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Development of a Screening Method and Device for the Detection of *Escherichia coli* from Agri-Food Production Environments and Fresh Produce

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This study was conducted to develop a screening method using Colilert-18 and a device for the detection of *E. coli* from agri-food production environments and fresh vegetables. The specificity and sensitivity of Colilert-18 by temperature (37°C and 44°C) were evaluated with 38 *E. coli* and 78 non-*E. coli* strains. The false-positive rate was 3.8% (3/78) and 0% (0/78) at 37°C and 44°C, respectively. The detection limit of *E. coli* at 37°C at <1.0 log CFU/250 ml was lower than that at 44°C. The efficiency of the developed device, which comprised an incubator equipped with a UV lamp to detect *E. coli* in the field, was evaluated by measuring the temperature and UV lamp brightness. The difference between the set temperature and actual temperature of the developed device was about 1.0°C. When applying the developed method and device to various samples, including utensils, gloves, irrigation water, seeds, and vegetables, there were no differences in detection rates of *E. coli* compared with the Korean Food Code method. For sanitary disposal of culture samples after experiments, the sterilization effect of sodium dichloroisocyanurate (NaDCC) tablets was assessed for use as a substitute for an autoclave. The addition of one tablet of NaDCC per 50 ml was sufficient to kill *E. coli* cultured in Colilert-18. These results show that the developed protocol and device can efficiently detect *E. coli* from agri-food production environments and vegetables.

Keywords: *E. coli*, Colilert-18, NaDCC, device, on-site detection

Introduction

The Centers for Disease Control and Prevention in the United States (CDC) and the European Food Safety Authority (EFSA) have reported an increase in foodborne illness associated with fresh produce in recent years [1, 2]. The latest numbers provided by EFSA indicate that, in Europe, outbreaks associated with food of non-animal origin corresponded to 5% of the total foodborne outbreaks in 2007, but this percentage increased to 30% in 2011 [3]. Increased consumption and larger scale production and distribution of produce over the past two decades are factors that might have contributed to the increase in the number of foodborne outbreaks [4]. Thus, it is important to monitor the sanitation of the water, gloves, and utensils used by workers, as well as the vegetables themselves, to control the microbial safety of fresh vegetables.

Escherichia coli, coliform bacteria, and Enterobacteriaceae are commonly used as indicator organisms of overall food hygiene conditions. Among these, *E. coli* is often preferred as an indicator because it is specific and most reliably reflects fecal origin. For the detection of *E. coli*, a method based on microbial enzyme profiles has been used as an attractive alternative to traditional methods. In this method,

two active substrates, *O*-nitrophenyl-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG), are combined to detect the bacterium. *E. coli* produces galactosidase, which hydrolyzes ONPG, producing a yellow color change. *E. coli* also produces β-glucuronidase, which hydrolyzes MUG to form a fluorescent compound that can be detected when viewed under an ultraviolet (UV) lamp [5]. The Colilert-18 defined-substrate technology system (Idexx Laboratories, Inc., USA) is intended to analyze total coliforms and *E. coli*. The Colilert-18 assay system is based on a defined substrate medium containing MUG and ONPG and is used for the one-step detection of both total coliforms and *E. coli* in water samples. The Colilert-18 assay system allows detection of *E. coli* within 18 h, and this rapid and simple method has been adopted for water analysis by the US Environmental Protection Agency [6] and the Korean Ministry of Environment [7]. The Colilert-18 assay system has been validated on different matrices, including raw meat, fresh-cut products, and water from marine and other sources [8–13]. Many researchers have reported that *E. coli* analysis based on enzyme reaction is very easy and efficient. To date, no studies have been published on the assessment and application of the method for samples from agricultural environments, including seeds, utensils, and workers' gloves, to monitor hygienic conditions.

In order to analyze *E. coli* using this method, specialized equipment, such as an incubator, UV lamp, and autoclave, is required. However, it is difficult to equip farms and post-harvest facilities with these devices because they are expensive and require a lot of space for installation. Thus, it is important to develop a device to detect *E. coli* inexpensively and easily, incorporating the technology to sterilize bacteria after experimental completion for sanitary disposal. For this reason, the development of a small-scale device equipped with a UV lamp and an incubator would simplify the equipment needed to detect *E. coli* in the agri-food industry. Moreover, a method of sanitary disposal using sodium dichloroisocyanurate (NaDCC) tablets, which have been used to disinfect drinking water, could replace an autoclave. NaDCC releases free available chlorine in the form of hypochlorous acid, an effective microbicide against a wide range of bacteria, viruses, and parasites [14]. To date, no studies have been published on the development and validation of a device for the detection of *E. coli* that includes technology for sanitary disposal of samples and can be used in agri-food industrial situations.

Therefore, the purpose of this study was to develop a device and screening method for the detection of *E. coli* that can be easily employed in agri-food environments.

Materials and Methods

Bacterial Strains and Growth Conditions

Thirty-eight *E. coli* strains and 78 non-target strains (listed in Table 1) were used in this study. These strains were obtained from the American Type Culture Collection (ATCC, USA), the Korean National Culture Collection for Pathogens (NCCP, Korea), the Korean Agricultural Culture Collection (KACC, Korea), the Korean Culture Center of Microorganisms (KCCM, Korea), and the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Korea). All strains were stored at −80°C in 20% glycerol.

