JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 7135–7140 7135
DOI:10.1021/jf1009654

Rapid Detection of Enterotoxigenic *Clostridium perfringens* in Meat Samples Using Immunomagnetic Separation Polymerase Chain Reaction (IMS−PCR)

Zheng-You Yang,[†,‡] Won-Bo Shim,[§] Kyeong-Yeol Kim,[‡] and Duck-Hwa Chung*[,‡,§]

[†]Department of Microbiology, College of Life Science, Key Laboratory for Agriculture Microbiology, Shandong Agricultural University, Taian 271018, China, [‡]Division of Applied Life Science, Graduate School of Gyeongsan National University, Jinju, Gyeongnam 660-701, Korea, and [§]Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Gyeongnam 660-701, Korea

Rapid detection of enterotoxigenic *Clostridium perfringens* in meat samples was accomplished with an immunomagnetic separation polymerase chain reaction (IMS−PCR). First, a monoclonal antibody (mAb) specific to *C. perfringens* was generated. The antibody showed strong binding to *C. perfringens* and no binding to non-*Clostridia* bacteria, except a weak cross-reaction to *Staphylococcus aureus* based on the enzyme-linked immunosorbent assay (ELISA). Then, magnetic beads were coated with the mAb, and the IMS−PCR system was developed. With the optimized conditions, the IMS−PCR assay was capable of detecting as few as 10 colony forming units (CFU)/g of *C. perfringens* cells in the meat sample within 10 h. Of the 116 collected samples (26 chicken samples, 20 beef samples, 30 pork samples, 20 fish samples, and 20 processed meat samples) examined with IMS−PCR, 36 (31%) were *C. perfringens*-positive samples and 2 (1.7%) were enterotoxigenic *C. perfringens*-positive samples. The IMS−PCR results gave a good agreement with the results obtained by conventional culture methods. In comparison to conventional culture methods, the IMS−PCR is a rapid and specific method and has potential use as a screening tool for enterotoxigenic *C. perfringens* in food samples.

KEYWORDS: Enterotoxigenic *Clostridium perfringens*; immunomagnetic separation PCR; monoclonal antibody; foodborne pathogen

INTRODUCTION

Clostridium perfringens is a Gram-positive, anaerobic, rod-shaped, spore-forming bacterium commonly found in the intestinal tract of humans and mammals, feces, and soil (*1*). On the basis of their ability to produce α, *β*, *ε*, and *ι* toxins, isolates of *C. perfringens* are classified into five types (types A−E) (*2*, *3*). Each *C. perfringens* type is associated with certain human or veterinary diseases. *C. perfringens* type A food poisoning has been known as one of the most common cause of foodborne disease in the United States (*4*−*6*), Europe (*7*, *8*), and Australia (*9*). Symptoms associated with *C. perfringens* type A food poisoning are diarrhea and severe abdominal pain. The symptoms are mediated by an enterotoxin (CPE), a 35 kDa single polypeptide produced during sporulation of the organism in the small intestine (*10*). *C. perfringens* lacks the ability to produce 13 of the 20 essential amino acids and is therefore associated with protein-rich food, and 75% of the foodborne outbreaks can be traced to meat and meat products (*11*). However, most of these isolates from food samples are non-enterotoxigenic strains; only about 5% of all *C. perfringens* isolates carry the gene (*cpe*) encoding enterotoxin (*12*, *13*). It is necessary to distinguish the enterotoxigenic organisms from the non-enterotoxigenic ones to confirm food poisoning by *C. perfringens*.

The previously described methods for detecting enterotoxigenic *C. perfringens* in food samples include enzyme-linked immunoassays (*14*), plasmid analyses (*15*−*17*), and gene probe methods (*18*, *19*). These techniques have been used in detection and identification schemes following cultivation and isolation of the target organism, which increases the time to obtain a positive identification, and thus lack the speed required for analysis of food products. A number of alternative detection methods based on amplification of *C. perfringens* DNA using the polymerase chain reaction (PCR) have been reported (*20*−*23*). However, most methods of this nature still rely on selective enrichment to both grow sufficient bacteria for detection and ensure viability.

The isolation of *C. perfringens* directly from food is an alternative to selective enrichment. This approach presents several challenges, including assessment of the viability of the microorganisms, physically detaching them from the food and separating them from food materials that may inhibit the PCR. An immunomagnetic separation (IMS) system has been frequently used for the isolation of pathogens from food homogenates (*24*−*26*). This method used magnetic beads coated with antibodies to extract and concentrate the target organism from the sample. In this way,

*To whom correspondence should be addressed. Telephone: +82-055-751-5480. Fax: +82-055-753-4630. E-mail: dhchung@gnu.ac.kr.

Published on Web 05/27/2010

Table 1. Reactive of mAb J4H9 to Different Organisms

	reaction		
	ELISA		
organism	killed cell[a]	live cell	western blot
C. perfringens			
FD-1041, type A[b]	+	+	+
ATCC 3624, type A[c]	+	+	+
ATCC 3626, type B	+	+	+
NCTC 3180, type C	+	+	+
NCTC 8346, type D	+	+	+
NCTC 6719, type E	+	+	+
Clostridium difficile ATCC 9689	−	−	−
Clostridium sporogenes ATCC 3584	−	−	−
Clostridium tertium ATCC 14573	−	−	−
Clostridium acetobutylicum ATCC 824	−	−	−
Escherichia coli O157:H7 ATCC 43984	−	−	−
Listeria monocytogenes ATCC 19113	−	−	−
Staphylococcus aureus ATCC 25923	+	+	+
Vibrio parahemolyticus ATCC 17802	−	−	−
Bacillus cereus ATCC 21366	−	−	−
Salmonella typhimurium ATCC 13311	−	−	−

[a] Formalin-killed cell or heat-killed cell. [b] *C. perfringens* FD-10141 is a enterotoxigenic-positive type A strain. [c] *C. perfringens* ATCC 3624 is a enterotoxigenic-negative type A strain.

