Prevalence and molecular characterization of *Escherichia coli* isolates during radish sprout production in the Republic of Korea

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Abstract
This study was conducted to investigate the prevalence of coliforms and *Escherichia coli* across radish sprout production facilities in the Republic of Korea and to characterize isolated *E. coli*. A total of 324 samples were collected from three different radish sprout production units in spring and summer. Overall, the levels of coliforms were significantly higher (> 3.75 log CFU/g) in radish sprouts during summer season than in spring. *E. coli* was detected in the pre-harvest (50%) and post-harvest (39%) sprouts and water samples (33%) from farms B and C. Of the 48 *E. coli* isolates, five *E. coli* isolates were positive for the *eaeA* gene, indicative of EPEC O110, two isolates were *eaeA*-positive (EPEC ONT) and one isolate was *ipaH*-positive (ETEC O71). These three *E. coli* strains were identified as novel sequence types by the multilocus sequence-typing assay based on the combination of allelic profiles. In addition, the EPEC O110 isolates obtained from the stream water stored in container of farm C were toxic to *Caenorhabditis elegans*. It is necessary to implement safety measures during the production of radish sprouts to prevent *E. coli* outbreaks.

Keywords: *Escherichia coli*, Radish sprout, Multilocus sequence typing (MLST), Toxicity

Introduction
Due to consumer demands for healthy and natural food products, seed sprouts with antioxidant and anti-tumor properties have gained worldwide popularity [1, 2]. This growing demand has been accompanied by an increase in bacterial outbreaks associated with sprouted seed products [3].

In the last four decades, several outbreaks linked with seed sprouts have been reported in several countries [4]. The world's largest foodborne outbreak was caused by *Escherichia coli* O157:H7, which involved more than 7,000 patients, was associated with white radish sprout consumption in Japan [5]. A massive outbreak of EHEC O104:H4 linked with the fenugreek sprouts caused illness in 6,829 patients and 53 deaths in Germany during the summer of 2011 [6]. In the same year, 29 people were infected with EHEC O26 after consuming raw clover sprouts served in the Jimmy John's restaurant, USA [7]. Moreover, a multistate EHEC O103 outbreak involving 51 people linked to clover sprouts was reported in the USA [8].

Microbial contamination in sprouts can occur from numerous sources, including contaminated seeds, soil, fertilizer, tools, and the water used during the pre- and post-harvest processing stages [9]. Preharvest sprout contamination may occur from substandard water, soil, and the close proximity of cattle rearing facilities [10], while postharvest contamination may occur during packing, transport, and storage [11, 12]. Coliforms are group of closely related harmless bacteria that are widespread throughout the environment including soil and water. The level of coliform bacteria indicates the microbial contamination state of the environment.

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quality of the food product and generally reflects the hygienic practices followed during food production or handling [13]. Laboratory-based monitoring of sprout producing units and the frequent safety evaluation of the farm tools and materials used during the production process can help to identify the source of contamination and prevent disease outbreaks. Thus, we collected samples from all the materials used in sprout production, including seeds, sprouts, water, farming, and packing tools from various sprout production companies and analyzed them for the presence of pathogenic E. coli. The isolated strains were discriminated into subgroups by serotyping and further characterized at the molecular level using multiplex polymerase chain reaction (PCR) and multilocus sequence typing (MLST) and genetic relatedness between the isolates. In addition, the toxicity of the isolates was determined using Caenorhabditis elegans. The results of this study provide information about the prevalence and characteristics of E. coli isolates recovered from radish sprout production environments.

**Materials and methods**

**Sampling**
Three hundred twenty-four samples, including radish seeds (n=18), preharvest sprouts (n=54), postharvest sprouts (n=18), ground water pumped from well (n=18), irrigation water i.e. water passed from well through pipes to sprouts directly (n=18), stream water (n=6). Agricultural materials nutrient solutions A (n=18), B (n=18) and A+B (n=18), cultivation equipments including nutrients storage tank (n=18), fabric (n=18), tray (n=18), basket (n=18) and postharvest equipments knife (n=18), cutting board (n=15), packing material (n=15) and glove (n=18), were collected twice from three radish sprout facilities located in Gyeonggi-do, Republic of Korea, during April–May and August 2016. The radish sprouts were cultivated hygienically in all the farms. The farms A and B used ground water and irrigation water for radish sprout cultivation while farm C used stream water along with ground water for irrigation without filtration or disinfection.

