Biocontrol Science, 2005, Vol. 10, No. 3, 91－96

Original

Simultaneous Enrichment Detection Method for Four Types of Pathogenic Bacteria in Food

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Received 4 January 2005/Accepted 5 April 2005

Recently, the incidence of foodborne disease outbreaks associated with contaminated meat, seafood and fresh produce has increased worldwide. Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7, *Salmonella* spp., *Listeria monocytogenes* and *Shigella* spp. have been responsible for recent outbreaks associated with the consumption of such contaminated food. Hence, suitable detection methods for monitoring the risk of microbial food contamination are necessary. These bacterial pathogens in food could be detected by enrichment culture in buffered peptone water (BPW) and multiplex PCR analysis of culture broth. After the detection of pathogens by multiplex PCR analysis, each pathogen-positive sample was cultured in an enriched selective broth. After the selective enrichment culture, the culture broth was plated on a selective agar plate to obtain colonies. EHEC (≧13 cells/25 g), *S. Enteritidis* (≧15 cells/25 g), *L. monocytogenes* (≧394 cells/25 g) and *S. sonnei* (≧2.4 cells/25 g) were detected and isolated. These pathogenic bacteria in frozen foods were also detected and isolated.

Key words : Simultaneous enrichment/Multiplex PCR/Pathogenic bacteria/Isolation.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7, *Salmonella* spp., *Listeria monocytogenes* and *Shigella* spp. are the major causative agents of food poisoning. Even a small number of these bacteria are sufficient to cause food poisoning in humans : $10^1 \sim 10^4$ cells (Blaser and Newman, 1982), 10^2 cells (Paton and Paton, 1998), 10^2 cells (Maruyama and Kokubo, 2000) and $10^1 \sim 10^2$ cells (Dupont et al., 1989), respectively. Although separate detection methods for EHEC, *Salmonella* spp., *L. monocytogenes* and *Shigella* spp. have been established, a simultaneous detection method for these four pathogens in food has not yet established. In stool samples, real-time PCR assays can simultaneously detect 8 of 17 pathogens (Fukushima et al., 2003). In patients with enteritis symptoms caused by pathogenic bacteria, a large number of bacteria can be detected easily in their stool. However, because pathogenic bacteria are usually present in contaminated food in small numbers, the detection method for bacteria in stool samples is not useful for detecting bacteria in contaminated food. Enrichment culture is required for the detection of bacteria in food. Three types of bacteria, namely EHEC, *Salmonella* spp., and *L. monocytogenes*, in vegetables and fruits were detected by our previously reported method (Miyahara et al., 2002). Furthermore, *S. sonnei* was detected by a similar enrichment culture (Miyahara and Konuma, 2002 and Miyahara et al., 2003). A combined detection method for EHEC, *Salmonella* spp., *L. monocytogenes* and *Shigella* spp. in contaminated food will be described in this paper. This detection method will be simple, accurate and economical. Colonies of causative pathogens can be obtained by further selective enrichment culture following the primary enrichment culture and plating on a selective agar plate.

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MATERIALS AND METHODS

Food samples

Cucumbers, cabbage, bean sprouts and ground beef were purchased from a retail store in Tokyo. Cucumbers, cabbage and bean sprouts were homogenized using a food processor.

Simultaneous enrichment method and multiplex PCR

Simultaneous enrichment detection method for four types of bacteria in food is shown in Fig. 1. Simultaneous enrichment cultivation in buffered peptone water (BPW) was performed first.

