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Prevalence and Characterization of Antimicrobial-Resistant *Escherichia coli* Isolated from Conventional and Organic Vegetables

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Abstract

To compare the characteristics and to identify the epidemiological relationships of *Escherichia coli* isolated from organic and conventional vegetables, the antimicrobial resistance and genetic properties of *E. coli* were investigated from 2010 to 2011. *E. coli* was isolated from 1 of 111 (0.9%) organic vegetables and from 20 of 225 (8.9%) conventional vegetables. The majority of strains were isolated from the surrounding farming environment ($n = 27/150$ vs. 49/97 in organic vs. conventional samples). The majority of the vegetable strains were isolated from the surrounding farming environments. *E. coli* isolated from organic vegetables showed very low antimicrobial resistance rates except for cephalothin, ranging from 0% to 17.9%, while the resistance rates to cephalothin (71%) were extremely high in both groups. *E. coli* isolates expressed various resistance genes, which most commonly included bla_{TEM}, *tet*(A), *str*A, *str*B, and *qnr*S. However, none of the isolates harbored *tet*(D), *tet*(E), *tet*(K), *tet*(L), *tet*(M), or *qnr*A. The transferability of *tet* gene, *tet*(A), and *tet*(B) was identified in tetracycline-resistant *E. coli*, and the genetic relationship was confirmed in a few cases from different sources. With regard to the lower antimicrobial resistance found in organic produce, this production mode seems able to considerably reduce the selection of antimicrobial-resistant bacteria on vegetables.

Introduction

Vegetables generally contain less protein and fat, but they are rich in vitamins, minerals, and phytochemicals, which contain antioxidant, antibacterial, antifungal, antiviral, and anticarcinogenic properties (CDC, 2013). Many people prefer organic vegetables to conventionally farmed vegetables due to an increased interest in good health. However, most vegetables are being consumed in a raw state without any heating. As a result, people are easily affected by foodborne pathogens, including antimicrobial-resistant bacteria, when vegetables are unsanitary. Recently, foodborne outbreaks have been frequently reported due to the consumption of raw vegetables and fruits. In Germany, in 2011, an *Escherichia coli* O104:H4 outbreak occurred in sprouts (ECDC, 2011). *E. coli* O104:H4 displayed the pathogenic characteristics of enterohemorrhagic and EAggEC enteroadhesive *E. coli*, was resistant to various antimicrobials, such as ampicillin and tetracycline, and also had extended-spectrum ß-lactamase (ESBL)-producing characteristics.

Tetracycline was the most commonly used (43–51%) antimicrobial agent as growth promoter or disease-prophylactic drug for livestock farming in Korea during 2002–2006 (KFDA, 2007). In 2008, the Korean Food and Drug Administration also reported high antimicrobial resistance of *E. coli* from the food animals and meats, even though the use of tetracycline had been decreased from 2006 (KFDA, 2008).

Various antimicrobials have been used to control disease in livestock, fish, and agriculture farming. Antimicrobial-resistant bacteria could be hazardous to public health. Research on antimicrobial-resistant bacteria has been conducted extensively in the fields of livestock farming and livestock products, and there was a lack of research in agricultural farming and agricultural products. Interests in organic food have led many researchers to study antimicrobial resistance to compare and contrast organic and conventional products. These studies were mostly focused on livestock, such as pork (Miranda *et al.*, 2008), poultry (Cui *et al.,* 2005; Luangtongkum *et al.,* 2006), and dairy products (Walk *et al.,* 2007). Most of the reports suggested that antimicrobial resistance in conventional products was higher than that in organic products. Selective pressure, which includes resistance developed in response to the excessive use of antimicrobials, could be a major reason. Now, research on antimicrobial resistance is carried out by monitoring antimicrobial-resistant bacteria as well as by studying antimicrobial-resistance determinants

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and their mechanisms, which should be targeted to reduce antimicrobial resistance. This suggests that antimicrobial-resistance determinants as well as antimicrobial-resistant bacteria should be targeted to reduce antimicrobial resistance (Karami *et al.,* 2006; Koo and Woo, 2011; Tamang *et al.,* 2012).

