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Comparative Analysis of Detection Methods for Food-borne Pathogens in Fresh-cut Agricultural Materials

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The consumption of fresh-cut agricultural materials is increasing due to increased public interest in health and the increase of single-person households. Most fresh-cut agricultural materials can be eaten without heating, thus easily exposing the consumer to food-borne pathogens. As a result, food-borne diseases are increasing worldwide. In the analysis of food-borne pathogens, it is important to detect the strains, but this is time consuming and laborious. Alternative detection methods that have been introduced, include polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), which is performed without prior culturing. Samples of fresh-cut agricultural materials, such as vegetables, were analyzed by the culture-based method. In 129 samples, non-pathogenic *Escherichia coli* (3.9%), *Bacillus cereus* (31.8%), *Clostridium perfringens* (5.4%), *Yersinia enterocolitica* (0.8%), and enterohemorrhagic *E. coli* (0.8%) were detected. Eight samples contaminated with bacteria were randomly selected, further analyzed by PCR-DGGE, and compared with the culture-based method. Two cases detected non-pathogenic *E. coli* by PCR-DGGE only, despite a lack of detection by the culture method. It was supposed there was possibility of sample loss during its 10-fold dilution for appropriate cultivation. In the detection of high-risk food-borne pathogens, it was found that the detection limit was lower in PCR-DGGE than in the culture-based method (10 CFU/g). This suggests that PCR-DGGE can be alternatively used to detect strains. On the other hand, low-risk food-borne pathogens seem to have higher detection limits in PCR-DGGE. Consequentially, this study contributes to the improvement of food-borne pathogen detection and the prevention of its related-diseases in fresh-cut agricultural materials.

Key words : Culture-based method, enterohemorrhagic *Escherichia coli,* food-borne pathogens, fresh-cut agricultural materials, PCR-DGGE

Introduction

The consumption of vegetables is increasing due to the improvement of dietary life and increased interest in health according to the development of the food industry. In addition, the trend of the food industry pursuing simplicity and small packaging has been formed due to the social structural change of increasing single-person households and accordingly demand for fresh-cut agricultural materials is increasing [13, 14]. Agricultural materials are classified into three categories as agricultural products, fresh-cut agricultural foods and fresh-cut agricultural products. Agricultural products that have not been processed in raw are necessary to trim before cooking. Fresh-cut agricultural foods and fresh-cut agricultural products are those that have been simply processed of agricultural products. Fresh-cut agricultural foods are defined in the Food Sanitation Act, which are managed by microbiological standards [16]. On the other hand, fresh-cut agricultural products are clearly similar to fresh-cut agricultural foods but are not managed, because of no microbiological standards under the Agricultural Products Quality Control Act. In Korea, fresh-cut agricultural products market grew by more than 30% annually and entered the markets of 1 trillion won in 2015, fresh-cut agricultural foods market grew 51.1% in the last 5 years [11].

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Since these foods and products are often consumed directly without heating as lettuces and perilla leaves, salads, etc, it is easily exposed to food-borne pathogens so that outbreaks of food-borne disease caused from contaminated fruits and vegetables are increasing worldwide. Especially in 2018, large-scale food-borne diseases occurred twice in the United States due to Romaine lettuce contaminated with enterohemorrhagic *Escherichia coli*, resulting in 5 and 62 deaths and 96 and 25 hospitalization, respectively [3]. In the United States in 2016-2018, mediated foods of agricultural products to cause food-borne diseases were identified as sprouts, frozen vegetables, packaged salads, leafy vegetables and romaine lettuce. *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* were the causative pathogens of these food-borne diseases [2]. All of these causative pathogens are high-risk food-borne pathogens. These pathogens account for a high proportion of food-borne pathogens related to agricultural materials worldwide, but it is worth noting that there are no microbiological standards for fresh-cut agricultural products in Korea. The high-risk food-borne pathogens detected in agricultural products in Korea were found to be *Campylobacter jejuni* and *L. monocytogenes* reported by Bae *et al*. [1] and *Salmonella* spp. by Hong *et al*. [5].