After BPW cultivation, multiplex PCR analysis was performed once. The preparation of DNA solution for PCR and the PCR method were described previously in our previous report (Miyahara et al., 2003). The primer sets used were as follows: O157 EVC-1/2 VT1 and 2 (amplicon 171 bp) (TaKaRa) for EHEC; SIN-1/2 for *inv*A of *Salmonella* (378 bp) (TaKaRa); prfA1 (5'-ACCAATGGGATCCACAAGA-3') and prfA2 (5'-CAGCTGAGCTATGTGCGAT-3') for *L. monocytogenes* (467 bp) (Bubert et al., 1997); and IPA-1/2 for *Shigella* (242 bp) (TaKaRa). The DNA solution was stored at 4℃. PCR amplification was performed using 1 μl of the solution and TaKaRa Ex Taq (TaKaRa, Kusatsu, Japan). The electrophoregram of the multiplex PCR products from the DNA solutions of EHEC, *Salmonella*, *Listeria* and *Shigella* on agarose gel is shown in Fig. 2.

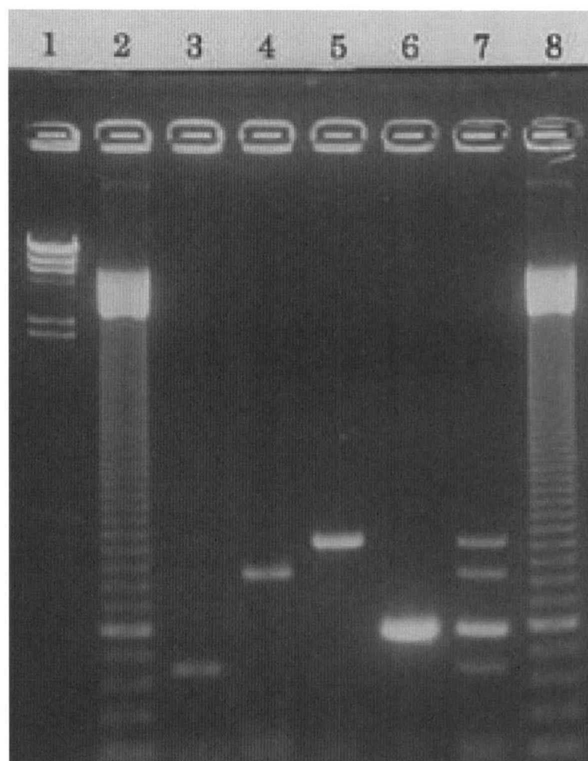

FIG. 2. Two percent agarose gel electrophoresis of multiplex PCR for EHEC, *Salmonella, Listeria* and *Shigella*. Lanes: 1, Molecular weight marker (Lambda DNA-HindIII digest; 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125 bp, respectively); 2 and 8, 50 bp ladder markers (250 bp: high light); 3, EHEC (PCR product, 171 bp); 4, *Salmonella* (378 bp); 5, *Listeria* (467 bp); 6, *Shigella* (242 bp); 7, mixture of four pathogens.

