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ARTICLE

Comparison of Upgraded Methods for Detecting Pathogenic *Escherichia coli* in Foods Using Centrifugation or Filtration

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Abstract

In the present study, centrifugation and filtration pretreatments were evaluated to decrease sample preparation time and to improve the sensitivity and specificity of multiplex polymerase chain reaction (PCR) for the detection of low levels of pathogenic *Escherichia coli* in various foods. Pathogenic *E. coli* (*E. coli* NCCP11142, *E. coli* NCCP14037, *E. coli* NCCP 14038, *E. coli* NCCP14039, and *E. coli* NCCP15661) was inoculated into pork, beef, and baby leafy vegetables at 1, 2, and 3 Log CFU/g. The samples were shaken 30 times (control), then centrifuged or filtered. DNA extracts from the samples were subjected to PCR using the Powerchek™ Diarrheal *E. coli* 8-plex Detection Kit. In the pork samples, no *E. coli* was detected in the control samples, while *E. coli* were detected in 100% of 3-Log CFU/g inoculated and centrifuged samples, and in 100% of 2 and 3-Log CFU/g inoculated, and filtered samples. In the beef samples, all control samples appeared to be *E. coli*-negative, while *E. coli* was detected in 50-75% of centrifuged samples, regardless of inoculated level, and in 100% of 2 and 3-Log CFU/g inoculated, and filtered samples. In baby leafy vegetables, *E. coli* were not detected in 25-50% of the control samples, while *E. coli* were detected in 0-25% of the centrifuged samples, and 75-100% of the filtered samples, depending on the inoculum amount. In conclusion, filtration pretreatment can be used to minimize sample preparation time, and improve the sensitivity and specificity of rapid detection of pathogenic *E. coli* in various foods.

Keywords *Escherichia coli*, sample pretreatment, centrifugation, filtration, rapid detection, polymerase chain reaction

Introduction

Escherichia coli are facultative anaerobic bacteria found in human intestines, and can be categorized into pathogenic and non-pathogenic *E. coli*. The clinical symptoms of pathogenic *E. coli* infection are diarrheal disease, urinary tract infection, sepsis, and meningitis (Nataro and Kaper, 1998). Pathogenic *E. coli* are categorized as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC), according to their pathogenic features (Kuhnert *et al*., 2000; Sidhu *et al*., 2013). In South Korea, there were 38 *E. coli* outbreaks in 2006 and 59 *E. coli* outbreaks in 2016 (MFDS, 2017). During 2004-2006, Lee *et al*. (2009) isolated EHEC, ETEC, and EPEC strains from pork (201/3000; 14.9%), beef (31/3000; 4.1%), and poultry (41/3000; 4.6%), purchased from meat processing facil-

ities, butcher shops, wholesale stores, and wet markets in several regions of South Korea. Recently, *E. coli* outbreaks resulting from vegetable consumptions have also been reported (CDC, 2017; Denis *et al.*, 2016; Hong *et al.*, 2012; Lee *et al.*, 2016; Wendel *et al.*, 2009). Consumption of baby leafy vegetables has increased, usually in the form of fresh-cut salad, which is vulnerable to contamination with pathogens from soil, water, and human handling (Beuchat *et al*., 1996; Tomás-Callejas *et al*., 2011).

The traditional method for isolation and identification of pathogens involves a lot of time and labor (Fukushima *et al.*, 2007). Rapid identification of pathogens is important to prevent foodborne illnesses, and a rapid detection method is therefore necessary, especially for raw foods (Ge and Meng, 2009; Naravaneni and Jamil, 2005). A rapid detection method should be sufficiently sensitive to detect low concentrations of pathogens, and should be time-efficient for immediate detection (Law *et al*., 2015). However, rapid detection methods are not usually accurate at low cell concentrations (Mandal *et al*., 2011). If the bacterial cells on foods can be concentrated by a physical method, rather than by enrichment, the speed of rapid detection methods may be improved (Stevens and Jaykus, 2004). Polymerase chain reaction (PCR) is a commonly used nucleic acid-based method, which has high sensitivity and specificity compared to that of immunological methods based on antibody-antigen reactions (Law *et al.*, 2015).

