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ARTICLE

Antimicrobial resistance, molecular, and phenotypic diversity of *Escherichia coli* isolates from fresh vegetable products in Korea

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Abstract *Escherichia coli* is generally considered as an indicator of antimicrobial resistance due to its genetic flexibility and adaptability. In the present study, antimicrobial resistance patterns, biofilm formation ability, and genotypes were assessed in *E. coli* isolates from fresh vegetable products in Korea. Among 120 isolates, 22 isolates (18.3 %) were resistant to one or more antimicrobials and 11 isolates were concurrently multidrug resistant against up to six antimicrobial agents. The highest resistance rate was detected on ampicillin (14.2 %), followed by piperacillin (11.7 %) and cefalotin (10.0 %). Rep-Polymerase chain reaction (PCR) revealed that five out of the 26 isolates were assigned to one cluster with a similarity >95 %, and strains carrying the same antimicrobial resistance profile had discrete rep-PCR patterns. A biofilm formation assay demonstrated that significantly larger amounts of biofilm were formed by an isolate, which showed no antimicrobial resistance. However, 60 % of the five intermediated biofilm-forming *E. coli* exhibited multidrug resistance against three or four antimicrobial agents. These results suggest that fresh vegetable products contaminated with *E. coli* are sources of antimicrobial resistance and other diverse virulence determinants. Therefore, the information provided in this study can be useful for improving food safety and public health.

Keywords Antimicrobial resistance · Biofilm · *E. coli* · Rep-Polymerase chain reaction · Vegetable products

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Introduction

Escherichia coli is a ubiquitous commensal bacterium in the intestinal tracts of humans and animals, as well as in soils and crops, such as raw vegetables (Khandaghi et al. 2010). Commensal *E. coli* strains are typically considered as indicators of antimicrobial resistance because the genetic flexibility and ability of this organism to adapt to constantly changing environments allow the acquisition of a large number of antimicrobial resistance mechanisms (Szmolka and Nagy 2013). By obtaining resistance genes, commensal *E. coli* strains develop resistant mutants to survive and maintain microbial homeostasis (Schroeder et al. 2002). This selective pressure favoring antimicrobial-resistant phenotypes results in a critical public health threat because the efficacy of antimicrobial treatment may be reduced. Moreover, the intra- and/or inter-species transfer of antimicrobial resistance determinants via transmissible plasmids accelerates the spreading of antimicrobial resistance (Wright 2007). The emergence and dissemination of antimicrobial resistance in bacteria are regarded as a serious public health issue and a food safety problem worldwide (Cohen 2000).

In most natural ecosystems, bacteria reside predominantly in sessile communities rather than as free-living planktonic cells. Motile planktonic cells attach to a surface, and the sessile cells aggregate and subsequently develop into matured biofilms (Hall-Stoodley et al. 2004). Previous studies have revealed that naturally formed biofilms on plant leaves comprise 10–80 % of total microbial populations (Morris et al. 1998; Lindow and Brandl 2003; Rayner et al. 2004; Erickson et al. 2010). Bacterial biofilm cells are usually more resistant to antimicrobial agents or sanitization than planktonic cells, so they are extremely difficult to

remove (Cerf et al. 2010; Bridier et al. 2011). The frequency of transformation, one of the important mechanisms for transfer of antimicrobial resistance determinants, is also enhanced in biofilm structures, which are close contact with high concentrations of DNA and rich in nutrients (Baur et al. 1996). Because biofilm structures are frequently encountered in many parts of the food industry environment (Arnold and Silvers 2000), the biofilm formation by foodborne pathogens including antimicrobial-resistant *E. coli* has become one of the important problems for food safety.

Food of non-animal origin (FoNAO) that is contaminated with antimicrobial-resistant *E. coli* is a major health concern because this type of food is frequently consumed raw (EFSA 2011). During agricultural production and harvesting, FoNAO can be infected with antimicrobial-resistant pathogens or commensals from animal and human sources. Delaquis et al. demonstrated that surface contamination of the edible tissues of FoNAO often occurs due to pathogen transfer from the soil or water (Delaquis et al. 2007). As FoNAO does not undergo any inactivation or preservation treatment during processing, consumers may be exposed directly to all of these resistant bacteria, including antimicrobial-resistant *E. coli*. Although outbreaks of foodborne illnesses caused by the consumption of FoNAO, such as fresh fruits, vegetables, and unpasteurized fruit juices, have dramatically increased in frequency and caused considerable mortality, morbidity, and financial losses in recent several decades (Beuchat 2002; Harapas et al. 2010; Scallan et al. 2011; Tzschoppe et al. 2011), FoNAO has rarely been examined as a possible source of human infection with antimicrobial-resistant *E. coli*. Moreover, little information on the antimicrobial resistance and biofilm formation ability of *E. coli* isolated from FoNAO is available to estimate the risk of human exposure to antimicrobial resistance determinants from the consumption of FoNAO. This study is focused on following characteristics of the *E. coli* isolates obtained from fresh vegetable products in Korea: (a) resistance to antimicrobial agents, (b) genetic relationships based on repetitive sequence-based polymerase chain reaction (rep-PCR) technology, and (c) ability to form biofilm.