Evaluation of Colilert-18 for the Detection of *E. coli* from Fresh Produce and Production Environments

Evaluation of the specificity of Colilert-18. To evaluate the specificity of the Colilert-18 reagent (IDEXX Laboratories), *E. coli* strains grown on tryptic soy agar (TSA; Oxoid, UK) were inoculated in 2 ml of tryptic soy broth (TSB; Oxoid) in a 24-well microtiter plate (SPL, Korea) and incubated for 16 h at 37°C. Some non-*E. coli* strains (*Salmonella* spp., *Cronobacter* spp., *Staphylococcus aureus*, *Shigella* spp.) and others were grown in 2 ml of TSB in 24-well microtiter plates for 24 h at 37°C and 30°C, respectively. Colilert-18 reagent was dissolved in 100 ml of sterile distilled water, and 2 ml of the Colilert-18 solution was added to 24-well microtiter plates. An inoculum (10 μl) of each of the 38 *E. coli* and 78 non-target bacteria was added individually to duplicate wells of the 24-well microtiter plates containing Colilet-18. Each plate was incubated at 37°C or 44°C for 18 h. The plates were then exposed to UV light (365 nm), and fluorescence and color changes were observed. Yellow-colored wells and those that fluoresced UV light were considered positive.

Evaluation of the sensitivity of Colilert-18. The detection limits for *E. coli* were assessed to determine the sensitivity of Colilert-18. Different volumes of Colilert 18 were required to detect *E. coli* from various samples, including working utensils, gloves, water, seeds, and produce. In general, utensil surfaces were sampled using a swab kits (3MTM pipette swab; 3M Korea Ltd) containing 10 ml of saline. Glove and hand samples were collected using the glove juice method; that is, the hand or glove was rinsed with 50 ml of saline in a sterilized pack. For water, 100 ml of sampled water was used in the test. For produce, 225 ml of saline was added to 25 g of each sample, and a total of 250 ml of the sample was used for the analysis. Thus, the sensitivity was evaluated in 10, 50, 100, and 250 ml of Colilert-18 solution. Seven *E. coli* strains (*E. coli* NCCP 13715 (enterohemorrhagic *Escherichia coli*), 13718 (enterotoxigenic *Escherichia coli*), 13719 (enteroinvasive *Escherichia coli*), 13721 (enterohemorrhagic *Escherichia coli*), and three strains (non-pathogenic *E. coli*) isolated from sprouts) were used to evaluate the sensitivity of Colilert-18. Each strain was cultured in 10 ml of TSB at 37°C for 16 h. The cultures were then centrifuged at 2,000 ×*g* for 10 min at 4°C. Cells were washed twice with 10 ml of phosphate-buffered saline (PBS; Oxoid Ltd.) and resuspended in 10 ml of PBS. These cell suspensions were serially diluted to

Table 1. Bacteria used for evaluation of the specificity of Colilert-18.

Group	Tested bacteria and source[1)]
Target strains (38)	Pathogenic *Escherichia coli* (NCCP 13715(EPEC), NCCP 13717(ETEC), NCCP 13718(ETEC), NCCP 13719(EIEC), NCCP 13721, ATCC 43890, ATCC 35150, ATCC 43889 (EHEC)) Non-pathogenic *Escherichia coli* (ATCC 25922, ATCC 35218, radish seed(3), radish sprout(12), water(8), growing container(2), wash tray, spin-dryer, worker's gloves)
Non-*Target strains* (78)	*Salmonella* Agona NCCP 12231, *Salmonella* Bovismorbificans NCCP 13691, *Salmonella* Braenderup NCCP 13697, *Salmonella* Corvallis NCCP 13705, *Salmonella* Derby NCCP 12238, *Salmonella* Dublin NCCP 12232, *Salmonella* Enteritidis (ATCC 13076, ATCC 4931), *Salmonella* Arizonae ATCC 13314, *Salmonella* Give NCCP 13696, *Salmonella* Heidelberg NCCP 13698, *Salmonella Houtenae* ATCC 43974, *Salmonella* Infantis NCCP 12233, *Salmonella* London NCCP 10357, *Salmonella* Mabandaka NCCP 13695, *Salmonella* Montevideo NCCP 10140, *Salmonella* Newport NCCP 12235, *Salmonella* Panama NCCP 13694, *Salmonella* Paratyphi (NCCP 12213, NCCP 14759), *Salmonella* Thompson NCCP 13578, *Salmonella* Typhi (ATCC 19946, NCCP 13699), *Salmonella* Typhimurium (ATCC 13311, ATCC 19586, ATCC 43174, DT 104), *Salmonella* Virchow NCCP 13688 *Shigella boydii* (ATCC 8700, ATCC 12031, ATCC 29928), *Shigella dysenteriae* ATCC 13313, *Shigella flexneri* (ATCC 12022, NCCP 11203), *Shigella sonnei* (ATCC 25981, NCCP 16122) *Staphylococcus aureus* (ATCC 13565, ATCC 23235, ATCC 27664) *Yersinia frederiksenii* NCCP 11121, *Yersinia enterocolitics* (NCCP 12713, NCCP 10830), *Listeria monocytogenes* (ATCC 19113, ATCC 19115, ATCC 19119), *Listeria innocua* CCARM 17, *Listeria ivanovii* CCARM 18 *Pantoea ananatis* KACC 10059, *Pantoea stewartii subsp. Indologenes* KACC 10540, *Providencia vermicola* KACC 12350, *Providencia stuartii* KACC 10188 *Enterobacter aerogenes* KACC 13732, *Enterobacter cloacae* KACC 10526, *Enterobacter* spp. (sprout) *Enterococcus faecalis* KACC 11304 *Acinetobacter* spp.(cabbage), *Acinetobacter calcoacetius* (sprout) *Cronobacter sakazakii* (ATCC 29544, barley, fried pepper) *Citrobacter braakii* (sprout), *Citrobacter freudii* KACC 11404 *Hafnia alvei* CCARM 13 *Klebsiella pneumoniae* (stream water(3), sprout(3)) *Kocuria kristinae* (carrot) *Ochrobacterium* spp. (Korean chive) *Pectobacterium carotovorum* KACC 10057, *Pseudomonas aeruginosa* (lettuce, pepper), *Pseudomonas chlororaophis* KACC10150, *Pseudomonas corrugate* KACC10141

