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**EVALUATION OF PCR AND DNA HYBRIDIZATION
PROTOCOLS FOR DETECTION OF VIABLE
ENTEROTOXIGENIC *CLOSTRIDIUM PERFRINGENS* IN
IRRADIATED BEEF**

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ABSTRACT

The sensitivity of DNA hybridization and polymerase chain reaction (PCR), was evaluated in irradiated cooked and raw beef samples. A membrane-based colony hybridization assay and a PCR protocol, both with specificity for the enterotoxin A gene of Clostridium perfringens, were compared with viable plate counts. The results of the colony hybridization procedure were in agreement with viable plate counts for detection and enumeration of enterotoxigenic C. perfringens. The PCR procedure combined a 4 h enrichment followed by a nucleic acid extraction step and assessed the amplification of 183 and 750 base pair enterotoxin gene targets. Detection of C. perfringens by PCR did not show a reliable correlation with viable plate counts or the colony hybridization assay. C. perfringens killed by irradiation were not detected by the plate count or colony hybridization methods; however, killed cells were detected with the PCR technique. By relying on the growth of viable cells for detection and/or enumeration, the colony hybridization and plate count methods provided a direct correlation with the presence of viable bacteria.

INTRODUCTION

Food irradiation provides an efficient and safe method for food preservation through the reduction or elimination of spoilage organisms and foodborne pathogens (Grant and Patterson 1992; Harewood *et al.* 1994; Josephson 1983). The effects of gamma irradiation in controlling vegetative and spore-forming food

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borne pathogens have been extensively reported (Gombas and Gomez 1978; Gomez *et al.* 1980; Grant and Patterson 1992; Harewood *et al.* 1994; Thayer *et al.* 1992; Thayer and Boyd 1994). Among these biological contaminants, spore-forming bacteria constitute one of the most difficult to eradicate due to their higher radiation resistance (Gombas and Gomez 1978; Gomez *et al.* 1980; Grant and Patterson 1992; Thayer and Boyd 1994).

Monitoring the microbiological safety of foodstuffs after radiation treatment relies on methods which detect viable organisms. Current preferences are for detection methods that provide rapid, sensitive, and specific results. The availability of choices for such methods are numerous. However, techniques relying on serological assays and nucleic acid-based methods are among those commonly used. Within the nucleic acid based methods, the polymerase chain reaction (PCR) and gene probe technology are the most widely used (Wolcott 1991). Combinations of one or more of these detection technologies have permitted levels of detection to single molecules (Sano *et al.* 1992). Nevertheless, an unresolved concern is the reliable correlation between viable cells and the detection signals obtained by these highly sensitive methodologies.

In the present study, we compared the plate count method to a colony hybridization assay and a polymerase chain reaction procedure for assessment of viability of irradiated *Clostridium perfringens* based on the detection of the bacterial DNA. The assessment of bacterial viability in cooked and raw ground beef was monitored after doses of gamma radiation up to 25 kGy.

MATERIALS AND METHODS

Culture and Growth Conditions

Clostridium perfringens strains NCTC 8238, NCTC 8239, and ATCC 10288 were used for enterotoxin detection. The potential for enterotoxin production and the presence of the enterotoxin gene were confirmed by a reversed passive latex agglutination assay (SPET-RPLA)(Oxoid Inc., Columbia, MD) and by PCR amplification, respectively. In the latter, the oligonucleotide primers PS1 (TGTAGAATATGGATTTGGAAT) and NS1 (AGCTGGGTTTGAGTTT-AATGC) were used for amplification of a 363 bp enterotoxin fragment (Baez and Juneja 1995). Stock cultures were maintained in cooked-meat medium (CMM) stored at 4C (Difco Laboratories, Detroit, MI). Vegetative cell cultures were grown by inoculating 0.1 mL of the stock culture into 10 mL of reduced fluid thioglycollate medium (Difco Laboratories, Detroit, MI). The inoculated medium was heat-shocked at 75C for 20 min followed by aerobic incubation at 37C for 18 h. For enumeration of vegetative cells, the cultures were serially diluted in 0.1% (w/v) peptone-water and plated onto tryptose-sulfite-cycloserine (TSC) agar overlaid with an additional 10 mL of TSC agar (Hauschild and Hilsheimer 1974).

The plates were incubated overnight at 37C in anaerobic jars (BBL GasPack Anaerobic Systems, Beckton Dickinson, Cockeysville, MD).

