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Inhibitory Effects of Collagen on the PCR for Detection of *Clostridium perfringens*

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It is essential to identify specific food components that inhibit PCR in order to increase the sensitivity of the PCR method for rapid detection of pathogens contaminating a food. We found that collagen, a major component of several foods, inhibited PCR. The inhibitory action of collagen on PCR could be partially reversed by adjusting the concentration of magnesium ion in the reaction mixture and by the use of various DNA extraction methods to remove the collagen from the DNA. Also, the source of thermostable DNA polymerase was affected by the presence of collagen. These results suggest the need to optimize the extraction and assay conditions for rapid detection of enterotoxigenic *Clostridium perfringens* by PCR with respect to the kind of food being analyzed.

Clostridium perfringens type A food poisoning is among the most common of the human gastrointestinal illnesses (3, 9). Symptoms associated with *C. perfringens* type A food poisoning are diarrhea and severe abdominal pain. The symptoms are mediated by an enterotoxin, a 35-kDa single polypeptide produced only during sporulation of the organism in the small intestine following ingestion of food contaminated with large numbers of vegetative cells (12, 13). *C. perfringens* is a typical gram-positive, rod-shaped, spore-forming, anaerobe commonly found in the intestines of humans and mammals. *C. perfringens* is a part of the microflora of soil, and many reports have revealed the widespread occurrence of this organism in raw and processed foods. However, most of these isolates are nonenterotoxigenic strains. Recent surveys suggest that only about 6% of all *C. perfringens* isolates carry the gene (*cpe*) encoding enterotoxin (8, 19). It therefore is necessary to distinguish the enterotoxigenic organisms from the nonenterotoxigenic ones to confirm food poisoning by *C. perfringens*.

Rapid and highly sensitive techniques based on PCR have been developed recently for the detection of foodborne pathogens (2, 7, 14, 17). A PCR-based detection system is highly sensitive and eliminates the need for enrichment culturing (17). PCR can be used to detect genes involved in the virulence of foodborne bacteria. However, the complex nature of food components offers unique challenges in the application of PCR to rapid detection of pathogens in a food (16). A variety of components—for example, heme and its metabolic products (15), acidic polysaccharides (5, 6), humic substances (18), bean sprout homogenates (7), oysters (7), and soft cheese (20)—have been shown to inhibit PCR amplification.

We have tried to develop a rapid PCR-based method for detection of enterotoxigenic *C. perfringens* contamination in a food by testing for the presence of the *cpe* gene directly without preenrichment. Four Korean ethnic foods, man-doo (a bun stuffed with seasoned meat and vegetables), soon-dae (a sausage made of beef and bean curd, stuffed in pig intestine), kim-bab (rice, meat, and vegetables wrapped with seaweed), and steamed pork hock, were tested. Food samples were artificially contaminated with enterotoxigenic *C. perfringens* type A strain NCTC 8239 (Hobb's serotype 3 [H3]) cells at densities ranging from 10^1 to 10^8 CFU/g of food. The food samples were homogenized in a Waring blender for 2 min in distilled water at a ratio of 1 g of food to 5 ml of water, and the cells were collected by filtration through Whatman no. 41 filter paper followed by centrifugation of the filtrate at 15,000 × *g* for 10 min. The pellet was used for preparation of a DNA template for PCR. Traditionally, DNA templates have been prepared by phenol-chloroform extraction. However, various easy and rapid DNA isolation methods have been developed to replace the time-consuming procedure of phenol-chloroform extraction and ethanol precipitation. We prepared a DNA template for PCR by using two commercially available DNA extraction kits, a QIAamp tissue kit (Qiagen, Hilden, Germany) and a GeneReleaser kit (Bio Ventures, Inc., Murfreesboro, Tenn.),

TABLE 1. Minimum number of *C. perfringens* cells detectable by PCR when DNA was prepared with a QIAamp kit or by phenol extraction from foods artificially contaminated with *C. perfringens*

Detection method	Min. no. of *C. perfringens* cells detectable in:				
	No food	Man-doo	Soon-dae	Kim-bab	Pork hock
QIAamp kit	30/ml	400/g	2.5×10^3/g	4.5×10^3/g	ND[a]
Phenol extraction	10/ml	10^4/g	1×10^3/g	1×10^3/g	1×10^4/g

[a] ND, none detected.