[1)]ATCC, American Type Culture Collection; NCCP, Korean National Culture Collection for Pathogens; KACC, Korean Agricultural Culture Collection; CCARM, Culture Collection of Antimicrobial Resistant Microbes.

$<10^1$/ml. One milliliter of each 10^1–10^3 cell suspension of *E. coli* was used to inoculate 9, 49, 99, and 249 ml of Colilert-18 solution, respectively. Inoculated Colilert-18 was incubated at 37°C and 44°C for 18 h, respectively. Color and fluorescence were observed using a UV light box (IDEXX Laboratories) after incubation.

Application of Colilert-18 for the detection of *E. coli* from fresh produce and irrigation water. The feasibility and optimum temperature for detecting *E. coli* using Colilert-18 were determined using vegetables and water. Baby leafy vegetables, radish sprouts, and stream water were used for the cultivation of napa cabbage, since there have been reports that *E. coli* is frequently detected in these samples. Vegetables, including baby leafy vegetables (n = 5) and radish sprouts (n = 5), were purchased from a local supermarket in Wanju, Jeollabuk-do Province. Two-liter water samples (n = 10) were collected from a stream near napa cabbage-growing farms located in Pyeonchang, Gangwon Province. Two detection methods, including that prescribed by the Korean Food Code and enzymatic analysis using Colilert-18, were used for this experiment. For vegetables, 100 g of each vegetable sample was placed in a sterile pack containing 500 ml of distilled water. Vegetable samples were homogenized using a stomacher (Bagmixer 400VW; Interscience, France) for 2 min at low speed. In the test using the Korean Food Code method, 125 ml aliquots of the rinsate were combined with 125 ml of 2× EC broth (Oxoid Ltd.) prior to incubation at 37°C or 44°C for 18 h. A loopful of each enrichment culture was then streaked onto eosin methylene blue (EMB) agar (Oxoid Ltd.). After incubation at 37°C for 24 h, the identity of suspected colonies was confirmed with VITEK (bioMerieux, France). In the enzymatic analysis using Colilert-18, 125 ml aliquots of the rinsate were combined with 125 ml of 2× Colilert-18 solution. After incubation at 37°C or 44°C for 18 h, the samples were exposed to UV light (365 nm). Aliquots (0.1 ml) of suspected positive samples were inoculated in 10 ml of EC broth and incubated at 44°C for 18 h. Enriched cultures were streaked onto EMB using a disposable loop. Plates were incubated at 37°C for 24 h, and typical colonies were picked and identified using VITEK. In the water experiment using EC broth, 100 ml of each water sample was filtered using a membrane filter (pore size: 0.45 μm, diameter 47 mm; Millipore,

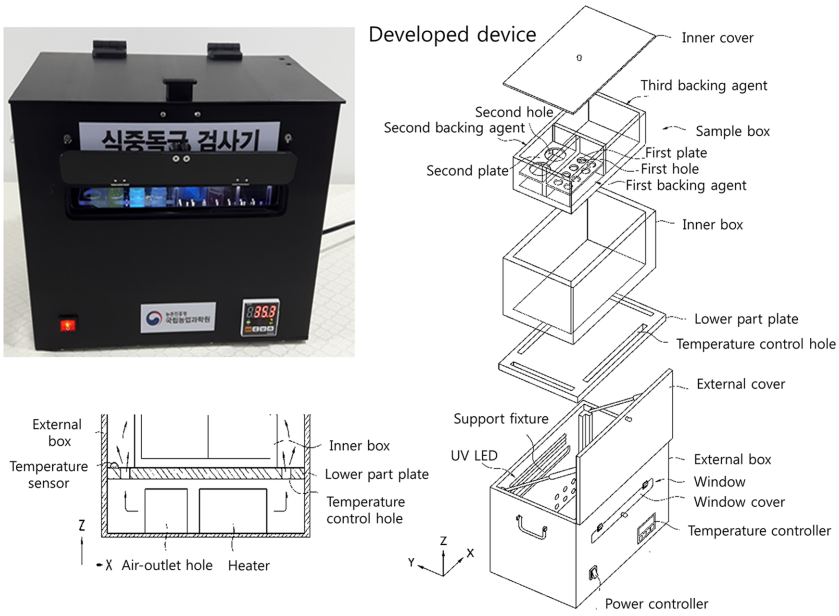

Fig. 1. Schematic diagram of the developed device.