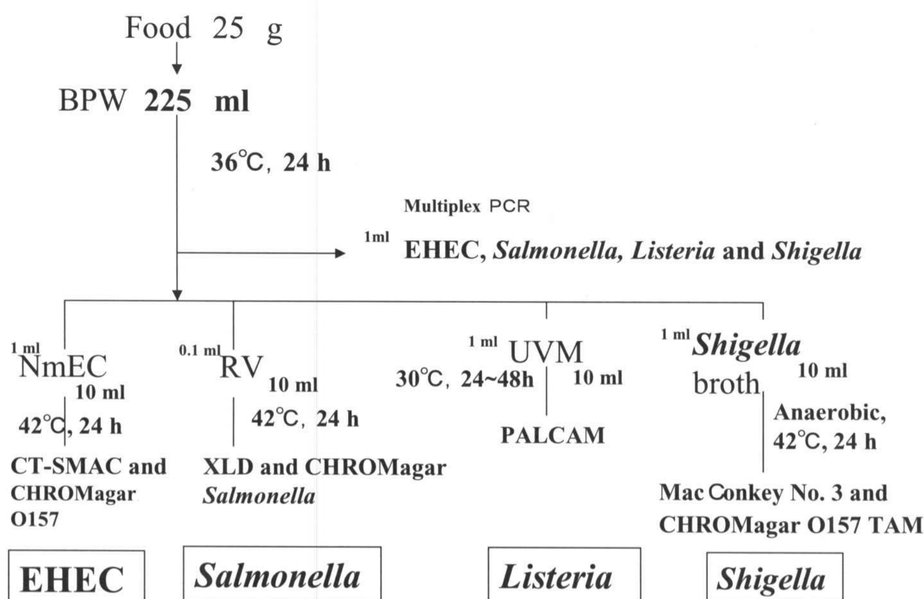

FIG. 1. Simultaneous enrichment detection method for pathogenic bacteria (EHEC, *Salmonella, Listeria* and *Shigella*) in food.

Selective culture

Selective cultivation was performed as shown in Fig. 1. NmEC for EHEC, Rappaport-Vassiliadis enrichment broth (RV) (OXOID, Hampshire, England) for *Salmonella*, University Vermont broth (UVM)-*Listeria* selective enrichment broth modified (MERCK, Darmstadt, Germany) for *Listeria*, and *Shigella* broth (MERCK) for *Shigella* were used as selective media. As an exception, *Shigella* broth was used under anaerobic conditions. Anaerobic conditions were achieved using AnaeroPack, an ageless package and a clip (Mitsubishi Gas Chemical Co./Sugiyamagen, Tokyo, Japan). After the selective cultivation, PCR with appropriate primers was performed. Fig. 1 shows the details of culture conditions.

Plating

After selective cultivation, a 10 μl loopful of the selective broth was streaked onto separation-selection agar plates. CHROMagar O157 TAM (TAM) (CHROMagar/Kanto Chemical, Tokyo, Japan) and sorbitol MacConkey agar containing 2.5mg/l potassium tellurite and 0.05mg/l cefixime (CT-SMAC) (Kanto Chemical) for EHEC, CHROMagar *Salmonella* (CHROMagar/ Kanto Chemical) and xylose lysine desoxycholate medium (XLD) (OXOID) for *Salmonella*, PALCAM-*Listeria*-selective agar with supplement (PALCAM) (MERCK) for *Listeria*, and TAM and MacConkey No.3 (OXOID) for *Shigella* were used for plating. Each plate was used for bacterial detection. Further plating was performed, when the result was not clear. PCR with selective primers was also performed to determine the enrichment conditions in the selective culture. All the plates were incubated at 35-37℃ for 20-24 h. As an exception, the PALCAM plates were incubated at 30℃ for 24-48 h. Suspected colonies on the plate were ascertained by colony PCR analysis.

Sensitivity test for EHEC

The simultaneous enrichment detection method is schematically shown in Fig. 1. As described in Fig. 4 of the study of Miyahara et al. (2002), EHEC was cultivated in BPW at 36℃ for 6h. One milliliter of the BPW culture broth was added to 10 ml of *Escherichia coli* broth containing novobiocin and bile acid (NmEC) (Eiken, Tokyo). This improved method was simplified as shown in Fig. 1. Twenty-four h in BPW was adopted for primary cultivation. The control 0.1 ml of physiological saline and EHEC (1.7 or 17CFU/0.1 ml) suspensions were inoculated into 25g of cucumber, respectively. Each group consisted of five inoculated samples. The EHEC suspension was prepared by overnight cultivation in tryptic soy broth at 36 ℃ and diluted with physiological saline. Inoculation size was calculated by the plating of a diluted suspension on tryptic soy agar (TSA).

Sensitivity test for *Listeria*

A *Listeria monocytogenes* suspension (1 ml) was inoculated into 25 g of each sample (cabbage) in a stomacher bag. One milliliter of *L. monocytogenes* suspension was inoculated into the bag. The numbers of inoculated cells were 6. 4, 32, 64, 394, 1778, 3556, 17780 and 35560 CFU/25 g of sample, respectively. One milliliter of the BPW cultivated broth was transferred to the UV broth. The details of the method are described above and in Fig.1.