Meat Preparation

Ground beef was obtained from local retail markets. Portions of 160 grams of the raw or cooked (autoclaved 121C/15 min) beef were aseptically weighed into polyethylene stomacher bags (SFB-0410; Spiral Biotech., Bethesda, MD) and inoculated with a combination of the three *C. perfringens* strains to obtain 6 log₁₀ CFU/g. Meat samples were also inoculated with sterile 0.1% (w/v) peptone water, as a negative control. The beef samples were homogenized for 5 min in a Stomacher Lab-Blender 400 (Tekmar Company, Cincinnati, OH). The homogenized beef samples were subdivided into 20 g portions, placed in individual polyethylene stomacher bags, and spread thinly to an approximate area of 10 × 10 cm. The bags were vacuum-sealed in a Multivac A300/16 (Multivac, Wolfertschwenden, West Germany), and vacuum sealed inside a second stomacher bag to reduce oxygen transmission to the samples. The meat samples were cooled to 4C before gamma irradiation.

Irradiation of Samples

A ¹³⁷Cs self-contained gamma-radiation source with a dose rate of 0.1054 kGy min⁻¹ was used. The dose rate was established using National Physical Laboratory (Middlesex, U.K.) dosimeters. Double-vacuum-packaged stomacher bags containing test samples were in a uniform portion of the radiation field. Samples were maintained at 4 ± 0.5C by injecting the gas phase from liquid nitrogen into the radiation chamber. Sample temperature was monitored during the radiation procedure. The entire experiment was repeated four times with duplicate analysis of all samples.

After radiation, each beef sample was diluted with 20 mL of filter-sterilized (0.2 µm nylon membrane filter, Nalgene Company, Rochester, NY) phosphate buffered saline (PBS, pH 7.4) containing 0.1% Tween 80 (Sigma Chemical Co., St. Louis, MO). The beef samples were homogenized for 1 min in a Stomacher Lab-Blender 400 (Tekmar Company, Cincinnati, OH). Meat homogenates (7 - 10 mL) were transferred to sterile 15 mL polystyrene conical screw cap tubes (Sarstedt, Inc., Princeton, NJ) for further dilution, plating, PCR enrichment or gene hybridization analysis.

PCR Amplification

One milliliter of the meat homogenate was transferred to 9 mL of CMM and incubated aerobically at 37C for 4 h. These enrichment conditions were demonstrated to increase the sensitivity of detection of *C. perfringens* to ≤ 1 log₁₀

CFU/g after 4 h enrichment in CMM (Baez *et al.* 1995; Baez *et al.* 1996). After incubation, 1 mL portions were collected for extraction of total chromosomal DNA before PCR amplification using a commercial G-NOME™ DNA isolation kit (Bio 101, Inc., La Jolla, CA). The precipitated DNA was resuspended in 100 µL of sterile distilled water and stored at -20C until further use.

Amplification reactions were performed in 50 µL volumes. The reaction mixture contained 3 µL of the extracted chromosomal DNA, 200 µM concentrations of dATP, dCTP, dGTP, dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 µM of each primer CPEPS (AGGAGATGGTTGGATATTAGG) and CPENS (ATATTGTCCGGCATCTAAGCT) for the 750 bp enterotoxin A fragment, INTPS (TGGTAATATCTCTGATGATGG) and INTNS (TTCTGTA-GCAGCAGCTAAATC) for the 183 bp enterotoxin A fragment, 1.5 mM MgCl₂, 0.01% gelatin and 1.25 units of *Taq* polymerase (Perkin Elmer, Norwalk, CT). The samples were subjected to 30 cycles of amplification in a Programmable Gene Amp PCR system model 9600 (Perkin Elmer). The amplification conditions consisted of denaturation at 94C for 30 s, annealing at 60C for 30 s, and extension at 74C for 1 min, with a cycle extension of 3 s per cycle. After 30 amplification cycles, a final extension incubation by holding the temperature at 74C for 10 min was allowed. An aliquot equivalent to 20% (10 µL) of the amplification reaction was electrophoresed through a 1% or 2% agarose gel for the 750 bp and 183 bp products, respectively. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/mL) for 15 to 20 min (Maniatis *et al.* 1982), and the amplification products were visualized using a FOTO/PREP I transilluminator (Fotodyne, Inc., New Berlin, WI.)

