J. Microbiol. Biotechnol. (2015), 25(9), 1460–1466
http://dx.doi.org/10.4014/jmb.1502.02034

jmb

Antibiotic Resistance and Virulence Potentials of Shiga Toxin-Producing *Escherichia coli* Isolates from Raw Meats of Slaughterhouses and Retail Markets in Korea

Hyun-jung Park[1], Jang Won Yoon[2], Eun-Jeong Heo[1], Eun-Kyoung Ko[3], Ki-Yeon Kim[4], Young-Jo Kim[1], Hyang-Jin Yoon[3], Sung-Hwan Wee[3], Yong Ho Park[4], and Jin San Moon[3]*

[1]*Ministry of Food and Drug Safety, Cheongwon 363-951, Republic of Korea*
[2]*College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 200-701, Republic of Korea*
[3]*Animal and Plant Quarantine Agency, Anyang 430-757, Republic of Korea*
[4]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, Republic of Korea*

Received: February 13, 2015
Revised: April 22, 2015
Accepted: June 18, 2015

First published online
June 22, 2015

**Corresponding author*
Phone: +82-31-467-4303;
Fax: +82-31-467-4321;
E-mail: moonjs727@korea.kr

pISSN 1017-7825, eISSN 1738-8872

In this study, the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) was investigated among raw meat or meat products from slaughterhouses and retail markets in South Korea, and their potential for antibiotic resistance and virulence was further analyzed. A total of 912 raw meats, including beef, pork, and chicken, were collected from 2008 to 2009. *E. coli* strains were frequently isolated in chicken meats (176/233, 75.9%), beef (102/217, 42.3%), and pork (109/235, 39.2%). Putative STEC isolates were further categorized, based on the presence or absence of the Shiga toxin *(stx)* genes, followed by standard O-serotyping. Polymerase chain reaction assays were used to detect the previously defined virulence genes in STEC, including Shiga toxins 1 and Shiga toxin 2 (*stx1 and 2*), enterohemolysin (*ehxA*), intimin (*eaeA*), STEC autoagglutination adhesion (*saa*), and subtilase cytotoxin (*subAB*). All carried both *stx1* and *eae* genes, but none of them had the *stx2*, *saa,* or *subAB* genes. Six (50.0%) STEC isolates possessed the *ehxA* gene, which is known to be encoded by the 60-megadalton virulence plasmid. Our antibiogram profiling demonstrated that some STEC strains, particularly pork and chicken isolates, displayed a multiple drug-resistance phenotype. RPLA analysis revealed that all the *stx1*-positive STEC isolates produced Stx1 only at the undetectable level. Altogether, these results imply that the locus of enterocyte and effacement (LEE)-positive strains STEC are predominant among raw meats or meat products from slaughterhouses or retail markets in Korea.

Keywords: Retail meat, Shiga toxin, Stx-producing *E. coli*, antibiogram, polymerase chain reaction

Introduction

Shiga toxin-producing *Escherichia coli* (STEC; also known as verotoxin-producing *E. coli*, VTEC) has emerged and is recognized as an important zoonotic food-borne pathogen and risk to public health [11, 29]. The microorganism produces Shiga toxins (Stxs), which belong to the AB_5-type bacterial toxin family with a potent cytopathic effect on mammalian cells [10]. In addition to toxin production, another virulence-associated factor expressed by STEC is an adhesin called intimin (the chromosomally encoded *eae* gene product), which is responsible for the intimate attachment of STEC to intestinal epithelial cells, causing attaching and effacing lesions in the intestinal mucosa [17, 47]. The microorganism also carries another virulence factor, EhxA (the enterohemorrhagic *E. coli* hemolysin), which acts as a pore-forming cytolysin on eukaryotic cells [40]. Recently, the STEC autoagglutinating adhesin (Saa)

has been characterized to be required for full virulence of STEC [16].

