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Identification and Molecular Characterization of Antimicrobial-Resistant Shiga Toxin–Producing *Escherichia coli* Isolated from Retail Meat Products

Ming-Cheng Li, Fang Wang, and Fan Li

Abstract

Ten (2.7%) Shiga toxin–producing *Escherichia coli* (STEC) were isolated from 370 samples of raw minced beef, mutton, pork, and chicken from the Jilin region of China; and additional 10 *E. coli* O157:H7 isolates were previously isolated from different Jilin regions. Seventeen of the isolates were multiresistant, exhibiting resistance to ampicillin, ciprofloxacin, tetracycline, sulfamethoxazole-trimethoprim, gentamycin, and streptomycin. Class 1 integrons were detected in nine (45.0%) of the STEC isolates and consisted of serogroups O157, O62, O113, O149, and O70. Integrons containing amplicons of a 0.5–1.5 or 1.0 kb gene cassette were found in seven (77.8%) of the integron-containing isolates. Sequencing analysis revealed that these gene cassettes encode genes conferring resistance to trimethoprim (*dfrA*1) and streptomycin (*aadA*1). The 0.5 kb cassette described here was found to encode a putative transporter peptide in the STEC. Seventeen isolates contained plasmids with different bands, and transfer by conjugation between strains of *E. coli* demonstrated that class 1 integrons located on mobile plasmids could contribute to the emergence and dissemination of antimicrobial resistance to ampicillin, gentamycin, streptomycin, and sulfamethoxazole-trimethoprim amongst STEC. These data revealed the high prevalence of antimicrobial-resistant STEC isolates in Jilin's surrounding regions, providing important and useful surveillance information reflecting antimicrobial selection pressure.

Introduction

Shiga toxin–producing *Escherichia coli* O157:H7 (STEC O157) is an important human enteric pathogen first described by Riley *et al.* in 1983, and it has become an increasingly common cause of severe gastrointestinal illness (Dontorou *et al.*, 2003). Since its discovery, this serovar has been responsible for hundreds of outbreaks throughout the world (Karmali, 1989). Domestic animals are a known reservoir of this pathogen, with >100 serotypes of STEC being found in the fecal flora of bovine, ovine, goats, pigs, cats, and chickens (Bettelheim *et al.*, 2003).

The resistance of STEC to an increasing number of antimicrobial agents is a well-recognized problem (Meng *et al.*, 1998). Several mechanisms involving mobile genetic elements, such as plasmids, transposons, and integrons, have been shown to contribute to the spread of antimicrobial resistance (Fluit and Schmitz, 1999).

Relatively little is known about the occurrence of class 1 integrons and the mechanisms of acquisition, and dissemination of antimicrobial resistance, among STEC isolates in China.

Thus, the aim of our study was to examine resistance profiles and to determine the prevalence of class 1 integrons and R-plasmids among STEC isolates recovered from bovine, ovine, poultry, and pig meat products from the Jilin region of China.

Materials and Methods

Collection of retail meat products, STEC isolation, serotyping, and standard strains

Food samples were purchased from a local supermarket and from randomly selected farmers' markets ($n = 12$) in the Jilin region, on different production days over a 1-year period (2006). The samples were grouped into two categories. The first category consisted of 220 samples of fresh ground meat containing beef ($n = 70$), mutton ($n = 50$), pork ($n = 50$), or chicken ($n = 50$). The second category consisted of 150 uncooked frozen prepackaged boneless cutlets or raw sausages containing beef ($n = 40$), mutton ($n = 30$), pork ($n = 30$), or chicken ($n = 50$).

All samples were handled aseptically. The fresh products were transported in sterile containers at 4°C and the frozen

Department of Pathogenobiology, Norman Bethune College of Medical Sciences, Jilin University, Changchun, Jilin, People's Republic of China.

products at −10°C. On delivery to the laboratory, all samples were processed. The bacterial strains were isolated according to methods listed in the Administration Bacteriological Analytical Manual (U.S. FDA, 1992).