Surfaces of nutrients storage tank, fabric, tray, basket, knife, cutting board and packing material were swabbed with commercial kit, and glove samples were collected using the glove-juice method [14]. Seeds and radish sprouts (200 geach) from the cultivation and postharvest steps were collected and placed in sterile plastic bags. For hydroponic farming, the farms diluted nutrient solutions A and B with water and delivered them through irrigation water to the sprouts two days post germination. Two-liter water and nutrient solutions were individually collected in sterile bottles.

**Analysis of coliform and Escherichia coli**
For the quantitative analysis of coliforms and E. coli, 25 g of seed and sprouts samples were placed in 225 ml of 0.1% peptone water (PW) (Oxoid, Basingstoke, UK) and homogenized in a BagMixer® (Interscience, Saint-Nom-la-Bretèche, France). Surface samples were homogenized using a vortex prior to dilution. These pretreated samples were serially diluted 1:10 in 0.1% PW and 1 ml aliquots were inoculated on 3 M™ Petrifilm™ E. coli/coliform count plates (3 M Microbiology, Minnesota, USA), followed by incubation at 37 °C for 24–48 h. Water samples were evaluated for coliforms and E. coli using the Colilert-18 detection kit from IDEXX laboratories (Westbrook, Maine, USA).

For the qualitative analysis of E. coli and E. coli O157:H7, 25 g of seed and sprouts were placed in 225 ml of E. coli broth (EC) (Oxoid) and modified tryptophan soy broth (mTSB) (Oxoid) for E. coli and E. coli O157:H7 detection, respectively, and homogenized in a BagMixer®. For the swab and glove samples, 1 ml of homogenate was dispensed into 9 ml of EC broth and mTSB individually. Water samples (250 ml) were filtered using a 0.45 μm membrane filter (Merck-Millipore, Carrigtwohill, Ireland). Each filter was placed in sterile bottles containing 40 ml of EC broth or mTSB and incubated at 44 °C and 37 °C for 24 h, respectively. The enriched cultures were streaked onto eosin-methylene blue agar plates (Oxoid) and CHROMagar™ E. coli O157 (CHROMagar™, Paris, France) plates for E. coli and E. coli O157:H7 using a disposable loop. After incubation at 37 °C for 24 h, typical colonies were picked and identified using the VITEK 2 system, an automated system that performs microbial identification and antibiotic susceptibility testing (bioMérieux-Vitek, USA). Additionally, the E. coli isolates were further identified and classified by 16s rRNA sequencing (Macrogen, Seoul, Korea) [15].

**Serotyping and virulence determination**
Colonies of E. coli and E. coli O157:H7 were serotyped using Escherichia coli O-antigen antisera kit (Joong Kyeom, Republic of Korea). Genomic DNA was extracted from the isolates and retention of virulence determinants (eaeA, bfpA, ipaH, aggR, vt1, vt2, lt, st) in the isolates were determined by PCR using the PowerChek™ Diarrheal E. coli 4-plex PCR Detection Premix I/Premix II kit (KogeneBioTech Co., Ltd., Republic of Korea) [16].

**Multi locus sequence typing (MLST)**
To determine clonal relationship of the E. coli isolates, genomic DNA of the isolates was prepared and the housekeeping genes (Table 3) were amplified by PCR. The resulting DNA fragments were purified and sequenced
(GnCBio Co. Ltd., Republic of Korea). Sequence types (STs) of the E. coli isolates were assigned using the Bacterial Isolate Genome Sequence Database (BIGSdb) tool from the PubMLST server.