Bacterial Enumeration

Vegetative cell numbers of *C. perfringens* in the inoculated meat samples were determined by plating appropriate 10-fold dilutions of the corresponding meat homogenate onto TSC agar. As described above, the diluted samples were plated in duplicate on TSC agar using a spiral plater (Spiral Systems, Model D, Cincinnati, OH), and also by inoculating 0.1 mL of the undiluted meat homogenate fluid onto TSC agar.

Background bacterial flora present in raw ground beef was also enumerated. Uninoculated meat supernatant were serially diluted and plated onto brain heart infusion (BHI) and plate count agar (Difco Laboratories, Detroit, MI), using a spiral plater, and also by inoculating 0.1 mL of the meat supernatant fluid as described previously.

Digoxigenin-labeled *C. perfringens* Enterotoxin (CPE) Probe

Detection and enumeration of *C. perfringens* from irradiated and nonirradiated samples was performed by a nonradioactive colony hybridization procedure

described earlier (Baez and Juneja 1995). Briefly, a 363-bp digoxigenin-labeled probe was generated by PCR amplification using the Genius Labeling and Detection kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The amplified products were detected by electrophoresis of 10 - 15 μ L of the reaction mixture through a 2% agarose gel in Tris-Acetate-EDTA (TAE) buffer followed by ethidium bromide staining. After development of the colony hybridization reactions, the *C. perfringens* colonies containing the enterotoxin gene were counted visually and the images were digitally recorded using a DAGE-MT1 model 68 TV camera equipped with a 50 mm macro lens and a neutral density 4 \times filter.

RESULTS AND DISCUSSION

The detection methods that differentiate viable from nonviable organisms are required because of the eradication or alteration of the microbial flora after irradiation of food. The present study compared anaerobic plate counts to two DNA-based methods for the detection of *C. perfringens* in cooked and raw beef containing high levels (10^6 CFU/g) of contamination. Doses of radiation at 1 kGy increments resulted in parallel decline in the number of viable *C. perfringens* cells from 6 \log_{10} CFU/g to 0 \log_{10} CFU/g. After 1-5 kGy radiation dose, survival rates in raw and cooked beef were not significantly different ($p > 0.05$) as demonstrated by anaerobic plate count (Table 1). However, while no cells were detected in raw beef after a 6 kGy radiation dose, low numbers were observed in cooked beef. The low numbers of *C. perfringens* cells in cooked beef were confirmed by colony hybridization (Table 1 and Fig. 1). It is unclear if after a dose of 6 kGy survival of *C. perfringens* could be attributed to a protective effect in the cooked beef, or if reduced fat and water content in the cooked beef acted as factor(s) inducing spore formation during the preparation of the samples and/or irradiation process. Interestingly, PCR results for either preparation (raw or cooked) showed amplification of the two PCR targets after the 4 h enrichment in CMM (data not shown). These findings are in agreement with our previous studies (Baez *et al.* 1995; Baez *et al.* 1996) in which a high sensitivity of the PCR technique was demonstrated; *C. perfringens* levels as low as 1 \log_{10} CFU/g were detected when CMM enrichment was extended to 4 h.

For assessment of the correlation between the two DNA-based methods and the anaerobic plate counts a radiation dose of 25 kGy was selected. This dose assured a complete elimination of viable cells or spore forms in the irradiated meat samples. Our results showed that the application of PCR is an unreliable approach to assess microbial viability. Figure 2A shows the amplification products for the 750 bp enterotoxin A fragment before irradiation. The level of *C. perfringens* in the artificially contaminated samples was estimated by anaerobic plate count at approximately 10^6 CFU/g. Amplification was obtained for each of the enterotoxigenic strains included in the study either before or after the 4 h CMM

TABLE 1.
SURVIVAL^a OF VEGETATIVE *C. PERFRINGENS* IN RAW AND COOKED BEEF AS
DETERMINED BY ANAEROBIC PLATE COUNT AND COLONY HYBRIDIZATION
METHODS

Radiation dose (kGy)	Anerobic Plate Count		Colony Hybridization	
	Cooked	Raw	Cooked	Raw
0	4.07×10^6	2.34×10^6	ND ^b	ND
1	2.75×10^6	1.91×10^6	ND	ND
2	1.70×10^6	1.10×10^6	ND	ND
3	6.03×10^5	2.82×10^5	ND	ND
4	1.00×10^5	2.19×10^4	ND	ND
5	1.98×10^3	7.83×10^2	1.60×10^3	ND
6	2.04×10^1	0	2.50×10^1	0
25	0	0	0	0

^aSurvival data expressed in CFU/g and are the means of four replicate experiments.