Since the concerns of food-borne pathogens are increasing, now it is time to suggest efficient detection methods together with microbiological management and countermeasures for the rapidly growing market in fresh-cut agricultural foods and products. For microbiological management and prevention of food-borne disease, it is important to analyze contamination of bacteria quickly and accurately. Thus, simple and rapid techniques have been developed toward microbiological methods. However, agricultural materials diverse in surface area, surface properties, and its pH make it difficult to analyze with uniform microbiological methods [7, 8]. In the analysis of food-borne pathogens, the culture process is popular, but the detection of strains takes more time relatively and requires a lot of effort [6]. So some methods comparable to culture-based methods without the culture process can be applied, such as the PCR-DGGE method.

Therefore, fresh-cut agricultural materials in markets were purchased in form of vegetables to analyze food-borne pathogens. In this study, it was intended to provide basic data for microbiological risk assessment and improvement of detection methods for food-borne pathogens. In addition, it aimed to recognize the risk of food-borne diseases related to fresh-cut agricultural foods and products and to provide a basis for preparing regulation of microbiological standards.

Materials and Methods

Sample collection

From February to August 2019, 26 fresh-cut agricultural foods and 103 fresh-cut agricultural products as vegetables from marts in Gyeongsangnam-do, Korea were collected and used as experimental samples.

Food-borne pathogens analyses by culture-based method

After taking 25 g of the sample into a sterile sample bag (TEMPO SACS, BioMerieux, Craponne, France), 225 ml of each enrichment broth was added and mixed. All samples were cultured according to each condition [15]. Genomic DNA was extracted by the boiling method [4]. Screening test was performed for eight species of food-borne pathogens (*Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni/coli*, *Yersinia enterocolitica*) [15]. Powerchek 20 pathogen Multiplex Real-time PCR kit (Kogenebiotech, Seoul, Korea) was used for real-time PCR. When each amplification was observed at a Ct value of 33 or less, their selective culture and isolation of strains were performed according to the microbiological test methods [16]. Identification was performed using an automated microbiological identification instrument (VITEK 2 compact, BioMerieux).

Sampling and genomic DNA extraction

PCR-DGGE was conducted using the randomly selected vegetables contaminated with food-borne pathogens. These 8 samples belonged to herbs, packaged salads, packaged sprout vegetables, and asparagus. One sample was fresh-cut agricultural food and seven were fresh-cut agricultural products.

Genomic DNA was extracted from vegetable samples using Genomic DNA extraction kit (FastDNA spin kit for soil, MP Biomedicals, Irvine, CA, USA). 500 mg of the sample was prepared, placed in a tube with beads, and homogenized with homogenizer (Precellys® 24, Bertin Technologies, Siège, France) for 40 seconds after adding 978 µl of sodium phosphate buffer and 122 µl of MT buffer, according to the manufacturer's method. Artificial markers for PCR-DGGE were made with 4 food-borne pathogens (*E. coli, B. cereus,*

C. perfringens, Y. enterocolitica) isolated and identified from the vegetables. Colonies were collected, put into sterile water, heated at 105℃ for 20 minutes, and centrifuged for 5 minutes at 13,000 rpm. Finally, the supernatants were used as genomic DNA of artificial markers.