Although the most common STEC serotype associated with human outbreaks is known to be the serotype O157:H7, there is a growing recognition that non-O157 STEC serotypes have also been responsible for several human illnesses. Supporting this notion, recent studies have reported that not only STEC O157:H7 but also 100 additional non-O157 STEC serotypes were isolated from beef-related foods or food products. Thus, non-O157 STEC is now recognized as a potential zoonotic pathogen worldwide [5, 9, 14, 44]. Moreover, USDA-FSIS announced that ground beef and its precursor products would be routinely analyzed for the non-O157 STEC serogroups O26, O45, O103, O111, O121, and O145 [44, 45] because these serogroups have been found responsible for 70% of the reported STEC-related illnesses in the US [4]. In Korea, pathogenic *E. coli*-related food-poisoning cases are more common than other pathogenic bacteria [21].

In general, most outbreaks of human infection by STEC occur through the ingestion of contaminated foods or drinking water, including undercooked meats, unpasteurized milk and dairy products, vegetables, or fruit juice. Direct contact with animal carriers or the environment can also cause the infection [27, 37]. Therefore, the identification of the sources of infection is an important step towards decreasing the prevalence of this pathogen as well as reducing the risk of infection of humans [1, 31].

Cattle are regarded as the main reservoir in many countries [28, 32, 33]. However, previous studies have reported that a variety of livestock, such as sheep, goats, pigs, and chickens, can also carry STEC in their gastrointestinal tracts [13, 26, 32, 34]. Therefore, STEC contamination among retail meat products is an important threat to public health and food safety. To date, little information is available on the prevalence of STEC from raw meat in South Korea, because STEC O157:H7 has only been legally prohibited in the standards for the processing and ingredients specifications of livestock products in Korea. Thus, the prevalence and virulence potentials of STEC isolates from raw beef, pork, and chicken meats from slaughterhouses and retail markets in Korea were evaluated in this study [35, 38].

Materials and Methods

Sample Collection and Preparation

A total of 912 meat products were collected in South Korea; 473 samples in 2008 and 439 samples in 2009 (Table 2). Samples were taken either from the meat surfaces from cattle, pigs, and chickens in slaughterhouses or from raw beef, pork, and chicken meats packaged in retail markets. Samples were handled according to the guideline in the Standards for Processing and Ingredients of Specifications Livestock Products in Korea [24]. Sponges moistened with buffered peptone water (BPW; Oxoid, Hampshire, UK) were wiped in the flank, brisket, and rump of bovine and swine carcasses. BPW was added into the sponge and stomached; the

Table 1. Primers used in this study.

Primer	Sequence (5′ to 3′)	Target gene	References
Saa_1 F	CGTGATGAACAGGCTATTGC	*saa*	Jenkins *et al.*
Saa_1 R	ATGGACATGCCTGTGGCAAC		[18]
saa_2 F	CCTCACATCTTCTGCAAATACC	*saa*	Paton *et al.*
saa_2 R	GTTGTCCTGCAGATTTTACCATCCAATGGACATG		[30]
stx1 F	ACCCTGTAACGAAGTTTGCG	*stx1*	Pradel *et al.*
stx1 R	ATCTCATGCGACTACTTGAC		[33]
Stx2 F	ATCCTATTCCCGGGAGTTTACG	*stx2*	Pradel *et al.*
Stx2 R	GCGTCATCGTATACACAGGAGC		[33]
ehxA F	GGTGCAGCAGAAAAAGTTGTAG	*ehxA*	Pradel *et al.*
ehxA R	TCTCGCCTGATAGTGTTTGGTA		[33]
eae F	GAACGGCAGAGGTTAATCTGC	*eae*	Pradel *et al.*
eae R	TCAATGAAGACGTTATAGCCC		[33]
SubA F	GTACGGACTAACAGGGAACTG	*subA*	Tozzoli *et al.*
SubA R	ATCGTCATATGCACCTCCG		[43]
SubB F	GTAGATAAAGTGACAGAAGGG	*subB*	Tozzoli *et al.*
SubB R	GCAAAAGCCTTCGTGTAGTC		[43]

solution was analyzed for detection of *E. coli*. For poultry, whole bird carcasses were placed into sterile bags and 400 ml of sterile BPW was added. The bag was thoroughly shaken. Both wiped-sponge and carcass-rinsing solutions were used as sample solutions for further analyses. In the case of packaged meat samples from retail markets, 25 g was taken and 9-fold of diluents (BPW) was added and stomached. All samples were analyzed within 36 h after collection.