USA). Then, filters were placed in sterile bottles containing EC broth and incubated at 37°C or 44°C for 18 h. Each enriched culture was streaked onto EMB using a disposable loop. Inoculated plates were incubated at 37°C for 24 h. For the enzymatic analysis using Colilert-18, 100 ml of water was added to a sterile bottle and one capsule of Colilert-18 powder sufficient for 100 ml was added to each sample. After incubation at 37°C or 44°C for 18 h, the samples were exposed to UV light (365 nm). Subsequent procedures were as described in the aforementioned vegetable sample test.

Development and Validation of a Device for the Detection of *E. coli* in Agri-Food Production Environments

Development and assessment of a device for the detection of *E. coli*. A device for the detection of *E. coli*, based on specific enzymatic activity, was developed for agri-food production environments where there is a lack of suitable instrumentation. The device is an incubator and UV light source combination (Fig. 1) measuring 35 × 20 × 30 cm in size with a temperature control range of 20°C–55°C. The inside of the instrument is equipped with a 365-nm UV LED lamp for detecting fluorescence after culturing positive *E. coli* samples in Colilert-18. In order to check the temperature control capability of the device, the internal actual temperature was measured using a thermometer with a built-in temperature recorder (μ10000 Recorder; Yokogawa, China). The brightness of the UV LED lamp was measured using an illuminometer (LUX meter, LX-101; Lutron, Taiwan).

Validation of the combination of Colilert-18 and the developed device using various samples. The performance of Colilert-18 using the developed device for detecting *E. coli* was assessed using various samples, including knives (n = 9), chopping boards (n = 9), gloves (n = 10), radish seeds (n = 5), alfalfa seeds (n = 5), baby leaf sprouts (n = 10), radish sprouts (n = 10), and water (n = 10). Equipment, including knives and chopping boards, and the gloves used to harvest sprouts, were collected from three radish sprout farms located in Uiwang, Gyeonggi-do Province. The knives and chopping boards were swabbed with a swab kit used to test for *E. coli* contamination, and glove samples were collected using the glove juice method. Vegetables and seeds were purchased from a local supermarket in Wanju, Jeollabuk-do Province, and 2 L water samples (n = 10) were collected from a stream near napa cabbage farms located in Pyeonchang, Gangwon Province. Both the Korean Food Code method and enzymatic analysis using Colilert-18 were used to validate the combination of Colilert-18 and our developed device. In tests using the Korean Food Code method, each sample was homogenized using a vortex and stomacher. For the knife and chopping board samples, 1 ml of each homogenate was dispensed into 9 ml of EC broth. For the gloves, 10 ml of homogenate was dispensed into 40 ml of 1.25× EC broth. For the vegetables and seeds, 100 g of each vegetable sample was placed in each sterile pack containing 500 ml of distilled water. Vegetable samples were homogenized using a stomacher for 2 min at low speed. A 125-ml aliquot of the rinsate was combined with 125 ml of 2× EC broth. For water, 100 ml samples of water were filtered using membrane filters, as described above, and then placed in sterile bottles containing EC broth. All pretreated samples were cultured at 37°C for 18 h in an incubator. A loopful of each culture was then streaked on EMB, and the plates were incubated at 37°C for 24 h. The morphology of each colony on EMB was observed and typical colonies were picked on EMB and identified using VITEK. In the enzymatic analysis using Colilert-18, a 1-ml aliquot of each knife and chopping board homogenate was dispensed into two 9-ml aliquots of Colilert-18,

respectively. For the gloves, 10 ml of each homogenate was added to two 40-ml aliquots of 1.25× Colilert-18. Vegetables and seeds were treated as described above, and 125-ml aliquots of each rinsate were individually combined with two 125-ml samples of 2× Colilert-18. For water, 100-ml samples were added to two sterile bottles with one capsule of Colilert-18 powder. Each pretreated sample was cultured in two instruments (an incubator and the device developed in this study) at 37°C for 18 h. The samples cultured in the incubator were exposed to UV light (365 nm) using a UV light box. The samples cultured in the developed device were exposed to the UV light within the device. Then, 0.1-ml aliquots of suspected positive samples were inoculated in 10 ml of EC broth and incubated at 44°C for 18 h. Enriched cultures were streaked onto EMB using a disposable loop. The plates were incubated at 37°C for 24 h, and typical colonies were picked on EMB and identified using VITEK.

