수의학석사학위논문

Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* (STEC) from cattle in South Korea between 2010 and 2011

2010년과 2011년 사이 국내 소에서 분리된 Shiga toxin-producing *Escherichia coli* (STEC)의 분포양상과 특성

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서울대학교 대학원

인수공통동물질병학 전공

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(Major: Zoonotic Diseases)

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A dissertation submitted to the faculty of the Graduate School of Seoul National University in partial fulfillment of the requirement for the degree of Master in Zoonotic Diseases

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Abstract

A total of 156 Shiga-like toxin producing *Escherichia coli* (STEC) were isolated from fecal samples of Korean native (100/568, 18%) and Holstein dairy cattle (56/524, 11%) in South Korea between September 2010 and July 2011. A total of 33% (52 isolates) of STEC harboring both *stx*1and *stx*2, and genes encoding enterohemolysin (*ehx*A) and autoagglutinating adhesion (*saa*) were detected by polymerase chain reaction in 83 (53%) and 65 (42%) isolates, respectively. By serotyping, 6 STEC strains from native cattle and 4 STEC strains from dairy cattle were identified as O26, O111, O104 and O157 which were O-serotypes involved in human disease. MLST and PFGE patterns revealed a various genetic diversity of the STEC strains and genetic differences between years. Antimicrobial susceptibility tests showed that the multidrug resistance rate was increased from 12% in 2010 to 42% in 2011. Difference between isolates of

year of 2010 and year of 2011 is suggested to be resulted from seasonal variations during sampling or other factors therefore continuous epidemiologic studies will be needed. More public health attention has to be required for dairy products as the risk of STEC, especially in dairy cattle, seem to be increased in this study.

Keywords: *Escherichia coli*, Shiga toxin, cattle, serotyping, multidrug resistance

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Contents

INTRODUCTION

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) has been the main food-borne pathogen causing illnesses that range from mild diarrhea to bloody diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome (HUS) worldwide [27]. STEC strains produce Shiga toxins (Stxs), including Stx1 and/or Stx2 which are essential virulence factors in STEC diseases [23].

In addition, STEC strains may also have several other virulence factors related to causing severe diseases such as intimin, STEC autoagglutinating adhesin (Saa), and enterohemolysin hlyA (EHEC-*hly*) which are encoded by *eaeA*, *saa*, and *ehxA*, respectively [25, 32, 40]. Intimin and auto-agglutinating adhesion are responsible for the intimate attachment to the intestinal surface and enterohemolysin is responsible for enterocyte damage [8, 25]. There are a large number of serotypes associated with human diseases but only a few serotypes (O26, O91, O103, O111 and O157) mainly cause severe human illnesses [12, 27, 32].

PFGE is a gold standard method of molecular subtyping to study clonality among STEC strains [9, 22] and MLST is the DNA sequence based molecular subtyping method based on the sequences of gene fragments from a number of different housekeeping loci [35]. Bovine STEC epidemiological studies at a

nationwide level and on a large scale have not been adequately addressed in South Korea to date [4, 18]. Therefore, we utilized PFGE and MLST to study clonality of STEC strains generally at a nationwide level and to determine the genetic relatedness of STEC isolates in South Korea.

The usefulness of antimicrobial therapy for STEC infections is unresolved but recent studies suggest that some antimicrobials, if administered early in the course of infection, may prevent disease progression to HUS [34]. Because STEC infections are not aggressively treated with antimicrobial therapy, many isolates may yet be susceptible to numerous antimicrobials. Therefore, antibiotic susceptibility patterns need to be continuously considered [33].

Since animals in good health, particularly healthy cattle, can harbor STEC, they are considered natural reservoirs of STEC; therefore, consumption of undercooked ground beef or dairy products contaminated with pathogens is the main cause of STEC infection. Recently, the risk of STEC infection has gradually been increasing because of the increased consumption of meat [15, 16]. National studies in South Korea show that enterohemorrhagic *E. coli* (EHEC) was detected from workers related to the farm industry as well as the slaughter houses, especially for people who had more opportunities to come into contact with cattle [13, 14]. Therefore, not only carcasses at the slaughter stage of the harvest level but also cattle at farms at the pre-slaughter stage should be

controlled to prevent STEC. However, in South Korea, few studies have investigated bovine STEC in cattle farms. The aims of this study were 1) to investigate the prevalence of STEC in Korean native and Holstein dairy cattle in South Korea, 2) to characterize their virulence genes, serotypes and antimicrobial susceptibility patterns and 3) to perform genetic comparisons using multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacterial isolation

Native cattle, Han-u, which is a breed of cattle raised in Korea and Holstein-Friesian cattle which is dairy cattle bred for the ability to produce large quantities of milk were selected from 20-month-old to 30-month-old in 54 farms (27 native cattle farms and 27 dairy cattle farms) of 6 regions of South Korea (A: GyeongGi, B: ChungCheong, C: JeolLa, D: GyeongSang, E: GangWon and F: JeJu, Table 1). Rectal swab samples were collected between September 2010 and July 2011. All farms did not break out foot and mouth disease (FMD) directly but were involved in the outbreak of FMD, except for C and F regions. During sampling between 2010 and 2011, overlapping farms (native cattle farms/ dairy cattle farms) were 4/3 in A, 4/3 in B, 3/4 in C, 1/1 in D, 5/0 in E and 2/0 in F. Samples were inoculated into trypticase soy broth (BD, Franklin Lakes, USA) with novobiocin 20 mg/L at 37℃ for 18 h and then were inoculated onto MacConkey (MAC) agar (BD) or sorbitol-MacConkey (SMAC) agar (BD) at 37℃ for 24 h. After incubation, pink colonies on MAC agar and pink or white colonies on SMAC agar were selected. Existence of glucose fermentation of presumptive colonies was verified by inoculating into Triple Sugar Iron Agar (BD) slant and presumptive colonies were finally confirmed with ViteK II

(Biomeriux, Durham, France). From selected colonies DNA was extracted using the Genomic DNA extraction kit (Quiagen, Hilden, Germany) and amplified by polymerase chain reaction (PCR) to confirm the 16S rRNA gene which is conserved in *E. coli* [31].

