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ARTICLE

Microbiological quality and characteristics of isolated *Escherichia coli* in irrigation water used in Napa cabbage cultivation

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Abstract To ensure the safety of Kimchi, the safety of Napa cabbage is the most important. Contaminated irrigation water can be a major cause of pathogens during growth of Napa cabbage. The purpose of this study was to investigate the microbial quality of irrigation water used in the cultivation of Napa cabbage. A total of 111 samples including surface water (n = 75) and groundwater (n = 36) collected from four different regions in Korea where Napa cabbage is intensively cultivated were analyzed for a fecal indicator (*Escherichia coli*) Moreover, 164 *E. coli* isolates from irrigation water were investigated for pathogenic characteristics including antibiotic resistance, pathogenic genes, serotype, and toxicity using *Caenorhabditis elegans*. *E. coli* was detected in 96% of surface water samples and 25% of groundwater samples. The level of *E. coli* in surface water (0.2–3.2 log MPN/100 mL) was higher than that in groundwater (0–2.0 log MPN/100 mL). When the 164 *E. coli* isolates were investigated concerning antibiotic resistance, resistance rates were 11.0%, 2.4%, 3.0%, 1.8%, 2.4%, 4.3%, and 3.0% for ampicillin, ampicillin/sulbactam, cefazolin, cefoxitin, gentamicin, levofloxacin, and trimethoprim/sulfamethoxazole, respectively. In addition, 10 (6.1%) of the isolates were positive for the *eaeA* gene, indicative of enteropathogenic *E. coli*. Eight of these 10 isolates were obtained from the surface water of the mountainous region II and were toxic to *C. elegans*. The results indicate the need to manage the microbial risk of irrigation water to enhance the safety of cultivated Napa cabbage.

Keywords *E. coli* · Napa cabbage · Irrigation

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Introduction

Kimchi was listed in the Codex Alimentarius in 2001 [1] and was ranked as one of the top four health foods in 2006 [2]. In Korea, the demand for Kimchi is increasing as the country's economy improves and group meals grow in popularity [3]. However, since Kimchi is a non-sterile, natural, fermented food that does not undergo sterilization during its manufacture, it is difficult to control contamination by foodborne bacteria unless the product is fully fermented [4]. Recent foodborne outbreaks associated with Kimchi have been reported [4]. To ensure the safety of Kimchi, the safety of Napa cabbage is paramount, since the cabbage is the main material of Kimchi. Potential sources of Napa cabbage contamination include substandard hygiene of personnel involved in cabbage harvest and processing, soil, animal feces, inadequately composted manure, and irrigation water [5, 6]. The latter is especially influential on the safety of Napa cabbage [7]. Irrigation water can be a major source of pathogens that persist during the growth of leafy vegetables [8–12]. Contaminated water was the reported source of intestinal pathogens in various recent crop-related outbreaks [13–16]. Several

government regulatory authorities have proposed guidelines and standards for microbial water quality to minimize the associated risks of irrigation water [17–19]. However, in the agricultural water quality standard of Korea, microorganisms are not considered in the grade 4 environmental water quality standards [20]. It is necessary to establish microbial quality standards for irrigation water to ensure water safety, and hence crop safety.

In many countries, *Escherichia coli* is used as a water quality standard. Most of the *E. coli* strains used as water microbial quality standards are not harmful to humans. However, some strains can cause foodborne illness [27, 28]. Foodborne outbreaks caused by Kimchi contaminated with pathogenic *E. coli* were reported in 2012. It is very important to evaluate whether *E. coli* isolated from agricultural water is likely to be a foodborne illness-causing strain. The determination of the virulence of *E. coli* often involves the detection of virulence genes using polymerase chain reaction (PCR). This approach is convenient to perform but is not always definitive, since the detection of even a pathogenic gene does not absolutely indicate pathogenicity [21, 22]. Therefore, pathogenic evaluation of *E. coli* requires in vivo toxicity evaluation as well the detection of pathogenic genes.