PCR of 16s rDNA and PCR for DGGE

PCR for PCR-DGGE was performed as followed. PCR Premix (Takara Bio, Shiga, Japan) was used for PCR with a total volume of 20 μl, including 10x *Taq* buffer 2 μl, dNTP 1.6 μl, DW 12.3 μl, template DNA 2 μl, 20 pmol of each primer and 1 unit of *Taq polymerase*. And the primers for PCR were used as shown in Table 1. The denaturation temperature conditions for 1st PCR were repeated for 30 cycles of 3 cycles of denaturation at 95℃ for 10 minutes and [denaturation at 95℃ for 30 seconds, annealing at 55℃ for 30 seconds, extension at 72℃ for 1 minute], followed by an extension reaction at 72℃ for 10 minutes. PCR products were confirmed by using automatic electrophoresis (DE/QIAxcel Advanced System, Qiagen). Subsequently, touchdown PCR for 2nd PCR was performed 20 cycles of denaturation at 95℃ for 10 minutes, [denaturation at 95℃ for 30 seconds, annealing at 65℃ for 30 seconds, extension at 75℃ for 30 seconds] and the annealing temperature was decreased by 0.5 every cycle until the touchdown temperature of 55℃ reached. Then additional 15 cycles were performed at an annealing temperature of 55℃. Finally, the reaction was extended for 10 minutes at 75℃.

DGGE analysis

DGGE was performed using the DCode system (Bio-Rad, Hercules, CA, USA), and the dimension of the gel was 16 cm × 15 cm × 1 mm. The denaturation gradient gel was made of 8% polyacrylamide gel consisting of a denaturant gradient of 30-50%. The electrophoresis was performed with a constant voltage of 70 V at 65℃ for about 13 hr [20 mM in 0.5X TAE bufferTris base, 10 mM acetic acid, 0.5 mM EDTA (pH 8.0)]. After electrophoresis, the gels were stained with ethidium bromide solution (10 μg/ml in DW) for 30 minutes, washed with DW, and observed under UV illumination.

Results

Food-borne pathogens detection by culture-based methods

Using 129 vegetables of agricultural materials, quantitative analyses were performed for non-pathogenic *Escherichia coli* was detected in 5 samples (3.9%), all of which were fresh-cut agricultural products (Table 2). The average number of non-pathogenic *E. coli* in fresh-cut agricultural products was 179 CFU/g (range: 5 to 700 CFU/g).

In analysis of low-risk food-borne pathogens, *Bacillus cereus* was detected in 41 samples (31.8%) at high level of detection rate (Table 3). The average in fresh-cut agricultural foods was 73 CFU/g (range: 20 to 100 CFU/g), while fresh-cut agricultural products was 940 CFU/g (range: 10 to 22,000 CFU/g). Although not high levels (3.1% of 129 total samples, 9.8% of 41 samples detected with *B. cereus*), four samples exceeded the 1,000 CFU/g, showing a unit of microbiological standard for managing fresh-cut agricultural foods under the Food Sanitation Act. But all of the four sam-

Table 1. List of primers for PCR-DGGE

Primer	Sequence (5′ → 3′)	Remark
Bact_011F	AGA GTT TGA TCC TGG CTC AG	1st PCR
Bact_1492R	ACG GCT ACC TTG TTA CGA CTT	1st PCR
Bact_011F (GC)	(GC-clamp)^3 AGA CTT TGA TCC TGG CTC AG	2nd PCR for DGGE
Bact_0536R	GW^{1}A TTA CCG CGG CK^{2}G CTG	2nd PCR for DGGE

W^1 is A or T, K^2 is G or T, (GC-clamp)^3 is CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG.

Table 2. Distribution of non-pathogenic *Escherichia coli* in agricultural materials (unit: CFU/g)

Class	n^2	Mean	Range	5-10	10-10^2	10^2-10^3
Fresh-cut agricultural foods (n^1=26)	0	0	0	0	0	0
Fresh-cut agricultural products (n^1=103)	5	179	5-700	2	1	2

n^1, Number of samples
n^2, Number of positive samples

Table 3. Distribution of *Bacillus cereus* in agricultural materials (unit: CFU/g)