Detection of STEC and various virulence genes

Presence of *stx*1 or *stx*2 genes in *E. coli* isolates was verified by PCR, using two oligonucleotide probes directed against the genes encoding for Shiga-like toxin (SLT), SLT-I and SLT-II or Stx1 and Stx2 [24]. Additional virulence genes, *saa*, *eaeA*, and *ehxA* were also tested by the multiplex PCR according to Paton *et al.*, [24].

Reverse passive latex agglutination (RPLA) test

The RPLA test was used for the detection of SLT-I and SLT-II produced by *E coli* cultured from fecal sample by RPLA kit (Oxoid, Basingstoke, United Kingdom). *E. coli* isolates were inoculated onto brain heart infusion agar (Oxoid) slopes and incubated at 37℃ for 18 to 20 h. After incubation, the colonies were suspended in 1mL of a 0.85% sodium chloride solution containing polymyxin B at a concentration of 5,000 unit/mL which induces extraction of toxin SLT-I and SLT-II. Extraction was continued for 30 min at 37℃ with shaking occasionally.

After extraction, the culture was centrifuged at 4,000 rpm for 20 min at 4℃ and the supernatant was used as a test sample. Twenty five μL of diluents was dispensed into each well of the V-bottom microplate and 25 μL of test sample supernatant was added to the first well of each column. Doubling dilutions were performed down each column, up to and including row 7. Twenty five μL of the test latex SLT-I and SLT-II, and control were added to each well in the first, second, and third column, respectively. The contents of each well were mixed well and the plate was left undisturbed at room temperature for 20-24 h. The sample in the well showing agglutination was determined as the SLT positive.

Serotyping

Determination of the O and H-antigen groups was carried out by the method described in the Denka Seiken protocol (DenkaSeiken, Tokyo, Japan). Set 1 was for *E. coli* Group O antigen serotyping (51 vials) and set 2 was for *E. coli* H antigen serotyping (22 vials). Group O antigen serotyping was performed by using the slide agglutination method. For H antigen-typing, first several cultures were performed by using a semi-fluid culture medium, then identified by using the test tube agglutination method.

MLST

MLST was performed according to the scheme of *E. coli* MLST site whose databases was managed by University College Cork. Ireland (http://mlst.ucc.ie/mlst/). The 7 housekeeping genes, *adk, fumC, gyrB, icd, mdh, purA* and *recA,* were amplified and the PCR product was sequenced to confirm sequence type (ST) [39]. Allelic profile of reference *E. coli* strain is GeneBank U00096.

PFGE

PFGE was performed for genetic comparison of STEC isolates. Briefly, bacterial plugs were digested by *Xba*I (New England BioLabs, Beverly, MA) and electrophoresis was performed with CHEF MAPPER III® (Bio-Rad Laboratories, Hercules, CA) following a previous study [31]. PFGE patterns were analyzed using Bionumerics version 6.5 (Applied Maths, Austin, TX) software. Similarity was calculated by unweighted pair group method with averaging (UPGMA) based on Dice similarity coefficiency with 2% of tolerance. More than three isolates with over 85% of similarity were suggested as one cluster group (CG).

Antimicrobial susceptibility test

A disk diffusion test was performed and verified by the agar dilution test according to Clinical and Laboratory Standards Institute (CLSI) guidelines [5, 6]

against 13 antibiotics of different antimicrobial classes using BD BBL Sensi-Disks (Becton, Dickinson and Company, Sparks, MD); carbenicillin, cefazolin, amoxicillin/clavulanate, ampicillin, imipenem, chloramphenicol, tetracycline, gentamicin, amikacin, streptomycin, neomycin, ciprofloxacin and nalidixic acid. The colistin resistance was tested by broth dilution technique. Multidrug resistant (MDR) *E. coli* were defined as the strain which was resistant against at least 3 different classes of antimicrobials.

Statistical analysis

Statistical comparisons between STEC strains isolated from native cattle and dairy cows were performed using the chi-square test with statistical significance at the 95% confidence level ($p < 0.05$) by SPSS program (SPSS Inc., Chicago, IL, USA).

RESULTS

Prevalence of STEC

A total of 1092 fecal swab samples were collected. From native cattle, 192 and 376 samples were collected in 2010 and 2011 and from dairy cattle, 122 and 402 samples were done in 2010 and 2011 respectively. *E. coli* isolates were considered as STEC strains when *stx*1 or *stx*2 genes were detected by multiplex PCR. The STEC colonization rates of native and dairy cows were 17.61% and 10.69%, respectively. The prevalence of STEC was from 20.38% (64/314) in 2010 to 11.82% (92/778) in 2011. The decrease of STEC colonization rate in native cattle was significant; from 29.68% in 2010 to 11.43% in 2011 ($p < 0.001$). On the other hand, that of dairy cows was increased from 5.73% in 2010 to 12.19% in 2011 ($p < 0.05$). As for the regional distribution, the A and E regions had higher prevalence rates than other parts of the country regardless of the species and the years except for dairy cattle in E region of 2010 (Table 1).

Table 1. Prevalence of Shiga toxin-producing *Escherichia coli* isolated from native and dairy cattle within South Korea in both 2010 and 2011

Years	2010						2011					
Species	NC*			DC*			NC			DC		
Region†	No. of samples (farms)	No. of STEC isolates	%	No. of samples (farms)	No. of STEC isolates	%	No. of samples (farms)	No. of STEC isolates	%	No. of samples (farms)	No. of STEC isolates	%
A	28 (4)	13	46	51 (4)	4	8	81 (6)	21	26	55 (6)	23	42
B	57 (4)	14	25	30 (4)	1	3	50 (4)	1	2	82 (6)	4	5
C	12 (3)	4	33	28 (4)	2	7	99 (7)	4	4	109 (7)	10	9
D	14 (1)	3	21	12 (1)	0	0	84 (3)	7	8	91 (3)	3	3
E	60 (5)	20	33	0 (0)	0	0	42 (5)	10	24	65 (2)	9	14
F	21 (2)	3	30	1 (1)	0	0	20 (2)	0	0	0 (0)	0	0
Total	192 (19)	57	30	122 (14)	7	6	376 (27)	43	11	402 (24)	49	12

*NC, native cattle; DC, dairy cattle.

†A, GyeongGi; B, ChungCheong; C, JeolLa; D, GyeongSang; E, GangWon and F: JeJu.