Sensitivity test for *Shigella*

A *Shigella sonnei* suspension (1 ml) was inoculated into 25 g of each sample (cabbage) in a stomacher bag. One ml of *Shigella sonnei* suspension was inoculated into the bag. The numbers of inoculated cells were 0.5, 2. 4, 4. 8, 24 and 48 CFU / 25g of sample, respectively. Although 0.5 ml of BPW cultivated broth had been transferred to 10 ml of *Shigella* broth in our previous report (Miyahara et al., 2003), 1.0 ml of BPW broth was transferred to *Shigella* broth in this report.

Simultaneous enrichment detection in food inoculated with pathogenic bacteria

The control (physiological saline, 1 ml) or bacteria (1 ml) suspension was inoculated into 25 g of food sample. Each control group and the bacterium-inoculated group consisted of five samples. The bacterial suspension was prepared by cultivating bacteria in tryptic soy broth overnight and by diluting the cultured broth with physiological saline. Inoculum concentration was calculated by plating the diluted suspension on tryptic soy agar (TSA). The number of viable bacteria in the food samples was determined by the standard plating count (SPC) method. When detection studies in nonfrozen food were performed, SPC was also determined in the nonfrozen food. When detection studies in frozen food was conducted, frozen control samples were also analyzed for SPC. All the samples were analyzed for four types of pathogenic bacteria by the method shown above and in Fig. 1. The number of inoculated bacteria is mentioned in the footnotes of Tables 4 to 7.

RESULTS AND DISCUSSION

As shown in Table 1, among the samples with EHEC at low or high inoculation doses, in the first PCR analysis, EHEC were detected in 3/5 and 5/5

TABLE 1. EHEC[a] detection and isolation from cucumbers inoculated with EHEC.

Inoculated pathogenic bacteria	Inoculated dose of EHEC CFU/25g	BPW-PCR[b]	NmEC-PCR[c]	Colony detection[d]
Control	–	0/5	0/5	N.T.[e]
EHEC	1.7	3/5	2/5	2/5
	17	5/5	5/5	5/5

The standard plating count of cucumbers used was 7.8×10^5CFU/g.
[a] Enterohaemorrhagic Escherichia coli
[b] Detection rate by PCR analysis from BPW.
[c] Detection rate by PCR analysis from NmEC.
[d] Suspected colonies were identified by PCR analysis.
[e] Not tested.

TABLE 2. *Listeria* detection and isolation from cabbage inoculated with *Listeria monocytogenes*.

Inoculated pathogenic bacteria	Inoculated dose of *Listeria* CFU/25g	BPW-PCR[a]	UVM-PCR[b]	Colony detection[c]
Control	–	–	N.T.[d]	N.T.
Listeria	6.4	0/5	0/5	0/5
	32	0/5	0/5	0/5
	64	0/5	0/5	0/5
Control	–	–	N.T.	N.T.
Listeria	394	5/5	4/5	5/5
	1778	5/5	5/5	5/5
	3556	5/5	5/5	5/5
	17780	5/5	5/5	5/5
	35560	5/5	5/5	5/5

The standard plating counts of cabbage used were 2.1×10^5 and 6.5×10^4CFU/g, respectively.
[a] Detection rate by PCR analysis from BPW.
[b] Detection rate by PCR analysis from UVM.
[c] Suspected colonies were identified by PCR analysis.
[d] Not tested.

TABLE 3. *Shigella sonnei* detection and isolation from cabbage inoculated with *Shigella sonnei*.

Inoculated pathogenic bacteria	Inoculated dose of *Shigella sonnei* CFU/25g	BPW-PCR[a]	*Shigella* broth[b]	Colony detection[c]
Control	–	0/5	N.T.	N.T.[d]
Shigella	0.5	1/5	1/5	1/5
	2.4	5/5	5/5	5/5
	4.8	5/5	5/5	5/5
	24	5/5	5/5	5/5
	48	5/5	5/5	5/5

The standard plating count of cabbage used was 6.5×10^4 CFU/g.
[a] Detection rate by PCR analysis from BPW.
[b] Detection rate by PCR analysis from *Shigella* broth.
[c] Suspected colonies were identified by PCR analysis.
[d] Not tested.