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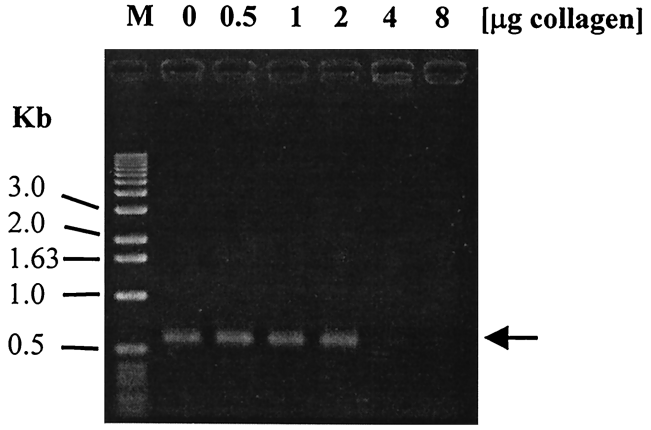

FIG. 1. Inhibition of PCR by type I collagen. Type I collagen from calf skin was added to 50 μl of PCR mix in the presence of 3.5 mM $MgCl_2$ at the concentrations indicated above the gel. The positions of molecular size markers (M) are shown on the left. The arrow indicates the position of the 599-bp DNA fragment.

according to the instructions of the manufacturers. Primers specific for the *C. perfringens* enterotoxin gene (*cpe*; GenBank accession no. X71844) were designed to include the promoter region and part of the structural *cpe* gene. These were cpe11 (5′-ACTTAGAGTATCTATAAACTTGATACTC-3′) and cpe12 (5′-TAAATTGTTACTAAGCATATTATAATTA ACATC-3′). The size of the PCR product made with these two primers was 599 bp.

The presence of various foods had inhibitory effects on PCR, as indicated in Table 1. We could detect as few as 30 cells/ml of *C. perfringens* culture, but the detection limit was increased in the presence of various foods. The detection limit was increased to 400 cells/g of man-doo, 2.5×10^3 cells/g of soondae, and 4.5×10^3 cells/g of kim-bab, respectively (Table 1). Interestingly, the inhibition by pork hock was so strong that even 10^5 *C. perfringens* cells per g of pork hock was insufficient for detection by this method. There was virtually no difference in the sensitivities of detection by PCR with GeneReleaser- and QIAamp-prepared DNA templates.

The inhibitory effect of pork hock on PCR was further studied. We tested the effect of collagen, one of the main components of pork hock, and found that it strongly inhibited PCR. Collagen is classified into a number of structurally and genetically distinct types (4), each of which showed a different degree of inhibition of the PCR. Type I collagen (Sigma type III) inhibited PCR if more than 2 μg was added to a reaction mixture and 3.5 mM $MgCl_2$ was used in the PCR buffer (Fig. 1). In contrast, type I collagen from human placenta (Sigma type VIII) did not show inhibition even at 8 μg/reaction. The inhibition of PCR by collagen was specific, since addition of an equivalent amount of bovine serum albumin to the PCR did not affect the reaction. Also, the inhibition of PCR by collagen was not specific to the particular primer set used for the detection of the *cpe* gene, since the primer set (plc2 [5′-TCCCC TTTCTAGATAACGATTAACAC-3′] and plc4 [5′-GTTA GCATGCTGTTTTCTAAGTTAAAACC-3′]) used for the detection of the *plc* gene of *C. perfringens* was also inhibited by collagen.

It has been shown that the function of thermostable DNA polymerases from different sources is inhibited differently by known PCR inhibitors (1). Collagen showed different degrees of inhibition of *Pwo* DNA polymerase. Generally, *Pwo* DNA polymerase was more sensitive to collagen than *Taq* DNA polymerase. These results suggest that the appropriate thermostable DNA polymerase should be used for sensitive PCR detection of pathogens contaminating a food.