IMS can eliminate the need for selective enrichment and reduce the PCR reaction-inhibitory substances in food simples; therefore, IMS can reduce the time required for conventional methods.

In the present study, we describe the direct detection of enterotoxigenic *C. perfringens* in meat samples by an IMS−PCR assay. We first produced a mouse monoclonal antibody (mAb) reactive with *C. perfringens* and then used IMS−PCR to rapidly detect and identify enterotoxigenic *C. perfringens* in meat samples.

MATERIALS AND METHODS

Bacterial Strains. All of the bacterial strains used in this study are listed in **Table 1**. The *Clostridium* strains were maintained in cooked meat medium (Difco, Sparks, MD) and stored at 4 °C. The other strains were maintained on agar slants or as stab cultures at 4 °C on medium containing trypticase soy broth (Difco, Sparks, MD), 0.6% yeast extract (Difco, Sparks, MD), and 1.5% Bacto Agar (Difco, Sparks, MD).

Production of mAb. For preparation of immunogens, enterotoxigenic *C. perfringens* strain FD-1041 cells were grown at anaerobic conditions in cooked meat medium at 37 °C for 20 h and then subcultured in brain−heart infusion broth (Difco, Sparks, MD) for 18 h. Cells were harvested by centrifugation at 4500*g* at 4 °C for 20 min and washed 3 times with phosphate-buffered saline (PBS, pH 7.2). The cell concentration was adjusted to 3×10^{10} cells/mL in sterile PBS.

In this study, two kinds of immunogens, whole cell immunogen and cell surface protein immunogen, were prepared. For preparation of whole cell immunogen, cells at the above-described concentration were pelleted by centrifugation. The pellets were suspended in an original volume of PBS containing 3.7% formaldehyde and kept at room temperature for 5 h. After the cells were washed 3 times, they were resuspended in PBS. Portions of the suspension were streaked on tryptose sulfite cycloserine (TSC) agar (Merck KGaA, Darmstadt, Germany) with egg yolk to confirm that no viable cells persisted. These preparations were adjusted in sterile PBS to obtain an OD_{600} of 1.4−1.5 and stored at −20 °C until used. For preparation of cell surface protein immunogen, cells at the above-described concentration were pelleted by centrifugation. The pellets resuspended in 4 M guanidine-HCl buffer (pH7.2) and incubated at 37 °C for 30 min. After centrifugation at 10000*g* for 20 min, the cell extracts were dialyzed exhaustively against sterile PBS at room temperature for 3 days and lyophilized for future use.

A total of 10 female, 7-week-old BALB/C mice were divided into two groups, where the first group was immunized with 2×10^{10} whole cell immunogen and second group was immunized with 500 μg of cell surface protein immunogen. The mice were first immunized by intraperitoneal injections of immunogens with an equal volume of Freund's complete adjuvant. The following intraperitoneal injections were performed with the same immunogens at 3 week intervals; three injections were made with Freund's incomplete adjuvant, and the last injection was made without adjuvant. To develop monoclonal antibodies, spleen cells obtained from the immunized mice showing a high titer in an indirect enzyme-linked immunosorbent assay (ELISA) were used for cell fusion with murine myeloma cells (P3-X63-Ag8.653) according to standard procedures (*27*). The fused cells producing antibodies against *C. perfringens* were screened by an indirect ELISA and cloned twice using the limiting dilution method. The cloned hybridoma cells were injected into BALB/C mice, and ascites was produced and collected. mAbs were purified from mice ascites by ammonium sulfate precipitation, followed by protein-G-affinity chromatography. The protein concentration of the purified mAb was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Development of the IMS System. The *C. perfringens*-specific mAb was used to coat superparamagnetic beads (Dynabeads M-280 Tosylactivated, Dynal Biotech ASA, Norway) as recommended by the manufacturer. The magnetic particles were recovered by a magnetic force, and the supernatant was discarded. The immunomagnetic beads were resuspended in PBS buffer containing 0.1% (w/v) bovine serum albumin (BSA) and stored at 4 °C.

For IMS, a 1 mL aliquot of fresh culture or suspended sample was transferred to a 1.5 mL microcentrifuge tube and the cells were harvested by centrifugation and then washed 3 times with sterile PBS. The pellets were suspended in 1 mL of PBS, and immunomagnetic beads were added to the suspended solution. After the solution was shaken at room temperature for 30 min in a rotating mixer, the immunomagetic beads were recovered by magnetic force. The supernatant was completely aspirated, and the beads were washed with 1 mL of sterile PBS. This washing step was repeated 1 time, and the beads were resuspended in sterile PBS for PCR or plating on TSC agar with egg yolk for colony counting.

