*. The *stx1c* variants in *eae*-positive STEC strains might be resulted from the dynamics of virulence genes. Of the *stx2* variants*, stx2a, stx2c,* and *stx2d* variants have been implicated in high STEC virulence (246, 272). While *stx2d* was not detected in the current study, the high prevalence of *stx2a,* and *stx2c* suggested the wide distribution of potentially pathogenic STEC strains in cattle farms. The *stx2g* variant was detected from five STEC non-O157 (three O15 and two O109 STEC). Previously, the *stx2g* variant has been identified from various sources, including cattle, beef or beef-containing products, and humans, suggesting a possible route of exposure of these STEC types via the food chain (267, 273).

To evaluate the virulence potentials of STEC strains isolated from cattle farms, the phenotypic and genotypic antimicrobial resistance features and the prevalence of virulence genes were investigated. In this study, all STEC isolates were susceptible to all tested antimicrobials, except for AMP, TE, and CTX. Resistance to AMP and TE in diverse sources, including cattle or beef products, have commonly been reported in previous studies (248, 249, 274), but resistance to CTX is uncommon, with only one isolate (of 722) from a bovine source being reported to date (275). CTX, a third generation cephalosporin, is used as an indicator to identify extended spectrum beta-lactamase (ESBL) production. Although ESBL production was not identified in this study (data not shown), the presence of CTX-resistant STEC indicates the need for implementing antimicrobial resistance control strategies to prevent the generation and spread of ESBL-STEC.

In addition, all the STEC strains that exhibited resistance to AMP, TE, and CTX were STEC non-O157 strains. Genotypic antimicrobial features also varied by its serotype. While antimicrobial resistance genes of *ampC*, *tetB*, *tetC*, and bla_{TEM} were only observed in STEC non-O157 strains, *tetD* was detected only in STEC O157 (34/35; 97.1%). These results suggest that antimicrobial resistance is higher in STEC non-O157 than in STEC O157, consistent with previous studies (274, 275).

The prevalence of virulence genes in each serotype was either 0% or 100%, except for *tir, espP*, and *iha*, indicating the sero-specific feature of virulence genes. To estimate the virulence potentials of STEC strains that might cause a risk to public

health, clustering analysis was performed based on the virulence gene profiles. Six clusters were generated, and sero-specific features were observed in each cluster. Cluster 1 was composed of O119 STEC, which has 100% prevalence of the well-known virulence factors *eae* and *ehxA*. The association between intimin (encoded by *eae*) and STEC virulence has been reported previously, and serogroup O119 has been detected in human infections (15, 22). This indicates that the STEC isolates in Cluster 1 might have the potential to cause human illness. Most of the other STEC strains were grouped in Cluster 4 (47/63, 74.6%), and these strains harbored most of the virulence genes at a high frequency, except for *subA* and *saa*. The *katP* and *stcE* gene products are believed to promote STEC virulence by assisting STEC colonization in the intestines and degrading the protective layers in the intestines, respectively (57, 256). A high prevalence of these two genes was reported for sero-pathogroups A and B, which are responsible for severe STEC illness (276). In this study, all of the O157 and O111 serotypes, which belonged to sero-pathotypes A and B, also belonged to Cluster 4, indicating the high virulence potential of the STEC in Cluster 4. Cluster 5 was characterized by the presence of *subA* and *saa*, and consisted of O8, O169, and NT STEC. *subA* is purported to increase STEC virulence. Saa also increases STEC virulence by assisting in adherence to host cells in *eae*-negative STEC (257, 277). On the other hand, the STEC in Clusters 2, 3, and 6 appeared to be less pathogenic to humans. High prevalence of *espP, iha*, and *ehxA* was reported

regardless of sero-pathotype, suggesting the absence of a strong association between these genes and STEC virulence (276).

PFGE analysis was performed to understand the clonal relatedness of STEC strains isolated from cattle farms located in different regions of the Gyeonggi province in Korea during 2012 to 2015. For the STEC O157 strains, those isolated from the same farm during the same sampling period had indistinguishable PFGE profiles except for a few isolates from farms 4, 7, 11, and 15, which showed one to three different bands. Considering that a single nucleotide mutation at a restriction enzyme site causes three fragment differences (278), a minor genetic variation may have occurred within the farm. In addition, STEC O157 from farm 4 showed high similarity between the 2012 and 2013 isolates. Phylogenetic analysis combined most isolates into group 3, which consisted of isolates from five farms in three different geographical locations. These results indicated the possible presence of a prototype of STEC O157 in the Gyeonggi province with a minor genetic variation, which led to within- and between-farm transmission during 2012 to 2013. However, STEC O157 isolates from farm 15 showed a higher degree of polymorphism; these isolates clustered in groups 1 and 2 (STEC strains isolated in 2014) and group 3 (STEC strains isolated in 2012). These results indicated that the prototype of STEC O157 in farm 15 might have changed in 2014. Because all the farms were located in the Gyeonggi province and the longest distance between farms was approximately 60 km, temporal effects may have been less important. While a high degree of genetic

diversity was observed in STEC non-O157, they were grouped together for strains with the same serotype. STEC O8, O15, O84, and O111 were isolated multiple times and shared genotypic similarity over the three-year period within the serogroup, implying that these STEC strains have endured and continue to survive, and causing within-farm transmission.

3.5. Conclusion

STEC prevalence differed greatly between farms, and temperature and rainfall affected the farm prevalence. A considerable number of STEC non-O157 stains were isolated, and different virulence and antimicrobial resistance features were observed between STEC O157 and non-O157 strains. While a high prevalence of virulence genes was observed in STEC O157 strains, the antimicrobial resistance rate was higher in STEC non-O157 strains. In addition, the *stx1c* variant was detected in *eae*-positive STEC, suggesting genetic dynamics among virulence genes in STEC isolates. Finally, PFGE analysis revealed the presence of a prototype STEC, which continues to evolve by genetic mutation and causes within- and between-farm transmission within the Gyeonggi province. Our results suggested that STEC from cattle have a high virulence potential and represent a threat to public health. Therefore, continual surveillance of both STEC O157 and non-O157 would be beneficial for controlling and preventing STEC illness.

Chapter IV.

The fecal microbial communities of dairy cattle shedding Shiga toxin-producing *Escherichia coli* or *Campylobacter jejuni*

Abstract

Cattle are a natural reservoir of Shiga tocin-producing *Escherichia coli* (STEC) and have recently been recognized as a major source of *Campylobacter jejuni* contamination. While several factors are known to be associated with bacterial colonization, the underlying microbial factors have not been clarified. Here, the fecal microbiota of dairy cattle (n = 24) was characterized using next-generation sequencing to elucidate the intestinal bacterial communities and the microbial diversity in relation to the presence of the foodborne pathogens STEC and *C. jejuni*. While no significant differences were observed in alpha-diversity between STEC-positive and STEC-negative samples, a high diversity index was observed in *C.*

jejuni-positive samples compared to that of *C. jejuni*-negative samples. From microbial community analysis, 16 phyla, 33 classes, 64 orders, 151 families, 547 genera, and 1709 species were identified. Among them, 9 phyla, 13 classes, 18 orders, 47 families, 148 genera, and 261 species were found to be the core microbiota in dairy cattle, covering 80.0~100.0% of the fecal microbial community. Diverse microbial communities were observed between cattle shedding foodborne pathogens and non-shedding cattle. *C. jejuni*-positive cattle had a higher relative abundance of *Bacteroidetes* ($p = 0.035$) and a lower relative abundance of *Firmicutes* ($p = 0.035$) compared to *C. jejuni*-negative cattle. In addition, while the relative abundance of 2 and 6 genera were significantly higher in cattle shedding STEC and *C. jejuni*, respectively, the relative abundance of 3 genera each were lower in STEC- and *C. jejuni*-negative cattle. However, these differences had a minor influence on the overall microbial community. These findings provide fundamental information on bacterial ecology in cattle feces and might be useful in developing strategies to reduce STEC or *C. jejuni* shedding in dairy cattle, thereby reducing the incidence of STEC infection and Campylobacteriosis in humans.