Detection of *stx1, stx 2* and other virulence genes

STEC isolates which carried only *stx*2 without *stx*1 were the highest in 2010 (Table 2). On the other hand, in 2011, the *stx*1 +/*stx*2 + STEC rate were increased in both of native and dairy cows (Table 2). Among other virulence genes, *saa* and *ehxA* genes were prevalent generally. By the species, *saa*, *ehxA* and *eaeA* genes were more detected in isolates from dairy cattle than in those from native ones (Table 3). On a year-on-year basis from 2010 to 2011, there was little difference in the percentage of all virulence genes in native cows but a sizeable increase of the percentages of *saa* gene and decrease of *eaeA* gene in dairy cows. This is because the increased number of STEC isolates in 2011 compare to in 2010 made population to be large, which means the result of virulence genes in dairy cattle were more reliable.

Detection of SLT production

There was correlation between the *stx* genotype and phenotype in this study. Most *stx* gene-detected STEC isolates produced of the toxin type that detected by RPLA test. One (6%) STEC isolate with *stx*1 +/*stx*2 –, 11 (13%) isolates with *stx*1 -/*stx*2 + and 4 (8%) STEC isolates with *stx*1 +/*stx*2 + did not reveal their phenotypes (Table 2).

Table 2. Genotypes and phenotypes of *stx* genes from Shiga toxin-producing *Escherichia coli* isolates in native and dairy cattle between 2010 and 2011

stx-genotype	No. of genotype detected				Total	No. of phenotype† expressed	Rate in accordance* (%)
	2010		2011				
	NC	DC	NC	DC			
*stx*1 +/*stx*2 -	6	2	5	4	17	16	94
*stx*1 -/*stx*2 +	41	5	20	21	87	76	87
*stx*1 +/*stx*2 +	10	0	18	24	52	48	92
Total	57	7	43	49	156	140	90

* Rate in accordance with the *stx* genotype and phenotype.

† Phenotype is determined by the reverse passive latex agglutination test.

Table 3. Characterization of virulence factor genes from Shiga toxin-producing *Escherichia coli* isolates in native and dairy cattle between 2010 and 2011

Virulence factor genes			No. of isolates (%)								
			LEE*			2010		2011		Total	
saa	*eae*A	*hly*A	Type	NC	DC	NC	DC	NC	DC	NC	DC
-	-	-	+	91(91)	47 (84)	28 (49)	3 (43)	22 (51)	17 (35)	50 (50)	20 (36)
-	-	+				1 (2)	0 (0)	1 (2)	2 (4)	2 (2)	2 (4)
+	-	+				22 (39)	2 (29)	17 (40)	23 (47)	39 (39)	25 (45)
-	+	-	-	9 (9)	9 (16)	0 (0)	0 (0)	1 (2)	1 (2)	1 (1)	1 (2)
-	+	+				6 (11)	2 (29)	2 (5)	6 (12)	8 (8)	8 (14)
Total				100	56	57	7	43	49	100	56

* LEE, locus for enterocyte effacement in pathogenicity island encoded by *eaeA*.

Serotyping

We have focused on the important serotypes in public health and safety in this study. In native cattle, O26 (3, 5%), O111 (1, 2%) and O157 (2, 4%) STEC were identified. All of these STEC were isolated in 2010. From dairy cows, O104 (1, 2%) and O157 (3, 6%) STEC were identified in 2011 (Table 4). 2 stains of O8:H19 from native cattle in 2010 and one strain of O8:H19 and O8:H21 from dairy cows in 2011 samples were identified. Those serotypes have caused hemolytic uremic syndrome before in other countries.. There was no O157:H7 and O104:H4 serotypes.

MLST

In 2010, MLST analysis showed 45 different STs including 34 newly designated STs. Commonly detected ST among native cows and dairy cows was ST223. The most prevalent ST was ST10 (5/64, 7.81%) followed by ST2101 (4/64, 6.25%) and ST297 (3/64, 4.69%). In the other hand, in 2011, due to increased STEC isolations of dairy cattle, various 32 STs were identified and different from 6 STs (except for ST223) among 7 STs in 2010 samples. The most frequent ST was ST2385 a newly assigned type accounted for 22.45% (11/49) and the remainder 22 STs occupied mostly 2-8%. As for MLST of native cattle

strains, ST10 was predominant (25.58%, 11/43) and 19 remainder STs were mostly 2-9% (Table 5).

Table 4. Frequency of different Shiga toxin-producing *Escherichia coli* O-Serotype from native and dairy cattle between 2010 and 2011

Species	Years	O-serotype (No. of strains)
NC	2010	6 (2) *, 8 (4), 15 (1), 26 (3), 111 (1), 125 (1), 146 (5) *, 157 (2), 112ac (2), N/T† (36)
	2011	1 (1), 6 (1) *, 44 (1), 146 (1) *, N/T (39)
DC	2010	N/T (7)
	2011	8 (3), 15 (1), 104 (1), 153 (1), 157 (3), 28ac (1), N/T (39)

†N/T, nontypable with antisera; * underlining O-serotypes were detected in both 2010 and 2011.

Table 5. Multilocus sequence typing analysis of Shiga toxin-producing *Escherichia coli* isolates recovered from Korean native and dairy cattle in both 2010 and 2011

Species	ST (No. isolates)				Total No. of isolates (%)
	NC		DC		
Year	2010	2011	2010	2011	
Group 1[a]	10 (5), 223 (2), 297 (3), 446 (2), 515 (2), 1623 (2), 2105* (1)	10 (11), 223 (1), 297 (3), 515 (4), 1623 (2), 2105* (1)	223 (1)	10 (3), 223 (1), 446 (2)	46 (29)
Group 2[b]	201 (2), 2031 (2), 2101* (4), 2102* (2), 2122* (2), 2132* (2), 209 (1), 278 (1), 677 (1), 2093* (1), 2094* (1), 2095* (1), 2096* (1), 2097* (1), 2098* (1), 2099* (1), 2100* (1), 2104* (1), 2106* (1), 2107* (1), 2108* (1), 2109* (1), 2116* (1), 2117* (1), 2118* (1), 2121* (1), 2123* (1), 2124* (1), 2125* (1), 2126*(1), 2130* (1), 2133* (1)	-	-	-	40 (26)
Group 3[c]	-		2091* (1), 2092* (1), 2103* (1), 2110* (1), 2119* (1), 2120* (1)	-	6 (4)