Animal models of toxicity provide critical information for assessing hazard and risk potential [23, 24]. However, many organizations, such as the Societies for Protection and Care of Animals, ethically oppose all forms of animal research [25]. Therefore, toxicity assessment using the *Caenorhabditis elegans* nematode has been introduced as an alternative to animal testing. The genetic complement of *C. elegans* is 60–80% similar to that of humans. However, advantageously, the unicellular *C. elegans* is less complex than multicellular mammalian organisms. *C. elegans* also has a short life cycle of about 3 days and has a non-hazardous biological system that can be easily maintained in the laboratory [26, 27]. In several studies, a correlation has been demonstrated between nematodes and pathogenic bacteria, and bacteria that are capable of killing mice [28, 29]. Hence, *C. elegans* has been used extensively in biological studies as an in vivo surrogate host. In particular, *C. elegans*–pathogen models have been used to explore the pathogenesis of foodborne pathogens including pathogenic *E. coli* and *Salmonella* spp. [30].

This study was conducted to investigate the microbial contamination level of irrigation water in several major Napa cabbage cultivation regions in Korea. *E. coli* isolated from irrigation water was investigated for characteristics of pathogenicity, including the presence of pathogenic genes and *C. elegans* toxicity.

Materials and methods

Investigation of microbiological quality

Sampling for investigation of microbiological quality of irrigation water To investigate microbiological quality, a total of 111 water samples including surface water (n = 75) and groundwater (n = 36) were collected from four major Napa cabbage cultivation regions in Korea (Table 1). Sampling was conducted during harvest period of Napa cabbage. Regions I, II, and III were mountainous, while site IV was a flat region. Surface water samples were collected from a stream, valley, and pond near the cabbage-growing farms. These samples were collected using a sterilized bucket and individually transferred to a sterilized screw-capped 2 L bottle. Groundwater samples were collected in 2 L sterilized bottles after the water was disembogued for 5 min. All samples were transported to the laboratory in an icebox within 6 h of collection for microbiological analysis.

Escherichia coli in irrigation water Enzymatic analysis for *E. coli* was performed using the Colilert-18 system (IDEXX Laboratories, Westbrook, ME, USA). Briefly, 10 mL of each water sample was serially diluted with 90 mL of sterilized water. One capsule of Colilert-18 powder was added to each diluted sample and dissolved by shaking. Each sample was poured into a quanti-tray (IDEXX Laboratories). Each quanti-tray was sealed using a heat sealer (IDEXX Laboratories) and incubated at 37 °C for 18 h. The trays were then exposed to ultraviolet (UV) light (365 nm) in a UV light box, and fluorescence and color changes were observed. Yellow-colored wells and those that fluoresced were considered positive. The number of *E. coli* in each 100-mL water sample was quantified using a most probable number (MPN) table provided by IDEXX.

Table 1 Irrigation water samples collected for testing microbial quality

Region	Sampling period	Source of water	Number
I	August	Surface water (stream, valley)	24
		Groundwater	6
II	August	Surface water (stream, valley)	21
		Groundwater	9
III	October	Surface water (stream, valley)	15
		Groundwater	12
IV	November	Surface water (ponds)	15
		Groundwater	9
Total sample		Surface water	75
		Groundwater	36

For isolation of *E. coli*, aliquots (0.1 mL) from five suspected positive wells of each quanti-tray were acquired using a syringe (Korea Vaccine, Seoul, Korea). Each aliquot was inoculated in 10 mL of EC broth (Oxoid Ltd., Basingstoke, UK). After 18-h incubation at 44 °C, enriched cultures were streaked on EMB using a disposable loop. Plates were incubated at 37 °C for 24 h, and typical colonies were picked and identified using a VITEK system (bioMerieux, l'Etoile, France).

Statistical analysis Quantitative analysis data of *E. coli* in water collected from four regions were transformed to log MPN per 100 mL. The data were analyzed by one-way analysis of variance (ANOVA) using SAS version 9.1 software (SAS Institute, Cary, NC, USA). All comparisons of means were performed using Duncan's multiple range test at an alpha = 0.05.

Characterization of *E. coli* isolated from water

Bacterial isolates The total of 164 strains comprised 67 from region I, 49 from region II, 22 from region III, and 29 from region IV.