Keywords: *Campylobacter*, Cattle, Foodborne pathogens, Gastrointestinal Ecology, On-farm food safety

4.1. Introduction

Foodborne illness is a significant public health concern worldwide. In the US alone, nearly 9.4 million cases of foodborne illness by 31 major pathogens occur each year, resulting in about 55,961 hospitalizations and 1,351 deaths (5). STEC is an important zoonotic pathogen causing mild to fatal complications (12, 13), and the incidence of Campylobacteriosis, caused by mainly *C. jejuni*, is also increasing worldwide (119). Domestic animals play an important role in the transmission of foodborne pathogens as asymptomatic carriers. Cattle are known to be a natural reservoir of STEC (62, 63) and have recently been recognized as the major source of *C. jejuni* contamination (157-159).

Research on the individual factors underlying the bacterial shedding of STEC or *Campylobacter* in cattle has been limited. Microbiota comprise an important individual factor, playing a critical role in animal health, physiology, productivity, and bacterial shedding (210, 220). Indigenous microbes may inhibit or promote the colonization of pathogens by competing for nutrition or by using the byproducts of indigenous bacteria (221, 222). It is reported that organic acids and volatile fatty acids and the presence of butyrate-producing bacteria might inhibit STEC shedding, and several bacterial species might promote or inhibit STEC shedding (223, 224). An increase in generic *E. coli* might inhibit *Campylobacter* colonization in mice

(225). However, these studies involved culture-based techniques, limiting the understanding of microbial ecology in cattle.

Recent advancements in molecular methodologies enable the investigation of microbial communities regardless of the culture methods, facilitating the characterization of the entire bacterial population in a sample. The microbial diversity in relation to STEC or *C. jejuni* shedding has been reported but not in dairy cattle (227, 230-232). Bacterial shift has been reported in *Campylobacter*-infected humans and mice (140, 225), and differences in bacterial communities have been reported in *Campylobacter*-shedding chickens (233). Moreover, while STEC infection may be asymptomatic in cattle, the presence of STEC might influence the composition of intestinal microbiota in beef cattle (223, 230). Limited information on this aspect is available for dairy cattle, the management of which differs from that of beef cattle. This necessitates the characterization of microbial populations in relation to foodborne pathogen shedding in dairy cattle. Furthermore, the influence of *Campylobacter* infection on cattle microbiota has not been investigated. Therefore, the fecal microbiota of dairy cattle was characterized using NGS to identify their intestinal bacterial communities and assess the microbial diversity in relation to the presence of STEC and *C. jejuni*.

4.2. Materials and methods

Fecal sampling and cattle information

A single dairy cattle farm at Gyeonggi-do, South Korea, was visited for sample collection from August 2012 to December 2014. All cattle were multiparous Holstein cows in milk, and were fed formulated feed mixture, roughage, and total mixed ration. Individual cattle information is presented in Table 4.1.

Feces were collected by rectal grab sampling. More than 20g of fresh feces were transported into the sterile bottle using sterile spatula and were immediately transported to the laboratory at 4°C. Some samples were subjected to bacterial isolation, while the remaining samples were stored at −70°C for microbial community analysis.

Table 4.1.Data summary of the basic information of 24 dairy cattle

No.	Coding[a]	Birth Month (YYMM)	Parity	Milking days	Milk yield for 305 days
1	0214_1208_S	2010-05	1	52	0
2	0214_1301_C	2010-05	1	200	9,665
3	0214_1408_S	2010-05	2	384	11,638
4	0214_1410_C	2010-05	2	414	11,638
5	0214_1412_N	2010-05	3	37	0
6	9554_1408_N	2009-10	2	188	12,514
7	9554_1410_S	2009-10	2	249	13,006
8	9554_1412_C	2009-10	2	311	13,479
9	9555_1408_S	2010-09	2	65	0
10	9555_1410_C	2010-09	2	126	12,299
11	9555_1412_SC	2010-09	2	188	12,078
12	0213_1408_S	2010-04	2	244	13,422
13	0213_1412_C	2010-04	2	314	13,683
14	3618_1408_S	2011-05	1	320	8,993
15	3618_1410_C	2011-05	1	381	8,993
16	8484_1408_S	2009-09	3	125	9,518
17	8484_1410_N	2009-09	3	186	10,725
18	7914_1408_S	2009-04	2	220	12,735
19	7911_1410_N	2009-04	3	99	9,180
20	8755_1410_C	2009-01	3	306	14,102
21	8756_1410_N	2009-01	3	353	14,219
22	7904_1410_N	2009-03	3	242	17,816
23	8494_1410_C	2009-11	3	108	10,376
24	1862_1410_N	2009-02	3	210	16,295

[a] Coding refers to the identification number of each cow followed by the sampling date (YYMM) and presence of foodborne pathogen (N, both STEC and *C. jejuni*-negative; C, *C. jejuni-positive*; S, STEC-positive; SC, positive for both STEC and *C. jejuni*)

STEC and *C. jejuni* isolation

STEC was isolated using the standard selective culture, IMS, and PCR-based culture method as described in the "Materials and Methods" section in Chapter II. For *C. jejuni* isolation, the standard culture method was used as described in the "Materials and Methods" section in Chapter I.

Sample selection for microbial community analysis

The farm was visited up to seven times during the period of the study. At each visit, 18–34 samples were randomly selected for fecal sampling, and 194 fecal samples were collected. The average fecal collection per cattle was 2.43 – 1.47 times (ranging from one to six times, median = 2). Among 194 fecal samples, 31 (16.0%) STEC isolates and 47 (24.2%) *C. jejuni* isolates were identified. Representative samples for microbial community analysis were first selected from the cattle that shed STEC and/or *C. jejuni* more than twice during the sampling period. The remaining samples were randomly selected from two groups of cattle: STEC shedders and *C. jejuni* shedders. For the selection of fecal samples from non-shedders, the feces from the same individuals chosen for STEC and/or *C. jejuni* shedders were first selected, if present, and the remaining cattle were randomly selected from the non-shedding cattle group. Finally, samples were selected from 7 non-shedders, 8 STEC shedders, 8 *C. jejuni* shedders, and 1 STEC and *C. jejuni* shedder (total, 24 representative samples).

Pyrosequencing and data analysis

Metagenomic DNA was extracted from 24 representative cows using the FastDNA SPIN extraction kit (MP Biomedicals, Santa Ana, CA, USA). The V1–V3 regions of 16S rRNA were amplified by PCR using a barcoded fusion primer (http://oklbb.ezbiocloud.net/content/1001). The PCR products were then purified using a PCR purification kit (Qiagen, CA, USA), quantified using the PicoGreen dsDNA Assay kit (Invitrogen, CA, USA), and sequenced using a 454 Junior system (Roche, Branford, CT, USA). Pyrosequencing was performed by ChunLab Inc. (Seoul, Korea). The sequences have been submitted to the NCBI Short Read Archive under accession no. SRP068080.

