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Specific Detection of Enteropathogen *Campylobacter jejuni* in Food Using a Polymerase Chain Reaction

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Abstract The use of the polymerase chain reaction (PCR) method was described using two sets of primers based on the *ceuN* gene (JEJ 1 and JEJ 2) which encodes a protein involved in siderophore transport and 16S rRNA gene (pA and pB) for the sensitive and specific detection of enteropathogen *Campylobacter jejuni*. Six oligonucleotides were utilized in an amplification experiment and PCR products of predicted sizes were generated from whole cells and boiled cell lysates at the same intensity. Two sets of the primer pairs, JEJ and pAB, were specific enough for all *C. jejuni* strains tested for the direct use of whole cells without DNA extraction or lysis steps. In the PCR using the pAB primer pair, the detection limit, as determined by the ethidium bromide staining of the amplification products on agarose gels, was at the level of 10^1 bacteria cells or less in both the pure culture and artificially inoculated milk and chicken enrichment samples, whereas the detection limit with the JEJ primer pair was relatively low, *i.e.* 10^3 cells or more in the same PCR samples. The PCR method using either a primer JEJ or pAB was both repeatable and specific for the detection of *C. jejuni* in food. This method is simply completed within 4 h.

Key words: *Campylobacter jejuni*, PCR, foods source, specific detection

Campylobacteria are gram-negative, spirally shaped microaerophilic bacteria and was originally identified as an animal pathogen long before being recognized as a human pathogen. Nowadays, *Campylobacter* spp. are widely accepted as one of the most important causes of acute diarrhea in humans all over the world [8, 10, 17, 26, 29]. Many outbreaks of *C. jejuni* are foodborne [7, 9, 27, 30]. A traditional enrichment culture method is basically reliable for detecting *C. jejuni* [1, 11, 15], however there is a potential to lose sensitivity due to the possible occurrence of viable but non-culturable forms (VBNC) and the growth conditions for the pathogen is complicated [6, 19, 25, 36].

Recently, DNA methods, such as DNA probes or PCR methods, have been increasingly studied for the rapid and specific detection of food borne bacterial pathogens including *C. jejuni* [21, 22, 31]. Mahadi *et al.* [18] has reported that application of multiplex PCR can be used for rapid screening of a large number of new isolates. However, the detection of *C. jejuni* by these methods in Korea has been rarely reported.

The PCR method is a faster, specific, and more sensitive method than the DNA probe method which relies on the use of radioactive labels. Therefore, in the present study, we attempted to use the PCR method for the specific detection of enteropathogen *C. jejuni* in food samples. We reviewed various primers which were previously reported [3, 4, 13, 14, 21-23, 31, 34] and chose two sets [13, 14] of the oligonucleotide primer pairs for practical use with the potential food vehicles of milk and raw chicken. One pair was JEJ 1 and 2, based on the *ceuN* gene which encodes the protein involved in siderophore transport and has the ability to differentiate *C. jejuni* from *C. coli* [14]. The other pair was primers pA and pB, based on the 16S rRNA gene which is well suited for all *Campylobacter* spp. [13]. Whole cells were directly applied to the PCR procedure to facilitate rapid detection without cell lysis treatment or DNA extraction. In this study, we evaluated the use of two sets of primers, and it was determined that the PCR technique along with these primers can be successfully applied to the direct detection of whole cells of *C. jejuni* in the mixed microflora of food samples.