Therefore, the objective of this study was to evaluate the efficiency of centrifugation and filtration pretreatment methods to decrease sample preparation time and to improve the sensitivity and specificity of multiplex PCR for the rapid detection of low concentrations of pathogenic *E. coli* in various foods.

Material and Methods

Inoculum preparation

A single colony of each pathogenic *E. coli* strain [*E. coli* NCCP11142, *E. coli* NCCP14037, *E. coli* NCCP 14038, *E. coli* NCCP14039, and *E. coli* NCCP15661] was cultured in 10 mL tryptic soy broth (TSB; Becton, Dickinson and Company, USA) at 37°C for 24 h. A 0.1 mL aliquot was then subcultured in 10 mL TSB at 37°C for 24 h. A five-strain mixture (25 mL) of pathogenic *E. coli* was centrifuged at 1,912 *g* and 4°C for 15 min, and the cell pellet was washed twice with phosphate-buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water) and then diluted serially to obtain 3, 4, and 5 Log CFU/mL.

Sample preparation and inoculation

Samples (pork, beef, and baby leafy vegetables) were purchased from a butcher shop and a market located in Seoul, South Korea in March, 2017. Pork (leg butt), beef (leg butt), and baby leafy vegetables were purchased and tested for the presence of *E. coli*. Twenty five grams of pork, 25 g of beef and 10 g of baby leafy vegetables were placed into a filter bag (3M, USA), and 0.1 mL of bacterial suspension was inoculated onto each sample to obtain 1, 2, and 3 Log CFU/g. A total of four samples (n=4) were prepared for each inoculum concentration. After inoculation, the samples were massaged 20 times to allow *E. coli* attachment.

E. coli enumeration

To enumerate *E. coli* cells in the inoculated samples, negative control samples and *E. coli* inoculated samples at 1, 2, and 3 Log CFU/g were resuspended with 50 mL 0.1% BPW in a filter bag, and shaken 30 times. One-milliliter aliquots of the diluents were then spread onto *E. coli*/Coliform Count Plate (Petrifilm™, 3M, USA). The plates were incubated at 37°C for 24 h and blue colonies with gas bubbles were then counted. Four replicates (n=4) were analyzed for bacterial concentrations and sample pretreatments.

Sample pretreatment

For each sample in bags as previously described, bacteria were allowed to be attached for 15 min, and then 50 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company) was added and shaken 30 times. The shaken samples were pretreated as follows: (i) control: shaken sample, (ii) centrifuged: 50 mL of the shaken sample was transferred to a conical tube and centrifuged at 1,912 *g* at 4°C for 15 min, and the cell pellet was then resuspended in 5 mL 0.1% BPW, and (iii) filtered: 50 mL of the shaken sample was filtered through filter paper (Hyundai micro Co., Ltd., Korea) and subsequently through a 0.45 μm membrane filter (Hyundai micro Co., Ltd.), followed by vortexing the membrane filter in 5 mL 0.1% BPW for 1 min.

DNA extraction and PCR

One-milliliter aliquots of control, centrifuged, and filtered samples were used for DNA extraction. DNA was

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extracted with Powerprep™ DNA Extraction Food and Feed Kit (KogeneBiotech, Korea) according to the manufacturer's protocol, and the DNA extracts were subjected to PCR with Powerchek™ Diarrheal *E. coli* 8-plex Detection Kit (KogeneBiotech) according to the manufacturer's protocol. Eight genes [heat-labile enterotoxin (LT; 530 bp), heat-stable enterotoxin (ST; 167 bp), *bfpA* (400 bp), *eaeA* (231 bp), *aggR* (757 bp), *stx*1 (637 bp), *stx*2 (297 bp), and *ipaH* (141 bp)] can be detected by this kit.

Statistical analysis

The significance was determined by Fisher's exact test ($p<0.05$) to analyze the positive proportions of PCR results among bacterial concentrations or pretreatments using SAS® version 9.4 (SAS Institute, USA).