Raw sequence data were filtered. Low quality reads (<25 bp or >300 bp) were removed and the primer sequences trimmed. Chimera sequences were excluded using the UCHUME algorithm. Alpha (α) and beta (β) diversity analyses were performed using the CLcommunity software (ChunLab Inc.). The reads were taxonomically assigned using the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/) based on 97% similarity to identify the OTUs. Rarefaction curves were illustrated after normalization to the minimum number of reads. α-Diversity indices were computed by 2 different methods: cluster database at high identity with tolerance (CD-HIT) and taxonomy-based clustering (TBC). The core microbiota were investigated by identifying taxa present in more than 70% of dairy cattle.

To evaluate the microbial diversity among 24 fecal samples, an UPGMA dendrogram was generated based on hierarchical clustering of the Fast UniFrac distance matrix.

Statistical analysis

Differences in the α-diversity and RA of each taxon were investigated between STEC- or *C. jejuni*-positive and negative, STEC-positive (n = 9) vs. STEC-negative (n = 15), and *C. jejuni*-positive (n = 9) vs. *C. jejuni*-negative (n = 15) cattle. The differences were analyzed using the two-tailed Mann–Whitney U test by SPSS statistics, version 22.0 (SPSS IBM, New York, NY, USA). Differences were considered significant at $p < 0.05$.

4.3. Results

α-Diversity of microbial communities

A total of 153,572 reads were obtained from the 24 dairy cattle (Table 4.2.). The Goods' coverage was 0.87 ± 0.05. The rarefaction curves for all 24 samples are shown in Figure 4.1. The α-diversity between STEC-positive and STEC-negative and *C. jejuni*-positive and *C. jejuni*-negative cattle did not differ significantly. The observed OTUs in non-shedders, STEC-shedders, and *C. jejuni*-shedders are presented in Table 4.3.

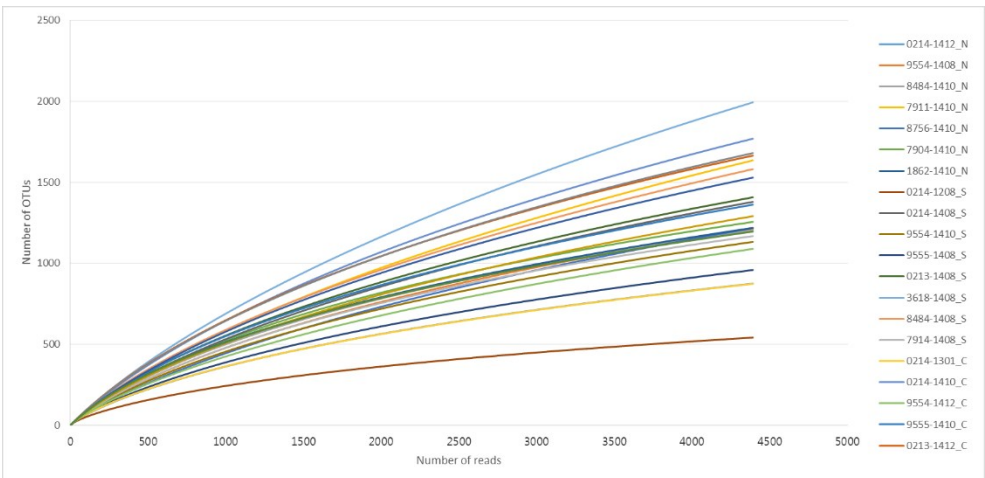


Figure 4.1. Rarefaction curves for fecal microbial communities of 24 dairy cattle. The curves were truncated after 4,217 reads (the minimum across all samples).

Table 4.2. Estimation of α-diversity for 24 dairy cattle using 2 different calculation methods

Target reads	Valid reads	OTUs	CD-HIT[a]			TBC[a]			Goods Lib. Coverage
			Ace	Chao1	Shannon	Ace	Chao1	Shannon	
0214-1412_N	4398	876	1976.07	1601.45	5.25	1979.27	1789.73	6.23	0.90
9554-1408_N	8400	1699	3667.76	2851.79	6.11	3763.43	3435.20	7.06	0.90
8484-1410_N	5130	1300	2632.39	2190.16	6.14	2626.35	2386.22	6.85	0.88
7911-1410_N	5482	1876	5913.67	4174.07	6.55	4109.55	3523.28	7.15	0.79
8756-1410_N	5133	1340	4061.84	2881.00	5.77	2972.59	2582.18	6.54	0.84
7904-1410_N	8011	1656	2533.71	2484.03	6.43	3642.50	3288.17	7.20	0.91
1862-1410_N	7930	1629	3444.23	2664.75	6.44	3687.44	3323.49	7.23	0.90
0214-1208_S	7053	675	1318.59	1123.56	4.55	2068.86	1882.57	6.09	0.96
0214-1408_S	8275	1920	3961.28	3228.44	6.34	4009.24	3653.00	7.24	0.89
9554-1410_S	7364	1471	2995.49	2396.85	5.90	3133.98	2827.33	6.92	0.90
9555-1408_S	8444	1335	2853.45	2166.65	5.51	3125.05	2836.84	6.76	0.93
0213-1408_S	7761	1891	3989.29	3132.46	6.53	4044.49	3645.85	7.31	0.88
3618-1408_S	4459	2014	6908.40	4395.69	7.05	4773.59	4074.63	7.38	0.71
8484-1408_S	5975	1896	5474.11	3798.26	6.67	3886.62	3473.28	7.22	0.82
7914-1408_S	4966	1241	2058.56	1929.35	5.96	2525.70	2282.26	6.74	0.88
0214-1301_C	7403	1132	2407.69	1897.51	5.41	2817.98	2569.53	6.55	0.93
0214-1410_C	7243	2352	5813.10	4313.78	6.96	4906.74	4449.11	7.51	0.82
9554-1412_C	7139	1413	3179.36	2520.38	5.76	3272.96	2949.78	6.83	0.90
9555-1410_C	6062	1614	3811.37	2955.89	6.50	3485.43	3143.32	7.18	0.86
0213-1412_C	4390	1665	4026.55	2994.50	6.91	3327.60	2924.33	7.31	0.79
3618-1410_C	6847	2128	4653.30	3644.32	7.02	4374.78	3841.54	7.49	0.84
8755-1410_C	4663	1335	3489.56	2540.40	6.21	2729.08	2460.08	6.79	0.84
8494-1410_C	6606	1914	4586.62	3320.44	6.61	3978.40	3583.43	7.26	0.85
9555-1412_SC	4438	1203	2571.60	2112.28	6.29	2491.49	2231.61	6.94	0.87

[a] α-diversity was calculated using 2 different calculation methods, cluster database at high identity with tolerance (CD-HIT) and taxonomy-based clustering (TBC)

Table 4.3. Estimation of α-diversity for 24 dairy cattle using 2 different calculation methods

Culture detection for STEC and *C. jejuni*	Valid read	CD-HIT[b]			TBC[b]			Goods' coverage
		OTUs	Chao 1 estimate	NP Shannon diversity index	OTUs	Chao 1 estimate	NP Shannon diversity index	
Both negative (n = 7)	6,354.86 ± 1,682.80	1,482.29 ± 335.34	2,692.47 ± 789.82	6.32 ± 0.45	2,084.29 ± 495.00	2,904.04 ± 658.60	7.19 ± 0.37	0.88 ± 0.04
STEC positive (n = 9)	6,526.11 ± 1,605.16	1,516.22 ± 448.87	2,698.17 ± 1,021.56	6.30 ± 0.78	2,144.00 ± 449.33	2,989.71 ± 760.92	7.26 ± 0.45	0.87 ± 0.07
***C. jejuni* positive (n = 9)**	6,087.89 ± 1,257.44	1,639.56 ± 420.57	2,922.17 ± 761.26	6.62 ± 0.57	2,207.67 ± 442.54	3,128.08 ± 715.62	7.41 ± 0.35	0.86 ± 0.04
Total (n = 24[a])	6,389.83 ± 1,436.14	1,565.63 ± 400.54	2,804.92 ± 848.60	6.42 ± 0.63	2,170.83 ± 440.21	3,048.20 ± 688.36	7.30 ± 0.40	0.87 ± 0.05

[a] One cow shed both STEC and *C. jejuni* and was counted in both the STEC-positive and *C. jejuni*-positive groups.