Group 4 [d]	-	17 (2), 21 (1), 26 (2), 154 (3), 1258 (1), 1308 (1), 2175* (1), 2385* (3), 2389* (1)		17 (1), 21 (2), 26 (1), 154 (1), 1258 (1), 1308 (1), 2175* (1), 2385* (11), 2389* (1)	35 (22)
Group 5 [e]	-	192 (1), 224 (1), 677 (1), 2384* (1), 2386* (1)		-	5 (3)
Group 6 [f]	-	-	-	11 (3), 58 (4), 101(2), 209 (1), 442 (1), 718 (3), 2008 (1), 2340* (1), 2387* (2), 2388* (4), 2390*(1)	23 (15)
none	-	-	(1)†	-	1 (1)

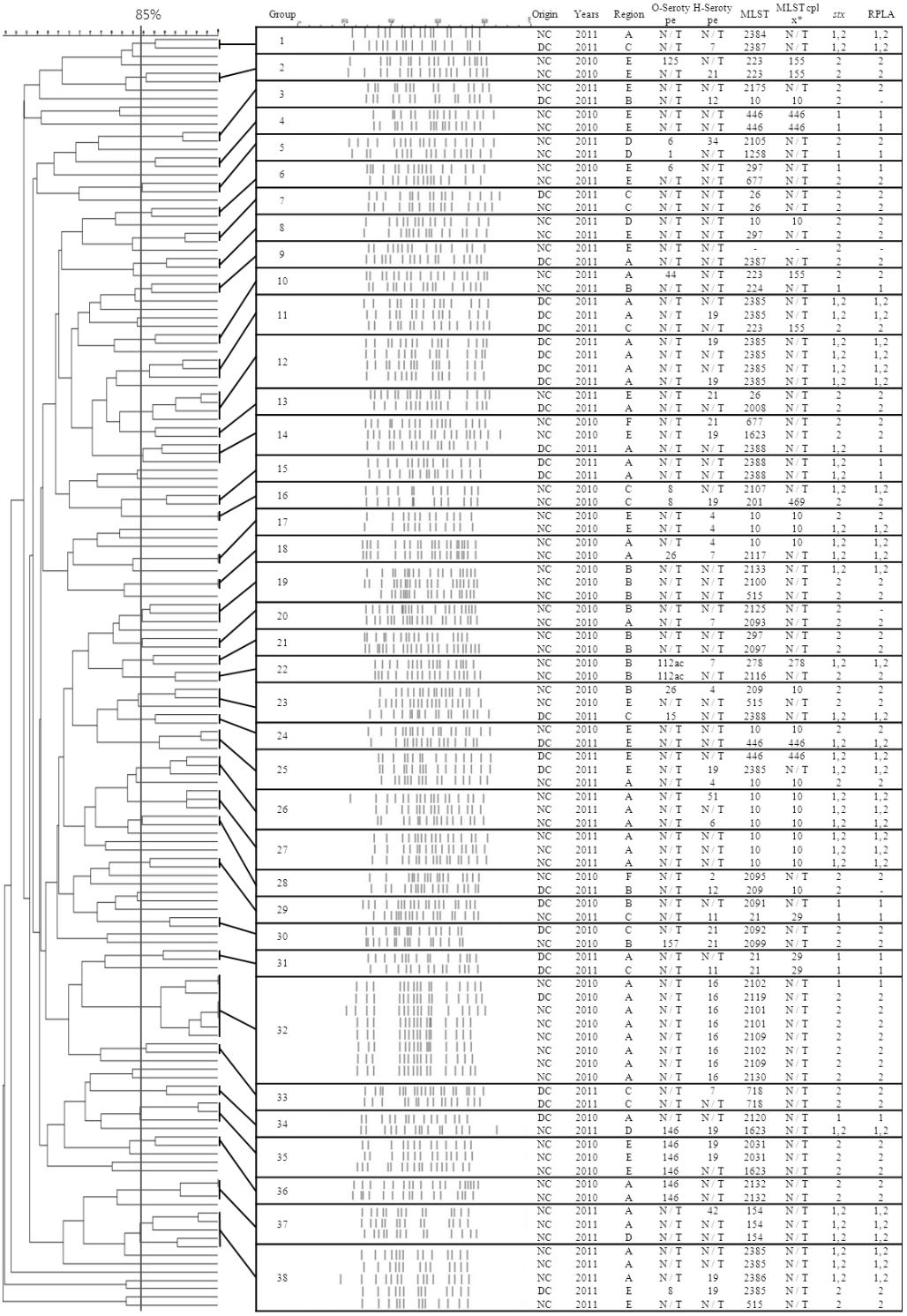
[a- f] STEC were divided into 6 groups according to sequence typings (STs); G1, overlapping STs between 2010 and 2011; G2, STs found only from native cattle in 2010; G3, STs found only dairy cattle in 2010; G4, overlapping STs between native and dairy cattle in 2011; G5, STs discovered only from native cattle in 2011; G6, STs discovered only from dairy cattle in 2011.

*New alleles and STs.

†ST of one Shiga toxin-producing *Escherichia coli* isolate in 2010 could not be identified.

PFGE

*Xba*I-digested DNA fragments of 156 STEC isolates generated 38 genetically related groups at 85% similarity (Figure 1). Individual PFGE patterns consisted of between 17 and 25 discernible DNA fragments ranging from 50 to 800 kb in size. There was no tendency for a specific group to be dominant throughout the country. Only 7 groups (G6, G14, G23, G24, G28, G29 and G34) contained STEC isolated from both 2010 and 2011 and the other groups consisted of STEC isolates from only one year of 2010 and 2011. The difference of species between dairy and native cows had no effect on PFGE patterns like the analysis of MLST, but PFGE patterns seemed to be affected by the regions. 11 groups (84 .6%) of all 13 groups of PFGE patterns in 2010 were originated by same regions. 7 groups (38.9 %) of all 18 groups in 2011 were derived from same areas and the rest groups contained STEC from more than two different regions in which only A overlapped the various other areas except for F.