When various types of collagen were added to *C. perfringens* culture before preparation of DNA by the use of a GeneReleaser or QIAamp kit, PCR was inhibited by the DNA preparation (data not shown). These results suggest that neither the

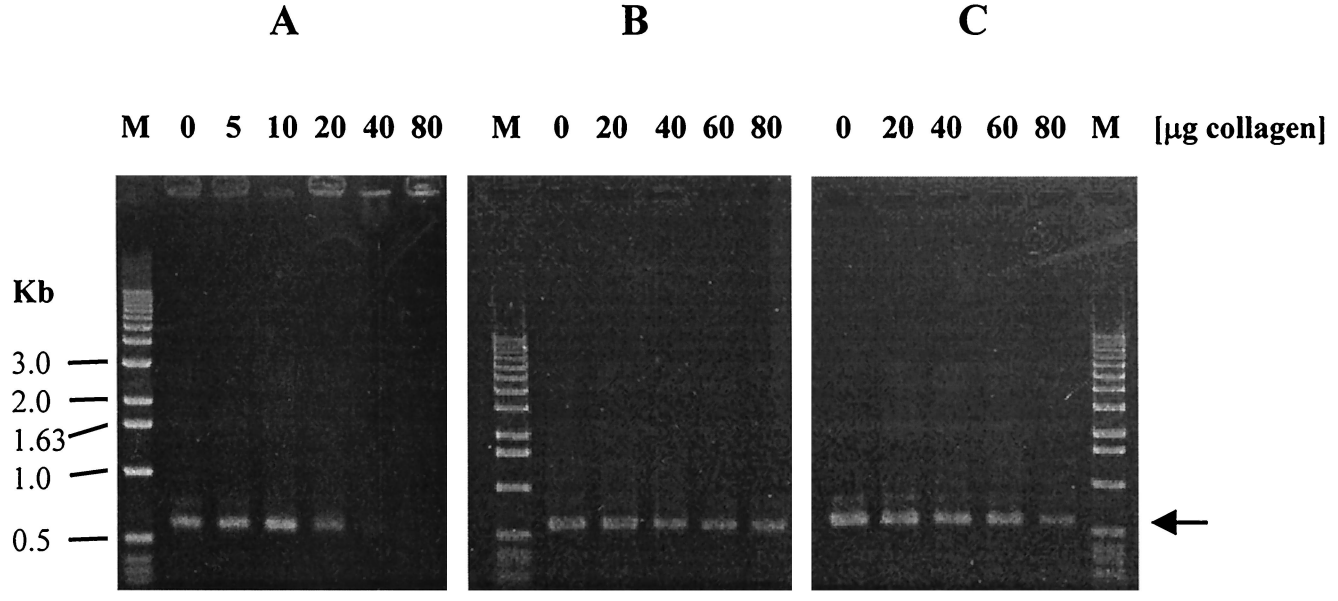

FIG. 2. Effects of type I collagen on PCR when DNA templates were prepared with a GeneReleaser kit (A), by phenol-chloroform extraction (B), or by NaI treatment (C) in the presence of collagen. One-tenth of the final volume of DNA preparation was used for each PCR, so that the actual amount of collagen present in each PCR was 1/10 the amount indicated at the top of each lane if collagen was not removed by the extraction method. The use of a QIAamp tissue kit produced results similar to those obtained with a GeneReleaser kit. The positions of molecular size markers (M) are indicated on the left. The arrow indicates the position of the 599-bp DNA fragment.

GeneReleaser nor the QIAamp procedure could remove the collagen from the DNA preparation. However, addition of more $MgCl_2$ to the reaction mixture relieved inhibition of PCR by various types of collagen to some degree, but the PCR product formed a broad band when a high concentration of $MgCl_2$ (more than 5 mM) was used (data not shown). We tested two additional DNA preparation methods (phenol extraction and NaI treatment) as described by Makino et al. (10, 11) in an attempt to remove collagen from the DNA preparation. Even though the presence of collagen inhibited the efficiency of DNA extraction by these methods, the degree of inhibition was much smaller when phenol extraction or NaI treatment was used than when a GeneReleaser or QIAamp kit was employed (Fig. 2). When we tested the phenol extraction and NaI methods for detection of *C. perfringens* contamination in a food, phenol extraction method worked well but the NaI method did not. We could not detect a PCR product when the *C. perfringens* DNA template was prepared by NaI treatment in the presence of the above-mentioned foods, which indicates that a PCR inhibitor possibly present in the food could not be removed by NaI treatment. These results indicate the need to evaluate DNA preparation methods and the type of thermal DNA polymerase if PCR-based methods are to be used for the direct detection of microorganisms in foods.

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