IMS Specificity and Efficiency Test. To determine the specificity and efficiency of the IMS system, different mixtures of *C. perfringens* with *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, and *S. aureus* were prepared and diluted to approximately 10^6 colony forming units (CFU)/mL in PBS. A total of 1 mL each of the mixtures was incubated with different volumes of immunomagnetic beads (10, 30, 50, 70, and 100 μL). After isolation by the IMS system, the final samples were decimally diluted and plated on each selective agar: TSC agar with egg yolk for *C. perfringens*, tellurite cefixime sorbitol MacConkey agar (TC-SMAC) for *E. coli* O157:H7, Hektoen enteric (HE) agar for *S. typhimurium*, Oxford agar for *L. monocytogenes*, and Baird-Parker agar for *S. aureus*. The colonies on the plates were counted after incubation at 37 °C for 24 h.

Determination of the Optimum Immunocapture Time. For maximal immunocapture efficiency, the incubation time of immunomagnetic beads with PBS and the meat suspension sample was optimized. For preparation of the meat suspension sample, 10 g of *C. perfringens*-negative chicken meat sample was placed in a sterile filter bag with 90 mL of 0.1% peptone water and blended in a stomacher. The meat suspension sample and PBS were then inoculated with *C. perfringens* FD-1041 to 1×10^6 CFU/mL. After centrifugation and washing 3 times, immunomagnetic beads were added to each 1 mL sample in a 1.5 mL microcentrifuge tube. The tubes were incubated at room temperature for 15, 30, and 60 min in a rotating mixer. After isolation by the IMS system, the final samples were decimally diluted and plated on TSC agar with egg yolk. The colonies on the plates were counted after incubation at 37 °C for 24 h.

PCR. For DNA extraction and duplex PCR, the immunomagnetic beads separated from samples were resuspended in 200 μL of sterile PBS and the DNA were extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The PCR for the detection of *C. perfringens cpa* and *cpe* genes was performed with primers that had been developed and validated (*28*, *29*). Primers *cpa*-F, 5′-GCTACATTCTATCTTGGAGA-3′, and *cpa*-R, 5′-TCCAACTGATGGATCATTAC-3′ (*28*), yield a 407 bp fragment for the *C. perfringens* α-toxin gene, and primers *cpe*-F, 5′-GGAGATGGTTGGATATTAGG-3′, and *cpe*-R, 5′-GGACCAGCAGTTGTAGATA-3′ (*29*), yield a 233 bp fragment for the *C. perfringens* enterotoxin gene. PCR was performed in a 50 μL reaction volume. The mixture contained 1× buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 0.01% gelatin, 2.5 mM $MgCl_2$,

and 100 μM each dNTP), 5 pmol of each primer, 1.25 units of Taq polymerase (TaKaRa, Japan), and 5 μL of extracted DNA. Amplification was carried out with a thermocycler apparatus (Perkin-Elmer Instruments, Norwalk, CT) for 30 cycles and was followed by a final 7 min extension at 72 °C. Each cycle consisted of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C. The PCR products were visualized by agrose gel electrophoresis and ethidium bromide staining.

Determination of IMS−PCR Sensitivity. To assay the sensitivity of IMS−PCR, chicken meat samples that were confirmed *C. perfringens*-negative by enrichment in modified iron milk medium were spiked with serial 10-fold dilutions from overnight cultures of *C. perfringens* FD-1041. Then, 90 mL of sterile 0.1% peptone water was added to each of the 10 g spiked samples in a sterile filter bag and homogenized for 2 min at low speed in a stomacher. The liquid portion was decanted (using the barrier film to retain large pieces of meat) into a sterile 100 mL centrifuge tube. The remaining particulate was removed by centrifugation for 2 min at 1600*g*. The supernatant fluid was recovered and decanted into another 100 mL tube, and the cells were recovered by centrifugation at 10000*g* for 10 min. The pellet was resuspended in 1 mL of sterile PBS and transferred to a 1.5 mL microcentrifuge tube. These samples were processed by the IMS−PCR as described above. A direct PCR without IMS on these samples was also performed.

Detection of Enterotoxigenic *C. perfringens* from Food Samples. A total of 116 food samples (26 chicken samples, 20 beef samples, 30 pork samples, 20 fish samples, and 20 processed meat samples) were collected from supermarkets and traditional markets located in Jinju (Gyeongnam province, Korea). Each sample was transported to the laboratory in an insulated box and analyzed immediately. A total of 10 g of each sample was aseptically transferred into a sterile filter bag with 90 mL of 0.1% peptone water. *C. perfringens* was isolated with the IMS system and tested by PCR, as described above. All of the food samples were also tested according to the protocol of a standard method, as described by Rhodehamel et al. (*30*). The *C. perfringens* isolates from meat samples were enriched in Duncan−Strong medium, as described by Lin et al. (*31*); the presence of CPE was determined by reversed passive latex agglutination (RPLA) using a commercially available kit (Oxoid, Ogdensburg, NY).