Group	Origin	Years	Region	O-Seroty pe	H-Seroty pe	MLST	MLST cpl x*	*stx*	RPLA
1	NC	2011	A	N / T	N / T	2384	N / T	1,2	1,2
	DC	2011	C	N / T	7	2387	N / T	1,2	1,2
2	NC	2010	E	125	N / T	223	155	2	2
	NC	2010	E	N / T	21	223	155	2	2
3	NC	2011	E	N / T	N / T	2175	N / T	2	2
	DC	2011	B	N / T	12	10	10	2	-
4	NC	2010	E	N / T	N / T	446	446	1	1
	NC	2010	E	N / T	N / T	446	446	1	1
5	NC	2011	D	6	34	2105	N / T	2	2
	NC	2011	D	1	N / T	1258	N / T	1	1
6	NC	2010	E	6	N / T	297	N / T	1	1
	NC	2011	E	N / T	N / T	677	N / T	2	2
7	DC	2011	C	N / T	N / T	26	N / T	2	2
	NC	2011	C	N / T	N / T	26	N / T	2	2
8	NC	2011	D	N / T	N / T	10	10	2	2
	NC	2011	E	N / T	N / T	297	N / T	2	2
9	NC	2011	E	N / T	N / T	-	-	2	-
	DC	2011	A	N / T	N / T	2387	N / T	2	2
10	NC	2011	A	44	N / T	223	155	2	2
	NC	2011	B	N / T	N / T	224	N / T	1	1
11	DC	2011	A	N / T	N / T	2385	N / T	1,2	1,2
	DC	2011	A	N / T	19	2385	N / T	1,2	1,2
	DC	2011	C	N / T	N / T	223	155	2	2
12	DC	2011	A	N / T	19	2385	N / T	1,2	1,2
	DC	2011	A	N / T	N / T	2385	N / T	1,2	1,2
	DC	2011	A	N / T	N / T	2385	N / T	1,2	1,2
	DC	2011	A	N / T	19	2385	N / T	1,2	1,2
13	NC	2011	E	N / T	21	26	N / T	2	2
	DC	2011	A	N / T	N / T	2008	N / T	2	2
14	NC	2010	F	N / T	21	677	N / T	2	2
	NC	2010	E	N / T	19	1623	N / T	2	2
	DC	2011	A	N / T	N / T	2388	N / T	1,2	1
15	DC	2011	A	N / T	N / T	2388	N / T	1,2	1
	DC	2011	A	N / T	N / T	2388	N / T	1,2	1
16	NC	2010	C	8	N / T	2107	N / T	1,2	1,2
	NC	2010	C	8	19	201	469	2	2
17	NC	2010	E	N / T	4	10	10	2	2
	NC	2010	E	N / T	4	10	10	1,2	1,2
18	NC	2010	A	N / T	4	10	10	1,2	1,2
	NC	2010	A	26	7	2117	N / T	1,2	1,2
19	NC	2010	B	N / T	N / T	2133	N / T	1,2	1,2
	NC	2010	B	N / T	N / T	2100	N / T	2	2
	NC	2010	B	N / T	N / T	515	N / T	2	2
20	NC	2010	B	N / T	N / T	2125	N / T	2	-
	NC	2010	A	N / T	7	2093	N / T	2	2
21	NC	2010	B	N / T	N / T	297	N / T	2	2
	NC	2010	B	N / T	N / T	2097	N / T	2	2
22	NC	2010	B	112ac	7	278	278	1,2	1,2
	NC	2010	B	112ac	N / T	2116	N / T	2	2
23	NC	2010	B	26	4	209	10	2	2
	NC	2010	E	N / T	N / T	515	N / T	2	2
	DC	2011	C	15	N / T	2388	N / T	1,2	1,2
24	NC	2010	E	N / T	N / T	10	10	2	2
	DC	2011	E	N / T	N / T	446	446	1,2	1,2
25	DC	2011	E	N / T	N / T	446	446	1,2	1,2
	DC	2011	E	N / T	19	2385	N / T	1,2	1,2
	NC	2011	A	N / T	4	10	10	2	2
26	NC	2011	A	N / T	51	10	10	1,2	1,2
	NC	2011	A	N / T	N / T	10	10	1,2	1,2
	NC	2011	A	N / T	6	10	10	1,2	1,2
27	NC	2011	A	N / T	N / T	10	10	1,2	1,2
	NC	2011	A	N / T	N / T	10	10	1,2	1,2
	NC	2011	A	N / T	N / T	10	10	1,2	1,2
28	NC	2010	F	N / T	2	2095	N / T	2	2
	DC	2011	B	N / T	12	209	10	2	-
29	DC	2010	B	N / T	N / T	2091	N / T	1	1
	NC	2011	C	N / T	11	21	29	1	1
30	DC	2010	C	N / T	21	2092	N / T	2	2
	NC	2010	B	157	21	2099	N / T	2	2
31	DC	2011	A	N / T	N / T	21	29	1	1
	DC	2011	C	N / T	11	21	29	1	1
32	NC	2010	A	N / T	16	2102	N / T	1	1
	DC	2010	A	N / T	16	2119	N / T	2	2
	NC	2010	A	N / T	16	2101	N / T	2	2
	NC	2010	A	N / T	16	2101	N / T	2	2
	NC	2010	A	N / T	16	2109	N / T	2	2
	NC	2010	A	N / T	16	2102	N / T	2	2
	NC	2010	A	N / T	16	2109	N / T	2	2
	NC	2010	A	N / T	16	2130	N / T	2	2
33	DC	2011	C	N / T	7	718	N / T	2	2
	DC	2011	C	N / T	N / T	718	N / T	2	2
34	DC	2010	A	N / T	N / T	2120	N / T	1	1
	NC	2011	D	146	19	1623	N / T	1,2	1,2
35	NC	2010	E	146	19	2031	N / T	2	2
	NC	2010	E	146	19	2031	N / T	2	2
	NC	2010	E	146	N / T	1623	N / T	2	2
36	NC	2010	A	146	N / T	2132	N / T	2	2
	NC	2010	A	146	N / T	2132	N / T	2	2
37	NC	2011	A	N / T	42	154	N / T	1,2	1,2
	NC	2011	A	N / T	N / T	154	N / T	1,2	1,2
	NC	2011	D	N / T	N / T	154	N / T	1,2	1,2
38	NC	2011	A	N / T	N / T	2385	N / T	1,2	1,2
	NC	2011	A	N / T	N / T	2385	N / T	1,2	1,2
	NC	2011	A	N / T	19	2386	N / T	1,2	1,2
	DC	2011	E	8	19	2385	N / T	2	2
	NC	2011	E	N / T	N / T	515	N / T	2	2

Figure 1. Characteristics of 38 groups based on PFGE (*Xba*I) patterns of 156 Shiga toxin-producing *Escherichia coli* isolated Korean native and dairy cattle from 6 parts of in the country between 2010 and 2011. A: GyeongGi, B: ChungCheong, C: JeolLa, D: GyeongSang, E: GangWon and F: JeJu. *MLST cplx is the clonal complex of MLST.