**Cytotoxicity assay**

The nematode Caenorhabditis elegans killing assay was carried out to evaluate the pathogenicity of E. coli isolates in compliance with Lee's method with modifications [17]. Overnight cultures of E. coli isolates possessing pathogenic genes (2 x 10⁹ CFU/ml) grown in Luria–Bertani broth at 37 °C for 18 h were spread on nematode growth medium (NGM). The nematodes were synchronized by hypochlorite bleaching and subsequently cultured on NGM in the presence of E. coli. Repeatedly, synchronized L1 larvae were transferred to a new plate containing E. coli OP50, allowing the production of L4 larvae serially. After three days, 20 L4/young adult hermaphrodites were placed on NGM, incubated at 25 °C for 45 min, and transferred to a new plate every single day. The survival of C. elegans without the presence of progeny and L4 nematodes was examined using the Kaplan–Meier method, and differences were determined with log-rank test.

**Results and discussion**

**Assessment of coliforms and E. coli contamination in radish seed sprouts production units**

The levels of coliforms and E. coli in the samples from sprouts, farming tools, and water from farms A, B, and C are presented in Tables 1 and 2. Of the 324 samples collected during spring and summer, the levels of coliforms were higher (>3.75 log CFU/g) in radish sprouts in summer than in spring. Coliforms were more prevalent in tray (4.96 ± 1.44), cutting board (5.62 ± 0.59) and glove (4.30 ± 0.00) from farm A and basket (5.57 ± 0.49), cutting board (5.40 ± 0.93) and glove (4.82 ± 0.11) from B during summer season compared to all the other samples including water (<2 log CFU/ml), agricultural materials (<3 log CFU/ml). Corresponding to our findings, several studies have reported increased microbial levels in postharvest sprouts than in seeds [18, 20]. The incidence of E. coli was significantly higher in the preharvest (50%) and postharvest (39%) sprouts and water samples (33%) from farms B and C. E. coli was detected in the basket (5%), glove (16%), and knife (11%) samples, while no E. coli were detected in the other samples.

Seeds usually acquire 10² to 10³ CFU/g of coliforms from the surrounding environment [21]. These bacterial populations can significantly increase during the sprouting process to as high as 10⁸ to 10¹¹ CFU/g [22, 23] without damaging the appearance of the final product [24]. Unaware of the contaminated seeds (0.90 ± 0.85 log CFU/g), farms B and C used the seeds for cultivation during the summer season, which resulted in a large number of coliforms in the pre- and post-harvest radish sprouts (3.75–4.92 log CFU/g).

Water can potentially cross-contaminate sprouts by transferring microbes from other sources during production and processing [25]. In this study, the seeds used for hydroponic germination in farms A and C during spring and summer were free of E. coli contamination. However, E. coli was detected in the preharvest and postharvest radish sprouts and water including ground water, irrigation water and stream water stored in container. Since water was the only source that was in contact with the seeds during sprouting, it is coherent that water transmitted E. coli to vulnerable seeds during irrigation and contaminated the sprouts. Even though knives and cutting boards were washed with water by the farms, they can still be contaminated during processing by transmitting E. coli from contaminated to non-contaminated fresh produce [26]. For instance, in this study, no coliforms were detected in the seeds and preharvest radish sprouts of farm A during the spring season, but during harvesting, the coliforms were transferred to the postharvest radish sprouts via contaminated knives (3.69 ± 0.03 log CFU/g) and gloves (2.84 ± 2.46 log CFU/g) used by the workers.

**Molecular characterization and virulence determination of E. coli isolates**

The E. coli isolates were discriminated into virulent and avirulent strains by identifying the presence of pathogenic genes in their genome. Of the 48-suspected E. coli isolates, only eight isolates from farms B and C were identified as pathogenic based on the results of serotyping and virulence determination tests (Table 3). Eight isolates belonged to the O-serogroup, five isolates and one isolate from water were assigned as serotypes O110 and O71, respectively, and the other two isolates were non-typeable O-antigens (ONT). Pathogenic E. coli possess certain combinations of virulence genes that cause food poisoning [27]. Based on the combination of virulence genes, E. coli strains can be classified as enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC), and enterohemorrhagic (EHEC) [28]. The O110 and ONT isolates possessed the virulence gene eaeA (E. coli attaching and effacing), while the O71 isolate contained the ipaH (invasion plasmid antigen H) gene, which designates these isolates as EPEC O110, EPEC ONT, and ETEC O71 (Table 3).