Analysis of antibiotic resistance of E. coli isolates Antibiotic resistance of the *E. coli* isolates was determined using the VITEK apparatus. Briefly, each isolated *E. coli* was cultured in nutrient agar (NA: Difco, Detroit, MI, USA) for 24 h at 37 °C. The bacteria were adjusted to a McFarland standard turbidity of 0.6 using 0.45% sodium chloride solution, and 145 μL of was diluted in 3 mL of 0.45% sodium chloride solution. Each diluted bacterial suspension was placed in the cassette of the VITEK apparatus along with and AST-N211 card (bioMerieux, France) coated with 17 antibiotics: ampicillin, amoxicillin/sulbactam, piperacillin/tazobactam, cefazolin, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, ertapenem, meropenem, amikacin, gentamicin, levofloxacin, tetracycline, and trimethoprim/sulfamethoxazole. Each cassette was put into the apparatus for analysis as detailed in the VITEK manual [31]. The results were determined according to the National Committee for Clinical and Laboratory Standards guideline [31]. *E. coli* ATCC 25922 and *E. coli* ATCC 35218 (American Type Culture Collection, Manassas, VA, USA) were used as the standards for antibiotic resistance.

Pathogenic genes of E. coli isolates from irrigation water were tested for the presence of eight pathogenic genes (LT, ST, VT1, VT2, *bfpA*, *aggR*, and *ipaH*) by polymerase chain reaction (PCR). The genomic DNA was isolated using a G-spin™ genomic DNA extraction kit (iNtRON Biotechnology, Sungnam, Korea) in accordance with the manufacturer's protocols. PCR was performed according to the manufacturer's method using the PowerChek™ Diarrheal *E. coli* 4-plex Detection Kit I and II (Kogenebiotech, Seoul, Korea). Briefly, 5 μL of each DNA was added to 15 μL of PCR premix that included reaction buffer, Taq polymerase, and primer. The thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) conditions were: 94 °C for 10 min; 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and extension at 72 °C for 10 min. After PCR, amplification products were confirmed by 1.5% agarose gel electrophoresis.

Serotyping of E. coli O-antigens O Serotyping was used to identify isolates belonging to commercially defined O serogroups. For determination of O-antigens, the TSA bacterial culture was suspended in 3 mL of normal saline and heated to 100 °C for 1 h. The boiled suspension was used as an antigenic mixture. One or two drops of specific O poly- or monovalent *E. coli* antisera (Joong Kyeom, Ansan, Korea) were mixed with the antigen preparation on a glass slide for 1 min and observed for agglutination.

In vivo *evaluation of toxicity of E. coli* Evaluation of the toxicity of *E. coli* possessing pathogenic genes was conducted using a *C. elegans* kill assay. *C. elegans* Bristol N2 wild-type strain (*Caenorhabditis* Genetics Center, University of Minnesota) was used. The assay was performed as previously described [32] with some modifications. Prior to the assay, the nematode worms were grown on agar plates of nematode growth medium (NGM) containing 3.0 g NaCl, 17.0 g agar, and 2.5 g peptone in 975 mL H_2O. During growth at 25 °C, the nematodes were fed *E. coli* OP50 [33]. For the killing assay, 10 *E. coli* isolates possessing pathogenic genes were grown overnight (18 h) in the Luria–Bertani (LB) broth (Oxoid) at 37 °C at 180 rpm. Twenty microliter aliquots of each culture (2×10^9 CFU/mL) were spread on NGM agar plates. Worms were synchronized by hypochlorite bleaching, allowed to hatch overnight, and were subsequently cultured on NGM plates in the presence of *E. coli* OP50. Synchronized L1 larvae were transferred to NGM plates in the presence of *E. coli* OP50 and allowed to develop to L4 larvae. On day 3 after synchronization, 20 L4/young adult hermaphrodites were placed on each plate, incubated at 25 °C for 45 min, and transferred to a new plate every each 24 h to determine the survival rates of the original worms without the presence of progeny. The worms were considered dead when they did not respond to touching with a platinum wire pick. Each experimental condition was performed in triplicate. Nematode survival was examined using the Kaplan–Meier method, and differences were determined with the log-rank test (STATA6 software; STATA, College Station, TX, USA).

Results and discussion

Recent cases of food poisoning caused by Kimchi in Korea have emphasized the safety of Kimchi [34]. Ensuring the safety of Kimchi depends critically on the safety of Napa cabbage, which is the main raw material of Kimchi [4, 34]. Napa cabbage can be contaminated with foodborne pathogens through various routes, including irrigation water, soil, and inadequately composted manure. Irrigation water can be a major source of pathogens that persist for the duration of the growth of Napa cabbage. This study evaluated the microbial safety of irrigation water used for cabbage irrigation in four major cabbage-producing regions of Korea.