Development of a method for sanitary disposal of cultured samples using NaDCC. For sanitary disposal of cultured samples at the end of the experiment, the microbial sterilization effect of NaDCC tablets (Aquatabs; Medentech Ltd, Ireland) was evaluated as a substitute for autoclave sterilization. Seven *E. coli* strains used in the aforementioned evaluation of the sensitivity of Colilert-18 were used, and cell suspensions were prepared as described above. One milliliter of 103 cell suspensions of *E. coli* was inoculated into 9, 49, 99, and 249 ml of Colilert-18 solution, respectively, and incubated at 37°C for 18 h. One to four NaDCC tablets (167 mg) were then individually added to the four different volume cultures (10, 50, 100, and 250 ml). After the Colilert-18 cultures had been treated with NaDCC for 1 h, 1 ml of each treated sample was inoculated into TSB and onto 3M Petrifilm *E. coli*/Coliform Count Plates (Petrifilm EC plates; 3M Microbiology, USA). Then, the inoculated tubes and films were incubated at 37°C for 24 h. The samples enriched in TSB were streaked onto TSA using a sterile disposable loop, and the plates were incubated at 37°C for 24 h. The presence of colonies on the Petrifilm EC and TSA plates was observed to assess the sterilization effect of NaDCC on bacteria cultured in Colilert-18. The performance of NaDCC was assessed in various samples, including knives (n = 9), chopping boards (n = 9), gloves (n = 10), radish seeds (n = 5), alfalfa seeds (n = 5), baby leaf sprouts (n = 10), radish sprouts (n = 10), and water (n = 10), all of which were cultured in Colilert-18. Then, one, one, two, and four NaDCC tablets (167 mg) were added to the utensil samples (10 ml), glove samples (50 ml), irrigation water samples (100 ml), and seed and vegetable samples (250 ml), respectively. Subsequent procedures were as described in the aforementioned tests of the microbial sterilization effect of NaDCC.

Results

Evaluation of Colilert-18 for the Detection of *E. coli* from Agri-Food Production Environments

Evaluation of the specificity of Colilert-18. To evaluate the specificity of Colilert-18, the growth of 38 *E. coli* and 78 non-*E. coli* was tested at 37°C and 44°C (Table 2). No significant differences in detection of the target bacteria were observed according to culturing temperature. Regardless of incubation temperature, 8.6% (3/38) *E. coli* strains, including *E. coli* O157:H7, showed no fluorecence due to ß-glucuronidase-negative strains. The positive reaction rates of non-*E. coli* were 3.8% (3/78) and 0% (0/78) at 37°C and 44°C, respectively. None of the non-*E. coli* strains cultured at 44°C showed positive yellow color changes and UV fluorescence. However, some *Shigella* spp., including *S. flexneri* and *S. sonnei*, showed false-positive reactions at 37°C. The *Shigella* spp. that showed false-positive results at 37°C did not grow well at 44°C. Growth of non-*E. coli* was markedly decreased by raising the temperature from 37°C to 44°C.

Table 2. Positive rates of *E. coli* and non-*E. coli* in Colilert-18 at 37°C and 44°C.

Strains	37°C	44°C
E. coli	91.4% (35/38)	91.4% (35/38)
Non-*E. coli*	3.8% (3/78)	0% (0/78)
False-positive bacteria	*Shigella flexneri* NCCP 11203 *Shigella sonnei* NCCP 16122 *Shigella sonnei* ATCC 25981	-

Evaluation of the sensitivity of Colilert-18. Differences were observed between different culture temperatures (Table 3). None of the tested volume and inoculation level combinations affected the sensitivity of Colilert-18 for the detection of *E. coli* at 37°C; however, the growth of *E. coli* in Colilert-18 was obviously suppressed at 44°C. Some of the tested *E. coli* strains were not detected in 50 and 100 ml cultures inoculated with less than 10^1 CFU at 44°C. In the case of 250 ml cultures, no strains grew in Colilert-18 inoculated with less than 10^1 CFU at 44°C. Furthermore, four *E. coli* strains inoculated at the level of 10^1 CFU/250 ml were suppressed at 44°C.

Application of Colilert-18 to produce and water samples. Since the specificity and sensitivity results were inconsistent, the Colilert-18 system was applied to various samples to determine the optimal temperature (Table 4). When isolating *E. coli* from irrigation water by culturing in EC broth and Colilert-18 at 37°C, the percentage of positive samples was 90% and 80%, respectively. After cultivating samples in EC broth and Colilert-18 at 44°C, *E. coli* was detected in 70% and 80% of irrigation water samples, respectively. In vegetables, there were differences in detection rates of *E. coli* between 37°C and 44°C. When culturing radish sprout samples in Colilert-18 broth at 37°C and 44°C, respectively, the positive sample rates were 80% and 60%. In addition, the detection rates of *E. coli* from baby leaf vegetables

Table 3. Detection limit of *E. coli* in various volumes of Colilert-18 at 37°C and 44°C.

Temperature	Strains	CFU/10 ml				CFU/50 ml				CFU/100 ml				CFU/250 ml			
		$<10^1$	10^1	10^2	10^3	$<10^1$	10^1	10^2	10^3	$<10^1$	10^1	10^2	10^3	$<10^1$	10^1	10^2	10^3
37°C	*E. coli* NCCP 13715	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli NCCP 13718	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli NCCP 13719	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli NCCP 13721	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli (isolated from radish sprout)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli (isolated from radish sprout)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	E. coli (isolated from radish sprout)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
44°C	*E. coli* NCCP 13715	+	+	+	+	-	+	+	+	-	+	+	+	-	-	+	+
	E. coli NCCP 13718	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
	E. coli NCCP 13719	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
	E. coli NCCP 13721	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+
	E. coli (isolated from radish sprout)	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	E. coli (isolated from radish sprout)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
	E. coli (isolated from radish sprout)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+

+ : Detected
- : Not detected

Table 4. Determination of the optimal temperature for detecting *E. coli* in produce and water.