Materials and methods

E. coli isolates

A total of 120 *E. coli* isolates were used to determine antimicrobial resistance, biofilm formation ability, and genetic diversity. *E. coli* isolates were obtained from 413 samples, which included mixed vegetable salads (128), sprouts and baby leaf salads (176) and unpasteurized fruit and vegetable samples (109) collected from retail markets in South Korea in 2011. Strain isolation and identification were described in previous study (Kim et al. 2014).

Antimicrobial susceptibility test

The antimicrobial susceptibility of the identified *E. coli* isolates was examined via the broth dilution test using the AST-N224 test card in a VITEK® 2 compact system (Biomerieux, France). The following antimicrobials were tested: ampicillin, amoxicillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cefalotin, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, tobramycin, levofloxacin, and sulfamethoxazole/trimethoprim. The qualitative interpretation (i.e., resistant or sensitive) was based on the breakpoints proposed in the CLSI standards (CLSI 2010). *E. coli* ATCC 10536 was used as a quality control organism in antimicrobial susceptibility determination.

Molecular typing

For rep-PCR, DNA was extracted from the *E. coli* isolates using the UltraClean microbial DNA isolation Kit (Mo Bio Laboratories, USA). All DNA solutions were standardized to a concentration of ca. 25 ng/μL. The DiversiLab *Escherichia* Kit (Biomerieux) was used for rep-PCR amplification of non-coding intergenic repetitive elements in the genomic DNA, according to the manufacturer's instructions. PCR was performed in a Mastercycler pro thermocycler using the following parameters: initial denaturation at 94 °C for 2 min; 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s; and a final extension at 70 °C for 90 s. The amplicons were analyzed using the DiversiLab system, which includes fragment separation using microfluidic chips and the Agilent B2100 Bioanalyzer (Agilent Technologies Inc., USA). Standard DNA markers, which were applied to normalize the sample runs, and the Chip kit molecular weight ladders were used. The results were analyzed using the DiversiLab software (version 3.3), which uses the Pearson correlation coefficient to determine distance matrices and the unweighted pair group method with arithmetic averages (UPGMA) to create dendrograms.

Biofilm formation

After incubation in brain heart infusion (BHI) broth (MERCK, Germany) at 37 °C until A_{600} of each strain reaches to 0.5, the culture of each strain was diluted 100-fold into fresh BHI medium. The 200 μL of cultures was transferred to each well of 96-well polystyrene microtiter plates (Nunc, Denmark), and biofilms were formed by incubating for 24 h at 37 °C. Once the

planktonic cells were removed, the biofilm cells on the wall were washed with PBS and then stained with a 1 % (wt/vol) crystal violet (CV) solution for 15 min at room temperature. The biofilms were quantitated by measuring the amount of CV eluted from the biofilms as the absorbance at 570 nm (A_{570}) (Kim et al. 2013).

Data analyses

Averages and standard errors of the mean were calculated from at least three independent experiments. Data were analyzed with Student's t tests using GraphPad software version 5.02 (Graphpad Software, USA). The significance of differences between experimental groups was accepted at a P value of <0.05.

Results

Antimicrobial resistance

Increasing antimicrobial resistance, particularly multidrug resistance, among Gram-negative bacteria represents a critical problem worldwide. To examine this possibility, the antimicrobial susceptibility of 120 *E. coli* isolates to seventeen antimicrobial agents was determined using the broth dilution test (Table 1). Twenty-two (18.3 %) of the 120 isolates were resistant to one or more of the antimicrobial agents tested. Resistance to ampicillin (14.2 % of the isolates), piperacillin (11.7 %), and cefalotin (10.0 %) was observed most often, and resistance to sulfamethoxazole/trimethoprim (5.0 %), levofloxacin (4.2 %), gentamicin (2.5 %), amoxicillin/clavulanic acid (1.7 %), cefoxitin (1.7 %), and cefotaxime (1.7 %) was observed less frequently. Intermediate susceptibility was observed for cefalotin (52.5 %), ampicillin (2.5 %), tobramycin (1.7 %), piperacillin (0.8 %), and cefotaxime (0.8 %). None of the strains were resistant to ceftazidime, cefepime, aztreonam, imipenem, meropenem, and amikacin.