[b] α-diversity was calculated using 2 different calculation methods, cluster database at high identity with tolerance (CD-HIT) and taxonomy-based clustering (TBC)

Core microbiota

From microbial community analysis, 16 phyla, 33 classes, 64 orders, 151 families, 547 genera, and 1709 species were identified, of which 9 phyla, 13 classes, 18 orders, 47 families, 148 genera, and 261 species were found to be the core microbiota in 24 dairy cattle. A box plot showing the RA of core microbiota (phyla and genera) in feces from 24 dairy cattle is depicted in Figure 4.2.

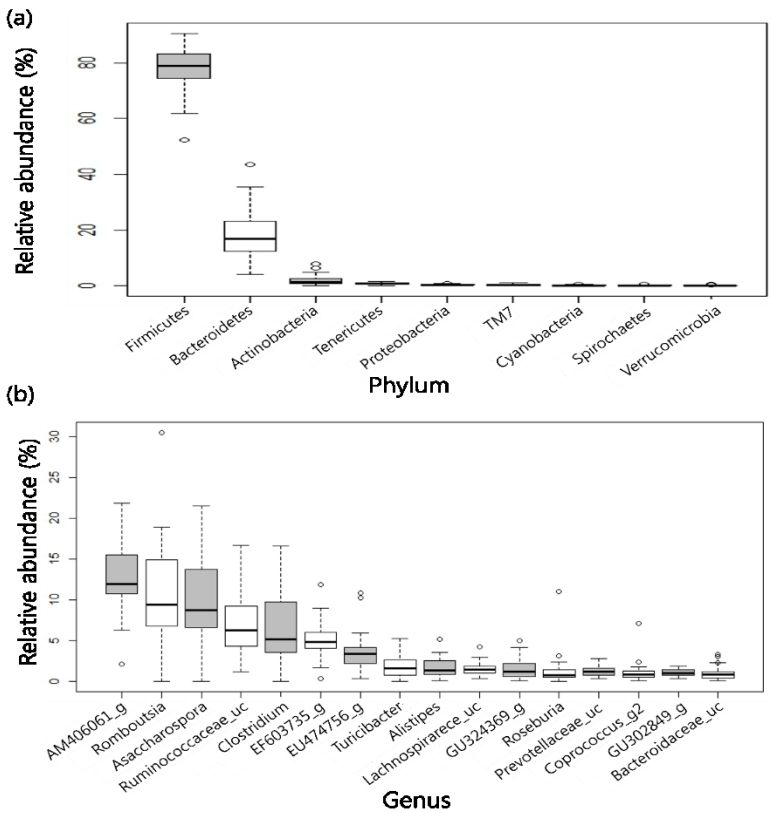


Figure 4.2. Box plot showing the RA of the core microbiota in feces from 24 dairy cattle.

A. Phyla
B. Geneera

Fecal bacterial communities of STEC-shedding cattle

In STEC-positive cattle, no significant differences were observed at phylum, class, or order level compared to the STEC-negative cattle. However, at the genus level, the RA of EU474837_g ($p = 0.015$) and EU475520_g ($p = 0.048$) were higher and those of family Clostridiales_us ($p = 0.035$) and genera Clostridiales_uc_g ($p = 0.035$), AB506319_g ($p = 0.018$), and DQ057459_g ($p = 0.048$) were lower in STEC-positive cattle than in STEC-negative cattle. A higher RA of *Ruminococcus* ($p = 0.064$) was observed in the STEC-positive group, but this was not significant (Fig. 4.3.A).

Fecal bacterial communities of *C. jejuni*-shedding cattle

C. jejuni-positive samples had a significantly lower RA of *Firmicutes* ($p = 0.035$) and higher RA of *Bacteroidetes* ($p = 0.035$) than *C. jejuni*-negative samples. *C. jejuni*-positive samples had a low RA of *Actinobacteria* and high RA of *Proteobacteria*, but this was not significant. The class *Bacteroidia* ($p = 0.035$); order *Bacteroidales* ($p = 0.035$); and families *Bacteroidaceae* ($p = 0.018$), EU464174_f ($p = 0.021$), and Clostridiales_uc ($p = 0.01$) were more abundant in *C. jejuni*-positive cattle, whereas the class *Clostridia* ($p = 0.025$) and order *Clostridiales* ($p = 0.025$) were more abundant in *C. jejuni*-negative cattle. At the genus level, EU474756_g ($p = 0.021$), EU845084_f_uc ($p = 0.021$), Clostridiales_uc_g ($p = 0.010$), EU465631_g ($p = 0.041$), DQ394632_g ($p = 0.010$), and HM630201_g ($p = 0.035$) were

significantly higher in *C. jejuni*-positive cattle, while AB239481_g (p = 0.012), AM500802_g (p = 0.030), and *Atopobium* (p = 0.030) were significantly lower (Fig. 4.3.B).

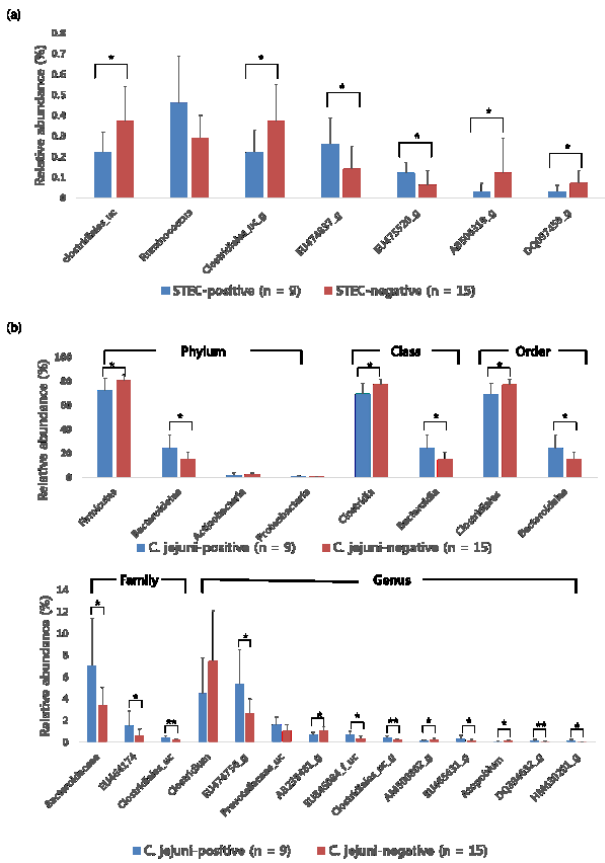


Figure 4.3. Differences in RA of taxa between cattle positive and negative for foodborne pathogens.