Isolation of *E. coli*

Twenty-five milliliters of the sample solutions was blended in 225 ml of Brilliant Green Bile Broth and incubated for 24–48 h at approximately 35–37°C. One loop of the enrichment culture was streaked onto Eosin Methylene Blue agar (Oxoid, UK). Plates were examined for typical *E. coli* colonies after an approximately 18 to 24 h incubation at 35–37°C. From each positive sample, typical colonies were transferred onto Tryptone Soy Agar (BD, US, USA) and incubated at 35–37°C for approximately 18–24 h. Each isolate was finally identified using the Vitek II Compact system (bioMerieux, France).

Detection of the Major Virulence Genes in STEC Isolates by Polymerase Chain Reaction (PCR)

To detect the previously defined virulence genes in the STEC isolates, PCR analyses were performed using the primers specific to the major STEC virulence genes that encode Shiga toxin (*stx1* and *2*), enterohemolysin (*ehxA*), intimin (*eaeA*), STEC autoagglutination adhesin (*saa*), and subtilase cytotoxin (*subAB*), as shown in Table 1 [18, 30, 33, 43]. Production of Stxs was quantified by a commercially available reverse phase latex agglutination (RPLA) kit (VTEC-RPLA, SEIKEN; Denka Seiken Co., Japan), and tested in accordance with the manufacturer's instructions.

O-Serotyping and Antibiogram Profiling of the STEC Isolates

The O-serotypes of STEC isolates were determined by using *E. coli* antisera "SEIKEN" set 1 (Denka Seiken Co.). An antibiogram of individual STEC isolates was examined by the determination of the minimal inhibitory concentrations (MIC) according to the guideline of the Clinical and Laboratory Standard Institute [7]. Using Sensititre plates (Trek Diagnostic System, UK), we diluted samples serially in broth and then incubated at 35°C to 37°C for 18 h. The MICs were read in an Auto Reader (Trek).

Detection of the Expanded Spectrum Beta-Lactamase (ESBL)

After incubation of peptone water with the sample solution, the enrichment broth was streaked on ESBL-bx agar plates (bioMerieux) at 37°C for approximately 18–24 h. Colonies on the ESBL-bx agar plates were subcultured onto blood agar (Asan Pharmaceutical, Seoul, Korea) plates and further identified using the Vitek II Compact system. To confirm the ESBL phenotype, a standard double-disk method was applied using cefotaxime/cefotaxime + clavulanic acid and ceftazidime/ceftazidime + clavulanic acid by E-test (AB Biodisk). The E-test with direct reading of the MIC was performed according to the manufacturer guidelines. The MICs of cefotaxime and ceftazidime, alone and in combination with clavulanic acid, were determined by broth microdilution according to CLSI guidelines using Trek microbroth dilution panels (Thermo Scientific). *E. coli* ATCC 25922 was used as a quality control. [7].

Results

Prevalence of STEC in Meat Products

In this study, a total of 387 (56.5%) and 82 (36.1%) *E. coli* strains were isolated from the 685 raw meat samples in slaughterhouses and 227 meat samples in retail markets, respectively, during the experimental period (Table 2). *E. coli* strains were frequently isolated in chicken meats (176/233, 75.9%), beef (102/217, 42.3%), and pork (109/235, 39.2%).

Serotype of *E. coli* and Virulence Gene of STEC Isolates

Twelve (2.6%) of the 469 *E. coli* isolates contained the *stx* genes, of which 11 isolates originated from the slaughterhouse samples (Table 2). The *stx* genes were found in the six pork samples, but in only two of the beef samples (Table 2). As shown in Table 3, 12 STEC isolates belonged to the 11 different O-serogroups, which were all non-O157 serotypes. All the isolates carried both *stx1* and *eae* genes, but none of them had the *stx2*, *saa*, or *subAB* genes. Six (50.0%) STEC isolates possessed the *ehxA* gene, which are known to be plasmid-encoded. However, RPLA analysis could not detect Stx1 production from all the STEC isolates

Table 2. Prevalence of STEC in meat products in Korea, 2008–2009.