^bND, not determined.

enrichment. Assessment of PCR amplification after the 25 kGy radiation dose showed efficient amplification for most of the samples (Fig. 2B, 2C). Irradiated meat homogenates were analyzed by PCR before or after the 4 h enrichment in CMM. Variations in amplification for the 750 bp sequence were observed for the enterotoxigenic *C. perfringens* strains exposed to the 25 kGy dose as shown in Fig. 2B. These differences in the efficiency of the PCR amplification could be attributed to DNA damage after exposure to radiation. Despite of the higher survival rate previously observed for the cells in cooked beef, no differences were observed in the nucleic acid amplification for the corresponding samples. The 1:10 dilution introduced when aliquots of the 25 kGy-irradiated meat homogenates were added to enrichment media did not influence the detection of nonviable cells as shown in Fig. 2B, lanes 4, 5, 8, 9, 12, and 13. Amplification products were obtained for the three strains tested independent of the cooked or raw beef origin or whether the irradiated meat homogenates were analyzed by PCR before or after incubation in CMM. The meat homogenates previously subjected to PCR amplification for the 750 bp target were also used for amplification of a smaller, 183 bp enterotoxin A fragment. Uniform amplification was observed for all the irradiated samples suggestive of less radiation damage sustained by the smaller sequence (Fig. 2C). No viable cells were recovered by the colony hybridization

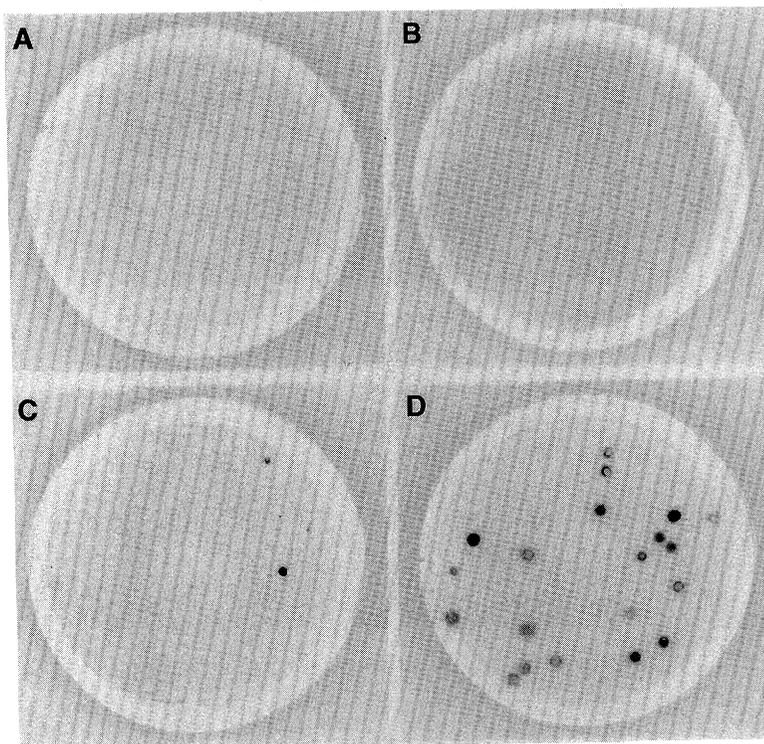


FIG. 1. REPRESENTATIVE FILTERS SHOWING COLONY HYBRIDIZATION OF IRRADIATED MEAT HOMOGENATES (6 KGY DOSE) CONTAINING *C. PERFRINGENS* VEGETATIVE CELLS PROBED WITH THE DIGOXIGENIN-LABELED ENTEROTOXIN GENE PROBE (A) raw beef, 10^{-1} dilution, (B) raw beef, undiluted meat homogenate, (C) Cooked beef, 10^{-1} dilution, (D) cooked beef, undiluted meat homogenate.

or the anaerobic plate count methods when the samples were enriched and analyzed after 4 and 24 h of incubation.