RESULTS

Characterization of mAb. A total of 1292 hybridomas were generated after six fusions. Each hybridoma was initially screened for reactivity against *C. perfringens* FD-1041 antigen preparations. On the basis of initially screening results, 24 hybridomas were chosen to retest against a panel of *Clostridia* and non-*Clostridia* cells to determine their specificity. Of the 24 hybridoma mAbs, mAb J4H9, which originated from cell surface protein immunogen immunized mice, showed the greatest degree of specificity, reacting with only *C. perfringens* (**Table 1**). None of the other bacteria tested gave positive results with mAb J4H9, except for the weak non-specific reaction obtained with whole cells of the protein-A-containing strain of *S. aureus* (**Figure 1**). Isotype analysis revealed that the isotype of mAb J4H9 was IgG1 with κ light chain. The remaining 23 hybridomas showed various reactivity profiles with four other *Clostridium* species (*Clostridium difficile*, *Clostridium sporogenes*, *Clostridium tertium*, and *Clostridium acetobutylicum*) and non-*Clostridia* cells (*E. coli*, *L. monocytogenes*, *S. aureus*, *V. parahemolyticus*, *B. cereus*, and *S. typhimurium*). The purified mAb J4H9 was studied by ELISA. The results showed that the mAb J4H9 reacted in ELISA with live cells, heat-killed cells, and formalin-killed cells of all *C. perfringens* strains tested and gave no cross-reactions with the other four *Clostridium* species tested (**Table 1**). In western blot analysis, mAb J4H9 recognized a protein with the molecular weight of 37 kDa present in all five toxin types of *C. perfringens* (**Figure 2**).

The mAb J4H9 reactive proteins in *C. perfringens* cell fractions were determined by the method described by Bhunia et al. (*35*), except that the cells were grown in 10 mL of brain−heart infusion broth (Difco, Sparks, MD) in anaerobic conditions for 18−24 h at 37 °C. The mAb J4H9 reaction to different cell fractions was monitored by ELISA. As shown in **Table 2**, about 0.09% of the mAb J4H9 reactive proteins are present extracellularly. The mAb J4H9 reactive proteins that can be washed out by PBS were calculated to be 9.8%. The intracellular fractions of the *C. perfringens* cell contained about 14% of the reactive protein

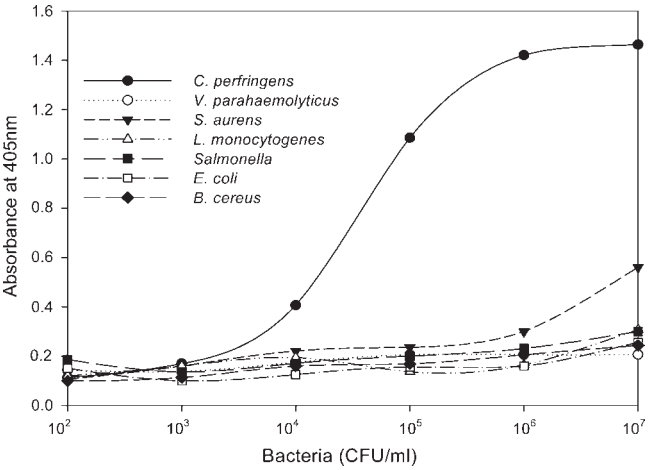

Figure 1. Rectivities of mAb J4H9 to different pathogenic bacteria using indirect ELISA.

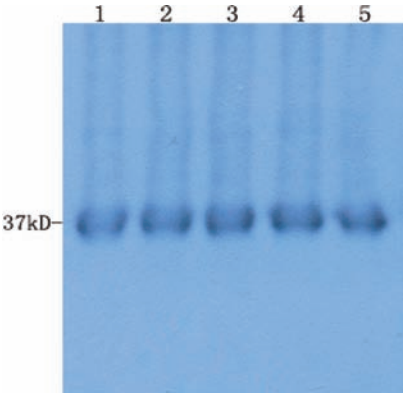

Figure 2. Western blot analysis of selected *C. perfringens*. Whole cell suspensions were heated at 100 °C for 15 min in PBS, mixed 1:1 in Laemmli sample buffer, separated by SDS−PAGE (4 and 12% discontinuous acrylamide), transferred to a Hybond polyvinylidene difluoride (PVDF) membrane, and immunoprobed with mAb J4H9. Lane 1, *C. perfringens* type A, FD-1041; lane 2, *C. perfringens* type B, ATCC 3626; lane 3, *C. perfringens* type C, NCTC 3180; lane 4, *C. perfringens* type D, NCTC 8346; and lane 5, *C. perfringens* type E, NCTC 6719.

Table 2. Determination of the Location of Specific Protein Reactions with mAb J4H9

fractions[a]	ELISA A_{405}[b]	percentage of the total[c]
cell-free culture supernatant	0.016 ± 0.001	0.09
PBS washing[d]		
first	0.098 ± 0.003	
second	0.056 ± 0.002	9.8
third	0.022 ± 0.005	
cytoplasm	0.257 ± 0.006	14
cell wall	1.346 ± 0.043	75
whole cell	1.438 ± 0.068	100

[a] About 10^8 cells/mL were used. [b] Mean of three experiments, each in duplicate. [c] Calculated by dividing individual A_{405} values by the sum of A_{405} values of all of the fractions. [d] Cell pellets were suspended in PBS and washed 3 times to remove loosely attached antigens.

Table 3. Specificity and Efficiency of the IMS System by Adding Different Volumes of Immunomagnetic Beads in a Mixed Culture

species	CFU/mL before IMS	isolation (CFU)[a] in bead volumes of 10 μL	30 μL	50 μL	70 μL	100 μL
C. perfringens	6.0×10^6	2.8×10^3	2.0×10^4	4.0×10^5	4.5×10^5	3.9×10^5
E. coli O157:H7	1.5×10^6	0	0	0	0	0
C. perfringens	6.0×10^6	2.9×10^3	2.5×10^4	3.6×10^5	3.1×10^5	3.5×10^5
S. typhimurium	2.0×10^6	0	0	0	0	0
C. perfringens	6.0×10^6	2.6×10^3	3.0×10^4	3.2×10^5	3.5×10^5	3.3×10^5
L. monocytogenes	1.5×10^6	0	0	0	0	0
C. perfringens	6.0×10^6	3.0×10^3	2.8×10^4	3.7×10^5	3.5×10^5	3.0×10^5
S. aureus	1.5×10^6	0	0	0	1×10^2	5×10^2

[a] Data are the means of three determinations after IMS.

antigens to mAb J4H9, and cell well fractions contain 75% of the reactive protein antigens to mAb J4H9. This result indicates that mAb J4H9 reactive antigens are located on the cell well.