Samples		Temperature	Standard methods			Improvement methods		
			Enrichment	Isolation	Identification	Enrichment	Isolation	Identification
			EC	EMB	VITEK	Colilert-18	EMB	VITEK
Water		37°C	-[a]	90%(9/10)	90%(9/10)	80%(8/10)	80%(8/10)	80%(8/10)
		44°C	-	70%(7/10)	70%(7/10)	80%(8/10)	80%(8/10)	80%(8/10)
Vegetables	Baby leafy vegetables	37°C	-	100%(5/5)	100%(5/5)	100%(5/5)	100%(5/5)	100%(5/5)
		44°C	-	80%(4/5)	80%(4/5)	100%(5/5)	100%(5/5)	100%(5/5)
	Radish sprouts	37°C	-	80%(4/5)	80%(4/5)	80%(4/5)	80%(4/5)	80%(4/5)
		44°C	-	60%(3/5)	60%(3/5)	60%(3/5)	60%(3/5)	60%(3/5)
Total		37°C	-	90%(18/20)	90%(18/20)	85%(17/20)	85%(17/20)	85%(17/20)
		44°C	-	70%(14/20)	70%(14/20)	80%(16/20)	80%(16/20)	80%(16/20)

[1)]Unable to confirm positive reaction.

Table 5. Differences between the actual temperatures and setting temperatures in the developed device.

Setting temperature (°C)	32	34	36	38	40	42	44	46
Actual temperature (°C)	30.1 ± 0.3	34.4 ± 0.2	35.2 ± 0.5	37.2 ± 0.2	40.9 ± 0.7	42.7 ± 0.7	44.0 ± 0.5	46.8 ± 0.4

cultured in EC broth at 44°C were lower than those at 37°C by 20%. Overall, higher *E. coli* detection rates were observed after culturing samples at 37°C compared with 44°C.

Development and Application of the Device for Detection of *E. coli*

Development and assessment of the device. Table 5 shows the results of the assessment of the developed device for the detection of *E. coli*. To check the temperature control capability of the device, the internal actual temperature was compared with the setting temperature (Table 5). The difference between the setting temperature and the actual temperature of the device was about 1.0°C. The temperature remained constant during 24 h regardless of the set temperature (data not shown). The brightness of the UV LED lamp was about 870 lux, and that was sufficient to confirm the fluorescence of the *E. coli*-positive samples.

Application of the combination of Colilert-18 and the developed device using various samples. The combination of Colilert-18 and the developed device was assessed using

Table 6. Validation of the combination of Colilert-18 and the developed device for the detection of *E. coli* in working utensils, seeds, vegetables, and water.

Samples		Standard method with incubator			Improvement method with incubator and UV box			Improvement method with developed device		
		EC	EMB	VITEK	Colilert	EMB	VITEK	Colilert	EMB	VITEK
Working utensils	Knife	-[a]	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)
	Chopping board	-	0.0% (0/9)	0.0% (0/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)	11.1% (1/9)
	Gloves	-	30.0% (3/10)	30.0% (3/10)	30% (3/10)	30% (3/10)	30% (3/10)	30.0% (3/10)	30.0% (3/10)	30.0% (3/10)
Seeds	Radish	-	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)
	Alfalfa	-	0.0% (0/5)	0.0% (0/5)	20.0% (1/5)	20.0% (1/5)	20.0% (1/5)	20.0% (1/5)	20.0% (1/5)	20.0% (1/5)
Vegetables	Baby leafy vegetables	-	100.0% (10/10)	100.0% (10/10)	100.0% (10/10)	100.0% (10/10)	100.0% (10/10)	100.0% (10/10)	100.0% (10/10)	100.0% (10/10)
	Radish sprouts	-	70.0% (7/10)	70.0% (7/10)	90% (9/10)	90% (9/10)	90% (9/10)	80.0% (8/10)	80.0% (8/10)	80.0% (8/10)
Water		-	90.0% (9/10)	90.0% (9/10)	80.0% (8/10)	80.0% (8/10)	80.0% (8/10)	80.0% (8/10)	80.0% (8/10)	80.0% (8/10)
Total			44.1% (30/68)	44.1% (30/68)	48.5% (33/68)	48.5% (33/68)	48.5% (33/68)	47.1% (32/68)	47.1% (32/68)	47.1% (32/68)

[a]Unable to confirm positive reaction.

various samples, including utensils, gloves, irrigation water, seeds, and vegetables (Table 6). From the results comparing the detection rates of *E. coli* between the Korean Food Code method and the Colilert-18 system, there were no differences in detection rates of *E. coli* in utensils, except for chopping boards. *E. coli* from irrigation water cultivated in EC broth had a higher detection rate than that of Colilert-18 by 10%. On the other hand, higher *E. coli* detection rates for seeds and vegetables were observed after cultivating samples in Colilert-18 compared with those in EC. Whereas the percentage of positive samples was 0% and 70% for alfalfa seeds and radish sprouts by the Korean Food Code method using EC and EMB, *E. coli* was detected in 20% and 90% of alfalfa seeds and radish sprouts using Colilert-18. Comparing the detection rate of *E. coli* in each sample by the equipment used showed no differences in *E. coli* detection rates between an incubator and the developed device. Thus, the device developed in this study is sufficient for use in agri-food production environments because of its accuracy and convenience.