Of the 22 resistant isolates, 11 showed resistance against more than three different classes of antimicrobials (i.e., multidrug resistant) and six different patterns of resistance were observed (Table 2). Two isolates were resistant to six antimicrobial agents (i.e., ampicillin, piperacillin, cefalotin, cefotaxime, levofloxacin, and sulfamethoxazole/trimethoprim). Three isolates which resistant to five antimicrobial agents (i.e., ampicillin, piperacillin, cefalotin, levofloxacin, and sulfamethoxazole/trimethoprim) were also detected.

Molecular typing

As shown in Fig. 1, molecular typing using rep-PCR assigned the 26 *E. coli* isolates, which were selected based on their origin of samples and antimicrobial resistance in order to cover the diverse *E. coli* isolates from fresh vegetable products, to five clusters consisting of two or more isolates with a similarity >95 %. The largest cluster contained four isolates (No. 1–4), suggesting that this clone might be circulating in both salads and fresh juice products (Fig. 1). *E. coli* isolates among the largest cluster showed no resistance to antimicrobials except one isolate having resistance to ampicillin, piperacillin, and sulfamethoxazole/trimethoprim. Strains carrying the same antimicrobial resistance profile had distinct rep-PCR patterns (isolate No. 15, 22), suggesting that the presence of antimicrobial resistance determinants is not related to the noncoding repetitive sequences that are interspersed throughout the bacterial genome. In addition, ten isolates (No. 12, 19–27) remained ungrouped and appeared to represent unique genotypes, which indicates a high degree of genetic diversity among these strains.

Table 1 Antimicrobial resistance profiles of 120 *E. coli* isolated from fresh cut salads, sprouts, baby leaf vegetable salads, and non-pasteurized fruit and vegetable juices collected from Korea

Antimicrobials	No. of isolates (%)		
	Resistant	Intermediate	Susceptible
AM	17 (14.2)	3 (2.5)	100 (83.3)
AMC	2 (1.7)	0 (0)	118 (98.3)
PIP	14 (11.7)	1 (0.8)	105 (87.5)
TZP	0 (0)	0 (0)	120 (100.0)
CF	12 (10.0)	63 (52.5)	45 (37.5)
FOX	2 (1.7)	0 (0)	118 (98.3)
CTX	2 (1.7)	1 (0.8)	117 (97.5)
CAZ	0 (0)	0 (0)	120 (100)
FEP	0 (0)	0 (0)	120 (100)
ATM	0 (0)	0 (0)	120 (100)
IPM	0 (0)	0 (0)	120 (100)
MEM	0 (0)	0 (0)	120 (100)
AN	0 (0)	0 (0)	120 (100)
GM	3 (2.5)	0 (0)	117 (97.5)
TM	0 (0)	2 (1.7)	118 (98.3)
LEV	5 (4.2)	0 (0)	115 (95.8)
SXT	6 (5.0)	0 (0)	114 (95.0)

AM Ampicillin, *AMC* amoxicillin/clavulanic acid, *PIP* Piperacillin, *TZP* Piperacillin/Tazobactam, *CF* Cefalotin, *FOX* Cefoxitin, *CTX* Cefotaxime, *CAZ* Ceftazidime, *FEP* Cefepime, *ATM* Aztreonam, *IPM* Imipenem, *MEM* Meropenem, *AN* Amikacin, *GM* Gentamicin, *TM* Tobramycin, *LEV* Levofloxacin, *SXT* Sulfamethoxazole/Trimethoprim

Biofilm formation

To determine whether genetically related strains have similar biofilm-forming capacities, biofilm formation by the 26 *E. coli* isolates was assessed using an assay based on CV staining.

Table 2 Drug resistance patterns of 120 *E. coli* isolates recovered from fresh cut salads, sprouts, baby leaf vegetable salads, and non-pasteurized fruit and vegetable juices collected from Korea

No. of antimicrobials	Drug resistance patterns	No. of isolates (%)
6	AM-PIP-CF-CTX-LEV-SXT	2 (1.7)
5	AM-PIP-CF-LEV-SXT	3 (2.5)
4	AM-AMC-CF-FOX	1 (0.8)
	AM-PIP-GM-SXT	3 (2.5)
3	AM-PIP-SXT	1 (0.8)
	AMC-CF-FOX	1 (0.8)
2	AM-CF	1 (0.8)
	AM-PIP	5 (4.2)
1	AM	1 (0.8)
	CF	4 (3.3)
0	None	98 (81.8)