The observations from our study were consistent with a previous report by Rowe and Towner (1994) where a reduction in the hybridization signal was obtained in contaminated samples ($\geq 10^5$ CFU/g) subjected to irradiation doses of 10 kGy; also, no viable cells could be detected following irradiation. Although the reported hybridization results were reduced in intensity, the obtained signals were distinguishable above the experimental background. In another study,

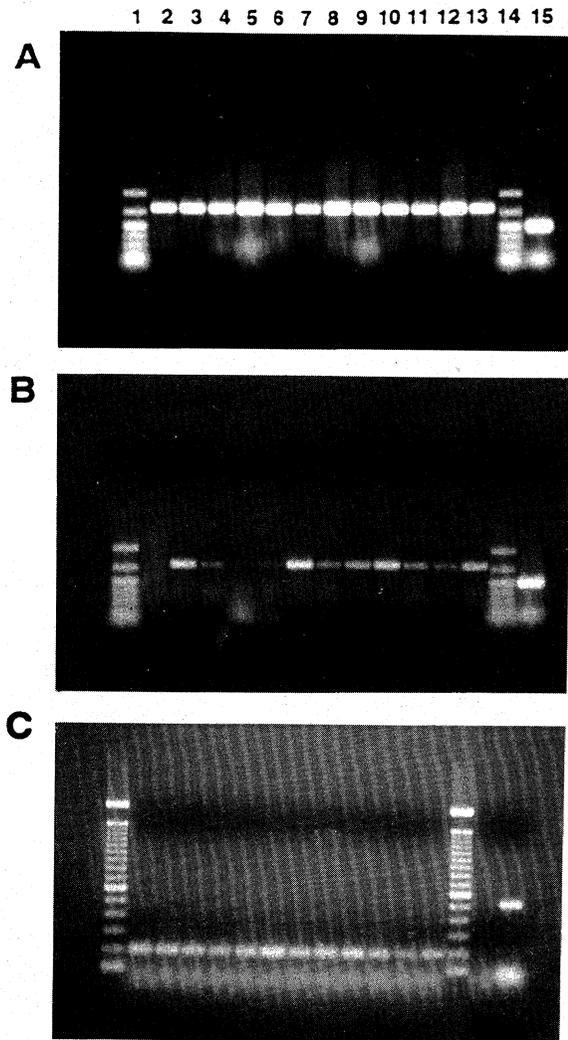


FIG. 2. AGAROSE GEL ELECTROPHORESIS OF PCR AMPLIFIED PRODUCTS FROM MEAT HOMOGENATE CONTAINING *C. PERFRINGENS* VEGETATIVE CELLS AFTER 25 KGy DOSE. (A) Primer pair CPEPS and CPENS was used for amplification of a 750 bp sequence of the enterotoxin A gene before 25 kGy radiation dose. (B) Primer pair CPEPS and CPENS was used for amplification of a 750 bp sequence of the enterotoxin A gene after 25 kGy radiation dose. (C) Primer pair INTPS and INTNS was used for amplification of a 183 bp sequence of the enterotoxin A gene. The panel shown is representative of the amplification results after beef samples were spiked with approximately $6-7 \log_{10}$ of the individual or mixed enterotoxigenic *C. perfringens* strains. Lane (1) molecular weight marker, (2-5) *C. perfringens* NCTC 8238: raw T_{0h} , cooked T_{0h} , raw T_{4h} , cooked T_{4h} , (6-9) *C. perfringens* NCTC 8239, (10-13) *C. perfringens* ATCC 10288, (14) molecular weight marker, (15) Lambda control reaction.

Masters *et al.* (1994) assessed the correlation between plate count and a gene probe-PCR technique for the detection of *Listeria monocytogenes* and *Escherichia coli* that were exposed to stress treatments such that no viable cells were recovered. Although no exposure to radiation was performed among the experimental conditions, the researchers concluded that PCR did not always correlate with loss of viability as amplifiable DNA sequences were detected. Our observations showed no significant differences for amplification of DNA from cooked or raw beef or when the DNA from the meat homogenates was extracted after 4 h incubation in CMM enrichment or when the extraction of nucleic acids was performed without the enrichment.

In summary, our results demonstrated that the colony hybridization and plate count methods, by detecting growth of viable cells, provided a direct correlation with survival of enterotoxigenic *C. perfringens*. Detection by PCR did not show a reliable correlation with the presence of viable cells in the irradiated samples as demonstrated by the results obtained from the 25 kGy treated samples. The latter suggests that, although the DNA present in irradiated-nonviable cells may sustain damage due to exposure to radiation, it is still amplifiable by PCR. The 750 bp DNA sequence appeared to sustain more damage due to radiation than the smaller 183 bp sequence as reflected by the efficiency of the amplification reaction after radiation.

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