Materials and Methods

Bacterial Strains and Culture Conditions

C. jejuni ATCC 33291, *Escherichia coli* ATCC 25922, and *Listeria monocytogenes* ATCC 19111 were purchased from

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the American Type Culture Collection. *C. jejuni* A74/C, W-1, and W-2 were obtained from USDA (U.S. Department of Agriculture) [28]. *Salmonella enteritidis* p1 was isolated in our laboratory. *C. jejuni* strains were cultured microaerophilically in FBP Supplemented Brucella Broth (FBP-SBB) at 42°C for 48 h in a 3.5 liter anaerobic jar with Campylobacter Microaerophilic System (Difco) [25]. The FBP-SBB consisted of 0.9 mM ferrous sulfate, 1.3 mM sodium metabisulfite, and 2.3 mM sodium pyruvate in Brucella Broth (Difco). Filtered antibiotics, vancomycin 15 mg, trimethoprim lactate 5 mg, polymyxin B 20,000 IU, cycloheximide 50 mg/l and 3% bovine calf serum (Hyclone, Logan, Utah, U.S.A.) were separately added into FBP-SBB after autoclaving. Either FBP-SBM (FBP-SBB with 2% agar plus 5% defibrinated sheep blood) or Campylobacter Selective agar (Lab. M Co.) were used for solid culture. *S. enteritidis* and *L. monocytogenes* were cultured aerobically in nutrient broth and Brain Heart Infusion broth at 37°C. *Clostridium perfringens* ATCC 13124 were cultured anaerobically at 37°C in Differential Reinforced Clostridial media (DRCM. Merk) in anaerobic chamber (Coy Laboratory Products Inc. Ann Arbor, U.S.A.)

Synthesis of Oligonucleotide Primers

Oligonucleotide primers were synthesized by a commercial company (Genotech, Taejon, Korea). The sequences of the oligonucleotide primer used in this study are shown in Table 1.

Sample Preparation for the PCR

Whole cells of *C. jejuni* were used without DNA extraction as the DNA template for the PCR. Liquid cultured cells were centrifuged, washed with sterile distilled water (SDW), and resuspended to a 1/10 volume of SDW. This cell suspension was then directly used for the PCR amplification [32]. Cell lysate was prepared by boiling the whole cell suspension at 100°C for 3 min [3]. For PCR assay of *C. jejuni* inoculated in food, chicken purchased from a local supermarket was minced and 10 g of sample containing skin and other meat parts were evenly suspended in 40 ml of FBP-SBB in a 100-ml Erlenmeyer flask. *C. jejuni* ATCC 33291 was microaerophilically grown in FBP-SBB for 48 h and was inoculated into the FBP-SBB containing chicken to a final concentration ranging from 2.8 to 2.8×10^5 CFU/ml. These chicken-SBB mixtures were placed in an incubator (42°C for 24 h) for enrichment. Subsamplings were carried out after 0, 4, 8, and 24 h incubation. In order to count the number of aerobic bacteria and *C. jejuni* cells during the enrichment procedure, a Plate Count agar (Difco) and Campylobacter Selective agar were used. In preparation for the PCR, 1 ml of the subsample was filtered using sterile filter paper (Whatman No. 1) and centrifuged at 10,000 rpm for 5 min. The pellet was washed twice with SDW and resuspended in 0.1 ml of SDW. In the case of the milk sample, 10 ml of milk purchased from a local supermarket was added to 40 ml of FBP-SBB, and C. *jejuni* was inoculated to the SBB with milk to a final concentration ranging from 1.3 to 1.3×10^5 CFU/ml pefore enrichment at 42°C for 24 h. At 0, 4, 8, and 24 h, the subsamples were taken to check the population of both *C. jejuni* and aerobic microflora. In preparation of the PCR, 1 ml of the milk-SBB enriched culture was subsampled and centrifuged at 10,000 rpm for 5 min, washed 3 times with SDW, and concentrated to 0.1 ml of SDW. Therefore, the cell number used for the PCR reaction in these case was 1/100 of the original culture broth per ml because 1 μl of 1/10 concentrated cells of the original solution was taken for the PCR tube. A suspension of whole cells was directly used

Table 1. Oligonucleotide primers used in this study.

No.	Primer	Sequences (5' → 3')	Target gene	PCR product	Reference
1	JEJ 1	CCTGCTACGGTGAAAGTTTTGC	*ceuE*	793 bp	[14]
	JEJ 2	GATCTTTTTGTTTTGTGCTGC			
2	p-1	GATGCTTCAGGGATGGCG	Flagellin	1300 bp	[3]
	p-2	TTTGTGATTCTGCTGCTTTAAC			
3	pA	GGAGGATGACACTTTTCGGAGC	16S rRNA	426 bp	[13]
	pB	ATTACTGAGATGACTAGCACCCC			
4	1	CCAAATCGGTTCAAGTTCAAATCAAAC	Flagellin	813 bp	[23]
	2	CCACTACCTACTGAAAATCCCGAACC			
5	C-1	CAAATAAAGTTAGAGGTAGAATGT	Randon chromosomal fragment	159 bp	[34]
	C-3	CCATAAGCACTAGCTAGCTGAT			
6	pg50	ATGGGATTTCGTATTAAC	*fla*A (flagellin)	450 bp	[22]
	pg3	GAACTTGAACCGATTTG			

as the DNA template for the PCR without further treatment.

