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Research Note

Prevalence and toxin type of *Clostridium perfringens* in beef from four different types of meat markets in Seoul, Korea

Dana Jeong, Dong-Hyeon Kim, Il-Byeong Kang, Jung-Whan Chon†, Hyunsook Kim[1], Ae-Son Om[1], Joo-Yeon Lee[2], Jin-San Moon[3], Deog-Hwan Oh[4], and Kun-Ho Seo*

Center for One Health, College of Veterinary Medicine, Konkuk University, Seoul 05029, Korea
[1]Department of Food & Nutrition, College of Human Ecology, Hanyang University, Seoul 04763, Korea
[2]Korea HACCP Accreditation and Services, Seoul 05248, Korea
[3]Veterinary Parmaceutical Management, Animal and Plant Quarantine Agency, Gimcheon, Gyeongbuk 39660, Korea
[4]Department of Food Science and Biotechnology, College of Agriculture and Life Sciences, Kangwon National University, Chuncheon, Gangwon 24341, Korea
†Jung-Whan Chon's current address: Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR 72079, USA

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*Corresponding Author
Tel: +82-2-450-4121
Fax: +82-2-450-3037
E-mail: bracstu3@konkuk.ac.kr

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Abstract Beef is the primary source of foodborne poisoning caused by *Clostridium perfringens*. We investigated the prevalence of *C. perfringens* in retail beef from four different types of meat markets in Seoul using a standard culture method and real-time PCR assay. From June to September 2015, 82 beef samples were collected from 6 department stores (*n*=12), 14 butcher shops (*n*=28), 16 traditional markets (*n*=32), and 5 supermarkets (*n*=10). The culture method and real-time PCR assay revealed that 4 (4.88%) and 10 (12.20%) samples were positive for *C. perfringens*, respectively. The beef purchased from the department store showed the highest prevalence (16.67%), followed by the traditional market (3.12%), butcher shop (3.57%), and supermarket (0%) (p>0.05). All isolates were type A and negative for the enterotoxin gene. In conclusion, the real-time PCR assay used in this study could be useful for rapid detection and screening of *C. perfringens* in beef.

Keywords: *Clostridium perfringens*, prevalence, beef, meat market type, toxin typing

Introduction

Clostridium perfringens is a foodborne pathogen that causes food poisoning and enteritis necroticans in humans (1,2). Based on the synthesis of four major lethal toxins—alpha (*cpa*), beta (*cpb*), epsilon (*etx*), and iota (*iap*)—this bacterium is classified into 5 toxigenic types: A, *cpa*-positive; B, *cpa*-, *cpb*-, and *iap*- positive; C, *cpa*- and *cpb*-positive; D, *cpa*- and *etx*-positive; E, *cpa*- and *iap*-positive (3). Each toxin type causes different forms of enteric infections and enterotoxaemia in various hosts (4). *C. perfringens* enterotoxin (*cpe*) is an important virulence factor related to the pathogenesis of foodborne poisoning in humans (5). Thus, the toxin type of *C. perfringens* strains could provide a better understanding of the pathogenicity of this bacterium and may be helpful in tracing contagious origin (6).

From 1998 to 2008, meat, especially beef, was found to be the most common causative food reported in *C. perfringens* outbreaks in the United States, suggesting that beef is the primary source of *C. perfringens* infection in cases of foodborne poisoning in humans (7). Although Korea has a zero-tolerance policy toward the presence of *C. perfringens* in meat products because of the possible severe consequences (8), the prevalence of this bacterium in beef, especially at the retail level, has been poorly studied to date.

The culture method is regarded as the standard method for the detection of *C. perfringens* from food samples, but it is time-consuming and labor-intensive (9). Furthermore, the detection performance of this method can be profoundly diminished by the presence of competing flora in foods (10). To overcome these drawbacks, we previously developed a real-time PCR assay for the detection and enumeration of *C. perfringens* in foods (9). However, the performance of this newly developed PCR assay has not been validated in field samples.

Hence, the aims of the current study were to: (i) investigate the prevalence of *C. perfringens* in beef from various types of meat markets, (ii) compare the performance of real-time PCR assay as early screening methods for detection of *C. perfringens* in field samples, and (iii) analyze the toxigenic types and presence of *cpe* gene in the isolates.