All biochemically identified nonsorbitol fermenting colonies were subjected to slide agglutination with the *E. coli* O157 Latex test kit and *E. coli* O- and H-specific antisera (Oxoid). The agglutinating colonies were confirmed using polymerase chain reaction (PCR) with primers for the *stx*1, *stx*2, *eae*, and *hly*A genes of *E. coli* O157 as previously described (Montenegro *et al.*, 1990).

Ten *E. coli* O 157 isolates were previously isolated in different Jilin regions; 7 from minced beef samples and 3 from minced chicken, mutton, and pork (Table 2).

Reference strains were included in all tests: *E. coli* C 600 Rif^r strain was used as a recipient in both conjugation and transformation experiments. Strain P 3749380 (Ridley and Threlfall, 1998) was used as a positive control for class 1 integron PCR. All strains were preserved in L-B broth containing 15% glycerol at −80°C for further analysis.

Antimicrobial susceptibility determination

Susceptibility to a panel of 17 antimicrobial agents (Sigma-Aldrich) was determined by an agar doubling dilution method similar to that of the Clinical and Laboratory Standards Institute (CLSI, 2005) criteria. Bacteria were grown overnight at 37°C in Mueller-Hinton (MH) broth, diluted 1/10 in normal saline (0.91% NaCl,) and inoculated using a multipoint inoculator onto the agar with a suitable concentration of antimicrobial agents. Antimicrobial resistance was determined according to their MICs. Control strains were *E. coli* ATCC 25922, *S.* ATCC 25923, and *Pseudomonase aeruginosa* ATCC 27853.

Plasmid content analysis and conjugation

Large plasmids were determined by alkaline lysis method described by Kado and Liu (1981) and analyzed by electrophoresis in 0.7% agarose gel. Small plasmids were extracted by the QIAprep Spin Miniprep Kit (Qiagen) in accordance with the manufacturer's instructions. Plasmid size was determined according to the supercoiled DNA ladder (Gibco BLL).

Conjugal transfer of all STEC isolates was performed on MH agar plates using *E. coli* C 600 as the recipient, as previously described (Gray *et al.*, 2006). Transconjugants were selected on MH agar containing rifampicin (400 g/mL) and sulfamethoxazole (200 μg/mL).

Detection of class 1 integrons using PCR

DNA templates, oligonucleotide primers, and PCR conditions (including positive and negative controls) to detect class 1 integrons were performed as previously described (Levesque *et al.*, 1995).

Cloning and DNA sequence analysis

PCR products for detection of class 1 integrons were electrophoresed on a 1.5% agarose gel. The DNA band of interest was ligated to pGEM-T Easy vector (Promega), transformed into JM109 competent cells, and the white clones were selected and sequenced. Nucleotide sequencing was performed directly on cloned fragments using an ABI Prism 377 DNA sequencer. Sequence homology was performed using the BLAST program available at the website of the National Center of Biotechnology Information.

Results

Prevalence and characterization of STEC

In 2006, 370 retail meat samples were analyzed, and 10 STEC non-O157 strains were isolated: 4 out of 70 (5.7%) from minced beef meat, 2 out of 50 (4.0%) from minced mutton, 1 out of 50 (2.0%) from pork meat, 2 out of 50 (4.0%) from minced chicken meat, and 1 out of 50 (2.0%) from raw chicken meat (Table 1). However, no STEC isolates were isolated from thawed meat cutlets. Serotyping of the non-O157 isolates showed that the 10 isolates belonged to eight different O groups and six different H groups. Results of the PCR assays for different virulence are presented in Table 2. All isolates from domestic animals possess *stx* genes.

Antimicrobial-resistant profiles of isolates

Twenty (100%) were resistant to sulfamethoxazole-trimethoprim, 16 (80.0%) to ciprofloxacin, 12 (60.0%) to tetracycline, 9 (45.0%) to ampicillin, followed by 6 (30.0%) to gentamycin, 6 (30.0%) to streptomycin, 4 (20.0%) to chloramphenicol, and 3 (15.0%) to cephalothin. Seventeen isolates were multiresistant and exhibited resistance to commonly used antimicrobial agents. All isolates were susceptible to the eight antimicrobial agents tested (Table 3).