Evaluation and application of NaDCC tablets to sterilize bacteria. Table 7 shows the results of the assessment of the sterilization effect of NaDCC tablets on the bacteria in Colilert-18 solution after the experiment. As the volume of Colilert-18 increased, more NaDCC was needed to kill the *E. coli* completely. Adding one tablet of NaDCC to 10 and 50 ml cultures killed all of the *E. coli*. However, one tablet was not sufficient to completely kill *E. coli* cultured in 100 and 250 ml of Colilert-18. When two tablets of NaDCC were added, *E. coli* cultured in 100 ml was completely killed. However, *E. coli* cultured in 250 ml was not sterilized by adding one to three tablets of NaDCC, but adding four tablets was enough to sterilize all *E. coli*. Therefore, it is recommended that one, one, two, and four tablets of NaDCC be added to sterilize *E. coli* cultured in 10, 50, 100, and 250 ml of Colilert-18, respectively.

To hygienically dispose of all cultured microorganisms, one, one, two, and four tablets of NaDCC were added to the utensil samples (10 ml), glove samples (50 ml), irrigation water samples (100 ml), and seed and vegetable samples (250 ml), respectively. No bacteria were detected in any of the samples (data not shown).

Discussion

Fresh-cut produce is increasingly being recognized as important vehicles of foodborne diseases [15]. To control the microbial safety of fresh vegetables, it is important to monitor the sanitation of the water, gloves, and utensils used by workers, as well as the vegetables. *E. coli* was used as the indicator organism of overall food hygiene conditions because it is specific and most reliably reflects fecal origin.

Table 7. Microbial sterilization effect of sodium dichloroisocyanurate tablets on *E. coli* culturing in various volumes of Colilert-18.

Strains	10 ml	50 ml	100 ml		250 ml			
	1 Tablet	1 Tablet	1 Tablet	2 Tablets	1 Tablet	2 Tablets	3 Tablets	4 Tablets
E. coli NCCP 13715	-	-	+	-	+	+	+	-
E. coli NCCP 13718	-	-	+	-	+	+	+	-
E. coli NCCP 13719	-	-	+	-	+	+	+	-
E. coli NCCP 13721	-	-	+	-	+	+	+	-
E. coli (radish sprout 1)	-	-	+	-	+	+	+	-
E. coli (radish sprout 2)	-	-	+	-	+	+	+	-
E. coli (radish sprout 3)	-	-	+	-	+	+	+	-

+ : Detected

- : Not detected

For the detection of *E. coli*, the Colilert-18 assay system based on microbial enzyme profiles has been used as an attractive alternative to traditional methods [16]. No studies have been published on the application of the Colilert-18 assay system for samples from agricultural environments. Thus, it is necessary to evaluate whether the Colilert-18 assay system could be used to monitor hygienic conditions of agricultural environments. The results of the evaluation of the specificity of Colilert-18 indicated that 8.6% (3/38) of *E. coli* stains, including *E. coli* O157:H7, showed no fluorescence owing to their inability to produce β-glucuronidase, and hydrolyze MUG [17]. Moreover, false-positive reaction was observed in some *Shigella* spp. at 37°C. Several studies have reported that *Shigella* spp. produce β-galactosidase and β-glucuronidase that can hydrolyze ONPG and MUG, respectively. Maheux *et al*. [18] reported *S. sonnei* strains that were β-galactosidase-positive in the Colilert test, and another study found that only 8.2% of *S. sonnei* strains were ONPG-negative [19]. According to a study by McDaniels *et al*. [20], positive results for β-glucuronidase were observed within members of the genus *Shigella*. It was presumed that these *Shigella* spp. were stressed by heat, since the reported temperature range for growth of *Shigella* spp. is 6–8°C to 45°C [21]. Small *et al*. [22] noted that bacteria died rapidly after the temperature increased was to 41°C.

In the results of evaluation of the sensitivity of Colilert-18, the Colilert-18 system was capable of detecting 1 CFU/250 ml of *E. coli* at 37°C. However, the growth of *E. coli* in Colilert-18 was obviously suppressed at 44°C. Other studies have reported that the Colilert system is a highly sensitive detection method that is capable of detecting 1 CFU/100 ml of *E. coli* [9, 11, 12]. It is known that 37°C is optimal for growth of *E. coli* because stressed *E. coli* cells can recover at this temperature [23]. Moreover, the growth rate of *E. coli* is slow at 44°C owing to thermal stress [24].

Since the specificity and sensitivity results in this study were inconsistent, the Colilert-18 system was applied to various samples to determine the optimal temperature. Higher *E. coli* detection rates were observed after culturing samples at 37°C compared with 44°C. A previous study reported that false-negative results may be a result of culturing conditions such as temperature. Alonso *et al*. [25] also reported that, although *E. coli* chromosomes contain the gene encoding β-glucuronidase, some strains may fail to express β-glucuronidase activity when incubated at 44.5°C. From the present results, it was presumed that production of β-glucuronidase and β-galactosidase by *E. coli* was not affected by the growth of non-target strains in samples at 37°C. *E. coli* was isolated in all samples that were positive for Colilert-18, because strains such as *Shigella* spp. that produce false-positives are rare in natural environments. For this reason, the manufacturer of Colilert recommends sample incubation at 37°C (IDEXX Laboratories, Inc.). These data suggest that enriching *E. coli* in samples at 37°C in Colilert and EC are appropriate to enhance the detection rates of *E. coli*. These results indicate that the sensitivity of Colilert-18 at 37°C is superior to that at 44°C at lower detection limits.