PCR Assay

PCR was performed using 1 μl of the cell suspension which had approximately 0- 10^6 CFU in a 20 μl volume of the PCR PreMix (Bioneer, Chongwon, Korea). The PCR mixture consisted of 1 U of thermostable DNA polymerase, 250 μM of each dNTP, 50 mM of Tris-HCl (pH 8.3), 40 mM of KCl, and 1.5 mM of $MgCl_2$. The mixture was covered with 20 μl of sterile mineral oil in each tube and the PCR was carried out in a BioRad Gene Cycler (Model No. 10167, Japan). The PCR cycle program of denature, annealing, and extension temperatures was comprised of one cycle of 5 min at 94°C, then 30 cycles of 30 sec at 94°C and 30 sec at 57°C (primer JEJ 1, 2), 60°C (primer p-1, p-2), 52°C (primer pA, pB), 50°C (primer 1, 2), 56°C (primer C-1, C-3), or 37°C (primer pg50, pg3), and 1 min at 72°C and one cycle of 5 min at 72°C. The PCR products (10 μl each) were analyzed using 1% agarose gel in a TAE buffer containing 0.5 μg/ml of ethidium bromide. The gel was visualized and photographed under UV light.

RESULTS

Selection of Primers for the PCR

General suitability of a PCR in the present study was evaluated for the specificities of six sets of primers using the whole cells of *C. jejuni* ATCC 33291 pure culture. As shown in Fig. 1, the six sets of the primer pairs generated six single predicted sizes of PCR products from the whole cells and boiled cell lysates. The pA and pB primer pair based on the 16S rRNA gene generated the strongest band (lane 3) among all the PCR products. The C-1 and C-3 primer pair based on a random chromosomal fragment [34] generated weak bands (lane 5) at the 159 bp position. Repeated experiments indicated no difference in band intensity between the whole cells and the boiled cell lysates. The JEJ 1, 2 and pA, pB primer pairs were used for the rapid and sensitive detection of *C. jejuni* from the artificially contaminated food source. That is because the JEJ pair can differentiate *C. jejuni* from other Campylobacter species such as *C. coli*, and the pA and pB pair can also generate the thickest bands which are specific for all *Campylobacter* spp. among tested primer samples. In a preliminary test, various cell lysis treatments to the *C. jejuni* including heating or freezing and thawing which released its DNA template, were not efficient in enhancing the PCR sensitivity (data not shown). Therefore, whole intact cells without the lysis were directly used as the DNA template throughout this PCR study.

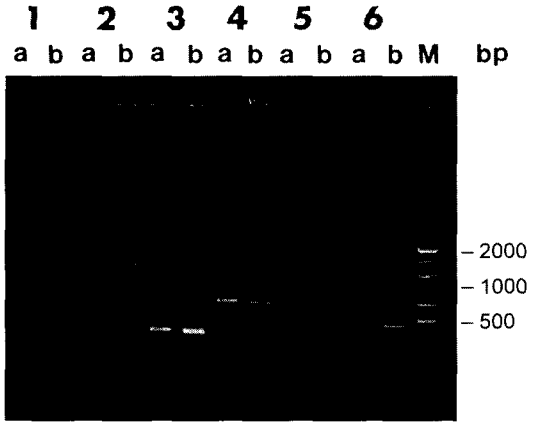

Fig. 1. Primer specificity for *C. jejuni* ATCC 33291.
PCR was performed with six different primer pairs 1 to 6 using 10^5 CFU of washed whole cells and the boiled cell lysates in 20 μl of total PCR reaction mixture. Lane 1, JEJ 1 and 2; lane 2, p-1 and p-2; lane 3, pA and pB; lane 4, 1 and 2; lane 5, C-1, C-2; lane 6, pg50 and pg3; lane a, whole cells; lane b, boiled cell lysates; M, DNA size marker.