The aim of this study is to assess the contamination with antimicrobial-resistant bacteria with regard to farming methods, in order to identify factors that might help to ultimately minimize public health hazards caused by pathogenic bacteria from organic vegetables, conventional vegetables, and their farming environments in the future.

Materials and Methods

Sample collection

Samples were collected from the most commonly consumed vegetables in Korea, including lettuce, sesame leaves, and sprouts, and common vegetable-farming environment components (i.e., soil, irrigation water, and liquid fertilizer). In total, 261 organic vegetable samples (111 from vegetables and 150 from farm environments) from 3 organic vegetable farms and 322 conventional vegetable samples (225 from vegetables and 97 from farm environments) from 5 conventional vegetable farms were collected from farms located in Gyeonggi, Chungcheongnam, and Chungcheongbuk provinces from 2010 to 2011. After 100 g of sample was randomly collected aseptically, the sample was immediately carried to the laboratory and refrigerated.

Isolation of E. coli

E. coli was detected and identified according to the Korea Food Code (2011). Twenty-five grams of each sample was inoculated into 225 mL of Difco™ EC Medium (Becton Dickinson, Sparks, MD) and enriched at 37°C for 24 h. The culture was inoculated onto BBL™ Eosin Methylene Blue Agar (Becton Dickinson) and incubated at 37°C for 24 h; then typical colonies were picked and identified using VITEK® (BioMeurieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by the disk-diffusion method in accordance with Clinical and Laboratory Standards Institute Guidelines (CLSI, 2010). Antimicrobial susceptibility testing was performed using 13 antimicrobials (all purchased from Becton Dickinson): ampicillin, piperacillin, cephalothin, cefazolin, cefamandole, streptomycin, gentamicin, amikacin, tobramycin, nalidixic acid, trimethoprim–sulfamethoxazole, chloramphenicol, and tetracycline.

Detection of antimicrobial resistance genes

Antimicrobial resistance genes were detected using polymerase chain reaction (PCR) of *E. coli* isolates resistant to tetracycline ($n = 37$ organic and 121 conventional), aminoglycoside ($n = 0/56$), sulfonamide ($n = 47/93$), β-lactam ($n = 28/28$), and quinolone ($n = 18/32$). Tested antimicrobial resistance genes were as follows: *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(K), *tet*(L), and *tet*(M) for tetracycline resistance (Koo and Woo, 2011); *str*A, *str*B (Lanz *et al.*, 2003), and *aad*A (Sáenz *et al.*, 2004) for aminoglycoside resistance; *sul*1, *sul*2 and *sul*3 for sulfonamides (Maynard *et al.*, 2003); bla_{TEM} for β-lactam resistance (Koo and Woo, 2012); and *qnr*A, *qnr*B_1, *qnr*B_4 (Kim *et al.*, 2011), *qnr*S (Park *et al.*, 2009), and *aac(6′)-Ib-cr* (Fihman *et al.*, 2008) for quinolone resistance.

PCR reactions had a total volume of 30 μL containing 5 μL of template DNA, 1 μL of forward and reverse primer (Macrogen, Seoul, Korea) (Supplementary Table S1; Supplementary Data is available online at www.liebertpub.com/fpd), Solg™ 2X Taq PCR Pre-Mix (SolGent Co., Ltd., Seoul, Korea), and distilled water. Using the MyCycler™ Thermal Cycler System (Bio-Rad Laboratories, Inc., Hercules, CA), PCR was performed over 30 cycles of denaturation at 94°C for 30 s, annealing at 55–60°C for 30 s, and extension at 72°C for 30 s. Purified water in equal volume with bacterial DNA were used as the negative control. The PCR product was run on a 1.0 % agarose LE (Georgia Chemistry, Hangzhou, China) gel, and the amplified product was analyzed using the Sequenase Version 2.0 DNA Sequencing Kit™ (USB Corporation, OH).