Escherichia coli was detected in 96% of the surface water samples and 25% of groundwater samples (Table 2). Surface water is more susceptible to microbial contamination than groundwater due to runoff, direct fecal sedimentation, and growth of bottom sediments [19, 35]. The level of *E. coli* in surface water and groundwater was 0.2–3.2 log MPN/100 mL and 0–2.0 log MPN/100 mL, respectively. The level of *E. coli* in surface waters from region I and III was significantly higher than those from region II and IV ($p < 0.05$). The difference in the level of *E. coli* among the regions was presumed to be affected by the locality and season. Region II was the highest altitude among the three mountainous areas, and sampling in region IV was conducted in the cold season of November. The number of *E. coli* from these two regions was relatively lower. Elsewhere, *E. coli* contamination of irrigation water used in lettuce cultivation in southern Brazil ranged from 2.1 to 5.4 log CFU/100 mL [10], which was higher than in this study. In irrigation water in Spain and Bilge, *E. coli* contamination ranged from 1.0 to 1.5 log CFU/100 mL, which was lower than the present values [8, 15]. The United States Food Safety Modernization Act (FSMA) requires that irrigation water that directly contacts the edible portion of the crop must have generic *E. coli* counts $\leq$ 126 CFU/mL [36]. Over half (52.0%) of the surface water samples we sampled exceeded the FSMA safety level. However, no water quality standards for irrigation water have been legislated in Korea. Water quality standards for irrigation water are urgently needed in Korea.

This study investigated the characteristics of 164 *E. coli* isolated from irrigation water. The results of resistance to the 17 antibiotics by the 164 isolates are presented in Tables 3 and 4. The overall resistance rate of *E. coli* was the highest in region I (28.8%), followed by region III (11%) and region IV (3.7%). The antibiotic resistance rates were 11.0%, 2.4%, 3.0%, 1.8%, 2.4%, 4.3%, and 3.0% for ampicillin, ampicillin/sulbactam, cefazolin, cefoxitin, gentamicin, levofloxacin, and trimethoprim/sulfamethoxazole, respectively. Of the *E. coli* isolated from surface water, 5.2% showed single resistance to ampicillin or cefazolin, and 8.4% showed resistance to multiple antibiotics. All multi-resistant strains were resistant to ampicillin. In addition, strains isolated from surface water of region I displayed resistance against five antibiotics (extended spectrum beta-lactam, ampicillin, gentamicin, levofloxacin, and trimethoprim/sulfamethoxazole). Moreover, one *E. coli* isolate from groundwater displayed resistance to three antibiotics (ampicillin, ampicillin/sulbactam, and trimethoprim/sulfamethoxazole). Sayah et al. [37] reported cephalothin resistance of *E. coli* isolated from the Red cedar watershed in the US. Chigor et al. [38] reported multi-drug resistance in *E. coli* and *E. coli* O157:H7 isolated from surface waters in Zaria, Nigeria. In another study, 73% of the 233 *E. coli* isolates from a region of water poisoning in the Dhaka area of Bangladesh displayed antibiotic resistance and 36% of the isolates displayed multi-drug resistance [39].

However, in this study, antibiotic resistance of *E. coli* isolates from irrigation water is relatively low compared to that isolated from livestock and aquatic production. Antibiotic resistance of *E. coli* isolated from livestock in Korea reportedly involves tetracycline, followed by ampicillin and streptomycin [40–42]. The highest rate of tetracycline resistance was also observed in *E. coli* isolated

Table 2 Contamination level and frequency of *E. coli* from irrigation water

Region	Surface water		Ground water	
	Average (log MPN/100 ml)	Frequency (%)	Average (log MPN/100 ml)	Frequency (%)
I	2.9 ± 0.5^{a}	100 (24/24)	$< 0.1^{b}$	17 (1/6)
II	1.7 ± 0.7^{c}	90 (19/21)	ND[b]	0 (0/9)
III	3.2 ± 0.5^{a}	100 (15/15)	2.0 ± 1.1^{a}	58 (7/12)
IV	1.9 ± 0.8^{b}	93 (14/15)	$< 0.1^{b}$	11 (1/9)
Total		96 (72/75)		25 (9/36)

ND not detection

The data are present as the mean ± SD of samples and the same letters are not significantly different among *E. coli* numbers in each region at $p < 0.05$ according to Duncan's test