Table 1. Isolation of Shiga Toxin–Producing *Escherichia coli* from Different Food in Jilin Region

		No. of positive samples (%)	
Food	*No. of samples*	*O157:H7*	*non-O157:H7*
Minced beef meat	70	0	4 (5.7)
Minced mutton meat	50	0	2 (4.0)
Minced pork meat	50	0	1 (2.0)
Minced chicken meat	50	0	2 (4.0)
Raw chicken sausages	50	0	1 (2.0)
Uncooked frozen beef cutlets	40	0	0
Uncooked frozen mutton cutlets	30	0	0
Uncooked frozen pork cutlets	30	0	0

Table 2. Characterization of R-Plasmid Profiles, Class 1 Integrons, Pathotypes, and Transferable Resistant Phenotypes Among Shiga Toxin–Producing *Escherichia coli* Isolates

Strain ID	*Serotype*	*Pathotype*			*Resistant pattern*	*Plasmid (kb)*	*Integron (kb)*	*Cassettes*	*Transferable phenotype*	*Source*
		stx	eae	hlyA						
EC 1	O157: H7	2	+	−	SXT	—			—	Beef
EC 2	O157: H7	2	−	+	AMP, SXT, S, C	92, 27	1.0	aadA1	AMP, SXT, S	Beef
EC 3	O157: H7	2	−	−	SXT	—	1.0 0.5	dfrA1 orf	—	Beef
EC 4	O157: H7	2	−	+	AMP, C, TE, SXT, GM, S	92, 27, 8.5, 5.8	1.0	aadA1	AMP, SXT, S	Beef
EC 5	O157: H7	1, 2	−	+	TE, SXT, CIP, GM, S	92, 27, 8.5, 5.8	1.0	aadA1	SXT, S	Beef
EC 6	O157: H7	2	−	+	SXT, C	92, 27			C	Beef
EC 7	O157: H7	2	+	+	SXT, CF	92, 27			CF	Beef
EC 8	O157: H7	2	−	+	TE, SXT, GM	92, 27			SXT, GM	Chicken
EC 9	O157: H7	1	+	+	TE, SXT	92, 27			SXT	Mutton
EC 10	O157: H7	1	+	−	SXT	—				Pork
EC 11	O113: H21	1	−	−	AMP, TE, SXT, S, CIP, GM	120, 25, 10	1.0	aadA1	AMP, SXT, S	Beef
EC 12	O22: H8	2	−	−	AMP, CIP, TE, SXT,	120, 25, 10			AMP, SXT	Beef
EC 13	O26: H11	1	−	−	TE, SXT, C	120, 25, 10	0.75	dfrA1	SXT, C	Beef
EC 14	O26: H11	1	−	−	SXT, GM	120, 25, 10			SXT, GM	Beef
EC 15	O26: H11	1	−	−	AMP, TE, SXT, CIP, C	120, 25, 10			AMP, SXT, C	Mutton
EC 16	O62: H32	1	−	−	AMP, TE, SXT, CIP, CF, C, AMC, S	120, 35, 5.8	1.5	aadA1 dfrA1	AMP, SXT, S	Pork
EC 17	O149: H12	2	−	−	AMP, TE, SXT, GM, S	120, 25, 10	1.0	aadA1	AMP, SXT, S	Mutton
EC 18	O70: H11	2	+	−	AMP, SXT, CIP, S	120, 25, 10	1.0	aadA1	AMP, SXT, S	Chicken
EC 19	O76: H32	1	+	−	TE, SXT, CF, GM	120, 85, 5.8			GM, SXT	Chicken
EC 20	O156: H47	1	+	−	TE, SXT, CIP, GM	120, 85, 5.8			SXT, GM	Chicken

ID, identification; AMP, ampicillin; CR, ceftriaxone; CF, cephalothin; AMC, ampicillin-clavulanic acid; C, chloramphenicol; K, kanamycin; S, streptomycin; GM, gentamycin; CIP, ciprofloxacin; TE, tetracycline; SXT, sulfamethoxazole-trimethoprim.