Specificities of the JEJ and pAB Primer Pairs

Specificity tests of JEJ (JEJ 1 and 2) and pAB (pA and B) primer pairs with *C. jejuni* were performed using four *C. jejuni* strains and three reference species. The specificities of these primers had been previously confirmed in other reports [13, 14], therefore, the specificities of the primers were only briefly tested with seven strains. As shown in Fig. 2A, the primer JEJ pair generated a specific band of 793 bp for the four *C. jejuni* strains (lanes 1, 2, 3, and 4) whereas not for the other reference species (lanes 5, 6, 7) and negative control (lane 8). The pA and pB primer pair also generated a specific band at 426 bp for the four of the *C. jejuni* strains, whereas no band for other species gave negative results (Fig. 2B). Both primers, JEJ and pAB

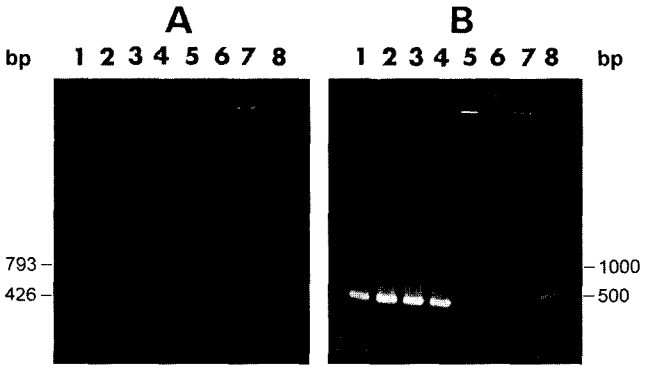

Fig. 2. Specificity test for *C. jejuni* 33291 by JEJ 1 and 2 primer (A) and pA and pB primer (B).
PCR was performed with 10 pmol primers using 10^5 CFU of washed whole cells in 20 μl PCR reaction mixture. A: Lane 1, *C. jejuni* ATCC 33291; lane 2, *C. jejuni* A74/C; lane 3, *C. jejuni* W-1; lane 4, *C. jejuni* W-2; lane 5, *E. coli* ATCC 25922; lane 6, *S. enteritidis* p1; lane 7, *Clostridium perfringens* ATCC 13124; lane 8, No DNA for negative control. B: lane 1, *C. jejuni* ATCC 33291; lane 2, *C. jejuni* A74/C; lane 3, *C. jejuni* W-1; lane 4, *C. jejuni* W-2; lane 5, *E. coli* ATCC 25922; lane 6, *S. enteritidis* p1, lane 7, *L. monocytogenes* ATCC 19111; lane 8, DNA size marker.

pairs, generated each single band of their corresponding sizes of DNA fragment in their positions. There were no false-positive or false-negative results.

Sensitivities of Primers, JEJ and pAB Primer Pairs

The sensitivity of JEJ and pAB primer pairs to *C. jejuni* were evaluated with the culture suspension of *C. jejuni* ATCC 33291 ranging from 3.0×10^6 to 3.0×10 cells (JEJ pair) and from 1.8×10^6 to 1.8×10^{-3} cells (pAB pair) in the PCR tube. The detection limit of the JEJ pair was 10^3 cells per PCR amplification reaction (lane 4 in Fig. 3A), whereas that of the pAB pair was at the level of 10^0 to 10^1 cells (lanes 6 and 7 in Fig 3B) per PCR tube. Therefore, the detection sensitivity of the pAB pair was approximately 100 fold higher than that of the JEJ 1 and 2 primer pair. The bands obtained at the level of 10^0 and 10^1 cells per PCR reactions with pAB primer were not clear, however, still detectable from further diluted samples and the negative control (lanes 8, 9, 10, 11, and 12). The PCR sensitivity level with pAB in this study was similar to the PCR for other bacteria ranging from 1 to 20 cells [20, 21, 32, 33]. Both primer sets were further used to directly detect *C. jejuni* by a PCR in a food source.