tet *gene transferability by conjugation*

The *tet* gene transferability conjugation test was performed on all *tet* gene–positive isolates using the broth mating method, with *E. coli* J53-AR (resistant to sodium azide) as a recipient strain (Jacoby and Han, 1996). Both recipient and donor cells were cultured in BBL™ Mueller Hinton II broth (Becton Dickinson) at 37°C overnight, mixed with each other at a 1:10 ratio (donor to recipient), and incubated for 3 h. The resulting mixture (0.2 mL) was spread onto the BBL™ MacConkey Agar (Becton Dickinson) plate with 8 mg/L tetracycline and 100 mg/L sodium azide (Junsei Chemical Co., Ltd., Chuo-ku, Tokyo, Japan) and incubated at 37°C for 24 h. Conjugation was confirmed by performing PCR.

Pulsed-field gel electrophoresis (PFGE)

PFGE was conducted using the CHEF-Mapper system (Bio-Rad Laboratories, Inc.) according to the PulseNet standardized protocol (Ribot *et al.*, 2006). DNA was digested with 20 U of *Xba* I (Roche Diagnostics, Indianapolis, IN) and separated on 1.0% low-melt agarose (Bio-Rad Laboratories, Inc.). The electrophoresis conditions were as follows: 6.0 V/cm, initial switch time of 2.2 s, final switch time of 54.2 s, and running time of 18 h at 14°C in 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM pH 8.0 EDTA). As a size marker, a lambda DNA ladder (Bio-Rad Laboratories, Inc.) was used. Genetic relationship analysis was performed using the Info-Quest FP software, version 4.5 (Bio-Rad Laboratories, Inc.).

Results

Prevalence of E. coli

In organic vegetables, *E. coli* was detected on only one sesame leaf (0.9%) and 27 (18%) organic farm environment samples. In conventional vegetables, *E. coli* was detected in 20 (8.8%) vegetable and 49 (50.5%) farm environment samples (Table 1). Compared to the detection in vegetables, *E. coli* was detected in a high number of farming environment samples such as soil, irrigation water, and liquid fertilizer.

Antimicrobial resistance profile

E. coli isolates from organic vegetables showed susceptibility values to 12 antimicrobial agents with very low resistance

Table 1. Prevalence of *Escherichia coli* in Organic Vegetable and Conventional Vegetable Samples

	Organic (n = *261*)				*Conventional* (n = *322*)			
Sample	*Vegetable* (n = *111*)		*Environment* (n = *150*)		*Vegetable* (n = *225*)		*Environment* (n = *97*)	
No. of positive samples (%)	1 (0.9%)		27 (18%)		20 (8.8%)		49 (50.5%)	
Sources	Sesame leaf	1	Fertilizer	1	Sesame leaf	14	Manure fertilizer	13
			Liquid fertilizer	11	Sprout	1	Stream	16
			Groundwater	1	Lettuce	5	Groundwater	3
			Soil	14			Soil	17

rate (0–17.9%). However, the resistance to cephalothin was extremely high (67.8%). In conventional vegetable isolates, the resistance rate was normally high compared to organic isolates, especially to cephalothin (71%) and cefazolin (63.8%). As shown in Figure 1, the antimicrobial resistance rates were significantly high in the isolates from conventional vegetables and organic vegetables. However, a similar resistance pattern was seen in vegetable isolates.

Resistance genes

The resistance genes bla_{TEM} ($n = 1$, 3.6%), *tet*(A) ($n = 3$, 10.7%), *str*A ($n = 3$, 10.7%), and *str*B ($n = 3$, 10.7%) were identified in organic vegetable isolates. bla_{TEM} ($n = 25$, 36.2%), *sul*1 ($n = 7$, 10.1%), *sul*2 ($n = 10$, 14.5%), *tet*A ($n = 15$, 21.7%), *tet*B ($n = 3$, 4.3%), *str*A ($n = 13$, 18.8%), and *str*B ($n = 13$, 18.8%) were detected in conventional vegetable isolates. *sul*3, *aad*A and *qnr*S were also found with low rate ($n = 1$, 1.4%). Ten isolates from conventional vegetables harbored more than four different resistance genes with different profiles (Table 2).

tet *gene transferability*

Transferability of tetracycline resistance genes was identified by conjugation in tetracycline-resistant isolates. Four tetracycline-resistant isolates from organic vegetables confirmed 100% transferability in *tet*(A). In 16 tetracycline-resistant strains from conventional vegetables, 73% were transferable *tet*(A) and 66% could transfer *tet*(B) horizontally.