Table 3 Analysis of antibiotics resistance of *E. coli* isolated from irrigation water according to regions

	I	II	III	IV	Total
1 drug	9.1 (6/66)	0 (0/49)	11 (2/22)	0 (0/27)	4.9 (8/164)
2 drugs	10.6 (7/66)	0 (0/49)	0 (0/22)	0 (0/27)	4.3 (7/164)
3 drugs	3.0 (2/66)	0 (0/49)	0 (0/22)	3.7 (1/27)	1.8 (3/164)
4 drugs	4.5 (3/66)	0 (0/49)	0 (0/22)	0 (0/27)	1.8 (3/164)
5 drugs	1.5 (1/66)	0 (0/49)	0 (0/22)	0 (0/27)	0.6 (1/164)
Total	28.8 (19/66)	0 (0/49)	11 (2/22)	3.7 (1/27)	13.4 (22/164)

Unit: % (No. of antibiotics resistance of *E. coli* / No. of tested *E. coli*)

Table 4 Analysis of antibiotics resistance of *E. coli* isolated from irrigation water according to water sources

	Antibiotics	Surface water	Ground water	Total
1 drug	Ampicillin	4 (6/155)	–	4 (6/164)
	Cefazolin	1 (2/155)	–	1 (2/164)
2 drugs	Ampicillin	1 (2/155)	–	1 (2/164)
	Ampicillin/sulbactam			
	Ampicillin	2 (3/155)	–	2 (3/164)
	Levofloxacin			
	Cefazolin	1 (2/155)	–	1 (2/164)
	Cefoxitin			
3 drugs	Ampicillin	1 (1/155)	–	1 (1/164)
	Cefazolin			
	Cefoxitin			
	ESBL	1 (1/155)	–	1 (1/164)
	Ampicillin			
	Ampicillin/sulbactam			
	Ampicillin	–	11 (1/9)	1 (1/164)
	Ampicillin/sulbactam			
	Trimethoprim/sulfamethoxazole			
4 drugs	Ampicillin	2 (3/155)	–	2 (3/164)
	Gentamicin			
	Levofloxacin			
	Trimethoprim/sulfamethoxazole			
5 drugs	ESBL	1 (1/155)	–	1 (1/164)
	Ampicillin			
	Gentamicin			
	Levofloxacin			
	Trimethoprim/sulfamethoxazole			
Total		13.5 (21/155)	11 (1/9)	13.4 (22/164)

Unit: % (No. of antibiotics resistance of *E. coli* / No. of tested *E. coli*)

from aquatic environments [43]. However, tetracycline-resistant isolates were not received in the present study. The reason may be the absence of livestock farms near the area where samples were collected. Other study also reported the lack of tetracycline-resistant strains in *E. coli* isolated from water, except from livestock areas [44].

Of the isolates, 6.1% (10/164) were positive for the *eaeA* gene, indicative of enteropathogenic *E. coli* (Table 5). Pathogenic genes were not detected in the other isolates. *E. coli* carrying the *eaeA* gene was detected in surface water mixed with ground and valley water collected from region II and valley water collected from region I. Ten *E. coli* isolates carrying the *eaeA* gene were serotyped. Four different serotypes (O121, O110, O156, and O171) were identified in the *E. coli* isolated from surface water in region II. Two *E. coli* isolates from regions II and I were not confirmed using O antisera. Cho et al. [45] described that an outbreak associated with consumption of Kimchi in

Table 5 Analysis of pathogenic gene and serotypes of *E. coli* isolated from irrigation water

Region	Pathogenic gene	Serotype	Isolates
I	EPEC (*eaeA*)	ONT	1
II	EPEC (*eaeA*)	O121	3
	EPEC (*eaeA*)	O110	1
	EPEC (*eaeA*)	ONT	1
	EPEC (*eaeA*)	O156	3
	EPEC (*eaeA*)	O171	1
Total			6.1% (10/164)