A. RA of taxa differing between STEC-positive and STEC-negative cattle. All the taxa significantly differed in RA between STEC-positive and STEC-negative cattle, except for *Ruminococcus* (p = 0.064).
B. RA of taxa differing between *C. jejuni*-positive and *C. jejuni*-negative cattle. All the taxa differed significantly for RA between *C. jejuni*-positive and *C. jejuni*-negative cattle, except 2 phyla (*Actinobacteria* and *Proteobacteria*) and 2 genus (*Clostridium* and Prevotellaceae_uc).

The error bar shows the standard deviation of the means. * $p < 0.05$, ** $p \leq 0.01$

Fecal bacterial communities of Cattle shedding both STEC and *C. jejuni*

Among 24 samples, only one sample (9555-1412_SC) was taken from cattle shedding both STEC and *C. jejuni*. Therefore, the concentration of taxa which showed significant difference in cattle shedding either STEC or *C. jejuni* were compared. The concentration of significantly different taxa in cattle shedding both STEC and *C. jejuni* had a similar patterns to that of the cattle shedding STEC and *C. jejuni* alone, respectively, except for two taxa, Clostridiales_uc in cattle shedding STEC and EU464174 in cattle shedding *C. jejuni* (Table 4.4.).

Table 4.4. Diversity of microbial communities in cattle shedding STEC and/or *C. jejuni*

Diversity of microbial communities in cattle shedding STEC

Rank	Taxa	Average RA of taxa (%)		p-value	RA of taxa (%)
		STEC-positive (n = 9)	STEC-negative (n = 15)		STEC and *C. jejuni*-positive (n = 1)
Family	Clostridiales_uc	0.22 ± 0.10	0.37 ± 0.17	0.035	0.36
Genus	Ruminococcus	0.46 ± 0.23	0.29 ± 0.11	0.064	0.74
	Clostridiales_uc_g	0.22 ± 0.11	0.37 ± 0.18	0.035	0.36
	EU474837_g	0.26 ±0.13	0.14 ± 0.11	0.015	0.34
	EU475520_g	0.12 ± 0.05	0.06 ± 0.07	0.048	0.14
	AB506319_g	0.03 ± 0.04	0.12 ±0.17	0.018	0.00
	DQ057459_g	0.03 ± 0.03	0.07 ± 0.06	0.048	0.02

Diversity of microbial communities in cattle shedding *C. jejuni*

Rank	Taxa	Average RA of taxa (%)		p-value	RA of taxa (%)
		C. jejuni-positive (n = 9)	*C. jejuni*-negative (n = 15)		STEC and *C. jejuni*-positive (n = 1)
Phylum	Firmicutes	72.16 ± 10.27	80.41 ± 5.20	0.035	61.72
	Bacteroidetes	24.06 ± 10.93	15.51 ± 5.97	0.035	35.53
	Actinobacteria	1.65 ± 2.41	2.24 ± 1.76	0.19	1.64
	Proteobacteria	0.83 ± 0.42	0.75 ± 0.25	0.22	0.70
Class	Clostridia	69.35 ± 9.17	77.25 ± 4.46	0.025	59.78
	Bacteroidia	24.05 ± 10.93	15.49 ± 5.98	0.035	35.53
Order	Clostridiales	69.26 ± 9.18	77.20 ± 4.45	0.025	59.78
	Bacteroidales	24.02 ± 10.90	15.47 ± 5.95	0.035	35.53
Family	Bacteroidaceae	7.06 ± 4.35	3.43 ± 1.65	0.018	14.08
	EU464174	1.48 ± 1.42	0.63 ± 0.59	0.021	0.47
	Clostridiales_uc	0.43 ± 0.19	0.24 ± 0.11	0.01	0.36
Genus	Clostridium	4.47 ± 3.29	7.47 ± 4.66	0.084	3.29
	EU474756_g	5.36 ± 3.20	2.67 ± 1.31	0.021	10.84
	Prevotellaceae_uc	1.59 ± 0.75	1.10 ± 0.53	0.084	1.76
	AB239481_g	0.68 ± 0.27	1.07 ± 0.36	0.012	0.54
	EU845084_f_uc	0.70 ± 0.33	0.37 ± 0.25	0.021	0.70
	Clostridiales_uc_g	0.43 ± 0.19	0.24 ± 0.11	0.01	0.36
	AM500802_g	0.13 ± 0.12	0.25 ± 0.15	0.03	0.25
	EU465631_g	0.32 ± 0.33	0.11 ± 0.17	0.041	0.29
	Atopobium	0.05 ± 0.07	0.13 ± 0.10	0.03	0.00
	DQ394632_g	0.13 ± 0.15	0.04 ± 0.06	0.01	0.14
	HM630201_g	0.13 ± 0.16	0.03 ± 0.03	0.035	0.11

Phylogenetic analysis

Four clusters were generated using UPGMA based on 97.5% similarity. While most of the samples belonged to cluster 1, single samples belonged to the remaining clusters: 0214_1208_S, in cluster 2; 1862_1410_C, cluster 3; and 0213_1412_C, cluster 4 (Fig. 4.4.).

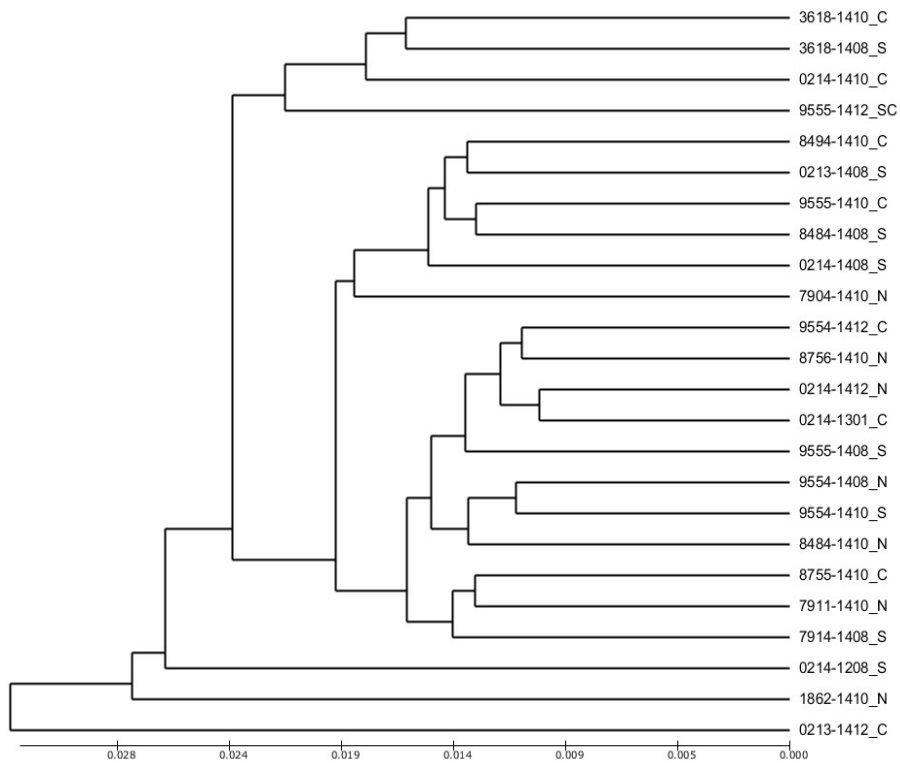


Figure 4.4. UPGMA dendrogram of fecal microbiota of 24 dairy cattle. Four clusters were generated with the UPGMA method based on 97.5% similarity. All the cattle belonged to cluster 1 except for 3 individuals, 0214-1208_S (cluster 2), 1862-1410_N (cluster 3), and 0213-1412_C (cluster 4). Sample ID refers to the identification number of each cow followed by the sampling date (YYMM) and presence of foodborne pathogen (N, both STEC and *C. jejuni*-negative; C, *C. jejuni-positive*; S, STEC-positive; SC, positive for both STEC and *C. jejun*