IMS System. For rapid isolation and concentration of *C. perfringens* in meat samples, mAb J4H9 was coated on magnetic beads and the IMS system was developed. The experiments to estimate the specificity and efficiency of the IMS system were carried out by adding different volumes of immunomagnetic beads in 1 mL mixtures of *C. perfringens* plus one of the following species: *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, or *S. aureus*. The colony forming units per milliliter were measured before and after IMS. As shown in **Table 3**, when 10, 30, and 50 μL volumes of immunomagnetic beads were used, only *C. perfringens* cells were captured by the immunomagnetic beads and separated from the mixture by the IMS system; there was no non-specific reaction with other bacteria. When 70 and 100 μL volumes of immunomagnetic beads were used, there was a non-specific reaction only with *S. aureus*, which reached 3 orders less than *C. perfringens* cells. Approximately 5% separation was observed when ≥50 μL of immunomagnetic beads was added to 1 mL of bacterial suspensions (**Table 3**). For overall economics, specificity, and efficiency, 50 μL volumes of immunomagnetic beads were used to capture *C. perfringens* cells from 1 mL test samples in all future experiments.

Determination of the Optimum Immunocapture Time. For maximal capture efficiency, immunomagnetic capture times of 15, 30, and 60 min after the addition of 50 μL immunomagnetic beads were invested. The number of *C. perfringens* cells recovered from PBS by IMS was not significantly affected by the immunomagnetic capture time; however, the number of *C. perfringens* recovered from the meat suspension was significantly affected by the immunomagnetic capture time. The percentage of recovery from meat suspension was very low, only approximately 0.5% for 15 min of immunomagnetic capture, and the percentage of recovery for 30 and 60 min of immunomagnetic capture was not found significantly different (7 and 6.5%, respectively). Therefore, the optimal immunomagnetic capture time for meat suspension samples was selected as 30 min.

Sensitivity of IMS−PCR. To establish the detection limit of the IMS−PCR, the meat sample was artificially inoculated with pure cultured *C. perfringens* FD-1041 in 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/g. After treatment in a stomacher, the samples were analyzed with the IMS−PCR and direct PCR without IMS. As shown in **Figure 3**, using IMS−PCR, the presence of 10 CFU/g of meat sample gave clear bands, while direct PCR without IMS was negative, with up to 1×10^6 *C. perfringens*/g of meat sample.

Detection of Enterotoxigenic *C. perfringens* from Food Samples. A total of 116 meat samples were analyzed by IMS−PCR. The enterotoxigenic *C. perfringens* present in the retail meat samples is show in **Table 4**. Enterotoxigenic *C. perfringens* (*plc*$^+$, *cpe*$^+$) was detected in 2 chicken meat samples by the IMS−PCR. No

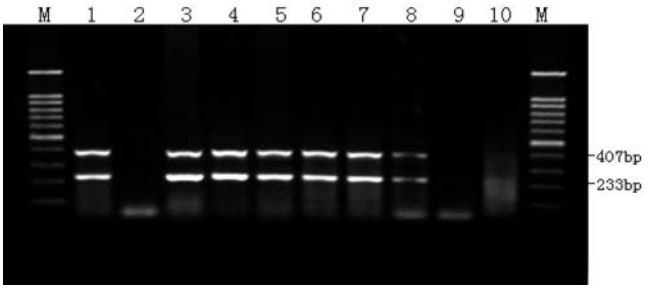

Figure 3. PCR amplicons generated from serial dilutions of *C. perfringens* FD-1041 onto chicken meat samples. Lane M, DNA markers (100 bp); lane 1, positive control; lane 2, negative control; lanes 3−9, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/g; lane 10, 10^6 pellet DNA amplified without IMS.

Table 4. Results of *C. perfringens* Screening in Meat Samples by IMS−PCR and Conventional Cell Culture Methods with Enterotoxin Detection by RPLA

sample	number of samples tested	number (%) of positive samples: IMS−PCR *plc*$^+$, *cpe*$^-$	IMS−PCR *plc*$^+$, *cpe*$^+$	conventional cell culture method	RPLA
chicken	26	17	2	19	2
beef	20	4	0	4	0
pork	30	9	0	9	0
fish	20	3	0	3	0
processed meat	20	1	0	1	0
total	116	34 (29.3)	2 (1.7)	36 (31)	2 (1.7)

entertoxingnetic *C. perfringens* was found in the other 114 meat samples tested. However, non-enterotoxigenic *C. perfringens* (*plc*$^+$, *cpe*$^-$) was found in more meat samples tested (**Table 4**). A total of 34 meat samples, including 17 chicken samples, 4 beef samples, 9 pork samples, 3 fish samples, and 1 processed meat sample, were detected containing non-enterotoxigenic *C. perfringens* by IMS−PCR. All of the meat samples were also analyzed by conventional culture methods. After identification of the *C. perfringens* isolates as described previously (*31*), 36 samples confirmed containing *C. perfringens*. After sporulation, *C. perfringens* isolates from 2 chicken samples were detected producing CPE by RPLA. The results agreed well with those obtained by IMS−PCR. The time sequence for IMS−PCR developed in this study included stomaching, centrifugation, IMS, DNA extraction, PCR amplification, and gel electrophoresis. Overall, the analysis can be finished within 10 h. In comparison to conventional culture methods, the IMS−PCR is a raped, specific method for the detection of enterotoxigenic *C. perfringens* in meat samples and has potential use as a screening tool for enterotoxigenic *C. perfringens* in food samples.