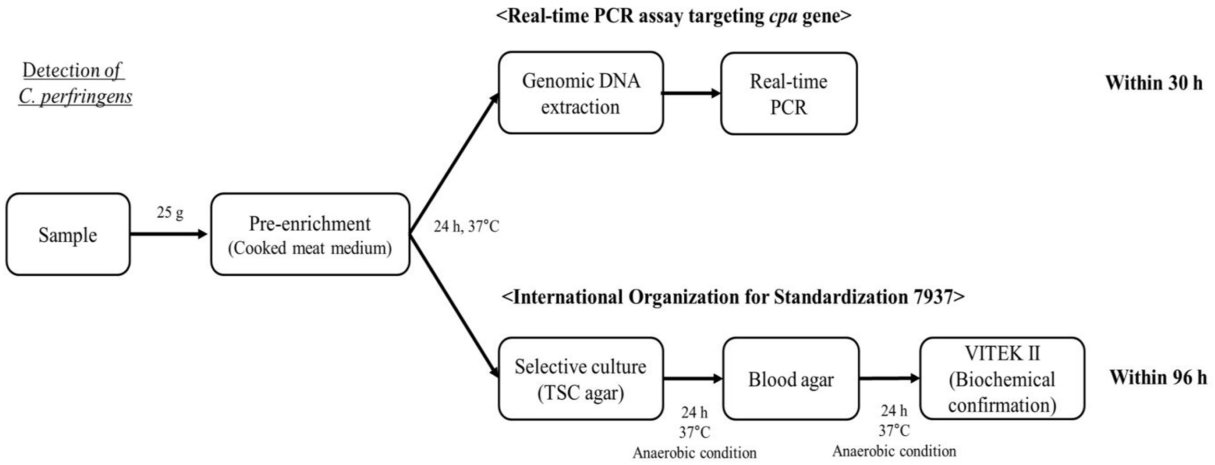

Fig. 1. Experimental procedures of this study

Materials and Methods

Beef samples In total, 82 beef samples were purchased from 6 department stores (large stores selling various goods including foods with a complex distribution structure), 14 butcher shops (small retail shops that perform secondary butchery to prepare and sell fresh cuts of meat), 16 traditional markets (meat wholesale markets that sell products directly from the manufacturers), and 5 supermarkets (large markets to which distribution centers directly supply processed meat products) from June to September 2015 in Seoul, South Korea (two samples per market). All beef samples were stored at 4°C immediately and analyzed within 6 h.

Detection of *C. perfringens* by the culture method *C. perfringens* was detected in beef by the culture methods presented by the International Organization for Standardization 7937 (11), with modifications (Fig. 1). Each beef sample (25 g) was placed in 225 mL of 0.1% peptone in water and homogenized for 30 s. After stomaching, 1 mL of 24-h enriched meat medium was inoculated into 9 mL of cooked meat medium (Oxoid, Basingstoke, UK) at 37°C for 24 h. The inoculated broth was streaked onto tryptose sulfite cycloserine agar (TSC; Oxoid) with 5% egg yolk emulsion (Oxoid), followed by inoculation at 37°C for 24 h under anaerobic conditions. Presumptive identification was based upon colony morphology, with black colonies with a white halo considered positive. Suspicious colonies were streaked onto 5% horse blood agar (Oxoid) and incubated at 37°C for 24 h under anaerobic conditions. Colonies on blood agar were selected for biochemical confirmation using the Vitek 2 system (bioMérieux, Marcy l'Etoile, France).

Genomic DNA extraction Bacterial genomic DNA was extracted as described by Kim *et al.* (10) with some modifications. The 1 mL of cooked meat medium was collected and centrifuged at 15,776×*g* for 3 min. The pellets were resuspended in 200 μL of PrepMan Ultra Reagent (Applied Biosystems, Foster City, CA, USA) and boiled for 10 min. The samples were centrifuged at 15,776×*g* for 3 min. The supernatant was used for the real-time PCR assay.

Real-time PCR assay The *cpa* gene was targeted using the primers and probe according to the method described in our previous study (9). The sequences (amplicon size, 85 bases) were as follows: forward primer: 5'-AAA AGA AAG ATT TGT AAG GCG CTT AT-3'; reverse primer: 5'-CCC AAG CGT AGA CTT TAG TTG ATG-3'; probe: 5'-FAM TGC CGC GCT AGC AAC TAG CCT ATG G -3'TAMRA. The extracted DNA (5 μL) was transferred into 20 μL of PCR mix consisting of 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), forward primer (2.5 μL, 900 nM), reverse primer (2.5 μL, 900 nM), and TaqMan probe (2.5 μL, 250 nM). The 96-microwell plate was sealed with optical adhesive covers (Applied Biosystems) and placed in an ABI 7500 (Applied Biosystems). The reaction was run at 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Ct values were analyzed using ABI 7500 software version 2.3 (Applied Biosystems).