Class	n^2	Mean	Range	10-10^2	10^2-10^3	10^3-10^4	>10^4
Fresh-cut agricultural foods (n^1=26)	3	73	20-100	1	2	0	0
Fresh-cut agricultural products (n^1=103)	38	940	10-22,000	15	19	3	1

n^1, Number of samples
n^2, Number of positive samples

ples belonged to fresh-cut agricultural products regulated by Agricultural Product Quality Control Act with no microbiological standard. Microbiological risk assessment studies report that the minimum amount of *B. cereus* infection during eating of vegetables is 10^5 to 10^7 CFU/g [17]. Table 4 showed the quantitative analysis of *Clostridium perfringens* detected in this study. *C. perfringens* was detected in 7 samples (5.4%) belonging to fresh-cut agricultural products. The average value was a low level of 23 CFU/g (range: 10 to 65 CFU/g) belonging to fresh-cut agricultural products. It has been reported that food-borne disease by *C. perfringens* requires a high level of bacteria to show symptoms (10^6-10^7 CFU/g) [9]. In case of fresh-cut agricultural foods, Food Sanitation Act restricts the numbers to 100 CFU/g or less of *C. perfringens*. On the other hand, *Staphylococcus aureus* was not detected in any samples both of fresh-cut agricultural foods and fresh-cut agricultural products (data not shown).

In analysis of high-risk food-borne pathogens, *Salmonella* spp., *Listeria monocytogenes,* and *Campylobacter jejuni/coli* were not detected in any samples of agricultural materials (data not shown). However *Yersinia enterocolitica* was detected in 1 sample (0.8%) of fresh-cut agricultural products, packaged salads, and enterohemorrhagic *Escherichia coli* was detected in 1 sample (0.8%) of fresh-cut agricultural products, parsnips. Fresh-cut agricultural products detected with *Y. enterocolitica* were packaged and sold with washing recommendation labeled on the pack. But it is likely to be overlooked due to immediate eating without any handling. It suggested that extra cares should be taken even if storing at refrigerated temperatures. Isolate of enterohemorrhagic *E. coli* from parsnips had VT1 toxin gene but its serotype was not O157:H7.

Food-borne pathogens detection by PCR-DGGE

Randomly selected eight samples contaminated with bacteria by culture-based method were further analyzed by PCR-DGGE. Table 5 showed sample types and their food-borne pathogens detection by culture-based method.

Enterohemorrhagic *E. coli* was detected in sample No. 1 by both culture-based method (160 CFU/g) and PCR-DGGE (Fig. 1). But non-pathogenic *E. coli* was not in PCR-DGGE of sample No. 5 and sample No. 6 that were detected by the culture method at 5 CFU/g (Fig. 1). It seemed that it was difficult to detect non-pathogenic *E. coli* due to the low level of bacteria. The detection limit of *E. coli* in the PCR-DGGE has been reported to be 10 CFU/g [10, 12]. On the other hand, the strain was recognized in PCR-DGGE of sample No. 4 and sample No. 7, even though no detection by the culture method (Fig. 1). In the quantitative culture meth-

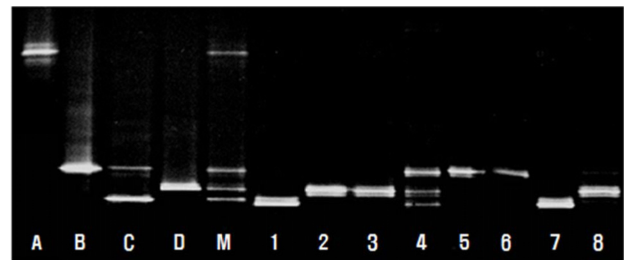

Fig. 1. Detection of food-borne pathogens by PCR-DGGE. DGGE of PCR products was performed on the 30% to 50% denaturing gradient gel. Lane A, B, C, D and M indicated the artificial marker made from isolated strains. Lane A: *Clostridium perfringens*, Lane B: *Bacillus cereus*, Lane C: *Escherichia coli*, Lane D: *Yersinia enterocolitica*, Lane M: markers (A+B+C+D), Lane 1-8: vegetable samples.