TABLE 4. Simultaneous enrichment analysis for four types of pathogenic bacteria.

Inoculated pathogenic bacteria	BPW-PCR[e]	Selection broth-PCR[f]	Colony-PCR[g]
– Cucumbers before freezing and thawing –			
Control	5/5[h]	5/5[i]	0/5
EHEC[a]	5/5	5/5	5/5
Salmonella[b]	5/5	5/5	5/5
Listeria[c]	2/5	5/5	4/5
Shigella[d]	4/5	5/5	4/5
– Cucumbers after freezing and thawing –			
Frozen control	0/5	N.T.[j]	N.T.
EHEC[a]	5/5	5/5	5/5
Salmonella[b]	4/5	5/5	5/5
Listeria[c]	3/5	0/5	4/5
Shigella[d]	5/5	5/5	4/5

The standard plating counts of cucumbers before and after freezing and thawing were 2.3×10^5 and 2.1×10^5 CFU/g, respectively.
[a] Inoculation size: 13 CFU/25g, enrichment broth: BPW, selection broth: NmEC.
[b] Inoculation size: 15 CFU/25g, enrichment broth: BPW, selection broth: RV.
[c] Inoculation size: 217CFU/25g, enrichment broth: BPW, selection broth: UVM.
[d] Inoculation size: 20 CFU/25g, enrichment broth: BPW, selection broth: *Shigella* broth.
[e] Detection rate by PCR analysis from BPW.
[f] Detection rate by PCR analysis from selective broth.
[g] Suspected colonies were identified by PCR analysis.
[h] EHEC PCR (±).
[i] EHEC PCR (±). However, selective culture broth showed no colony on EHEC selective agar.
[j] Not tested.

samples, respectively. In the second PCR analysis, EHEC were detected in 2/5 and 5/5 samples, respectively. Violet colonies, which were suspected to be EHEC, appeared respectively in 2/5 and 5/5 samples of CHROMagar O157 plates. The suspected colonies on the plates were confirmed as EHEC by colony PCR analysis. EHEC as few as 17 CFU/25g can be detected clearly by this method.

As shown in Table 2, as few as 394 CFU of *L. monocytogenes*/25g can be detected clearly by this method.

As shown in Table 3, as few as 2.4 CFU of *Shigella sonnei*/25g can be detected clearly by this method.

Four types of pathogenic bacteria in food were detected and isolated by the simultaneous enrichment detection method as shown in Tables 4 to 7. Table 4 shows the results for inoculated cucumbers. EHEC and *Salmonella* could be detected clearly. As few as 13 and 15 CFU of EHEC and *Salmonella* Enteritidis / 25g could be detected clearly by this method, respectively. Table 5 shows the results for inoculated bean

TABLE 5. Simultaneous enrichment analysis for four types of pathogenic bacteria.

Inoculated pathogenic bacteria	BPW-PCR	Selection broth-PCR	Colony detection
– Bean spouts before freezing and thawing –			
Control	4/5[e]	0/5	N.T.
EHEC[a]	5/5	5/5	5/5
Salmonella[b]	5/5	5/5	5/5
Listeria[c]	0/5	5/5	4/5
Shigella[d]	4/5	5/5	4/5
– Bean sprouts after freezing and thawing –			
Frozen control	0/5	N.T.	N.T.
EHEC[a]	5/5	5/5	5/5
Salmonella[b]	5/5	5/5	5/5
Listeria[c]	2/5	0/5	1/5
Shigella[d]	2/5	4/5	0/5

The standard plating counts of bean sprouts before and after freezing and thawing were 1.6×10^6 and 9.2×10^5 CFU/g, respectively.

[a] Inoculation size: 183 CFU/25g, enrichment broth: BPW, selection broth: NmEC.