AM Ampicillin, *AMC* Amoxicillin/Clavulanic Acid, *PIP* Piperacillin, *CF* Cefalotin, *FOX* Cefoxitin, *CTX* Cefotaxime, *GM* Gentamicin, *LEV* Levofloxacin, *SXT* Sulfamethoxazole/Trimethoprim

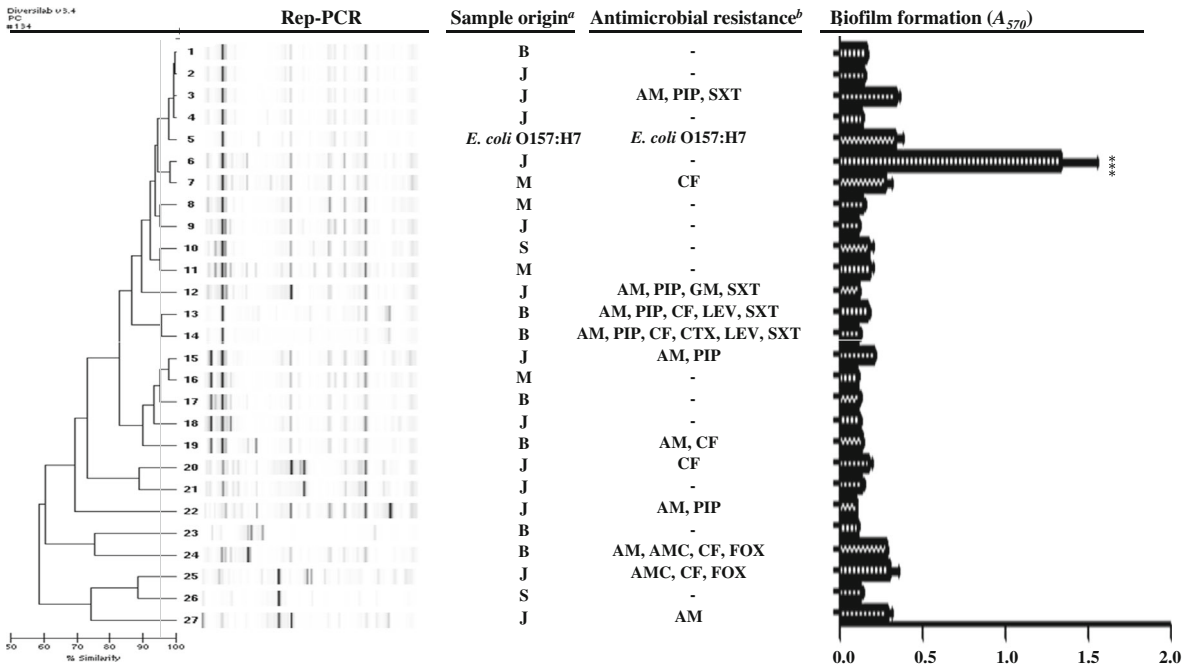

Fig. 1 PCR-generated dendrogram, virtual gel image, and similarity matrix of the 26 *E. coli* isolates typed using rep-PCR. The sample origin, antimicrobial resistance, and biofilm formation activity of each strain are shown in the right panel. ***$p < 0.001$, relative to the biofilm formed by wild-type *E. coli* O157:H7 (ATCC43895) under the same conditions. [a]*B* baby leaf vegetable salads; *S* sprout vegetable salads; *M* mixed vegetable salads; *J* fresh juices (fruit and vegetable). [b]*AM* ampicillin; *AMC* amoxicillin/clavulanic acid; *PIP* piperacillin; *CF* cefalotin; *FOX* cefoxitin; *CTX* cefotaxime; *GM* gentamicin; *LEV* levofloxacin; *SXT* sulfamethoxazole/trimethoprim

Although no correlation was observed between genetic subtype and the amount of biofilm accumulation, significantly high amounts of biofilms were formed by one isolate with an A_{570} of 1.34 (No. 6). Of the remaining *E. coli* isolates, five showed intermediate biofilm formation, with A_{570} values in the range of 0.25–0.35 (No. 3, 7, 24, 25, 27). Among *E. coli* with intermediate biofilm formation, three isolates (60 %) were resistant to three or four antimicrobial agents. The isolate with multiple antimicrobial resistant against more than five antimicrobial agents (No. 13, 14) showed low biofilm formation ability. These results indicate that the isolates differ in the regulation of their biofilm phenotypes, even when the isolates belong to the same cluster.