Table 4. Distribution of *Clostridium perfringens* in agricultural materials (unit: CFU/g)

Class	n^2	Mean	Range	5-10	10-10^2
Fresh-cut agricultural foods (n^1=26)	0	0	0	0	0
Fresh-cut agricultural products (n^1=103)	7	23	10-65	4	3

n^1, Number of samples
n^2, Number of positive samples

Table 5. Comparison of strains detected by culture-based method and PCR-DGGE

Sample No.	Class	Type	Strains detected by culture (CFU/g)				Strains detected by PCR-DGGE			
			EC	BC	CP	YE	EC	BC	CP	YE
1	P[1]	Herbs	+[3] (160)	+ (50)	-	-	+[3]	-	-	-
2	P[1]	Herbs	-	+ (40)	+ (10)	-	-	-	-	+
3	F[2]	Packaged salads	-	+ (60)	-	-	-	-	-	+
4	P[1]	Packaged sprout vegetables	-	+ (100)	+ (25)	-	+[4]	+	-	+
5	P[1]	Packaged sprout vegetables	+[4] (5)	+ (100)	-	-	-	+	-	-
6	P[1]	Asparagus	+[4] (5)	+ (100)	-	-	-	+	-	-
7	P[1]	Herbs	-	-	+ (60)	-	+[4]	-	-	-
8	P[1]	Packaged salads	-	-	-	+[5]	-	-	-	+

EC, *Escherichia coli,* BC, *Bacillus cereus,* CP, *Clostridium perfringens,* YE, *Yersinia enterocolitica*
1, Fresh-cut agricultural products
2, Fresh-cut agricultural foods
3, Enterohemorrhagic *Escherichia coli*
4, Non-pathogenic *Escherichia coli*
5, *Yersinia enterocolitica* was not quantitatively analyzed

od, samples were diluted by 10-fold stepwise without direct enrichment and possibility of loss occurred during the dilution process.

Among the 6 samples contaminated with *B. cereus,* PCR-DGGE detected it in three samples (No. 4~6) at 100 CFU/g or more (Fig. 1). But samples (No. 1~3) at 40~60 CFU/g were not detected in PCR-DGGE analysis (Fig. 1). The detection limit of *B. cereus* in PCR-DGGE was reported to be 1,000 CFU/g [10, 12]. Our results showed a similar pattern to those of previous studies [10, 12].

In three samples at 10~60 CFU/g of *C. perfringens,* the strain was not detected in PCR-DGGE (Fig. 1). It was reported that the detection limit of *C. perfringens* in PCR-DGGE is 1,000 CFU/g [10, 12] and therefore it was not detected by PCR-DGGE in three *C. perfringens* positive samples.

Y. enterocolitica was recognized in PCR-DGGE of sample No. 8 contaminated with *Y. enterocolitica*. But *Y. enterocolitica* was detected in PCR-DGGE of sample No. 2, No. 3 and No. 4, even though no detection in the culture method (Fig. 1), which were packaged salads, packaged sprout vegetables, and herbs. It seemed that it was difficult to identify a low level of bacteria by culture-based method.

Discussion

The consumption of fresh-cut agricultural foods and products is increasing and food-borne diseases are increasing worldwide. The purpose of this study was to investigate the contamination level of fresh-cut agricultural materials distributed in Korea and provide data for microbiological risk assessment and microbiological standard setting. Also, we tried to contribute to the improvement of the food-borne pathogen detection method by comparing the culture-based method and PCR-DGGE.

PCR-DGGE was conducted on randomly selected eight vegetables contaminated with many food-borne pathogens detected by the culture method. These vegetables belonged to herbs, packaged salads, packaged sprout vegetables, and asparagus, which can be eaten immediately without heating. There was some difference in the bacterial species detected by between the culture-based method and PCR-DGGE (Table 5). Enterohemorrhagic *Escherichia coli* was detected by both detection methods in one sample. Non-pathogenic *E. coli* was in two samples, which not detected by culture. It suggested that the strain at level of 160 CFU/g quantitatively in the culture method is possible to detect in PCR-DGGE [10, 12]. It seemed that it is easier to detect the species by PCR-DGGE than by the culture method. *Bacillus cereus* was identified by PCR-DGGE only in three samples detected at 100 CFU/g or more. *Clostridium perfringens* were detected at 10~60 CFU/g in the culture-based method, but did not in PCR-DGGE. In prior studies, the detection limit of PCR-DGGE was reported to be 1,000 CFU/g of *C. perfringens* and 1,000 CFU/g of *B. cereus* [10, 12]. This study showed a similar pattern to the detection limit of previous studies. On the other hand, although *Yersinia enterocolitica* was not detected in the culture method, three sample cases were recognized in PCR-DGGE only.