Antimicrobial susceptibility test

The result of antimicrobial susceptibility test in 2010 and 2011 indicated that resistances to tetracycline, streptomycin, ampicillin and carbenicillin were the most frequent and with changing the order slightly, there was a marked increase in antibiotic resistance rate in 2011 (Figure 2). Antibiotic resistance rate of STEC isolates which resisted more than one drug were 14.3% in dairy cows and 45.6% in native cattle in 2010 but grew at the faster rate (73.5% in dairy cows and 79.1% in native cattle). The rate of MDR which resists more than three antibiotics also greatly increased from 14.29% and 12.28% to 51.16% and 28.57% in dairy cows and native cattle, respectively. Resistance rates against carbenicillin, amoxicillin/clavulanate, ampicillin, neomycin, amikacin and streptomycin represented a more than three-fold increase over 2010-2011. The analytical results of carbenicillin, amoxicillin/clavulanate, ampicillin and neomycin were $p < 0.001$ and colistin, amikacin and streptomycin were $p < 0.05$.

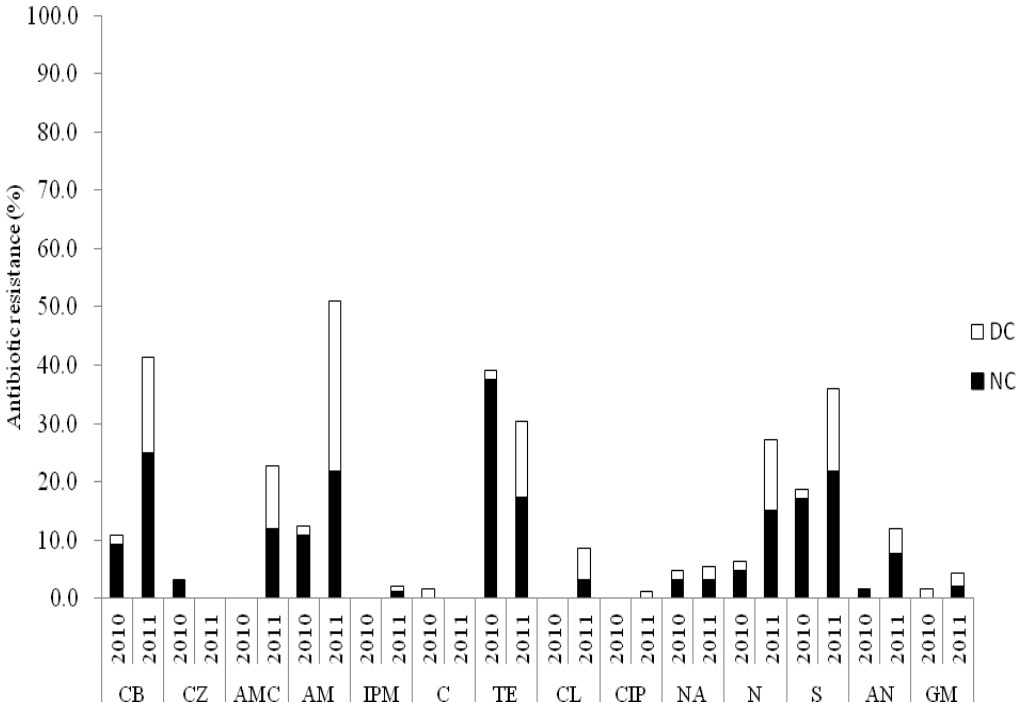


Figure 2. The antimicrobial resistant rate between 2010 and 2011. Black bars represent the antimicrobial resistant rate of Korean native cattle isolates and white bars represent the antimicrobial resistant rate of dairy cattle isolates. CB, carbenicillin; CZ, cefazolin; AmC, amoxicillin/clavulanate; AM, ampicillin; IPM, imipenem; C, chloramphenicol; Te, tetracycline; Cl, colistin; GM, gentamicin; AN, amikacin; S, streptomycin; N, neomycin; CIP, ciprofloxacin; NA, nalidixic acid

DISCUSSION

In this large-scale, nationwide study, the overall STEC prevalence isolated from the rectal swabs of healthy adult cows was 20.38% in 2010 and 11.82% in 2011. From previous studies in South Korea, the STEC prevalence was 10.5% from diarrhea samples of calves [19] and 9.5% from samples of cattle feces [4]. These results were lower than our results in 2010 but similar to those in 2011. The prevalence of STEC in cattle has also been described in several studies from different countries. Compared with our results, the STEC prevalences of Vietnam (26%), Japan (37.5%), France (34%), and Spain (35%) were higher [3, 11, 29, 37] and that of Germany (17.0%) was similar [20]. However, the STEC prevalence of the United States (8.4%) was lower than the prevalence in this study [38]. The use of different detection methods, the rapid development of new methods, and differences in diet and cattle species make it difficult to compare the STEC prevalence rates reported for various geographic areas [17].

In terms of the STEC prevalence according to the kinds of cattle, although native cattle (17.61%) had a higher STEC prevalence than dairy cattle (10.69%), the rate of increase in dairy cows was more than twice that in native cows for 2 years. These different aspects according to cattle species could be affected by changes in the environment and management practices and by improvement in

the feed used for each kind of cow [15]. With regard to the regional distribution of STEC, the STEC detection rates in the A and E regions were higher than in the other regions regardless of the kind of cow and the period of time except for dairy cattle in the E region of 2010. There are some regional characteristics that caused high infection of STEC in cattle in that area. First of all, the A region is immediately adjacent to the Seoul metropolitan area; there is less space to raise many cattle in than in the other areas. This situation causes many farms to become an intensive type of farm concentrated in the neighboring areas. The higher the density of cattle becomes, the more contaminated the environment becomes with STEC, and such contaminated soil and water expose animals to the possibility of STEC infection [10]. Second, the E region is the best-preserved area environmentally among the six regions in South Korea, which means there are many wild animals and many opportunities for cows to have contact with them. The wildlife sharing the surrounding land of farms with the cattle could potentially shed STEC along with the cattle, contributing to the transmission of STEC to the herd [15].