4.4. Discussion

In this study, fecal microbial communities were investigated from 24 dairy cattle using NGS techniques. The similarity of our Goods' coverage value (86.7% ± 5.3%) with that of a previous report (86.9% ± 3.3%) (230) suggests that metagenomic samples represent the actual bacterial communities of dairy cattle. Furthermore, the number of OTUs generated were 2.6 times higher than that reported in beef cattle (227) and 3.2 times higher than that reported in dairy cattle (279). The DIs in this study were 6.2 ± 0.6 and 7.0 ± 0.3 using CD-HIT and TBC methods, respectively; no significant differences in DIs were observed between cattle shedding STEC or *C. jejuni*. However, there is no consensus on the diversity of bacterial communities in STEC-shedding cattle. One study reported higher DIs in beef cattle with a high shedding of STEC O157 compared to non-shedders (230), whereas the DIs were higher in non-STEC shedders in another study (223). In yet another study, a higher DI was found in high-shedders than in low-shedders (280) a different report found no significant differences in DI with respect to shedding level (231). Differences in DI between *C. jejuni*-positive and *C. jejuni*-negative cattle have not been reported, but higher DIs in *C. jejuni*-positive groups have been found in chickens (233). Similarly, the increasing tendency of DIs in *C. jejuni*-positive cattle was observed in this study.

Among 16 phyla identified by high-throughput sequencing, 9 were found to be the core microbiota in dairy cattle, regardless of the presence of the STEC or *C. jejuni*. *Firmicutes* and *Bacteroidetes* were predominant in all samples, accounting for 96.0% of the bacterial composition. The predominance of these 2 phyla has been reported in mammalian samples, indicating their vital role in the mammalian gut (219, 226, 227). The average RAs of *Firmicutes* and *Bacteroidetes* were 77.3% and 18.7%, respectively, similar to those of dairy cattle (228) but different from those of beef cattle (227, 230). In beef cattle, a lower concentration of *Firmicutes* (52.6~62.8%) and higher concentration of *Bacteroidetes* (29.5~42.1%) were reported (227, 230).

Of 547 genera identified in this study, 148 were found to be core genera, accounting for more than 90% (90.70–99.10%) of the microbial community. Thus, less than one-third of the taxa account for most of the fecal bacterial community in dairy cattle. Among the top 10 predominant genera in this study, *Clostridium* and *Alistipes* are considered ubiquitous genera in dairy cattle (281), and *Peptostreptococcaceae* (the family containing *Romboutsia* and *Asaccharospora*) and *Turicibacter* are core taxa in beef cattle (282), indicating that both beef and dairy cattle might share the same bacterial taxa but with different RAs (227).

The microbial impact on STEC shedding is an interesting aspect. Among known genera, *Ruminococcus* was associated with high STEC shedding (but not significantly), corresponding to a study comparing high-shedders and low-shedders

of STEC (Fig. 4.3.) (280). Several genera have also been reported to be associated with STEC shedding in cattle, but the association does not seem consistent. Zhao et al. suggested that *Alistipes* might inhibit STEC shedding (223), while Xu et al. reported *Alistipes* to be more abundant in cattle shedding high numbers of STEC (230). The RA of *Prevotella* was more in cattle shedding low numbers of STEC than in high-shedders (280), whereas the opposite was found for high-shedders and non-shedders in another study (230). In this study, differences in the RA of *Alistipes* or *Prevotella* were not observed in cattle regardless of the presence of STEC. This might be ascribed to the use of dairy cattle in this study, because the RA of *Prevotella* differs between beef and dairy cattle (227).

A comparison of intestinal microbial diversity between dairy cattle with and without *C. jejuni* revealed that the colonization of *C. jejuni* was not linked to microbial composition (232). However, that study was performed at the herd level rather than for individual cattle. On comparing microbiota between *C. jejuni*-positive and *C. jejuni*-negative cattle, the bacterial composition at the phylum level was similar to that of chicken cecal samples with and without *Campylobacter* (Fig. 4.3.) (233). However, because the comparisons were made between 2 different species, a bias may exist.

Interestingly, the microbial composition of cattle shedding both STEC and *C. jejuni* had a similar features that observed in cattle shedding STEC and *C. jejuni* alone, respectively. Because only one sample was shedding both STEC and *C. jejuni*,

it is hard to draw some finding. However, these results were still interesting enough to hypothesize that the presence of STEC and *C. jejuni* might affect microbial communities independently. This hypothesis is plausible considering the distinct growth condition of STEC and *C. jejuni*. While STEC are a facultative anaerobic organisms enabling to survive in a relatively wide range of environment, *Campylobacter* requires strict microaerophilic conditions for their growth limiting their survival. Commensalism of *Campylobacter* with protozoa, *Tetrapymena pyriformis* or bacteria, *Psudomonas* spp. has been reported to resist from atomospheric damage (283, 284), indicating that microbial interaction might be one of the important pathway for pathogenesis of foodborne pathogens. Continual research on the microbial communities and their interaction between indigenous bacteria and pathogens would be milestone for developing food safety measures.

Hierarchical clustering showed that most samples were clustered together, indicating that fecal microbiota of dairy cattle are highly similar. In the clustering, microbial diversity was not related to the shedding STEC or *C. jejuni*. This may be because the differences in RAs of taxa between pathogen-positive and pathogen-negative individuals were too low to affect microbial diversity. Similarly, minor influences of fecal microbiota on STEC shedding in beef cattle have also been reported (231). Microbial diversity might be less affected by the presence of pathogens because the colonization of STEC or *C. jejuni* in cattle causes asymptomatic infection, and all tested cattle were clinically healthy. Considering that

all the samples were collected from the same farm, where animals were managed in a similar manner, the microbial composition might be influenced to a greater extent by management factors rather than individual factors.

4.5. Conclusion

In this study, the fecal microbiota of dairy cattle shedding STEC or *C. jejuni* was addressed. This is the first study to investigate the fecal microbial diversity in *C. jejuni*-shedding cattle and STEC-shedding dairy cattle. The core microbiota of dairy cattle revealed that a relatively small number of bacterial species covered most fecal microbiota, indicating their vital role in the intestinal microbial community. In addition, *C. jejuni*-positive cattle seemed to have more diverse microbiota compared to *C. jejuni*-negative cattle. Furthermore, several taxa were identified that had a distinguishable RA according to the shedding of foodborne pathogens STEC or *C. jejuni*, suggesting the possibility to investigate the interaction between pathogens and indigenous bacteria. In-depth investigations of the fecal microbial population would provide essential data for controlling STEC or *C. jejuni* infection at the farm level. This would have potential applications in the development of strategies to prevent foodborne illnesses in humans.

General conclusion

This study was performed to identify the prevalence and virulence potential of foodborne pathogen, and to suggest measure to reduce foodborne pathogens in cattle farm by using a newly adapted techniques, LAMP and NGS.