PFGE

E. coli strains had various correlation profiles. Even among the conventional farm isolates, greatly diverse patterns were identified with low similarity (data not shown). *E. coli* isolates from organic farms showed various PFGE patterns as shown in Figure 2. Among them, three *E. coli* isolates from organic vegetables showed 100% similarity: two isolates originated from vegetable farm soil and one from a sesame-leaf sample. Particularly, one soil isolate and one sesame-leaf isolate showed identical PFGE patterns as well as antimicrobial resistance profiles.

Discussion

Along with an increase in the overall interest in health, the consumption of raw food and demand for minimally processed food are on the rise. Food-poisoning outbreaks related to raw vegetables have been gradually increasing. Most of the reported gastrointestinal disease outbreaks caused by fresh produce were bacteria related, particularly the Enterobacteriaceae family (Falomir *et al.,* 2010). *E. coli* can contaminate vegetables through several routes. Infected people who contact vegetables without washing their hands, animal droppings, and food processing could all be sources of bacterial contamination

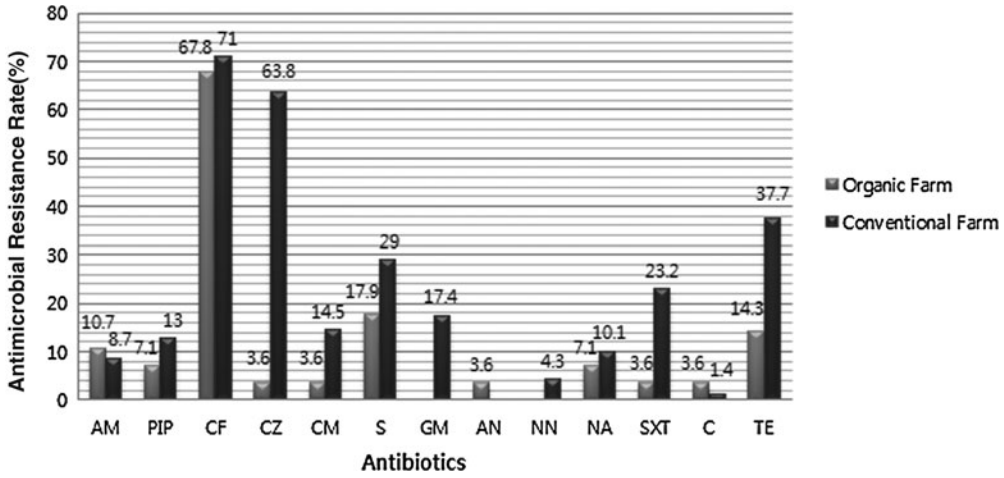

FIG. 1. Antimicrobial resistance of *Escherichia coli* isolates. AM, ampicillin; PIP, piperacillin; CF, cephalothin; CZ, cefazolin; CM, cefamandole; S, streptomycin; GM, gentamicin; AN, amikacin; NN, tobramycin; NA, nalidixic acid; SXT, trimethoprim–sulfamethoxazole; C, chloramphenicol; TE, tetracycline.