In the PCR results for the detection of the *stx* genes, *stx*1-/*stx*2+ was the predominant type among the STEC isolates from two kinds of cows in 2010. This result coincided with previous reports [3] that STEC harboring the *stx*2 gene is prevalent in adult cows [3]. However, the *stx*1-/*stx*2+ STEC isolates in 2011

decreased while STEC carrying *stx*1+/*stx*2+ isolates increased. This may be because of seasonal variations in the sampling period or because of several other factors (changes of environment or feeding and management) between the two sampling times, changing the population of the STEC organisms but further study is necessary.

An immunoassay for the *in vitro* detection of Shiga (Vero) toxins Stx1 and Stx2 (VTEC-Screen "Seiken") was performed to compare with the results from the *stx*-gene specific PCR for the detection of STEC. Although PCR is suitable for a general diagnosis of STEC, the RPLA assay has also a good specificity for the detection of Stx-positive stool samples and shows the toxin expression of *stx* genes in practice and not just checking for their existence on the chromosomal DNA of bacteria [2]. The STEC-screen detected 95 (95%) of the 100 STEC isolates in native cattle and 45 (80.36%) of the 56 STEC isolates in dairy cows (Table 2). The sensitivities of the RPLA were less than that of the PCR, which was similar to published results, but there were no false positives [2]. According to the toxin types, there was a false negative sample of Stx1 but there were 15 false negative samples of Stx2. Beutin *et al*. explained that some stx2 and stx2 variant strains (i.e., Stxd-Ount and Stx2e/Stx2ev) produce only small amounts of toxin and they cannot make enough toxins to be detected by the RPLA assay [2]. Therefore, additional experiments are required to assess whether the suspected

samples express their toxins because some strains have levels of toxins that are too low to be detected or there are performance issues related to cross reactivity when performing the assay.

Pathogenic STEC strains not only produce Shiga toxins but also can produce other virulence factors that may cause more severe human illnesses [12]. Among the many related factors, this study focused on intimin, auto-agglutinating adhesion and the plasmid-encoded enterohemolysin. These virulence factors of STEC strains, which are considered to be highly virulent in humans, are necessary to generate the attaching and effacing lesions (A/E lesions) to adhere to enterocytes properly. Intimin encoded by *eaeA*, which is included in the "pathogenicity island" called the locus for enterocyte effacement (LEE) is involved in producing the A/E lesion [40]. Therefore, the LEE, which is commonly confirmed by the detection of the *eaeA* gene, appears to confer enhanced virulence; however, the LEE is not essential for pathogenesis because many cases of severe STEC disease, including HUS and occasional outbreaks, were caused by LEE-negative strains [26]. Paton suggested that *saa* is one of the markers in LEE-negative STEC as an ill-defined subset like *eaeA* in LEE-positive STEC capable of causing life-threatening disease in humans [25]. The result in this study shows that no STEC isolates possessed both *eaeA* and *saa* at once, and *saa* (41%) was detected more than *eaeA* (12%) regardless of the period

of time and species, which means LEE-negative STEC strains have preponderance over LEE-positive STEC in South Korea [25]. It was shown that there is a correlation between the presence of *saa* and *ehxA* for certain STEC strains in a previous study [25] and their result was in agreement with our result. All of the isolates positive for *saa* were positive for *ehxA* while 16 isolates positive for *ehxA* were negative for *saa*. Among the 16 isolates positive for *ehxA,* 13 isolates were also positive for *eaeA;* therefore, at least 13 isolates positive for *ehxA* were contained in LEE positive STEC strains. Considering the possible correlation between *saa* and *ehxA,* this means that a plasmid - encoded RTX (repeats in toxin) toxin, which carries *ehxA,* is different between the plasmids of the LEE-positive and LEE-negative STECs [25]. According to the prevalence of the three virulence factors, since all virulence genes were found more in dairy cows than native cows, the more severe illness in humans is connected with STEC infection from dairy cattle.

The detection of major serotypes related to severe human diseases was performed but only a few strains represented the O26, O104, O111 and O157 strains. In 2010, 3 (5%) isolates of O26, 1 (2%) isolate of O111, and 2 (4%) isolates of O157 were detected from only native cattle; however, in 2011, 1 (2%) isolate of O104 and 3 (6%) isolates of O157 were detected from only dairy cattle. In a previous study in South Korea, the same O-serotypes were found but the

detection rates were different (O157, 42%; O26, 42%; O111, 1%) [4]. It may be due to different sampling methods and animal species. Our results on the major serotypes (O26, O104, O111, O157) were similar with those in Japan (O157, 1.1%; O26, 9.8%; O111, 1.1%; O104, 2.2%) [17]. Along with the results for the virulence factors, we should take notice of the increase in dangerous serotypes in dairy cattle recently.

STEC isolates in 2010 and 2011 had various different STs and a lot of new STs were found. This is because the majority of the available database contents cover *E. coli* strains from Europe, North America, Africa and Asia [28] but only a few strains from South Korea. In a previous report in South Korea, from the MLST results among 3 pathogenic *E. coli* isolates, one was ST101 and the others were new STs: ST1815 and ST1820 [18]. However, the present study carried out MLST on 156 STEC isolates from large-scale sampling of healthy cows in the whole country and more kinds of new STs were discovered. With regard to the kinds of cattle, there was only one overlapping ST, ST223, between the native and dairy cows in 2010 but 11 STs overlapped in 2011, which could mean there was no preferable ST for different kinds of cattle. As far as the regional aspect of STs, although ST10 was detected throughout the country except for in the F region for over 2 years and since appearing only in the A and E regions narrowly in 2010 and not being the most frequently detected ST especially in 2011, it is

reasonable that there is no predominant ST throughout the country at present. As previously stated, there is no prevalent ST in South Korea; additionally, many STs had changed from 2010 to 2011, which could be due to seasonal variations or changes of environment or feeding and management in the last 2 years. We assume that seasonal differences including ambient temperature, rainfall and seasonally driven factors such as the insect population affect the change in STEC strain pools [1]. Another assumption is that environmental alterations of farms such as reinforcement of sanitation controls and changes in the raising system prevented the continuous infection of STEC strains and selected for other STEC strains.