Table 3. Antimicrobial Resistance of Shiga Toxin–Producing *Escherichia coli* Isolates from Animals Meat Products

Antibiotics	*No. of STEC isolates resistant to antimicrobial agents (%)*
Ampicillin	45.0
Ceftriaxone	0
Cefotaxime	0
Ceftazidime	0
Cephalothin	15.0
Cefazolin	0
Aztreonam	0
Imipenem	0
Ampicillin-clavulanic acid	0
Chloramphenicol	25.0
Kanamycin	0
Streptomycin	30.0
Gentamycin	30.0
Ciprofloxacin	80.0
Tetracycline	60.0
Sulfamethoxazole-trimethoprim	100.0
Ticarcillin-clavulanic acid	0

STEC, shiga toxin–producing *Escherichia coli.*

R-plasmid profiles and transferabitity of resistance properties

Seventeen isolates were shown to contain plasmid DNA by agarose gel electrophoresis; there was a big variation in the plasmid sizes observed. The plasmids ranged from 120 to 5.8 kb (Table 2). Seven isolates of O157 possess an ~92-kb plasmid, called pO157, which encodes several virulence-associated genes. However, three isolates of O157 did not contain pO157. Conjugation experiments with *E. coli* C600 as the recipient confirmed that all transferred plasmids ranging in size from 85 to 25 kb were conjugative and transferred from the donor bacteria to *E. coli* C600 at a frequency of 10^{-3} to 10^{-5} per recipient. The resistance phenotypes were transferred and expressed in *E. coli* C 600 transformants, as presented in Table 2.

Integrons

Nine (45.0%) of the STEC isolates contained class 1 integrons (Table 2). Integrons with amplicons of 0.5–1.5 kb in length, and with a 1.0 kb gene cassette, were found in seven (77.8%) of these nine integron-containing isolates. Class 1 integrons were found in isolates of serogroups O157, O62, O133, O149, and O70. DNA sequence analysis revealed that the strain EC 16 contains a 1.5 kb amplimer carrying *dfrA*1 and *aadA1* cassettes identical to those described in GenBank accession number AJ 419168; 6 of the 1000 bp amplimers carried *aadA1* cassettes identical to those described in GenBank accession number AB126601; 11 of the 0.75 kb amplimers carried a *dfrA*1 cassette identical to that described in GenBank accession number AF 382145; and 3 of the 1.0 and 0.5 kb amplimers carried a *dfrA*1 cassette and an empty cassette (without any inserted cassettes). This ORF closely matched a similar sequence found in *Shigella sonnei* (accession no. CP000038), which encodes a putative transporter peptide.

Discussion

A number of reports from different countries report the presence of *E. coli* O157 isolates in a variety of foods of domestic animal origin. Chinen *et al.* (2001) reported that 4.8% of 83 fresh pork sausage and 3.3% of 30 dry sausage samples examined in Argentina were contaminated with *E. coli* O157. Mattar and Vasquez (1998) reported that 8.7% of hamburger samples tested were contaminated with *E. coli* O157 in Colombia. Zhou *et al.* (2002) reported that 5.0% of 40 beef samples and 3.3% pork samples were contaminated with *E. coli* O157, and STEC was isolated from 10% to 20% of the domestic animals tested including cattle, goats, and chickens in the Changchun area.

In our study, 2.7% of 370 samples of raw minced beef, mutton, pork, and chicken from the Jilian region were contaminated with STEC non-O157. The prevalence of STEC was significantly higher in beef and mutton minced meat than in pork minced meat. However, STEC was isolated from a high percentage of foods of chicken origin in the region and also in other regions of China due to the rearing style. The farmers preferred to scatter their fowls in the fields, and all fowls like to hunt for grains or insects in the feces from domestic animals. Thus, STEC can be transferred from enteric fecal flora in food-producing animals to the fowls via the food chain. This study demonstrated that meat products were contaminated by STEC in the Jilin area.