ONT o-nontypeable

Korea in 2012 was caused by enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC) isolated from Kimchi and a patient. In addition, ETEC was categorized as either the O99 or O120 serotype, and EAEC was belonged to the O120 serotype. Pulsed-field gel electrophoresis verified the close link between pathogenic *E. coli* from Kimchi and the patient. Thus, the *E. coli* that caused the 2012 outbreak was different from the *E. coli* isolated in this study. The *eaeA*-positive strains detected in this study seemingly pose less risk, but may be pathogenic. An ongoing study is investigating the specific molecular pathogenesis of *E. coli* isolated from the irrigation water mixed with groundwater and valley water. *C. elegans* was used to verify the virulence of isolated *eaeA*-positive bacteria. As shown in Fig. 1, *E. coli* strains isolated from the irrigation water mixed with groundwater and valley water of region II significantly decreased the survival of *C. elegans* compared with non-pathogenic *E. coli* OP50 ($p < 0.05$). Merkx-Jacques et al. [46] reported pathogenic gene-positive *E. coli* isolated from 143 water samples in the South Nation River in Canada that were tested for toxicity using *C. elegans*; *E. coli*-harboring toxic genes were pathogenic. However, some *E. coli* having a toxicity gene did not show any toxicity. These results suggest that the toxicity test of *C. elegans* can be evaluated indirectly for *E. coli*, which has the potential to be toxic for humans.

The irrigation water used in major Napa cabbage-producing regions was found to be inadequate in the case of mountainous areas. In addition, most of the pathogenic gene-positive strains of *E. coli* were detected in mountainous areas.

Several recent articles have reported that wildlife has been identified as a possible source of the waterborne pathogens, including *E. coli*. The access of wild animals to the mountainous area is easier than that to the plain area [47, 48]. Therefore, the load of microorganisms in agricultural water from mountainous regions is high. Moreover, there is increasing evidence of contamination of agricultural products due to irrigation water; however, there is insufficient information on the microbial quality of agricultural water [49]. Thus, the plans for improving the quality and safety of irrigation water for agricultural products should be urgently needed. First approach is to establish periodic monitoring system to identify sources of potential pathogens. Second approach is to develop and extend physical or chemical sterilization system that is easy

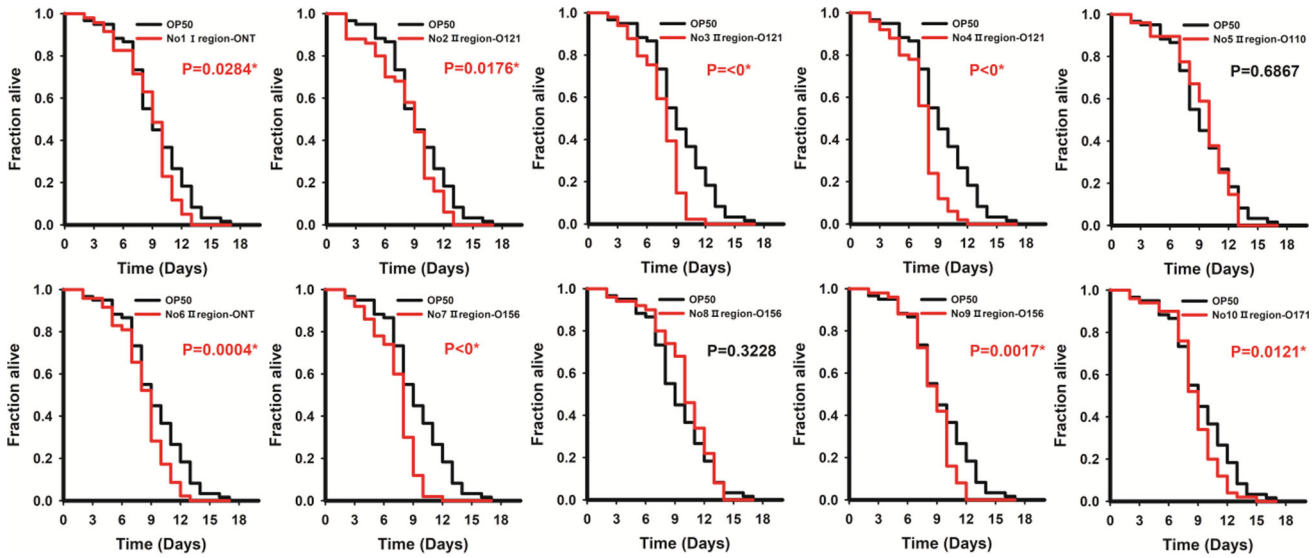

Fig. 1 Analysis of pathogenicity of *E. coli* isolated from irrigation water using nematode

to apply in the field. Finally, criteria for the microbial quality of agricultural water should be established.

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