DISCUSSION

Routine identification of *C. perfringens* in food samples requires characterization by lengthy procedures, which take 2−5 days or more and involve the use of selective enrichment medium, biochemical tests, and final confirmation of enterotoxigenic strains by culturing in Duncan−Strong sporulation medium combined with serological analysis (*31*). This is costly and laborious, and the results are not obtained soon enough to influence the release of the finished food product. Rapid and highly sensitive techniques based on PCR have been developed recently for the detection of foodborne phathogens (*20*−*23*, *29*). A PCR-based detection system is highly sensitive and eliminates the need for enrichment culturing (*32*). However, the complex nature of food components offers unique challenges in the application of PCR for rapid detection of pathogens in food (*33*). Moreover, the collection of low numbers of pathogenic bacterial cells from a complex food matrix poses a difficult challenge for successful detection of those bacteria by any sensitive detection methods. Thus, a clean and efficient sample preparation is crucial, which should not only yield high numbers of target bacteria but also minimize the amount of interfering food constituents. In recent years, many studies on the IMS method from food samples have been reported (*24*−*26*, *34*). This method used magnetic beads coated with antibodies to extract and concentrate the target organism from the sample. It is a very effective tool for the isolation of target cells from food components or other matrixes. Toward the goal of developing a rapid IMS−PCR for detection of enterotoxigenic *C. perfringens* in meat samples, mAb J4H9 was developed in this study. It was shown to react with live or killed *C. perfringens* cells, as shown by ELISA. Specificity for *C. perfringens* was confirmed by ELISA testing of mAb J4H9 against a comprehensive panel of *Clostridia* and non-*Clostridia* organisms, many of which often grow in meat products. mAb J4H9 failed to react significantly with any of the non-*C. perfringens* cells. The reactivity of the mAb J4H9 with *S. aureus* was shown to be due to non-specific reactivity with protein A (*35*). This reaction was explained by the fact that *S. aureus* often expresses protein A in the cell wall and that this protein has a strong affinity to IgG. The isotype of mAb J4H9 developed in this study is IgG1. It can be concluded that mAb J4H9 specifically recognizes a genus-specific *C. perfringens* antigen.

Using the mAb J4H9, the IMS−PCR method was developed and optimized for detection of *C. perfringens* from meat samples. Working with real food samples, we had to overcome the adverse influence of background bacteria on the sensitivity of the IMS−PCR assay. Prior to natural samples, we tested a designed mixture of bacteria (**Table 3**). The non-specific cross-reaction was only found with *S. aureus* by IMS. However, this is not a significant problem for identification of enterotoxigenic *C. perfringens* by IMS−PCR because specific primers of *C. perfringens* were used in PCR. Because mAb J4H9 recognizes all of the five toxin types of *C. perfringens*, we used two pairs of PCR primers to identify enterotoxigenic *C. perfringens* in meat samples. One pair of primers was derived from the phospholipase C gene present all strains of *C. perfringens* (*28*). The other pair was derived from the enterotoxin gene found predominantly in *C. perfringens* strains associated with outbreaks of food poisoning (*29*). The IMS−PCR reported here is a rapid and sensitive diagnostic technique; enterotoxigenetic *C. perfringens* is detectable in meat samples at a concentration of 10 CFU/g within 10 h. Using sandwich ELISA, McCourt et al. (*36*) were able to detect 10^2−10^6 CFU/mL *C. perfringens* cells from field cases of necrotic enteritis of poultry. Wise and Siragusa (*19*) developed a real-time PCR for quantitative detection of *C. perfringens* in the broiler fowl gastrointestinal tract; the consistent limit of detection with ileal samples was approximately 10^2 CFU/g of ileal material but only about 10^4 CFU/g of cecal samples. Shimizu et al. (*18*) developed a fluorescent *in situ* hybridization in combination with a filter cultivation method for enumeration of viable *C. perfringens*; the detection limit of this method was 10^2 CFU/g, after a 6 h period of anaerobic cultivation on TSC-selective medium. Augustynowicz et al. (*21*) developed a duplex PCR for detection of enterotoxigenic *C. perfringens*; the detection limit was 10−20 bacterial cells. The sensitivity was same as our method, but it needed culture in reinforced clostsridial broth before PCR.

Using the IMS−PCR, enterotoxigenic *C. perfringens* was found in randomly collected chicken meat samples, whereas no enterotoxigenic *C. perfringens* was detected in the beef, pork, fish, and processed meat samples. The incidence of entertoxigenic *C. perfringens* in chicken meat samples was high in comparison to other meat samples. It has been reported that the incidence and amount of enterotoxigenic *C. perfringens* in the intestinal contents of chicken was higher than that of cattle or swine (*37*). Chicken carcasses may be contaminated with enterotoxigenic *C. perfringens* by their intestinal contents during the slaughter process, which results in the contamination of the retail meat.