Results and Discussion

The absence of *E. coli* in the initial pork, beef, and baby leafy vegetable samples was confirmed by a quantitative experiment (detection limit: 0.5 Log CFU/g for pork and beef, and 0.8 Log CFU/g for baby leafy vegetables). To evaluate the effect of centrifugation and filtration on decreasing sample preparation time and improving the sensitivity of PCR, pathogenic *E. coli* were inoculated into the samples at 1, 2, and 3 Log CFU/g, and targeted *E. coli* concentrations were obtained (Table 1). The presence of pathogenic *E. coli* was detected by Powerchek™ Diarrheal *E. coli* 8-plex Detection Kit. The kit can detect eight genes, including heat-labile enterotoxin gene (LT; 530 bp), heat-stable enterotoxin gene (ST; 167 bp), *bfpA* (400 bp), *eaeA* (231 bp), *aggR* (757 bp), *stx*1 (637 bp), *stx*2 (297 bp), and *ipaH* (141 bp). *E. coli* genes were detected in the inoculum using the kit. Five of the tested *E. coli* strains possessed the genes *eaeA* (*E. coli* NCCP 11142, 14037, 14038, and 15661), *aggR* (*E. coli* NCCP 14039), and *bfpA* (*E. coli* NCCP 15661).

After pretreatment of inoculated samples, PCR was performed to compare the effect of sample pretreatment on the time taken to detect pathogenic *E. coli*. In pork samples, pathogenic *E. coli* were not detected in the control samples at any of the inoculum concentrations (data not shown). Centrifuged pork samples were all *E. coli*-positive (100%; 4/4) at 3 Log CFU/g, while no *E. coli*-positive samples were observed at 1 and 2 Log CFU/g ($p<0.05$) (Fig. 1). For filtered pork samples, *E. coli* was detected in all samples (100%; 4/4) at 3 Log CFU/g and 2 Log CFU/g (100%; 4/4), but at 1 Log CFU/g, no *E. coli* was detected in any of the samples ($p<0.05$) (Fig. 2). This result indicates that filtration pretreatment can decrease sample preparation time, compared to enrichment because filtration takes less than 30 min, but enrichment takes 24 h

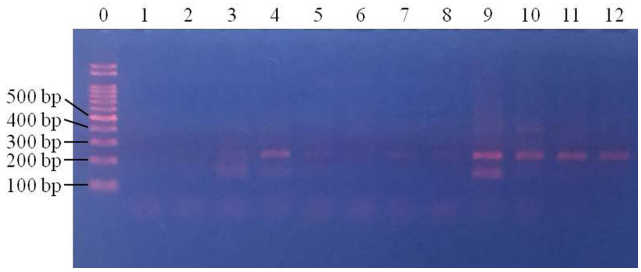

Fig. 1. Detection of pathogenic *Escherichia coli* in pork samples by multiplex PCR after sample pretreatment by centrifugation. Lane 0: 100-bp ladder; lane 1-4: 1 Log CFU/g inoculated samples; lane 5-8: 2 Log CFU/g inoculated samples; lane 9-12: 3 Log CFU/g inoculated samples.

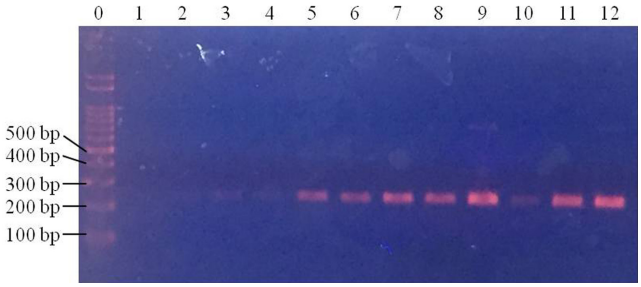

Fig. 2. Detection of pathogenic *Escherichia coli* in pork samples by multiplex PCR after sample pretreatment by filtration. Lane 0: 100-bp ladder; lane 1-4: 1 Log CFU/g inoculated samples; lane 5-8: 2 Log CFU/g inoculated samples; lane 9-12: 3 Log CFU/g inoculated samples.