Table 2. Identified Antimicrobial Resistance Gene Profiles in *Escherichia coli* Isolates from Each Origin

Resistance gene pattern	*Organic vegetables (%)*	*Conventional vegetables (%)*
tet(A)	2[a] (7.1)	15 (21.7)
*str*B	1 (3.6)	0 (0)
tet(A)-*str*A	1 (3.6)	0 (0)
*str*A-*str*B	2 (7.1)	3 (4.3)
bla_{TEM}	1 (3.6)	21 (30.4)
sul2	0	2 (2.9)
tet(B)	0	1 (1.4)
qnrS	0	1 (1.4)
tet(B)-bla_{TEM}	0	1 (1.4)
tet(B)-bla_{TEM}-*str*A-*strB*	0	1 (1.4)
strA-strB-aadA-sul3	0	1 (1.4)
strA-strB-sul2-bla_{TEM}	0	1 (1.4)
strA-strB-sul1-sul2-bla_{TEM}	0	7 (10.1)
Total	7 (25.0)	54 (78.3)

[a]Number of isolates.

(Wilson, 2014). In Korea, since the year 2000, major food-poisoning outbreak causes have been connected to the consumption of raw foods such as fish, shellfish, and fresh vegetables (MFDS, 2013).

There have been few reports on the characterization of antimicrobial-resistant bacteria isolated from vegetables. Antimicrobial resistance of commensal bacteria can be regarded as an indicator to estimate the prevalence of antimicrobial resistance in foodborne pathogens. Ruimy *et al.* (2010) tested 393 products and identified serious contamination with multidrug-resistant, Gram-negative bacteria, and most of the isolates were derived from soil and farming environments. In addition, Popowska *et al.* (2012) suggested that agricultural soil is a possible reservoir of resistant bacteria and that both the prudent use of antibiotics and regulations at the national level are required. Burjaq and Shehabi (2013) detected 17 (27.8%) multiantibiotic-resistant *E. coli* isolates from leafy vegetables. Another previous study detected that antibiotic-resistant endophytic bacteria were widely recov-