In conclusion, the IMS−PCR method is a rapid, specific, and sensitive detection method for enterotoxigenic *C. perfringens* in meat samples. The IMS−PCR method developed in this study has potential as a rapid screening tool for enterotoxigenic *C. perfringens* contaminating food samples.

LITERATURE CITED

(1) Brynestad, S.; Granum, P. E. *Clostridium perfringens* and foodborne infections. *Int. J. Food Microbiol.* **2002**, *74*, 195–202.

(2) McClane, B. A.; Uzal, F. A.; Miyakawa, M. F.; Lyerly, D.; Wilkins, T. The enterotoxic *Clostridia*. In *The Prokaryotes*; Dworkin, M., Falkow, S., Rosenburg, E., Schlcifer, K. H., Stackebrandt, E., Eds.; Springer-Verlag: New York, 2006; Vol. 4, pp 698−752.

(3) Petit, L.; Gilbert, M.; Popoff, M. R. *Clostridium perfringens*: Toxinotype and genotype. *Trends Microbiol.* **1999**, *7*, 104–110.

(4) Lin, Y. T.; Labbe, R. L. Enterotoxigenicity and genetic relatedness of *Clostridium perfringens* isolates from retail foods in the United States. *Appl. Environ. Microbiol.* **2003**, *69*, 1642–1646.

(5) Centers for Disease Control and Prevention. *Surveillance for Foodborne-Disease Outbreaks—U.S. 1998−1992*. Centers for Disease Control and Prevention, Atlanta, GA, 1996.

(6) Centers for Disease Control and Prevention. *Surveillance for Foodborne-Disease Outbreaks—U.S. 1993−1997*. Centers for Disease Control and Prevention, Atlanta, GA, 2000.

(7) Abubakar, I.; Irvine, L.; Aldus, C. F.; Wyatt, G. M.; Fordham, R.; Schelenz, S.; Shepstone, L.; Howe, A.; Peck, M. P.; Hunter, R. A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. *Health Technol. Assess.* **2007**, *11*, 1–216.

(8) Adak, G. K.; Long, S. M.; O'Brien, S. J. Trends in indigenous foodborne disease and deaths England and Wales: 1992 to 2000. *Gut* **2002**, *51*, 832–841.

(9) Dalton, C. B.; Gregory, J.; Kirk, M. D; Stafford, R. J.; Givney, R.; Kraa, E.; Gould, D. Foodborne disease outbreaks in Australia, 1995 to 2000. *Commun. Dis. Intell.* **2004**, *28*, 211–224.

(10) McClane, B. C. *Clostridium perfringens*. In *Food Microbiology Fundamentals and Frontiers*; Doyle, M. P., Beuchat, L. R., Montville, T. J., Eds.; ASM Press: Washington, D.C., 1997; pp 305−326.

(11) Johnson, S.; Gerding, D. Enterotoxemic infections. In *The Clostridia: Molecular Biology and Pathogenesis*; Rood, J., McClane, B. A., Songer, J. G., Titball, R. W., Eds.; Academic Press: London, U.K., 1997; pp 117−140.

(12) Daube, G.; Simon, P.; Limbourg, B.; Manteca, C.; Mainil, J.; Kaeckenbeeck, A. Hybridization of 2,659 *Clostridium perfringens* isolates with gene probes for seven toxins (α, β, ε, ι, θ, μ and enterotoxin) and for sialidase. *Am. J. Vet. Res.* **1996**, *57*, 496–501.