Escherichia coli strains with serotype O110 were previously isolated from various sources in different countries, including healthy adults in Japan [29], dairy samples in France [30], and ovine strains in Spain [31]. However, all
Table 1 Evaluating the frequency of coliforms (log CFU/g) on radish seed sprouts and their production environment

| Source                      | No. of samples | Sample                  | A (Spring) | Summer | B (Spring) | Summer | C (Spring) | Summer | Total     |
|-----------------------------|----------------|-------------------------|------------|--------|------------|--------|------------|--------|-----------|
| Seeds and Sprouts           | 18             | Seeds                   | ND         | ND     | 0.90 ± 0.85| ND     | 0.49 ± 0.85| ND     | 0.23 ± 0.38|
|                             | 18             | 2nd Day sprouts         | ND         | 1.54 ± 0.21 | ND     | 4.92 ± 0.51| ND     | 3.75 ± 0.05| 1.7 ± 2.15|
|                             | 18             | 4th Day sprouts         | ND         | ND     | 4.83 ± 0.02| ND     | 4.08 ± 0.25| ND     | 1.49 ± 2.31|
|                             | 18             | 6th Day sprouts         | ND         | ND     | 4.77 ± 0.11| ND     | 4.60 ± 0.10| ND     | 1.56 ± 2.42|
|                             | 18             | Packaged sprout         | 4.65 ± 0.24| ND     | 4.21 ± 0.34| ND     | 3.99 ± 0.09| ND     | 2.14 ± 2.36|
| Water                       | 18             | Ground water            | ND         | 0.67 ± 1.15| ND     | ND         | ND     | 0.11 ± 0.27|
|                             | 18             | Irrigation water        | ND         | ND     | ND         | ND     | 0.98 ± 1.71| ND     | 0.16 ± 0.4 |
|                             | 6              | Stream water stored in container | NS | NS | NS | NS | 1.09 ± 1.88 | ND | 0.18 ± 0.44 |
| Agricultural materials      | 18             | Nutrient solution A     | ND         | ND     | 1.37 ± 2.38| ND     | ND         | ND     | 0.22 ± 0.56|
|                             | 18             | Nutrient solution B     | ND         | ND     | ND         | ND     | ND         | ND     | 0           |
|                             | 18             | Diluted nutrient solution A + B | ND | 3.08 ± 0.07 | ND | ND | ND | ND | 0.51 ± 1.26|
| Equipment                   | Cultivation    | Nutrient solution storage tank | ND | ND | 0.77 ± 1.33 | ND | ND | ND | 0.13 ± 0.31|
|                             | 18             | Fabric                  | ND         | 1.23 ± 2.14| ND     | 2.00 ± 0.00| ND     | 0.54 ± 0.87|
|                             | 18             | Tray                    | 0.40 ± 0.70| 4.96 ± 1.44 | ND     | 4.34 ± 0.35| ND     | 0.22 ± 0.38| ND | 1.65 ± 2.33|
|                             | 18             | Basket                  | ND         | 1.70 ± 3.10| ND     | 5.57 ± 0.49| ND     | 0.97 ± 0.85| ND | 1.37 ± 2.17|
| Post-harvest               | 18             | Knife                   | 3.69 ± 0.03| 3.48 ± 0.26 | 3.07 ± 2.73| 3.86 ± 0.49| 3.59 ± 0.57| 0.67 ± 1.15| ND | 3.06 ± 1.2 |
|                             | 15             | Cutting board           | NS         | 5.62 ± 0.59| ND     | 5.40 ± 0.93| ND     | 3.91 ± 0.19| ND | 2.49 ± 2.79|
|                             | 15             | Packing material        | NS         | 1.49 ± 1.31| ND     | 1.60 ± 2.78| ND     | 0.23 ± 0.41| ND | 0.55 ± 0.77|
|                             | 18             | Glove                   | 2.84 ± 2.46| 4.30 ± 0.00 | ND     | 4.82 ± 0.11| ND     | 4.12 ± 0.29| ND | 2.68 ± 2.18|
|                             | Total          |                         | 0.61 ± 1.42| 1.48 ± 1.89 | 0.23 ± 0.76| 2.53 ± 2.26| 0.53 ± 1.18| 1.19 ± 1.82| ND | 1.09 ± 0.99|