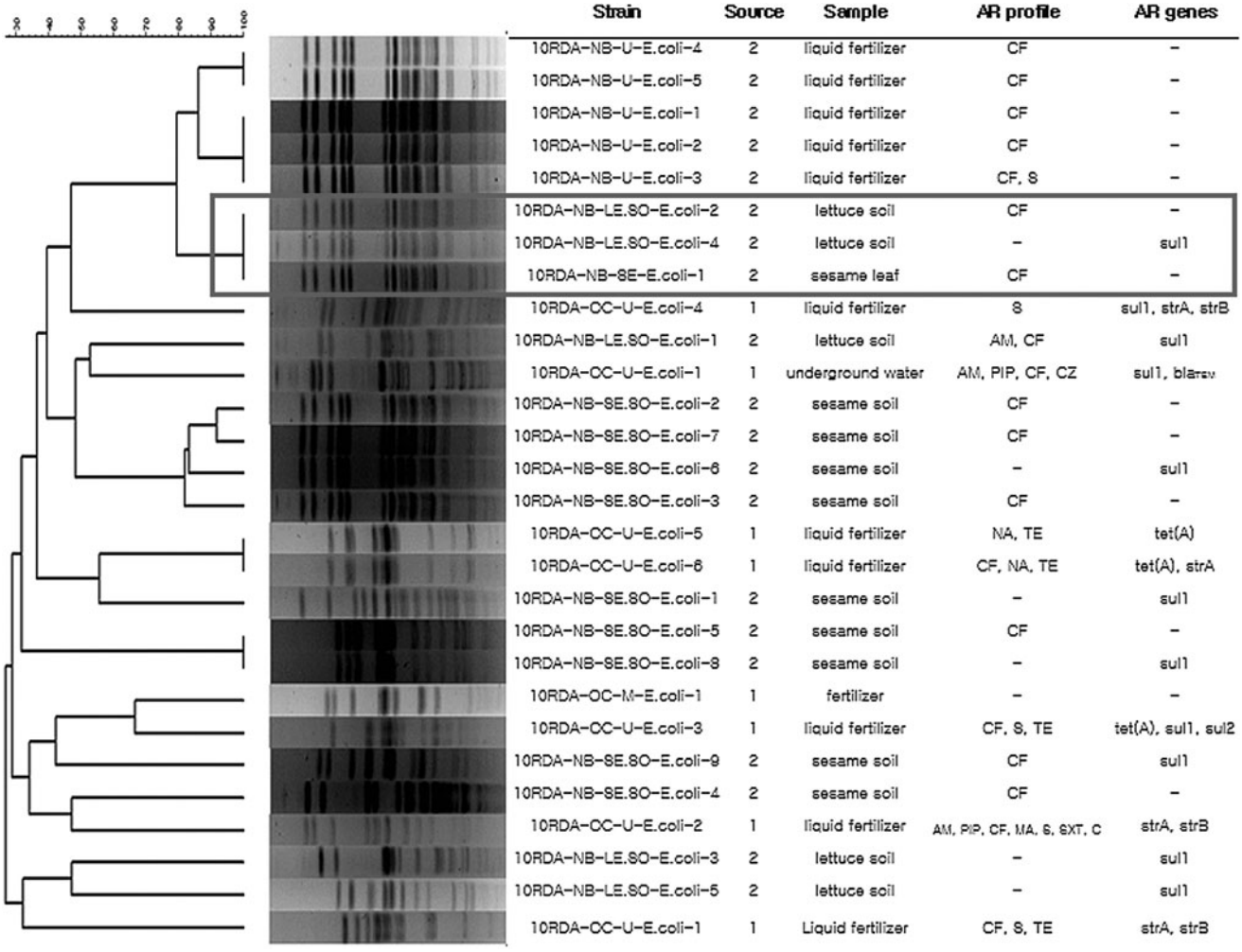

FIG. 2. Dendrogram of pulsed-field gel electrophoresis (PFGE) profiles in *Escherichia coli* isolates from organic vegetables. *E. coli* showing 100% similarity is indicated with a marked box: Two isolates originated from vegetable-farm soil and one from a sesame-leaf sample. Particularly, one soil isolate and one sesame-leaf isolate showed identical PFGE patterns as well as antimicrobial resistance profiles. Although the three strains were isolated from the same farm, vegetable and soil samples were collected from different cultivation areas. *Source: Organic farm number. [a]AR profile, antimicrobial resistance profile. [b]AR genes, antimicrobial resistance genes. For abbreviations, see Figure 1 legend.

ered from celery, *pak choi* (also known as *bok choy*), and cucumber fertilized with chicken manure (Yang *et al.*, 2014).

In this study, *E. coli* was isolated from 6.3% of vegetables and 30.8% of environmental samples. The results supported the importance of safety management regulations for cultivation environments that produce vegetables. In organic vegetables, the detection rate of *E. coli* and the isolation of resistant strains were low, though we could not confirm whether or not animal manure was or was not used as fertilizer on the organic farms. However, if incomplete fermented animal manure was used as fertilizer in organic farming, the safety of organic vegetables could not be guaranteed. As a result, the bacterial contamination is hard to eliminate by simply washing the vegetables with water. In the study by Bermúdez-Aguirre and Barbosa-Cánovas (2013), the disinfection of fresh produce needs the combination of several nonthermal sanitation treatments. Therefore, to reduce the hazard caused by foodborne pathogens and antimicrobial-resistant bacteria in vegetables, the introduction of hygienic cultivation manuals such as Good Agriculture Practice needs to be considered.

E. coli isolates in this study also commonly harbored *tet*(A), *str*A, *str*B, bla_{TEM}, and *qnr*S genes, while *tet*(D), *tet*(E), *tet*(K), *tet*(L), *tet*(M), and *qnr*B4 were not detected. As mentioned earlier, due to tetracycline resistance in Korea being considered severe, the *tet* gene had been screened in tetracycline-resistant isolates. Koo and Woo (2011) reported *tet*(A) to be the predominant gene expressed in tetracycline-resistant Gram-negative bacteria isolated from various samples, such as swine and livestock environments. The mechanism of tetracycline resistance is widely assumed to involve the upregulation of efflux pumps encoded by the *tet* gene located in the plasmid (Karami *et al*., 2006). In another report, *E. coli* isolates from meat samples in Canada primarily harbored *tet*(A), *tet*(B), and *tet*(C) genes (Sheikh *et al.,* 2012). In accordance with previous reports, this study showed similar prevalence of the *tet* resistance genes. Bryan *et al*. (2004) indicated that *tet*(B) is highly detected in *E. coli* isolates from 12 animal and human sources, followed by *tet*(A), *tet*(C), *tet*(D), and *tet*(M), thus creating the first report of *tet*(M) gene expression. In China, the *tet*(M) gene was identified in *E. coli* isolates from sick ducks (Hu *et al.,* 2013). Therefore, it is suggested that the wide spread of resistance genes shows the diversity of bacterial species as well as the regions, sources, and patterns of antimicrobial agent use.