For rapid and sensitive detection of STEC and *C. jejuni*, the LAMP assay was developed, revealing the high sensitivity and specificity of LAMP assay. Moreover, the developed LAMP assay was able to quantify, which could be applied to measure the level of contamination of STEC and *C. jejuni* to identify high-shedder. *C. jejuni* is one of the fastidious bacteria to culture and isolate, which prevalence is often underestimate. In addition, *C. jejuni* grows slowly on media, requiring at least 5 days to isolate. The use of LAMP assay for screening from the enrichment broth culture, the faster detection would be possible. Moreover, LAMP would provide higher detection rate of *C. jejuni* by preventing *C. jejuni* from oxygen exposure during confirmation test. The real-time mLAMP assay for shiga toxin genes of STEC was first introduced. The mLAMP was effective for detection of both STEC O157 and non-O157 isolates, as well as STEC in the enrichment broth culture. The high detection rate of LAMP assay from enrichment broth samples indicates the potential utility of this assay as a screening method for detecting STEC or *C. jejuni* in cattle

farm samples. Further studies including application of the LAMP assay on various sources like pigs, poultry, or food would play an important role in the prevention of contamination in the food chain, thereby reducing the future risk of human infection.

From the third study, prevalence of STEC in cattle farm and their virulence potentials were investigated. STEC prevalence differed greatly between farms, and temperature and rainfall affected the farm prevalence. A considerable number of STEC non-O157 stains were isolated, and different virulence and antimicrobial resistance features were observed between STEC O157 and non-O157 strains. While a high prevalence of virulence genes was observed in STEC O157 strains, the antimicrobial resistance rate was higher in STEC non-O157 strains. In addition, the *stx1c* variant was detected in *eae*-positive STEC, suggesting genetic dynamics among virulence genes in STEC isolates. Finally, PFGE analysis revealed the presence of a prototype STEC, which continues to evolve by genetic mutation and causes within- and between-farm transmission within the Gyeonggi province. Our results suggested that STEC from cattle have a high virulence potential and represent a threat to public health. Therefore, continual surveillance of both STEC O157 and non-O157 would be beneficial for controlling and preventing STEC illness.

From the final study, the bovine gut microbiota in relation to the presence of the foodborne zoonotic pathogens, STEC and *C. jejuni* was identified. The microbial diversity was observed between cattle shedding foodborne pathogens and non-shedding cattle. The presence of STEC had a minor effect on microbial community

compared to that of the *C. jejuni*. While few genera showed a significant difference in RA according to the presence of STEC or *C. jejuni*, their role in microbial ecology towards pathogen growth remains to be elucidated. Considering that super-shedder contributes for more than 90% of bacterial shedding, control measures targeted to super-shedder would be highly effective. Understanding epidemiology and determinants of super-shedding would be future goal to control foodborne pathogens. As one of the important individual factor, investigation of microbiota of cattle would provide fundamental information on bacterial ecology in cattle feces, and would be useful to develop strategies for controlling bacterial shedding.

Overall, these findings emphasis the need of continual identifying foodborne pathogens and provide possible application of an adapted techniques for an effective control of foodborne pathogens. Further studies may be needed for an extended subject following food-process line to increase food safety and reduce human disease risk.

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국문 초록

젖소에서 Shiga toxin-producing *E. coli* 와 *C. jejuni* 의 분자진단 및 분자역학 연구

동 희 진

(지도교수: 조성범, D.V.M., Ph.D.)

서울대학교 대학원
협동과정 인수공통동물질병학 전공

식품매개성전염병은 공중보건학적 측면에서 중요한 이슈이며, 전체 위장염의 25~30%가 식품매개병원체에 의해 발생되는 것으로 보고되고 있다. 세균성 식품매개성 병원체 중, 시가독소생성대장균 (STEC)은 출혈성 설사, 출혈성대장염, 및 용혈성요독증후군 등 치사율이 높은 질병을 일으키는 원인균이다. 또한 최근에는 *E. coli* O104:H4 에 의해 유럽 다수 국가에서 대규모 유행이 발생한 바 있다. 이와 같이 위험성이 가장 잘 알려진 *E. coli* O157 외에도 다양한 혈청형의 STEC 에 의한 문제가 심각하며 세계적으로 이에 대한 관심이 집중되고 있다. 또한 캠필로박터 제주니 (*C. jejuni*)는 유럽, 북미 등의 선진국에서 세균성

설사의 중요한 원인체로 주목받고 있다. 사람에서의 감염을 최소화하기 위하여 STEC 및 *C. jejuni* 의 신속한 검출, 병원성 등의 병원체 특성 분석 등이 필요하다. 특히 소는 STEC 의 자연숙주로 알려져 있으며, 최근 *C. jejuni* 오염의 주요한 원인으로 보고되고 있다. 소에서 STEC 의 배출양상을 분석한 최근의 연구에서, 대부분의 소에서 약 10^2 CFU/ml 의 STEC 을 배출하는 반면, 일부 소에서 10^4 CFU/ml 이상인 고농도의 STEC 을 배출함을 확인하였고, 이들이 STEC 수퍼 전파 및 발생율 증가에 큰 영향을 미침을 확인하였다. 하지만 super-shedding 의 발생역학 및 위험요인에 대한 연구는 미미한 실정이며, 장내미생물총이 중요한 개체 요인 중 하나로 인식되고 있다. 따라서 본 연구에서는 감염의 초기 단계인 소농장샘플을 대상으로, 첫째, STEC 과 *C. jejuni* 의 신속정확한 검출을 위한 등온증폭법 개발에 대한 연구를 진행하였고, 둘째, 소에서 STEC 의 발생율 및 독성포텐셜에 대해 연구하였다. 마지막으로 bacterial shedding 과 관련된 미생물학적 요인을 확인하기 위하여 STEC 과 *C. jejuni* 를 배출하는 소에서 미생물 군집 분석을 실시하였다.

첫번째 연구는 *C. jejuini* 특이적인 *hipO* 유전자를 타겟으로 한 실시간등온증폭법 개발과 관한 것이다. 총 84 주의 *C. jejuini* 와 41 주의 non-*C. jejuini* 에 적용했을 때 100%의 민감도 및 특이도를 보였으며,

검출한계는 10 fg/μl 였고, 정량화가 가능 (R^2 = 0.9133) 한 것으로 확인되었다. 등온증폭법을 2012 년~2013 년에 총 51 주의 소농장샘플에서 분리한 *C. jejuini* 분리주에 적용했을 때 모든 분리주에서 *hipO* 유전자를 30 분 이내 (평균 10.8 분)에 확인할 수 있었다. *C. jejuini* 에 자연감염된 소농장샘플의 1 차 증균액을 끓임법으로 추출한 DNA 에 등온증폭법 및 중합효소연쇄반응법 (PCR)을 이용하여 유전자를 확인한 결과 각 84.4% 및 35.5%의 민감도를 보여 등온증폭법의 민감도가 높은 것을 확인하였으며, 이는 등온증폭법이 PCR 에 비해 샘플에 섞여있는 다양한 저해제에 더 적은 영향을 받기 때문인 것으로 보여진다. 캠필로박터증의 제어를 위해 *C. jejuini* 의 효과적인 검출이 중요하지만 기존의 배지배양법은 산소에 취약하며, 배지상에서 구분할 수 있는 표현형이 없기 때문에 검출에 어려움이 있었다. 등온증폭법을 활용하여 효과적인 *C. jejuini* 검출을 할 수 있으며 이를 통해 소농장에서의 오염여부를 모니터링하여 이후 식품으로의 유입을 조기 방지하고, 궁극적으로 사람에서의 캠필로박터증 감소에 기여할 수 있을 것으로 기대된다.