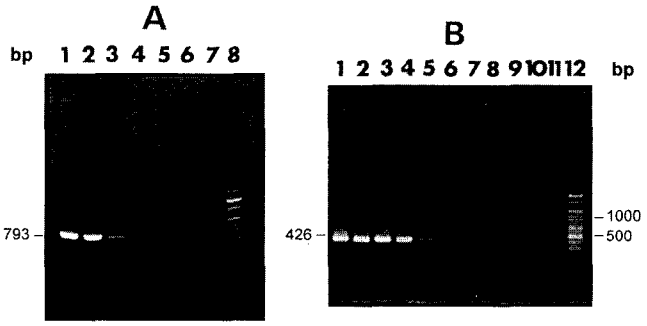

Fig. 3. Sensitivity test of *C. jejuni* 33291 by JEJ 1 and 2 primer (A) and pA and pB primer pair (B).
PCR was performed with 10 pmol primers using 3×10^6 to 3.0×10 CFU (A) and 1.8×10^6 to 1.8×10^{-3} CFU (B) washed whole cells in 20 µl PCR reaction mixture. A. Lane 1 to 6, 1.8×10^5 to 1.8×10 CFU; lane 7, No DNA for negative control; lane 8, DNA size marker. B. lane 1 to 10, 3.0×10^6 to 1.8×10^{-3} CFU; lane 11, No DNA for negative control; lane 12, DNA size marker.

Detection of *C. jejuni* in Inoculated Milk

The comparative sensitivities of the JEJ and pAB pairs to detect *C. jejuni* in artificially contaminated milk are presented in Table 2. At time zero, before an enrichment incubation at 42°C, any amplimer was not produced by amplification with the JEJ primer from the *C. jejuni* inoculated milk samples containing from 1.3 to 1.3×10^5 CFU/ml. However, in the same samples, the pAB pair generated a specific 426 bp fragment from an initial inoculation of 1.3×10^2 to 1.3×10^5 CFU/ml of the milk enrichment samples. After enrichment incubation for 24 h, the number of *C. jejuni* increased from levels of 10^1 to 10^5 CFU to 10^3 to 10^6 CFU/ml of the milk sample. The population of other aerobic bacteria in this milk enrichment culture was checked on a Plate Count agar at 37°C. Only 2-3 colonies appeared in the non-diluted milk enrichment sample after overnight culture. Accordingly, the growth of undesirable aerobic bacteria was not a problem in this milk enrichment sample. After 24 h enrichment in three samples with an initial inoculum of 1.3×10^3, 10^4, and 10^5 CFU of *C. jejuni*/ml using the JEJ primers a proper PCR amplimer of 793 bp was generated, whereas, a 426 bp amplimer was yielded by using pAB primer pair at an initial inoculum level of 10^0 to 10^5 CFU of *C. jejuni*/ml. The electrophoresis results at 8 h and at 24 h are described in Fig. 4. The detection limit using the JEJ pair was approximately 10^4 cells per PCR tube from 1.3×10^6, 3.3×10^6, 4.3×10^6 CFU/ml milk culture at 24 h, as shown in Table 2, whereas the

Table 2. Effect of enrichment time on the amplification of PCR products and the population for *C. jejuni* in milk enrichment sample using JEJ 1, 2 and pA, B primer pairs.

Initial inoculum (CFU/ml of SBB[a] with milk)	PCR band amplification								Population of *C. jejuni* (CFU/ml)			
	Incubation (h)								Incubation (h)			
	JEJ 1 and 2				pA and pB				0	4	8	24
	0	4	8	24	0	4	8	24				
0	-[b]	-	-	-	-	-	-	-	0[d]	0	0	0
1.3×10^0	-	-	-	-	-	-	-	△	0	2.0×10^1	0	0
1.3×10^1	-	-	-	-	-	-	-	+	0	6.0×10^1	1.6×10^2	2.3×10^3
1.3×10^2	-	-	-	-	△[c]	△	△	+	8.0×10^1	1.1×10^3	5.2×10^3	1.8×10^5
1.3×10^3	-	-	-	+	+	+	+	+	8.6×10^2	7.2×10^3	5.2×10^4	1.3×10^6
1.3×10^4	-	-	-	+	+	+	+	+	1.2×10^4	2.8×10^4	3.2×10^5	3.3×10^6
1.3×10^5	-	-	-	+	+	+	+	+	1.3×10^5	2.8×10^5	2.2×10^6	4.3×10^6