Detection of toxin genes by PCR amplification For toxin genotyping of isolates, the primer pair sequences and cycling conditions were applied according to Baum *et al.* (6). The sequences were as follows: *cpa* (amplicon size, 900 bp); forward primer: 5'-AGT CTA CGC TTG GGA TGG AA-3'; reverse primer: 5'-TTT CCT GGG TTG TCC ATT TC-3', *cpb* (amplicon size, 611 bp); forward primer: 5'-TCC TTT CTT GAG GGA GGA TAA A-3'; reverse primer: 5'-TGA ACC TCC TAT TTT GTA TCC CA-3', *etx* (amplicon size, 396 bp); forward primer: 5'-TGG GAA CTT CGA TAC AAG CA-3'; reverse primer: 5'-TTA ACT CAT CTC CCA TAA CTG CAC-3', *iap* (amplicon size, 293 bp); forward primer: 5'-AAA CGC ATT AAA GCT CAC ACC-3'; reverse primer: 5'-CTG CAT AAC CTG GAA TGG CT-3', *cpe* (amplicon size, 506 bp); forward primer: 5'-GGG GAA CCC TCA GTA GTT TCA-3'; reverse primer: 5'-ACC AGC TGG ATT TGA GTT TAA TG-3'. Each PCR amplification reaction was performed using 20 μL of a reaction mixture consisting of FRENCHE PCR PreMix (iNtRON Biotechnology, Seongnam, Korea),

50 ng of template DNA, and each primer at 500 nmol/L. In total, 5 μL of the amplified PCR product was analyzed with electrophoresis on a 1.5% agarose gel containing 50 μL of SafeView™ (Applied Biological Material Inc., Richmond, Canada) per liter. The amplified sequences were examined under ultraviolet light using a BioRad Molecular Imager® GelDocTM XR (BioRad Laboratories, Hercules, CA, USA).

Statistical analysis The number of positive samples was analyzed statistically using Instat Software (GraphPad Software, San Diego, CA, USA). Differences between the two methods were examined for significance using the two-sided chi-square test. Differences among the market types were analyzed using the two-sided Fisher's exact test. $p<0.05$ was considered statistically significant.

Results and Discussion

Prevalence of *C. perfringens* in beef determined using the culture method and real-time PCR assay The prevalence of *C. perfringens* in beef samples purchased from four types of meat markets determined by the standard culture method and real-time PCR assay is presented in Table 1. Among the 82 beef samples, 4 (4.88%) and 10 (12.20%) were positive for *C. perfringens* by the culture method and real-time PCR assay, respectively. There were no significant differences in the prevalence of *C. perfringens* in beef collected from different types of retail markets with either method ($p>0.05$).

To date, few studies have investigated the prevalence of *C. perfringens* in beef. Chae *et al*. (12) reported that the prevalence of *C. perfringens* in bovine carcasses was 3.3% (30/925) in Korea. Tizhe *et al*. (4) reported that 3.5% of beef samples tested (14/400) were positive for *C. perfringens* in Nigeria. Our results were similar to those of the previous studies. However, considering that beef is the top source of *C. perfringens* foodborne illness in humans, it is interesting that the prevalence of this bacterium in beef is not higher than that in other meats including pork and chicken, in which the prevalence ranges from 35 to 97% (14,15).

To the best of our knowledge, this is the first study to investigate the prevalence of *C. perfringens* in beef purchased from various types of meat markets in Seoul, Korea. Meat can be gradually contaminated as it passes through various distribution, storage, and processing stages (7). In Korea, meat handlers of department stores typically process more meat products than those at traditional market and supermarkets, which increases the opportunity for cross-contamination of foodborne pathogens from hands, utensils, and the environment (16,17). A previous study showed that that department stores exhibited higher levels of *E. coli* contamination than other market types (16). In addition, the butcher shop had the shortest distribution process among the four market types because meat was directly supplied from individual farms (16,17). Our results are consistent with the previous studies in that the beef purchased from the department store showed the highest prevalence of *C. perfringens* among all market types, although there were no significant differences. On the basis of our results that there were no significant differences among market types, it could be postulated that the initial contamination of *C. perfringens* in beef is more important than contamination in subsequent procedures. Because of its anaerobic and spore-forming nature, the initial contamination level of *C. perfringens* in beef could be maintained consistently to final consumption (18). Therefore, it would be most important to control the contamination of *C. perfringens* in the initial steps of beef production.