두번째 연구 역시 등온증폭법을 활용한 STEC 의 검출기법 개발에 관한 내용이다. 다양한 연구를 통해 등온증폭법이 민감하고 신속한 유전자증폭기법임이 확인되었지만, 꽃양배추모양 (cauliflower-like)의

최종생산물이 생성되기 때문에 다중유전자에 적용이 어려웠다. 본 연구에서는 두 가지 시가독소 유전자를 타겟으로 실시간다중등온증폭법을 개발하였으며, 두 개의 유전자는 서로 다른 T_m 값 (*stx1* 은 85.03 ± 0.54°C, *stx2* 는 87.47 ± 0.35°C) 을 기반으로 감별할 수 있었다. 다중등온증폭법은 100%의 민감도 및 특이도를 보였으며, 검출한계는 10 fg/μl 였고, 정량화가 가능 (R^2 = 0.9313) 한 것으로 확인되었다. 소농장에서 배지배양법, 면역자기분리법, 및 PCR 을 활용한 배지배양법의 3 가지 방법으로 분리한 각 12 주의 STEC O157, 17 주의 STEC O157, 및 11 주의 non-O157 에 적용했을 때 모든 분리주에서 시가독소유전자를 30 분 이내에 확인할 수 있었다. 특히 STEC non-O157 의 경우 배지에서 확인 가능한 특이표현형이 없기 때문에 배지배양법으로 검출하는데 어려움이 있으며 따라서 유전자 증폭 기법 등이 동반된 배지배양법이 이용되어왔다. 본 연구에서 개발된 다중등온증폭법은 STEC 을 검출하며, 동시에 시가독소형을 판명할 수 있어 STEC non-O157 의 검출에도 효과적으로 활용될 수 있다. 특히 샘플의 1 차 증균액에서 높은 민감도를 보인 다중등온증폭법은 소농장샘플에서 STEC 을 조기 검출하고, 모니터링하는데에도 활용될 수 있을 것이다.

세번째 연구에서는 소농장에서 STEC 의 발생률과 분리균주의 병원성 분석, 유전적 아형분석을 실시하였다. 소 농장의 STEC 발생률은 농장마다 큰 차이를 보였으며 기온 및 강우가 STEC 발생률에 영향을 미치는 것으로 확인되었다. 또한 STEC non-O157 이 40% 이상 검출되어 STEC non-O157 에 대한 모니터링도 지속적으로 할 필요성이 제기된다. 특히 독성유전자 분포 및 항생제 내성 양상은 STEC 의 혈청형에 따라 차이를 보였는데, STEC O157 의 경우 대부분의 독성 유전자를 높은 비율로 가지고 있었으며, STEC non-O157 의 경우 STEC O157 에 비해 항생제 내성률이 높았다. 시가독소형의 variants 를 확인한 결과 HUS 등 심각한 질환과 연관성이 높은 *stx2*a 및 *stx2*c 가 높은 비율로 존재하였으며, 소 특이 변형인 *stx2*g 가 O15 및 O109 혈청형에서 확인되었다. 또한 eae 양성 STEC 에서 stx1c variant 가 처음으로 확인되었따. PFGE 를 통한 유전적 아형 분석 결과 하나의 prototype 의 STEC O157 이 경기도 지역에 분포하고 있을 것으로 추정되며, 이 prototype 이 지속적인 농장내, 농장간 감염을 일으키는 것으로 추정된다. 본 연구는 소에서 분리된 STEC 이 높은 병원성을 가지고 있으며, 공중보건학적인 위해를 일으킬 가능성이 있음을 시사하며, 따라서 O157 및 STEC non-O157 의 지속적인 모니터링이 필요할 것이다.

마지막 연구는 STEC 과 *C. jejuni* 가 소의 장내미생물총에 미치는 영향에 대해 다루었다. 식품매개성 병원체가 숙주에서 세균집락을 형성하는데 미치는 다양한 요인에 대한 연구가 진행되어왔지만 미생물적 요인에 대한 연구는 미미한 실정이다. 본 연구에서는 차세대염기서열 (NGS) 분석 기법을 이용하여 STEC 또는 *C. jejuni* 배출과 관련된 총 24 마리의 소 분변 미생물총 분석을 실시하였다. 본 연구를 통해 총 9 문, 13 강, 18 목, 47 과, 148 속, 261 종의 핵심미생물종을 확인하였으며, STEC 을 배출한 소에서 상대적 존재비가 2 개 속에서 유의적으로 높았으며, 3 개 속에서 유의적으로 낮았다. 하지만 STEC 배출유무는 미생물종 다양성에 유의적인 차이를 나타내지 않아 STEC 은 소의 장내미생물총에 미미한 영향을 미치는 것으로 판단되었다. 반면, *C. jejuni* 를 배출한 소에서는 그렇지 않은 소에 비해 미생물종 다양성이 높았으며, *Bacteroidetes* 에서 더 높은 상대적존재비(p = 0.035), *Firmicutes* 에서 더 낮은 상대적 존재비 (p = 0.035) 를 보였다. 속 단위에서도 각각 6 개, 3 개 속의 상대적 존재비가 *C. jejuni* 를 배출한 소에서 유의적으로 높거나, 낮았다. 비록 STEC 및 *C. jejuni* 배출에 따른 다양한 미생물 군집이 관찰되었지만, 전반적인 미생물군집에 미치는 식품매개성 병원체의 영향은 미미한 것으로 확인되었다.

소에서 STEC 및 *C. jejuni* 의 빠르고 정확한 검출은 도축 후 음식생산라인으로 유입되는 병원체의 감소를 위해 특히 중요하다. 또한 본 연구에서 개발된 등온증폭법을 소뿐 아니라 돼지, 닭, 오리 등 다양한 산업동물의 확대 적용, 도축, 식육처리, 가공 등 음식생산라인의 단계별 적용 등을 통해 보다 효과적인 병원체 제어 및 예방이 가능할 것으로 기대된다. 본 연구 결과를 통해 소농장에서 STEC 의 혈청형에 따라 독성포텐셜에 차이가 있음을 확인하였다. 위험성이 잘 알려진 STEC O157 외에도 다양한 혈청형의 STEC 이 분리되었으며, 특히 STEC non-O157 의 경우 STEC O157 에 비해 높은 항생제 내성률을 보였다. 또한 병원성 유전자 프로파일링 결과 STEC O84, O108, O111 이 STEC O157 과 같은 그룹으로 높은 독성포텐셜이 있음을 확인하였다. 국내외적으로 STEC non-O157 에 의한 감염 및 유행 발생사례가 증가하고 있다. 따라서 국내에서도 지속적인 모니터링을 통해 STEC 제어 방안을 구축할 필요가 있을 것이다. 마지막으로 본 연구를 통해 STEC 및 *C. jejuni* 존재에 따른 다양한 미생물 군집이 확인되었다. 미생물 군집은 병원체 생존, 증식, 배출과 관련된 주요한 개체요인으로써 병원체를 제어를 위해 장내 미생물총의 생태학 및 상호관계에 대한 연구는 매우 유용한 정보를 제공해 줄 것으로 기대된다. 특히 병원체를 지속적으로 배출하거나 고농도로 배출하는 super-

shedder 의 제어는 병원체를 효과적으로 제어할 수 있는 효과적인 방법이다. 본 연구 기법은 발생역학 및 관련 요인 분석, 소 분변에서의 병원체 생태학을 이해하는데 중요한 정보가 될 것이다.

핵심어: 식품매개 인수공통전염병, 시가독소생성대장균, 캠필로박터제주니, 장내미생물총, 젖소

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