The 10 isolates belonged to eight different O groups and six different H groups. However, there were five STEC strains identified in this study that were also reported in patients with hemolytic uremic syndrome (HUS O22, O26, O113), which occurred in various countries worldwide (Beutin *et al.*, 2004).

Indeed published data for STEC isolates from various foods and veterinary sources indicated a tendency toward higher resistance rates to these antibiotics than in the previous years. Meng *et al.* (1998) found that among 125 STEC isolates from animals, food, and humans, 24% were resistant to at least one antibiotic and 19% were resistant to three or more antibiotics. The authors concluded that the STEC isolates from a different origin were genetically related and that antimicrobial therapy in these animals often included tetracycline and ciprofloxacin or other antibiotics resulting in higher resistance rates. They are a potential reservoir for antimicrobial resistance genes and play important roles in the ecology of antimicrobial resistance of bacterial populations (Shin *et al.*, 2005).

This prevalence of class 1 integrons found in both O157 and non-O157 in the present study was higher than in a previous study of STEC isolates (Ruby *et al.*, 2005), where a higher prevalence of O157 ($n = 5$) than non-O157 ($n = 4$) was observed. Eight (88.9%) of the nine class 1 integron-containing STEC isolates were resistant to three or more antimicrobials. These findings are similar to other studies, which indicated that cassettes for aminoglycoside and trimethoprim resistance were common in class 1 integrons (Ruby *et al.*, 2005). Each of nine STEC donors, except isolate EC 3, transferred class 1 integrons to an *E. coli* recipient strain *in vitro*. These strains carried one gene cassette yet were resistant to two or more antimicrobials, suggesting that elements besides class 1 integrons were involved in antimicrobial resistance among the STEC isolates.

In this study, we first reported the molecular characterization of a class 1 integron in isolate EC 3. The conjugation

experiment proved that the strain did not contain any transferable plasmid. We assumed that the class 1 integron was integrated into the chromosome. Recent findings of numerous "empty" integrons in bacteria species suggested that resistance gene cassettes might be excised in the absence of selective antibiotic pressures (Carattoli, 2001).

Plasmid analysis indicated an occurrence rate of 85% (17/20), with three plasmid profiles. The serotype-specific plasmid of 92 kb plasmid (pO157) of *E. coli* O157 was detected in seven strains of O157 examined in this work. However, three strains of *E. coli* O157 did not harbor pO157, and the *hly*A gene was not found in these isolates, which were positive for *stx*. The absence of pO157 observed in these isolates could be explained by the fact that these isolates do not represent clinical isolates. Bielaszewska *et al.* (2000) reported that plasmid-encoded virulence factors may not be essential for pathogenesis or they may have been lost during infection or storage. The findings indicated that these STEC isolates from the Jilin region are low-virulence variants.

Transferable R-plasmids between 85 and 25 kb in size were detected in the transconjugants. The observation that resistance phenotypes for ampicillin, streptomycin, gentamycin, and sulfamethoxazole were transferred, suggesting that resistance genes in addition to *aadA*1 and *dfrA1* were present on either the same plasmids or different plasmids.

This study has demonstrated that the use of antimicrobial agents is strongly associated with the prevalence of antimicrobial resistance in STEC isolates from food products of animal origins. The genetic content, combination, and emergence frequency of these gene cassettes may reflect the antibiotic selective pressure in this specific geographical region, providing useful antimicrobial surveillance information for the rational and effective use of antimicrobial agents.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Fan Li, M.D., Ph.D.
Department of Pathogenobiology
Norman Bethune College of Medical Sciences
Jilin University
No. 126, Xinmin St.
Changchun
Jilin 130021
People's Republic of China

E-mail: lifan@jilin.edu.cn