a: SBB, FPB-supplemented Brucella Broth.
b: -, 793 bp or 426 bp amplified product not seen; +, 793 bp or 426 bp amplified product seen.
c: Faint band.
d: No colony from 0.1 ml of undiluted sample.

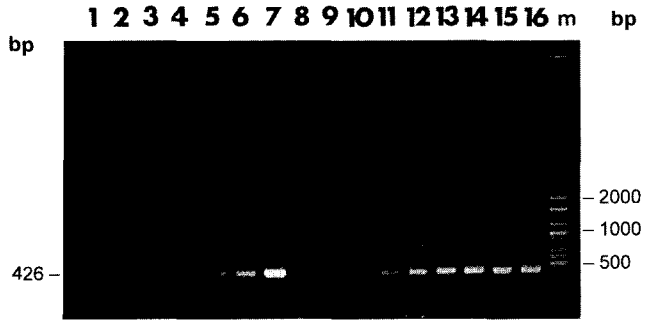

Fig. 4. Agarose gel electrophoresis showing detection limit of *C. jejuni* in inoculated milk using pA and pB primers after 8 and 24 h enrichment incubation.
The enriched milk sample was pelleted for PCR and the final number of *C. jejuni* in the milk enrichment sample were expressed in Table 2. Lanes 1 to 7, an initial inoculation 0 to 1.3×10^5 CFU/ml after 8 h; lane 8, blank; lane 9 to 15, an initial inoculation 0 to 1.3×10^5 CFU/ml after 24 h; lane 16, positive control with about 10^5 CFU of *C. jejuni* cells.

detection limit using the pAB pair was at level of 10^0 to 10^1 cells per PCR tube from 8.0×10, 8.6×10^2 CFU/ml milk culture at time zero, and 1.1×10^3, 7.2×10^3 CFU/ml milk culture at 4 h well shown in Table 2. The detection levels were almost the same as that observed in the PCR using pAB in pure *C. jejuni* culture. The detection limit slightly decreased at milk enrichment sample in PCR with JEJ pair.

Detection of *C. jejuni* in Inoculated Chicken.
PCR were carried out for the detection of *C. jejuni* in inoculated chicken using the JEJ and pAB primer pairs, and the population ratios of *C. jejuni* and other aerobic microflora are described in Table 3. The growth of aerobic microflora and *C. jejuni* were examined at 0, 4, 8, and 24 h. After 24 h enrichment, an initial inoculation level of 10^3, 10^4, and 10^5 CFU/ml reached to 10^5, 10^6, 10^7 CFU/ml, respectively, and 793 bp PCR products were generated by amplification using the JEJ primer. This means that the detection limit using the JEJ primer pair was at level of 10^3 CFU in the PCR tube. While using the pAB pair, an initial level of 10^1 to 10^5 of *C. jejuni*/ml reached to 10^3 to 10^7 CFU/ml after 24 h and 426 bp PCR products were generated from all samples. Therefore, the detection limit using the pAB pair was at level of 10^1. In contrast to the milk samples, an initial population of aerobic microflora was 10^3 CFU/ml in the chicken sample at time zero and it reached from 10^8 to 10^9 CFU/ml after a 24 h incubation. The chicken enrichment samples were all a mixture of FPB-SBB, solid chicken meat, released fragment components, and various species of bacteria; however the detection sensitivity of *C. jejuni* using the PCR method as demonstrated by the two sets of primers in this study was not interfered with.