Discussion

In this study, antimicrobial resistance profiles were identified for *E. coli* isolates from fresh vegetable products which were consumed without further cooking. Of the 120 *E. coli* isolates

characterized in this study, approximately 20 % displayed resistance to one or more antimicrobials, including ampicillin, piperacillin, cefalotin, sulfamethoxazole/trimethoprim, levofloxacin, gentamicin, amoxicillin/clavulanic acid, cefoxitin, cefotaxime, and tobramycin. Resistance to ampicillin, piperacillin, and cefalotin was observed most often. These data are consistent with a number of previous studies that were conducted with *E. coli* isolates from commercial and cooked food and fish and seafood in Korea (Ryu et al. 2012a, b). *E. coli* isolates showing ampicillin resistance were also detected at a higher frequency by other investigators (Holvoet et al. 2013; Skočková et al. 2013). With the low incidence of antimicrobial-resistant *E. coli* in fresh vegetable products, the risk of human exposure to antimicrobial-resistant bacteria from the consumption of fresh vegetable products might be low. In addition, the antimicrobial resistance observed in this study was much lower than that observed among hospital isolates in Korea. Of the *E. coli* isolates from hospital, 69 % were resistant to ampicillin, 8 % were resistant to cefoxitin, 36 % were resistant to ciprofloxacin/levofloxacin, and 35 % were resistant to trimethoprim–sulfamethoxazole (Yong et al. 2014). However, the exact level of human exposure should be assessed considering consumer's practice of fresh vegetable consumption and consumption amounts using the quantitative risk assessment tools.

The spread of mobile genetic elements (i.e., plasmids, transposons, and integrons) through horizontal gene transfer has been reported to result in antimicrobial-resistant phenotypes (Barlow 2009; Soledad Ramírez et al. 2010), leading to the multiple antimicrobial resistance observed in this study (Carattoli 2013). Therefore, the principal concern related to the presence of multiple antimicrobial-resistant *E. coli* in the food supply is the pathogenic potential of bacteria in the context of the horizontal transfer of genes associated with antimicrobial resistance; thus, genes from *E. coli* may be transferred to pathogenic microorganisms in the human gastrointestinal tract. Moreover, it is worth noting that FoNAO may act as a reservoir for multiple-resistant bacteria and facilitate the dissemination of resistance genes because fresh vegetable products, such as salad mix and unpasteurized juices, are commonly consumed without further cooking (EFSA 2011).

Biofilms are surface-associated microbial communities, and biofilm formation provides bacteria with protection from a variety of stresses in the environment, including antimicrobial agents and host immune defense systems during infection (Hall-Stoodley and Stoodley 2005). Our results revealed one strain isolated from fresh vegetable juices produces considerable amounts of biofilm, suggesting that other multiple antimicrobial-resistant *E. coli* isolates may reside in the biofilm, making these bacteria difficult to eradicate. Alternatively, rapid de novo evolution of heritable variation can occur in the biofilm (Tyerman et al. 2013), which may result in elevated tolerance to multiple antimicrobials. In either case, *E. coli* isolates that exhibit enhanced biofilm formation can be considered as one of the most important causes of food poisoning outbreaks. Moreover, *E. coli* isolates, which are resistant to more than three antimicrobial agents capable of produce intermediate level of biofilm, were identified in this study. These *E. coli* cannot be easily removed by conventional washing process of fresh vegetable products and can be exposed to human with high probability than *E. coli* of low biofilm formation ability. Although generic *E. coli* itself is considered as hygiene indicator rather than pathogen, the *E. coli* having multiple drug resistance as well as biofilm formation ability can be an important problem for food safety and public health. Intervention technologies to remove biofilm-forming bacteria should be developed using food isolates having typical characteristics.

Although we were unable to sufficiently demonstrate the intraspecies diversity and genetic relationships among the antimicrobial resistance, and biofilm formation activity of *E. coli* isolates, the combined results led us to conclude that such genetic determinants are located in different chromosomal regions. The combined rep-PCR profiles obtained using several primers are likely to provide more discriminative and complex fingerprint patterns; thus, the source of the horizontal spread of antimicrobial determinants to other pathogens can be identified. In addition, a steadfast link between virulence genes and phenotypic characteristics might be established using combined rep-PCR.

In conclusion, this study revealed the antimicrobial resistance patterns, genetic relationships, and biofilm formation ability of *E. coli* isolates from FoNAO in Korea. The data presented here extend our understanding of the diverse genotypes and phenotypes of *E. coli* isolates. Given the importance of antimicrobial-resistant and biofilm-forming *E. coli* in food safety as well as in public health, the results of this study could provide useful information that supports risk assessment and management, thereby developing an intervention technology for antimicrobial-resistant *E. coli*.

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