Table 1. Pathogenic *Escherichia coli* cell counts (mean ± standard deviation) in pork, beef, and baby leafy vegetable samples

Sample	Inoculated *Escherichia coli* level			
	Not inoculated	1 Log CFU/g	2 Log CFU/g	3 Log CFU/g
Pork	< 0.5[1)]	1.3 ± 0.1	2.1 ± 0.0	3.2 ± 0.0
Beef	< 0.5	0.8 ± 0.5	2.2 ± 0.1	3.2 ± 0.1
Baby leafy vegetables	< 0.8[2)]	1.3 ± 0.3	2.4 ± 0.1	3.1 ± 0.2

[1),2)]: detection limit

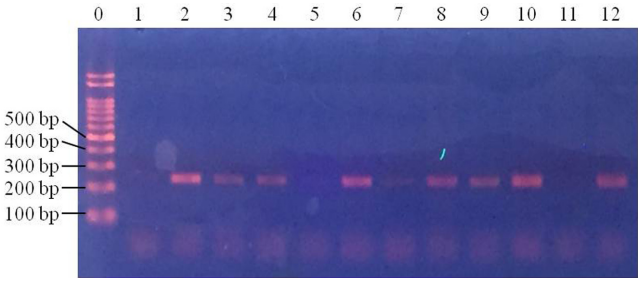

Fig. 3. Detection of pathogenic *Escherichia coli* in beef samples by multiplex PCR after pretreatment by centrifugation. Lane 0: 100-bp ladder; lane 1-4: 1 Log CFU/g inoculated samples; lane 5-8: 2 Log CFU/g inoculated samples; lane 9-12: 3 Log CFU/g inoculated samples.

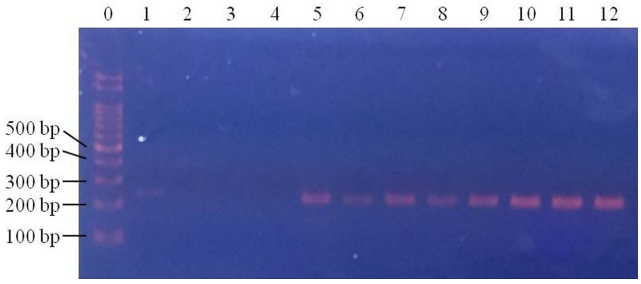

Fig. 4. Detection of pathogenic *Escherichia coli* in beef samples by multiplex PCR after pretreatment by filtration. Lane 0: 100-bp ladder; lane 1-4: 1 Log CFU/g inoculated samples; lane 5-8: 2 Log CFU/g inoculated samples; lane 9-12: 3 Log CFU/g inoculated samples.

as well as improving the sensitivity of PCR when detecting *E. coli* in pork.

In beef samples, *E. coli* was not detected in control samples (data not shown). Of the four centrifuged samples, *E. coli* was detected in three (75%; 3/4) samples at 1 Log CFU/g (Fig. 3). *E. coli* was detected in two of the centrifuged samples at 2 Log CFU/g (50%; 2/4), and in three samples at 3 Log CFU/g (75%; 3/4) (Fig. 3). In the filtered samples, *E. coli* was detected at 2 and 3 Log CFU/g, but not at 1 Log CFU/g ($p<0.05$) (Fig. 4). This result suggests that even though centrifugation can allow the detection of *E. coli* at 1 Log CFU/g in beef, the detection rates were not 100% at any concentrations. On the other hand, filtration allowed the detection of 2 and 3 Log CFU/g *E. coli* in 100% of the samples. Thus, filtration pretreatment can support rapid pathogenic *E. coli* detection in beef, using PCR.