As such, in the culture-based method, detection of strains takes more time relatively as 5-7 days and requires a lot

of effort but PCR-DGGE is possible within 3-4 days. Also, In the case of high-risk food-borne pathogens, the detection limit was lower in the PCR-DGGE method (10 CFU/g) than in the culture-based method, meaning easier detection. Based on the detection limit of PCR-DGGE, it was expected that it can be used to identify the species of high-risk food-borne pathogens with a low detection limit. Therefore, it suggested that PCR-DGGE can be used as an alternative method for detecting harmful microorganisms in vegetables. High-risk food-borne pathogens can cause food-borne diseases even in low levels of bacteria (10 CFU/g or less) and may cause death, complications, and sequelae when infected, so special attention is required. It is necessary to recognize the possibility of contamination of vegetables caused by high-risk food-borne pathogens. It was also suggested that sufficient washing recommendations and hygiene management should be done. This study will contribute to the improvement of food-borne pathogen detection methods and the prevention of food-borne disease outbreaks caused by fresh-cut agricultural materials.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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건강에 대한 관심 증대와 1인 가구 증가라는 사회구조적인 변화로 이용하기 편리한 농산물에 대한 소비가 증가하고 있다. 대부분의 신선 농산물은 가열하지 않고 섭취하는 경우가 많기 때문에 식품 매개 병원체에 쉽게 노출될 수 있어 세계적으로 과채류가 원인인 식중독 사고의 보고가 증가하고 있다. 이에 본 연구에서는 신선 농산물의 미생물학적 품질을 평가하고 식중독균 검출 방법을 비교 분석하고자 하였다. 신선 농산물 중 채소류 129건을 구입하여 배양기반 방법으로 식중독균을 분석한 결과, non-pathogenic *Escherichia coli* (3.9%), *Bacillus cereus* (31.8%), *Clostridium perfringens* (5.4%), *Yersinia enterocolitica* (0.8%), enterohemorrhagic *E. coli* (0.8%)가 검출되었다. 이러한 식중독균의 분석에는 증균 배양과정이 중요하게 작용을 하며 균주의 순수 분리 및 확인 동정에까지 상대적으로 많은 시간과 노력이 요구된다. 따라서 증균 배양의 과정 없이 식중독균을 신속하게 검출 할 수 있는 PCR-DGGE를 수행하여 배양 기반의 분석법과 비교하였다. 비병원성 대장균은 배양 기반 방법에서 검출되지 않았음에도 PCR-DGGE에서는 검출된 경우가 2건이 있었다. 본 연구에서 사용한 대장균 정량 분석방법은 시료를 10배 희석한 후 배양하는 과정에서 시료의 손실 가능성과 검출 한계가 높은 단점으로 PCR-DGGE가 균종의 확인에 더욱 용이할 것으로 보였다. 저위해성 식중독균은 배양 기반 방법보다 PCR-DGGE에서 검출 한계가 높은 것으로 보였다. 고위해성 식중독균은 배양 기반 방법보다 PCR-DGGE (10 CFU/g)에서 검출 한계가 낮아 균종 확인과 검출에 용이하다고 판단되었고 이를 통해 채소류에서 고위해성 식중독균의 잠재적 위험성을 확인하였다. 본 연구의 결과는 신선 농산물의 미생물 위해 평가와 기준 설정을 위한 기초 자료로 활용될 수 있으며 신선 농산물 관련 식중독균 검출 방법의 개선과 식중독 발생 예방에 기여할 것으로 기대한다.