Table 1. Prevalence of *C. perfringens* in beef from various types of meat markets, as determined via the standard culture method and real-time PCR assay

Market type	No. of positives/ No. of total samples (%)		*p* value [2)]
	Culture methods (ISO10001)	Real-time PCR targeting *cpa* gene[1)]	
Department store	2/12 (16.67)	3/12 (25.00)	0.6152
Butcher shop	1/28 (3.57)	2/28 (7.14)	0.5529
Traditional market	1/32 (3.12)	4/32 (12.50)	0.3516
Supermarket	0/10 (0.00)	1/10 (10.00)	0.3049
Total	4/82 (4.88)	10/82 (12.2)	0.1623
p value [3)]	0.297	0.453	-

[1)]Real-time PCR assay targeting *C. perfringens* alpha toxin gene (*cpa*) was conducted following Chon *et al.* (9).
[2)]Significant differences between the culture method and real-time PCR assay were analyzed using the two-sided chi-square test ($p<0.05$).
[3)]Significant differences among the market types were analyzed using the two-sided Fisher's exact test ($p<0.05$).

Comparing real-time PCR assay using field samples The newly developed real-time PCR assay was validated in naturally contaminated beef. Although there were no significant differences between the

Table 2. Toxin gene profiling of the beef isolates of this study

Isolate	Source	Toxin gene[1)]				Toxin type	*cpe* gene (enterotoxin)
		cpa	*cpb*	*etx*	*iap*		
DN1501	Department store	+	-	-	-	A	-
DN1502	Department store	+	-	-	-	A	-
DN1503	Traditional market	+	-	-	-	A	-
DN1504	Butcher shop	+	-	-	-	A	-

[1)]*C. perfringens* alpha (*cpa*), beta (*cpb*), epsilon (*etx*), and iota (*iap*) toxin genes

culture method and real-time PCR assay in terms of the determined prevalence of *C. perfringens*, the PCR assay results indicated a higher positive rate (Table 1, p=0.1605, Fisher's exact test). These results suggest the possibility of false positive results for real-time PCR or false negative results for the culture method; however, all positive samples in the culture method were found to be positive via real-time PCR, indicating that the novel real-time PCR assay could be used as an early screening method for detecting *C. perfringens* in beef samples.

The average level of background microflora of the 82 beef samples was 4.53±2.67 log CFU per gram, suggesting that the real-time PCR assay could detect the targeted organism even when it was hidden by other background organisms in the culture media. The same phenomenon was observed in the real-time PCR assays for the detection of *Listeria monocytogenes* and *Lactobacillus kefiranofaciens* in food samples with high levels of background microflora (10,19). In addition, the results of the real-time PCR assay for detection of *C. perfringens* could be obtained within 30 h, whereas the conventional culture method requires 96 h (Fig. 1). Considering the sensitivity and reduced test time, the real-time PCR assay could be a useful screening tool for detection of *C. perfringens* in food samples.

Toxin typing of the beef isolates The results of toxin genotyping of four beef isolates are presented in Table 2. All isolates were type A, showing positive results only for the *cpa* gene. In addition, all isolates were negative for the *cpe* gene.

C. perfringens type A is the most common type implicated in *C. perfringens* foodborne poisoning among the five toxigenic types in Japan, Europe, and the United States (15,20). In this study, all isolates were type A and *cpe*-negative. This result is similar to those in previous studies, in which the proportion of type A among *C. perfringens* strains isolated from chicken liver, calves, and ground beef was 86-100% (14,15,21). In fact, only 0-5.7% of beef isolates were found to be *cpe*-positive in Japan (15). Smedley *et al*. (22) also reported that *C. perfringens* enterotoxin is produced by less than 5% of all *C. perfringens* strains. Given that clinical signs, including diarrhea and cramping of the abdomen, are caused by the *C. perfringens* type A strain that is notably positive for enterotoxin production (5,20), commercial beef in Korea could thus be considered to be of relatively low risk in terms of the presence of highly pathogenic *C. perfringens*.

In summary, we investigated the prevalence of *C. perfringens* in four market types and validated the novel real-time PCR assay by comparison with the conventional method. Among 82 beef samples, four (4.88%) and 10 (12.2%) were positive for *C. perfringens* by the culture method and real-time PCR assay, respectively. Real-time PCR showed high performance with more positive results than the culture method. Moreover, there were no significant differences in the positive rate for *C. perfringens* among the different market types. In the toxin typing, all isolates were identified as Type A and *cpe*-negative.

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Disclosure The authors declare no conflict of interest.

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