E. coli is a part of the common intestinal flora, which is regarded as harmless to humans. However, antibiotic resistance genes can be transferred from intestinal bacteria such as commensal *E. coli* to pathogenic bacteria, such as *E. coli* O157:H7 or *Salmonella* spp., and consequently result in critical health problems. Since many antimicrobial resistance determinants are located in the transmissible gene cassettes, gene transfer is possible between the same types as well as different types of bacteria (Wang *et al.,* 2012). In this study, 4 organic vegetable isolates showed 100% transferability in *tet*(A), whereas the transferability of 16 strains from conventional vegetables was confirmed to be 73% in *tet*(A) and 66% in *tet*(B). It was determined that the *tet* gene could be easily transferred regardless of its origins. Therefore, the prevalence and transferability of *tet* genes conferring tetracycline resistance should be monitored regularly.

Moreover, the antimicrobial resistance gene bla_{TEM} was the most frequently detected resistance gene in both conventional and organic vegetables. Classical SHV-1, TEM-1, and TEM-2 enzymes are known as dominant plasmid-mediated β-lactamases. It was reported that various ESBL types such as TEM, SHV, and CTX-M could be genetically linked to other antimicrobial resistances, especially to fluoroquinolones, aminoglycosides, and sulfonamides (Coque *et al.,* 2008).

Tamang *et al.* (2012) investigated plasmid-mediated quinolone-resistance determinants in *E. coli* from food-producing animals in Korea, and detected the *qnr*B4, *qnr*S1, and *aac*(6′)-*lb-cr* genes. In our study, only *qnr*S was confirmed in *E. coli* from farm produce.

According to certified organic farming practice in Korea, there is little or no use of antimicrobials and agricultural chemicals to produce environmentally friendly fresh vegetables. This might be why comparative analysis of the isolates from conventional and organic vegetables suggested that *E. coli* isolated from conventional vegetables showed more resistance to various antimicrobials and higher resistance rates than organic vegetable isolates. Although the three strains that showed a 100% genetic relationship were isolated from the same farm, vegetable and soil samples were collected from different cultivation areas. Presumably, the resistant *E. coli* was spread to the farm by the same unconfirmed route.

Among domestic food-producing animals in Korea, antimicrobial use was the highest in swine, followed by fish, chickens, and cows. Although tetracyclines were the most commonly used antibiotics, the amount has rapidly decreased from 752,386 kg in 2001 to 308,206 kg in 2011, according to the comprehensive antimicrobial reduction policies established by the National Antimicrobial Resistant Management Program (NARMP).

Therefore, for the reduction of antimicrobial use and prevention of antimicrobial resistance, it is important to assure the sanitation of vegetable farms and establish continuous monitoring and national policies on antimicrobial resistance and antibiotic use.

Conclusions

The antimicrobial resistance rate was higher and various antimicrobial resistance genes were harbored in conventional farm produce than in organic vegetables. It has been suggested that the high use of antimicrobials in livestock and handling the animal manure as fertilizer could cause high resistance due to selective pressure in the isolates. The lower antimicrobial resistance in organic produce may suggest that this production method might be able to reduce the occurrence of antibiotic-resistant bacteria on vegetables. Therefore, to reduce antimicrobial resistance and decrease the infection risk in humans, this study suggests that good agriculture practices should be properly introduced in farming environments. In addition, it is important to establish continuous monitoring and national policies on antimicrobial resistance and antibiotic use.

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Disclosure Statement

No competing financial interests exist.

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