DISCUSSION

In the present study, we have evaluated the PCR method for the detection of *C. jejuni* in food based on the JEJ and pAB primer pairs. The appearance of PCR amplimers on agarose gel was both consistent and of the correct size in all tested samples, indicating that the use of the primers was effective for the detection of *C. jejuni*. It has been reported that the JEJ primer pair, chosen for this study, has the advantage of being able to differentiate between *C. jejuni* and *C. coli* [14]. However, the minimum numbers of the positive results were 10^3 for the pure culture, 10^4 CFU for the milk enrichment sample, and 10^3 CFU for the chicken enrichment sample per PCR assay in this experiment. The detection limit using the JEJ primer was slightly enhanced when food enrichment sample was subjected to an enrichment incubation (Table 2). However, the detection limit was not sensitive enough for the general detection of *C. jejuni* in food.

Table 3. Effect of enrichment time on the amplification of PCR products and the ratio of the population for *C. jejuni* versus aerobic microflora in chicken enrichment sample using JEJ 1, 2 and pA, B primer pairs.

Initial inoculum (CFU/ml of SBB[a] with chicken)	PCR band amplification								Ratio of *C. jejuni*/aerobic population (CFU/ml)			
	Incubation (h)								Incubation (h)			
	JEJ 1 and 2				pA and pB				0	4	8	24
	0	4	8	24	0	4	8	24				
0	-[b]	-	-	-	-	-	-	-	$0/10^3$	$0/10^3$	$0/10^4$	$0/10^9$
2.8×10^0	-	-	-	-	-	-	-	-	$0/10^3$	$0/10^3$	$0/10^4$	$0/10^9$
2.8×10^1	-	-	-	-	-	-	-	+	$10^1/10^3$	$0/10^4$	$0/10^5$	$10^3/10^9$
2.8×10^2	-	-	-	-	-	-	+	+	$10^2/10^3$	$10^2/10^4$	$10^3/10^4$	$10^5/10^8$
2.8×10^3	-	-	-	+	-	+	+	+	$10^3/10^3$	$10^3/10^4$	$10^2/10^4$	$10^5/10^8$
2.8×10^4	-	-	-	+	△[c]	+	+	+	$10^3/10^4$	$10^4/10^4$	$10^4/10^6$	$10^6/10^8$
2.8×10^5	-	-	-	+	+	+	+	+	$10^5/10^3$	$10^5/10^4$	$10^5/10^5$	$10^7/10^8$

a: SBB, FPB-supplemented Brucella Broth.
b: -, 793 bp or 426 bp amplified product not seen; +, 793 bp or 426 bp amplified product seen.
c: Faint band.

Alternatively, when using the pAB primer, which is based on the 16S rRNA gene, the detection limits were at levels of 10^0 to 10^1 cells for pure culture, and at level of 10^1 cells for the milk and chicken enrichment samples. The detection limit using the pAB primer was not affected by enrichment incubation, which means that PCR amplification with the pAB primer was not interfered by the change in the food composition or other unwanted microflora as a result of the enrichment incubation of a food sample. The results obtained using pAB showed the normal number of bacteria, 1-20 cells of sensitivity, as in other PCR studies [32, 33]. Therefore, it is expected that the JEJ primer would be applicable for the differentiation of isolated *C. jejuni* strains in food samples, whereas the pAB primer would be preferable for use with enriched food samples.

The problem lies in that the number of foodborne pathogens which cause diseases are often present in food at much lower levels than the general PCR detection limitation in food [21, 24]. Therefore, at this 1-10 cells per PCR assay level, direct detection by a PCR is impossible. There are many techniques in lowering the detection limit of the PCR, such as the use of r-RNA as a target gene [16], the nested PCR [35], the combination of the PCR with a short culture [12, 13], the development of an extraction method of DNA template [3, 34], and filtration [2]. Unfortunately, to the best of our knowledge, no practical PCR-based method for the direct detection of *C. jejuni* from food is available until now. Although detection limits have been enhanced by the development of various PCR-based methods, the role of the VBNC or dead cells should be elucidated to understand whether or not the damaged but PCR detectable cells are related to the virulence of campylobacteriosis [6, 19].

PCR is a very potential method to detect *C. jejuni* which causes disease with low number of cells, 10^2-10^9 [5, 24]. Further studies are needed to enhance sensitivity in food samples even though there are many obstacles yet to be solved.

Acknowledgments

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