In baby leafy vegetables, *E. coli* were detected in the control samples in 25% of 1 Log CFU/g samples, 25% of 2 Log CFU/g samples and 50% of 3 Log CFU/g samples

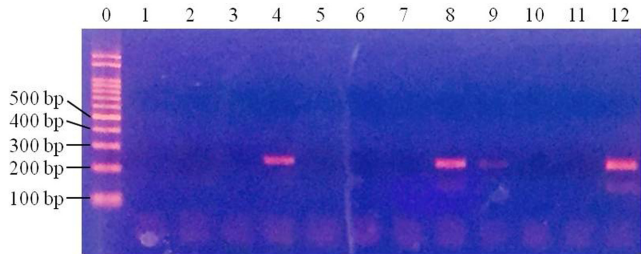

Fig. 5. Detection of pathogenic *Escherichia coli* in baby leafy vegetable samples by multiplex PCR for control. Lane 0: 100-bp ladder; lane 1-4: 1 Log CFU/g inoculated samples; lane 5-8: 2 Log CFU/g inoculated samples; lane 9-12: 3 Log CFU/g inoculated samples.

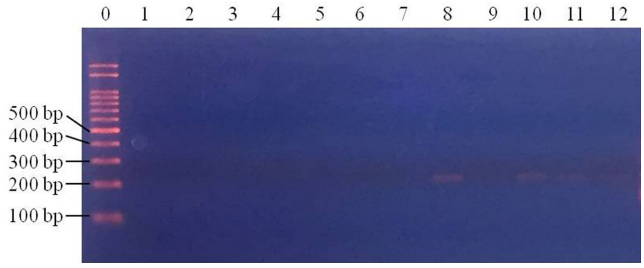

Fig. 6. Detection of pathogenic *Escherichia coli* in baby leafy vegetable samples by multiplex PCR after pretreatment by centrifugation. Lane 0: 100-bp ladder; lane 1-4: 1 Log CFU/g inoculated samples; lane 5-8: 2 Log CFU/g inoculated samples; lane 9-12: 3 Log CFU/g inoculated samples.

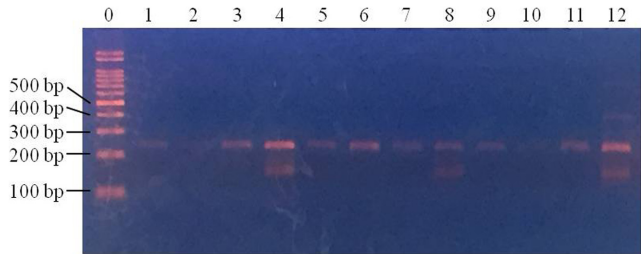

Fig. 7. Detection of pathogenic *Escherichia coli* in baby leafy vegetable samples by multiplex PCR after pretreatment by filtration. Lane 0: 100-bp ladder; lane 1-4: 1 Log CFU/g inoculated samples; lane 5-8: 2 Log CFU/g inoculated samples; lane 9-12: 3 Log CFU/g inoculated samples.

(Fig. 5). In samples subjected to centrifugation, *E. coli* was detected in 0% of 1 Log CFU/g samples, 25% of 2 Log CFU/g samples and 25% of 3 Log CFU/g samples (Fig. 6). Following filtration, *E. coli* was detected in 75% of 1 Log CFU/g, 100% of 2 Log CFU/g, and 100% of 3 Log CFU/g samples (Fig. 7). This indicates that filtration is a suitable pretreatment, which contributes to rapid detection of pathogenic *E. coli* by PCR.

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Enrichment, although time-consuming, is necessary prior to detection of pathogens by PCR, especially for low concentrations of bacteria (Fukushima *et al.*, 2007; Stevens and Jaykus, 2004). During enrichment, other bacteria are also enriched, especially in meat that contains more endogenous bacteria than vegetables, and the characteristics of the food matrix could affect the PCR assay (Bhunia, 2014; Wang and Salazar, 2016). PCR has high sensitivity and high specificity for the detection of foodborne pathogens (Law *et al.*, 2015), but in combination with enrichment, it cannot be a rapid detection method. Therefore, pretreatment for rapid detection using PCR is critical as described, and according to the results from our study, it can be concluded that filtration pretreatment can replace enrichment prior to PCR for the detection of pathogenic *E. coli* in pork, beef, and baby leafy vegetables, and can thus save time when detecting even low concentrations of *E. coli.*

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