(13) Kokai-Kun, J. F.; Songer, J. G.; Czeczulin, J. R.; Chen, F.; McClane, B. A. Comparison of western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *J. Clin. Microbiol.* **1994**, *32*, 2533–2539.
(14) Cudjoe, K. S.; Thorsen, L. I.; Sorensen, T.; Reseland, J.; Olsvik, O.; Granum, P. E. Detection of *Clostridium perfringens* type A enterotoxin in faecal and food samples using immunomagnetic separation (IMS)−ELISA. *Int. J. Food Microbiol.* **1991**, *12*, 313–322.
(15) Eisgruber, H.; Wiedmann, M.; Stoole, A. Use of plasmid profiling as a typing method for epidemiologically related *Clostridium perfringens* isolates from food poisoning cases and outbreaks. *Lett. Appl. Microbiol.* **1995**, *20*, 290–294.
(16) Schalch, B.; Sperner, B.; Eisgruber, H.; Stoole, A. Molecular methods for the analysis of *Clostridium perfringens* relevant to food hygiene. *FEMS Immunol. Med. Microbiol.* **1999**, *24*, 281–286.
(17) Mahony, D. E.; Ahmed, R.; Jackson, S. G. Multiple typing techniques applied to a *Clostridium perfringens* food poisoning outbreak. *J. Appl. Bacteriol.* **1992**, *72*, 309–314.
(18) Shimizu, S.; Ootsubo, M.; Kubosawa, Y. J.; Fuchizawa, I.; Kawai, Y.; Yamazaki, K. Fluorescent *in situ* hybridization combination with filter cultivation (FISHFC) method for specific detection and enumeration of viable *Clostridium perfringens* strains. *Food Microbiol.* **2009**, *26*, 425–431.
(19) Wise, M. G.; Siragusa, G. R. Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. *Appl. Environ. Microbiol.* **2005**, *71*, 3911–3916.
(20) Tansuphasiri, U.; Chanyasanha, C.; Cheaochantanakij, N. An enrichment broth culture-duplex PCR combination assay for the rapid detection of enterotoxigenic *Clostridium perfringens* in fecal specimens. *Southeast Asian J. Trop. Med. Public Health* **2005**, *36* (5), 1229–1238.
(21) Augustynowicz, E.; Gzyl, A.; Slusarczyk, J. Detection of enterotoxigenic *Clostridium perfringens* with a duplex PCR. *J. Med. Microbiol.* **2002**, *51* (2), 169–172.
(22) Magliulo, M.; Simoni, P.; Guardigli, M.; Michelini, E.; Luciani, M.; Lelli, R.; Roda, A. A rapid multiplexed chemiluminescent immunoassay for the detection of *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Salmonella typhimurium*, and *Listeria monocytogenes* pathogen bacteria. *J. Agric. Food Chem.* **2007**, *55* (13), 4933–4939.
(23) Kim, S.; Labbe, R. G.; Ryu, S. Inhibitory effects of collagen on the PCR for detection of *Clostridium perfringens*. *Appl. Environ. Microbiol.* **2000**, *66*, 1213–1215.
(24) Hudson, J. A.; Lake, R. J.; Savill, M. G.; Scholes, P.; McCormick, R. E. Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. *J. Appl. Microbiol.* **2001**, *90*, 614–621.
(25) Alarcón, B.; García-Cañas, V.; Cifuentes, A.; González, R.; Aznar, R. Simultaneous and sensitive detection of three foodborne pathogens by multiplex PCR, capillary gel electrophoresis, and laser-induced fluorescence. *J. Agric. Food Chem.* **2004**, *52* (23), 7180–7186.
(26) Fitzmaurice, J.; Duffy, G.; Kilbride, B.; Sheridan, J. J.; Carroll, C.; Maher, M. Comparison of a membrane surface adhesion recovery method with an IMS method for use in a polymerase chain reaction method to detect *Escherichia coli* O157:H7 in minced beef. *J. Microbiol. Methods* **2004**, *59*, 243–252.
(27) Yang, Z.-Y.; Kolosava, A. Y.; Shim, W.-B.; Chung, D.-H. Development of monoclonal antibodies against pirimiphos-methyl and their application to IC-ELISA. *J. Agric. Food Chem.* **2006**, *54* (13), 4551–4556.
(28) Kristian, M; Ahrens, P. Comparison of toxicity neutralization-, ELISA- and PCR tests for typing of *Clostridium perfringens* and detection of the enterotoxin gene by PCR. *Anaerobe* **1996**, *2*, 103–110.
(29) Meer, R. R.; Songer, J. G. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *Am. J. Vet. Res.* **1997**, *58*, 702–705.
(30) Rhodehamel, E. J.; Harmon, S. M. *Clostridium perfringens*. In *Bacteriological Analytical Manual*, 8th ed.; U.S. Food and Drug Administration, Association of Official Analytical Chemists (AOAC) International: Gaithersburg, MD, 2001; Chapter 16, Revision A.
(31) Lin, Y. T.; Labbe, R. Enterotoxigenicity and genetic relatedness of *Clostridium perfringens* isolates from retail foods in the united states. *Appl. Environ. Microbiol.* **2003**, *69*, 1642–1646.
(32) Swanimathan, B.; Feng, P. Rapid detection of food-borne pathogenic bacteria. *Annu. Rev. Microbiol.* **1994**, *58*, 2292–2295.
(33) Kim, S.; Labbe, R. G.; Ryu, S. Inhibitory effects of collagen on the PCR for detection of *Clostridium perfringens*. *Appl. Environ. Microbiol.* **2000**, *66*, 1213–1215.
(34) Ueda, S.; Maruyama, T.; Kuwabara, Y. Detection of *Listeria monocytogenes* from food samples by PCR after IMS-plating. *Biocontrol Sci.* **2006**, *11*, 129–134.
(35) Bhunia, A. K.; Ball, P. H.; Fuad, A. T.; Kurz, B. W.; Emerson, J. W.; Johnson, M. G. Development and characterization of a monoclonal antibody specific for *Listeria monocytogenes* and *Listeria innocua*. *Appl. Environ. Microbiol.* **1991**, *59*, 3176–3184.
(36) McCout, M. T.; Finlay, D. A.; Laird, C.; Smyth, J. A.; Bell, C.; Ball, H. J. Sandwich ELISA detection of *Clostridium perfringens* cells and α-toxin from field cases of necrotic enteritis of poultry. *Vet. Microbiol.* **2005**, *106*, 259–264.
(37) Miwa, N.; Nishina, T.; Kubo, S.; Honda, H. Most probable numbers of enterotoxigenic *C. perfringens* in intestinal contents of domestic livestock detected by nested PCR. *J. Vet. Med. Sci.* **1997**, *59*, 557–560.

Received for review March 13, 2010. Revised manuscript received May 12, 2010. Accepted May 17, 2010. This research was supported by a Grant from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (03-PJ1-PG1-CH11-0003). Z.-Y. Yang was supported by the Brain Korea 21 Program from the Ministry of Education, Republic of Korea, and the Research Foundation of Shandong Agricultural University and National Natural Science Foundation (grant no. 3097205), People's Republic of China. W.-B. Shim and K.-Y. Kim were supported by the Brain Korea 21 Program from the Ministry of Education, Republic of Korea.