In this study, DNA from 156 STEC isolates were digested with *Xba*I and the PFGE patterns generated 38 clusters with 85% similarity. There was no specific STEC which spread across the country in the clusters. Of the 38 groups, only 5 groups (G6, G14, G23, G24, G28, G29, G34; 18.4%) contained STEC strains from both 2010 and 2011 and the others covered STEC strains from each year. This low similarity between the 2010 STEC and 2011 STEC strains means low genetic relatedness with each other, which agrees with the MLST result that there were not continuous infections of STEC strains all year round but had changed. In addition, there was no preference for STEC strains found in specific kinds of cattle but regional and spatial effects were important in the PFGE

grouping. STEC from the same farm or region was mainly grouped together in the present study and Cobbold and Desmarchelier suggested that contact between the animals and various materials, such as drinking and feeding troughs, enables STEC to be closely disseminated by horizontal transmission [7]. Of all the regions, STEC patterns from the A region overlapped with other regions. This is because there are vigorous exchanges between the A region in the capital area and other areas. This study used two methods to investigate the epidemiology of STEC but could not compare the PFGE with MLST because of a number of new STs. Therefore, in order to research genetic relatedness among the STEC strains on a large scale nationally, both methods can be meaningful.

The result of the antimicrobial susceptibility tests in this study showed a similar tendency to that of other countries in that streptomycin and tetracycline resistances were prevalent among cattle [21, 23] and this was also similar to an earlier domestic study in that most STEC isolates were resistant to carbenicillin and ampicillin [4, 19]. Tetracycline, streptomycin, ampicillin and carbenicillin resistances were frequent regardless of the species. The prevalences of resistance to these frequent antibiotics except for tetracycline increased in 2011. It is noteworthy that the percentage of antibiotic resistance to not only one drug but to multiple drugs increased considerably in 2011 compared to the previous year. As previously mentioned, it was assumed that this result was also affected by

seasonal variations or changes of environment or feeding and management. Since summer has more applications of antimicrobial interventions than autumn, STEC from the summer sampling in 2011 was more resistant to most antibiotics. As damage control for the prevention of secondary bacterial infection following viral disease, many kinds of antibiotics might be excessively and indiscriminately used. This would give rise to the risk of livestock production with residual antibiotics present and lead to the emergence of drug resistant bacteria [36]. The increasing rate of antimicrobial resistance can cause public health problems; therefore, continuous epidemiological inspection will be needed.

To reduce the risk of STEC infection and improve food safety, we investigated the characteristics of STEC in South Korea at the farm level and discovered the STEC pool had changed between 2010 and 2011. In addition, the risk of STEC infection increased more in dairy cows than in native cattle and the antibiotic resistance of STEC isolates also increased over two years. This result might be attributed to seasonal variations during the sampling or to changes of environment or feeding and management from 2010 to 2011. Therefore, we should pay attention to the STEC distribution among dairy cattle and continuous epidemiologic studies on the factors influencing the changes in antibiotic resistance and the STEC pool over the past two years are needed.

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국문초록

2010 년과 2011 년 사이 국내 소에서 분리된 Shiga toxin-producing *Escherichia coli* (STEC)의 분포양상과 특성

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Shiga toxin-producing *Escherichia coli* (STEC)은 세계적으로 미약한 설사 증상에서부터 혈성 설사, 출혈성대장염 (hemorrhagic colitis), 용혈성 요독증후군 (Hemolytic Uremic Syndrome)까지 다양한 증상을 유발하는 식품 매개 병원체로 여겨져 왔다. 특히 소의 경우 건강한 상태에서도 STEC 을 보유할 수 있기 때문에 STEC 의 자연 보유숙주로 생각되며, 병원체로 오염된 육회 또는 유제품의 섭취는 STEC 감염의 주요한 원인이 된다. 본 연구에서는 2010 년과 2011 년 사이 도축 전 단계인 농장에서의 한우와 유우의 STEC 분포양상을 파악하고 이들의 병원성 유전자, 혈청형, 항생제 감수성에 대한 조사와 함께 분자유전학적인 검사를 시행하였다.

총 156 개의 Shiga toxin-producing *Escherichia coli* (STEC)이 2010 년 9 월부터 2011 년 7 월 사이에 국내 한우 (100/568, 18%)와 유우 (56/524, 11%)의 분변 샘플로부터 분리되었다.

전체 STEC 의 33% (52 주)는 stx1 과 stx2 유전자를 동시에 갖고 있었고, *ehx*A 와 *saa* 유전자는 PCR 결과 83 (53%)주와 65 (42%)주에서 각각 검출되었다. 혈청형의 경우, 한우에서 6 주, 유우에서 4 주가 사람에서 질병을 일으킬 수 있는 O-혈청형인 O26, O111, O104, O157 인 것으로 확인되었다. MLST 와 PFGE 패턴은 모두에서 STEC 균주 간의 유전학적 다양성이 나타났고 연도간에도 유전적인 차이를 드러냈다. 항생제 감수성 검사에서는 다재내성률이 2010 년에 12%에서 2011 년에 42%으로 증가함을 보였다. carbenicillin, amoxicillin/clavulanate, ampicillin, colistin, neomycin and amikacin carbenicillin, amoxicillin/clavulanate, ampicillin, colistin, neomycin, amikacin 에 대한 내성율도 2010 년에 비해 2011 에 3 배 이상 증가하였다.

이와 같이 2010 과 2011 년에 분리된 균주의 차이는 2010 년과 2011 년에 시행한 샘플링 시기의 계절적 차이나 환경과 사양관리의 변화와 같은 요인들로 인한 2011 년 초에 국내에서 발생한 구제역의 발병을 통제하기 위한 대규모의 도살로 인한 결과로 사료된다. 이 연구에서

증가한 것으로 보이는 STEC 의 위험성, 특히 유우에서의 위험성이 증가함에 따라 유제품에 대한 공중 보건적인 관심이 요구되어야 할 것이다.

주요어: *Escherichia coli,* Shiga toxin, cattle, serotyping, multidrug resistance

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