We developed and validated a device for the detection of *E. coli* in the field. The capability of temperature control and brightness of the UV LED lamp were sufficient to detect *E. coli* in samples. Several researchers have developed devices for the detection of *E. coli* using Colilert. The device developed by Yeom [26] comprised a UV lamp, color sensor, and data analyzer. Their method was developed to estimate the number of *E. coli* according to the degree of color after separate culture of the sample. Lee [27] also developed a device in which a water sample is dispensed into a container containing the medium, by an automatic pump within the device, and then incubated and checked for the presence of *E. coli* by the UV detector. However, there were disadvantages to using these devices in industrial

agri-food production environments, including the requirement for a separate culturing process and the fact that they could not be applied to various sample types, only water. The device developed in the present study, which can be applied to various samples, is inexpensive and convenient to use, making it suitable for testing for *E. coli* in the field.

When applied to various samples, such as knives, chopping boards, gloves, radish seeds, alfalfa seeds, baby leaf sprouts, radish sprouts, and water, the combination of Colilert-18 and the developed device gave higher rates of positive results, compared with a standard method (Korean Food Code). The accuracy of the Colilert system can be impacted by both the aforementioned microbiota within the matrix and the matrix itself. For example, the acidity in fruit juice impairs the fluorescence of 4-methylumbelliferone [28]. Thus, several studies have validated the Colilert system on different matrices, such as raw meat, fresh-cut products, and marine and other types of water samples [8–13]. Our findings supported the results of Julian *et al*. [28] that 98.9% of presumptive *E. coli* isolated from hands, soil, and fecal samples using the Colilert reagent was finally confirmed as *E. coli*. Kawasaki *et al*. [11] reported that *E. coli* was detectable from vegetables using the DOX system with Colilert-18. Venkateswaran *et al*. [5] showed that the level of recovery of *E. coli* as determined by the Colilert system (83%) was comparable to that obtained using the US Food and Drug Administration method (87%). We conclude that Colilert-18 in combination with our developed device is appropriate for the detection of *E. coli* from various samples, including hands, working utensils, water, seeds, and vegetables.

For the sterilization of bacteria in Colilert-18 solution after the experiment, we assessed the microbial sterilization effect of NaDCC, tablets which have been used in emergencies as an alternative to sodium hypochlorite to kill contaminating bacteria in drinking water [14, 29]. In our case, NaDCC tablets were considered as an effective solution to sterilize the post-test samples thoroughly, quickly, and also cheaply. According to the instruction manual with NaDCC, one 167 mg tablet should be added to 25 L of water to disinfect the drinking water. However, a modification of the dose (1, 1, 2, and 4 tablets for 10, 50, 100, and 250 ml *E. coli* cultures, respectively) was required after the test to kill *E. coli* in Colilert-18 owing to the high numbers of bacteria generated during the test processing. Compounds such as sodium hypochlorite or NaDCC disinfect water by releasing free available chlorine in the form of hypochlorous acid [30]. Aquatabs™ tablets containing 3.5 mg of NaDCC in an effervescent base can remove 1.8 to 2.8 logs of bacteria from raw water [31]. In our case, the bacteria density in the test may reach the level of 7 logs. Therefore, our experiments indicated that a higher dose of NaDCC was required to remove the bacteria completely.

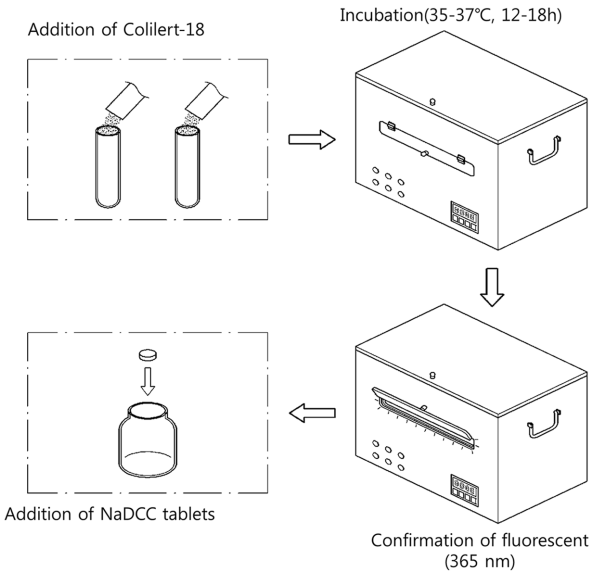

Fig. 2. Experimental process using the combination of Colilert-18 and the developed device.

In summary, Colilert-18 was confirmed to be suitable for the detection of *E. coli* from various samples, including utensils, gloves, irrigation water, seeds, and vegetables. The device developed in the present study can be used in combination with Colilert-18, can be applied to various samples, and is inexpensive and convenient to use, making it suitable for detecting *E. coli* in the field. One tablet (167 mg) of NaDCC per 50 ml was sufficient to kill *E. coli* in samples assessed in the protocol. Thus, it is possible to monitor hygienic conditions in agri-food production environments using the screening protocol (Fig. 2) and the device developed in the present study.

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