* All values shown in this table are the mean ± standard deviation of three independent experiments. *The values for each sample mentioned in this table are the average of three replicates. *ND not detected, *NS not sampled.
Table 2 Evaluating the frequency of *Escherichia coli* detection on radish seed sprouts and their production environment

| Source                     | No. of samples | Sample          | A           | B           | C           | Total         |
|---------------------------|----------------|-----------------|-------------|-------------|-------------|---------------|
|                           |                |                 | Spring      | Summer      | Spring      | Summer        |               |
| Seeds and Sprouts         | 18             | Seeds           | 0          | 0           | 0           | 3/3 (100%)    | 0             | 0             | 3/18 (16%)    |
|                           | 18             | 2nd Day sprouts | 0          | 1/3 (33%)   | 1/3 (33%)   | 2/3 (66%)     | 3/3 (100%)    | 1/3 (33%)     | 8/18 (44%)    |
|                           | 18             | 4th Day sprouts | 0          | 0           | 3/3 (100%)  | 1/3 (33%)     | 3/3 (100%)    | 2/3 (66%)     | 9/18 (50%)    |
|                           | 18             | 6th Day sprouts | 0          | 0           | 0           | 0             | 3/3 (100%)    | 0             | 3/18 (16%)    |
|                           | 18             | Packaged sprout | 0          | 1/3 (33%)   | 1/3 (33%)   | 2/3 (66%)     | 0             | 3/3 (100%)    | 7/18 (39%)    |
| Water                     | 18             | Ground water    | 0          | 0           | 0           | 0             | 3/3 (100%)    | 3/3 (100%)    | 6/18 (33%)    |
|                           | 18             | Irrigation water| 0          | 1/3 (33%)   | 1/3 (33%)   | 0             | 1/3 (33%)     | 0             | 3/18 (16%)    |
|                           | 6              | Stream water stored in container | NS | NS | NS | NS | 3/3 (100%) | 0 | 3/6 (50%) |
| Agricultural Materials    | 18             | Nutrient solution A | 0 | 0 | 0 | 0 | 0 | 0 | 0/18 (0%) |
|                           | 18             | Nutrient solution B | 0 | 0 | 0 | 0 | 0 | 0 | 0/18 (0%) |
|                           | 18             | Diluted nutrient solution A + B | 0 | 0 | 0 | 0 | 0 | 0 | 0/18 (0%) |
| Equipment Cultivation     | 18             | Nutrient solution storage tank | 0 | 0 | 0 | 0 | 0 | 0 | 0/18 (0%) |
|                           | 18             | Fabric          | 0          | 0           | 0           | 0             | 0             | 0             | 0/18 (0%)    |
|                           | 18             | Tray            | 0          | 0           | 0           | 0             | 0             | 0             | 0/18 (0%)    |
|                           | 18             | Basket          | 0          | 0           | 0           | 0             | 1/3 (33%)     | 0             | 1/18 (5%)    |
| Post-harvest              | 18             | Knife           | 0          | 2/3 (66%)   | 0           | 0             | 0             | 0             | 2/18 (11%)   |
|                           | 15             | Cutting board   | NS         | 0           | 0           | 0             | 0             | 0             | 0/15 (0%)    |
|                           | 15             | Packing material | NS        | 0           | 0           | 0             | 0             | 0             | 0/15 (0%)    |
|                           | 18             | Glove           | 0          | 0           | 0           | 0             | 3/3 (100%)    | 0             | 3/18 (16%)    |
| Total                     | 0              |                 | 0          | 5/57 (9%)   | 6/57 (10%)  | 8/57 (14%)    | 17/57 (30%)   | 12/57 (21%)   | 48/324 (14%)  |