[b] Inoculation size: 171 CFU/25g, enrichment broth: BPW, selection broth: RV.

[c] Inoculation size: 17 CFU/25g, enrichment broth: BPW, selection broth: UVM.

[d] Inoculation size: 52 CFU/25g, enrichment broth: BPW, selection broth: *Shigella* broth.

[e] *Salmonella* PCR (+). However, further selective culture broth showed no *Salmonella* by PCR analysis and no colony on *Salmonella* selective agar.

TABLE 6. Simultaneous enrichment analysis for four types of pathogenic bacteria.

– Ground beef after freezing and thawing –

Inoculated pathogenic bacteria	BPW-PCR[e]	Selection broth-PCR	Colony-PCR
Frozen control	0/5	N.T.	N.T.
EHEC[a]	4/5	5/5	5/5
Salmonella[b]	5/5	5/5	5/5
Listeria[c]	2/5	0/5	5/5
Shigella[d]	4/5	4/5	1/5

The standard plating count of ground beef after freezing and thawing was 1.6×10^4 CFU/g.

[a] Inoculation size: 139 CFU/25g, enrichment broth: BPW, selection broth: NmEC.

[b] Inoculation size: 132 CFU/25g, enrichment broth: BPW, selection broth: RV.

[c] Inoculation size: 352 CFU/25g, enrichment broth: BPW, selection broth: UVM.

[d] Inoculation size: 44 CFU/25g, enrichment broth:BPW, selection broth: Shigella broth.

[e] DNA solution was collected with a TaKaRa kit (GenTLEkun) to prevent blood contamination.

TABLE 7. Simultaneous enrichment analysis for four types of pathogenic bacteria.

– Cabbage after freezing and thawing –

Inoculated pathogenic bacteria	BPW-PCR	Selection broth-PCR	Colony-PCR
Frozen control	0/5	N.T.	N.T.
EHEC[a]	0/5	0/5	1/5
Salmonella[b]	2/5	5/5	5/5
Listeria[c]	0/5	0/5	4/5
Shigella[d]	4/5	1/5	1/5

The standard plating count of cabbage after freezing and thawing was 1.0×10^4 CFU/g.

[a] Inoculation size: 68 CFU/25 g, enrichment broth: BPW, selection broth: NmEC.

[b] Inoculation size: 79 CFU/25 g, enrichment broth: BPW, selection broth: RV.

[c] Inoculation size: 850 CFU/25 g, enrichment broth: BPW, selection broth: UVM.

[d] Inoculation size: 148 CFU/25 g, enrichment broth: BPW, selection broth: Shigella broth.

sprouts. EHEC and *Salmonella* could be detected and isolated clearly. Frozen *Listeria* inoculated under a limited detection dose (17 CFU/25g) could hardly be detected. Frozen *Shigella* (52 CFU/25g) could not be isolated. Tables 6 and 7 shows the results for ground beef and cabbage inoculated with four types of pathogenic bacteria and frozen, respectively. In both cases, inoculated and frozen EHEC and *Salmonella* could be detected clearly. Frozen *Listeria* could not be clearly detected by multiplex PCR and selection broth-PCR analyses but isolated colonies could be identified by PCR. Frozen *Shigella* could be detected by PCR analysis but could not be isolated clearly.

In conclusion, EHEC ($\geqq$ 13 cells/25g), *S.* Enteritidis ($\geqq$15 cells/25g), *L. monocytogenes* ($\geqq$ 394 cells/25g) and *S. sonnei* ($\geqq$2.4 cells/25g) in food were detected by BPW enrichment culture and multiplex PCR analysis, and isolated by further selective enrichment culture and plating on isolation selective agar plate. However, *Listeria* and *Shigella* in frozen food were difficult to detect. This method is convenient and economical for the simultaneous monitoring of these four pathogens in a large number of food samples.

Our ongoing research includes attempts to deal with samples of several different kinds of food contaminated by a small number of bacterial cells.

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