Species	Slaughterhouse					Retail market				
	Total sample	Number of *E. coli* Isolates (%)		Number of STEC isolates (%)		Total sample	Number of *E. coli* Isolates (%)		Number of STEC isolates (%)	
Beef	217	102	(47.0)	1	(1.0)	83	25	(30.1)	1	(4.0)
Pork	235	109	(46.4)	6	(5.5)	99	22	(22.2)	0	(0.0)
Chicken	233	176	(75.5)	4	(2.3)	45	35	(77.8)	0	(0.0)
Total	685	387	(56.5)	11	(2.8)	227	82	(36.1)	1	(1.2)

Table 3. Serotype of *E. coli* and the presence of the major virulence genes in STEC isolates.

Sample (n = 12)	Species	Virulence gene							O type
		Stx1	*Stx2*	*eae A*	*ehx A*	*saa*	*subA*	*subB*	
B47	Beef	+	-	+	+	-	-	-	O126
B53	Beef	+	-	+	-	-	-	-	O55
S11	Pork	+	-	+	+	-	-	-	O27
S35	Pork	+	-	+	-	-	-	-	O168
S40	Pork	+	-	+	+	-	-	-	O55
S57	Pork	+	-	+	+	-	-	-	O114
S58	Pork	+	-	+	+	-	-	-	O86a
S59	Pork	+	-	+	-	-	-	-	O1
C10	Chicken	+	-	+	-	-	-	-	O128
C6	Chicken	+	-	+	+	-	-	-	O6
C42	Chicken	+	-	+	-	-	-	-	O18
C46	Chicken	+	-	+	-	-	-	-	O125

although they were all positive for the PCR with the *stx1*-specific primers, which implies that all the STEC isolates might produce Stx1 only at the basal level.

Antibiotic Resistance Profiles of STEC Isolates

Through the 15 antibiogram profiles, the MIC level of most STEC strains was more than 4 μg/ml. Interestingly, chicken isolates of STEC showed higher resistance to the antibiotics evaluated in this study than did beef isolates (data not shown). Most of the *E. coli* strains had antibiotic resistance to ampicillin (10/12; 83.3%), tetracycline (8/12; 83.3%), and streptomycin (11/12; 91.7%) (Table 4). Some STEC strains, especially pork and chicken isolates, displayed a multiple drug-resistance phenotype. Among them, the three strains of *E. coli* from pork meats showed resistance (64 μg/ml) to florfenicol (Table 4). However, there were no ESBL producers among all the STEC isolates (Table 4).

Discussion

The ability of STEC to cause disease is related to the production of one or more Stxs and other virulence factors such as such as intimin (*eae*), Saa (*saa*), and EhxA (*ehxA*). By detection of these virulence genes, STEC can be further characterized [15–17, 40]. Twelve (2.6%) STEC isolates in the present study all carried the *stx1* gene and *eae* gene, but none of them had the *stx2*, *saa*, and *subAB* genes. Six (50.0%) STEC isolates also possessed the *ehxA* gene. It was contrary to other previous results reported. Lee *et al*. [20] reported that 39 pathogenic *E. coli* were isolated between 2004 and 2006, of which 14 isolates were categorized into enterohemorrhagic *E. coli* (EHEC) in Korea.

The prevalence of STEC isolates in the previous study was 4.1%, 14.9%, and 4.6% in retail beef, pork, and poultry meats, respectively [20]. However, the prevalence of STEC isolates in this study was 28.0%, 26.0%, and 45.0% in beef, pork, and poultry meats, respectively, which turned out to be higher than that observed in the previous study [20]. In contrast, Choi *et al*. [6] reported that there were no *E. coli* isolates with the *stx* genes, although the prevalence of *E. coli* isolates from retail pork and poultry meats was similar to our results. Meanwhile, it was reported in the US that STEC isolates collected from 1983 to 2002 possessed *stx1* (61%), *stx2* (22%), *eae* (84%), and *ehxA* (86%) genes [4]. They also reported that 61% of human non-O157 STEC isolates contained *stx1* alone, 21% had *stx2* alone, 18% carried both *stx1* and *stx2*, and 84% of those human isolates harbored *eae* [4]. The difference in the prevalence of virulence genes from STEC between the US and Korea may reflect the nature of STEC isolates, the conditions of the raising and husbandry practices for animal, climates, and/or variations in STEC isolates from one geographical region to another [8, 12, 34].