Total—number of *E. coli* positive / number of tested samples. *The values for each sample mentioned in this table are the average of three replicates. *NS not sampled
the O110 isolates possessed either one of the Shiga-toxin genes (stx1 and stx2), but none were eaeA-positive. The eaeA gene codes for the surface protein Intimin, a virulence factor usually found in EPEC (O127:H6) and EHEC (O157:H7) strains [32]. Intimins localize to the outer membrane of E. coli cells and promote intimate bacterial adhesion to the intestinal epithelia, resulting in the formation of effacing lesions that are necessary for causing EPEC and EHEC diarrhea [33]. The ETEC O71 serotype strains were isolated from patients with enteric illness in Brazil [34] and Vietnam [35]. The similarity between the strains was the presence of the eaeA gene; however, the strain isolated in our study was eaeA-negative and ipaH-positive (Table 3). The ipaH gene is a type 3 effector that facilitates the penetration of pathogens into intestinal cells [36]. The MLST database revealed three different STs that did not exactly match with other STs in the database (Table 3), indicating that the isolates could be new subtypes.

Evaluation of cytotoxicity of E. coli isolates using Caenorhabditis elegans

The cytotoxicity of the E. coli isolates was determined by comparing the survival rate of isolate-exposed C. elegans with that of those exposed to E. coli OP50. The survival rate of those exposed to the E. coli isolate EPEC O110 was significantly reduced from day 8 compared to E. coli OP50, which showed a reduction on day 12, indicating that the isolate EPEC O110 is pathogenic (Fig. 1). The isolates EPEC ONT and ETEC O71 caused survival rates similar to those of E. coli OP50, indicating that the isolates were non-pathogenic. Merkx-Jacques et al. (2013) demonstrated that the E. coli isolates that were predicted to be pathogenic by genotyping assays were able to kill C. elegans [37]. Correspondingly, in our study, only one of the eight isolates was a toxin producing E. coli.

**Conclusion**

The aim of this study was to identify the source of E. coli contamination in commercial radish production units and emphasize the need to minimize contamination by following good agricultural practices during sprout production and good hygienic practices during processing, packaging, and shipping. Genotyping and toxicity studies revealed that the pathogenic E. coli strain EPEC O110 was more prevalent in the stream water stored in the containers from farm C. The contaminated water may have potentially transferred pathogenic E. coli to the germinated seeds and sprouts during production and processing. In addition, the knives, cutting boards, trays and

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**Table 3** Serological and genotypic characterization of Escherichia coli isolates

| E. coli isolate | Farm | Season | Source | No. of isolates | Virulence genes | E. coli allelic profile (MLST) | ST |
|-----------------|------|--------|--------|----------------|----------------|-----------------------------|----|
| EPEC O110       | C    | Spring | Container water | 5 | + | 1 23 11 9 2 144 46 137 | New1 |
| EPEC ONT        | C    | Spring | Container water | 2 | + | 1 23 11 9 2 144 46 137 | New2 |
| ETEC O71        | B    | Summer | Irrigation water | 1 | - | 11 35 4 52 125 146 141 2 | New3 |

* The numbers in the MLST column represent the number of alleles per locus; ST sequence types.
gloves used during the processing of sprouts may have transmitted the pathogens through cross-contamination. The prevalence of diarrhea causing E. coli in the water that is stored in the container in radish sprout production units accentuates the need to sanitize stream water before irrigation and routinely clean the containers and other equipments at regular intervals.

Authors' contributions
NR and JHY contributed to the writing of the manuscript and performed the majority of data analysis. BY, NBH, WK, HK performed experiments and prepared raw materials. BYP contributed to the discussion of experimental results. SRK planned and led this research. All authors read and approved the final manuscript.

Funding
This study was funded by the National Institution of Agricultural Science, Rural Development Administration, Republic of Korea.

Availability of data and materials
All the data analyzed during this study are included in this article.

Competing interests
The authors declare that they have no competing interests.

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Received: 23 November 2020 Accepted: 5 February 2021
Published online: 08 March 2021

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