Several studies have examined the prevalence of STEC in swine, which seems to be a major source of STEC isolates from raw meats and meat products from our study. STEC was detected in 22% of pigs from Switzerland [19], 30.2% of pigs from Germany [46], 4.5% of pigs from Argentina [23], and 0.4% of pigs from South Africa [22]. Epidemiological studies suggest that non-O157 strains cause 20% to 50% of STEC infections, which cause approximately 169,000 illnesses annually in the US [39]. More than 70% of non-

Table 4. Antibiotic resistance profiles of STEC isolates.

Antimicrobial agent	No. of STEC isolates at indicated MIC (μg/ml)											Breakpoints (μg/ml)[a]		
	128	64	32	16	8	4	2	1	0.5	0.25	0.125	S	I	R
Amoxacillin/clavulanic acid				2	7	3						≤ 8/4	16/8	≥ 32/16
Ampicillin		10				1	1					≤ 8	16	≥ 32
Cephlothin			3	2	4	2	1					≤ 8	16	≥ 32
Cefoxitin			1	1	2	3	5					≤ 8	16	≥ 32
Ceftiofur								2	10			≤ 2[b]	4[b]	≥ 8[b]
Gentamicin		2	1	1				8				≤ 4	8	16
Neomycin			4				8					-[d]	-	-
Streptomycin	8	3		1								≤ 32[b]	N/A[b,c]	≥ 64[b]
Nalidixic acid	4				3		5					≤ 16	-	≥ 32
Ciprofloxacin				4				1		1	6	≤ 1	2	≥ 4
Colistin					1	11						-	-	-
Chloramphenicol		4			4	3	1					≤ 8	16	≥ 32
Florfenicol		3			2	3	4					-	-	-
Tetracycline	5	2	1				4					≤ 4	8	≥ 16
Trimethoprim/sulfamethoxazole						8				3	1	≤ 2/38	-	4/76

[a]CLSI breakpoints for *Escherichia coli*.

[b]CIPARS breakpoints for *Escherichia coli*.

[c]Not applicable.

[d]No breakpoints in CLSI table and CIPARS table.

O157 STEC infections are caused by serogroups O26, O103, O111, O145, O91, O113, O128, O45, and O121 [2, 13]. Recently, it has been suggested that non-O157 STEC strains are a major problem in many European countries [7]. The difference in the prevalence of STEC in terms of reported cases, major serotypes, carriage of major virulence genes, and antibiogram profiles might be due to samples of animal species, husbandry practices (such as stocking densities, types of feed offered), and prevailing climatic conditions, which likely account for the variations in STEC isolates from one geographical region to another [8, 12, 34].

As antibiotics are used against pathogenic infection, the emergency and spread of antibiotic resistance become an important problem in the clinical practice. According to a previous report, sulfisoxazole has the most common antimicrobial resistance, followed by tetracycline, streptomycin, ampicillin, trimethoprim, chloramphenicol, and neomycin [25]. Moreover, it has been reported that over 50% of their isolates displayed antimicrobial resistance against sulfamethoxazole, cephalothin, and tetracycline, and 20% of them against ampicillin and gentamicin [41]. The acquisition of antibiotic resistance in both commensal and pathogenic strains has become an important public health issue [36]. The development and persistence of antibiotic resistance in commensal and non-pathogenic bacteria are worldwide concerns, because they are thought to act as a reservoir of resistance genes capable of transferring those genes to other food-borne and zoonotic pathogens [10, 23]. In particular, retail foods, especially meat and meat products, may be an important vehicle for community-wide dissemination of antimicrobial-resistant *E. coli* and extra-intestinal pathogenic *E. coli* [42]. In this study, 50% of *E. coli* isolates showed the highest MICs against streptomycin and tetracycline. According to this result, most of the STEC isolates are considered to have high resistance to streptomycin and tetracycline in Korea.

In conclusion, our finding suggested a low level of STEC found from raw meats or meat products from slaughterhouses or retail markets in Korea, and that the locus of enterocyte and effacement (LEE)-positive STEC is predominant. Further studies are needed to determine their biological significance during host infection and the capabilities for propagation of their antibiotic resistance.

Acknowledgments

This study was funded by the budget for veterinary research and development of the Animal and Plant

Quarantine Agency (H-F503-2005-07-02). J.W.Y. was supported by a 2014 Research Grant from Kangwon National University.

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