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MEMBRANE FILTRATION IMMUNOSTAINING TECHNIQUE FOR DETECTION AND QUANTITATION OF ENTEROTOXIGENIC *CLOSTRIDIUM PERFRINGENS* IN PRECOOKED BEEF[1]

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ABSTRACT

A membrane filtration-immunostaining technique was developed for detection and direct enumeration of enterotoxigenic Clostridium perfringens *in beef. The procedure combined a microcolony technique with an indirect alkaline phosphatase conjugated antibody visualization system. Precooked ground beef samples containing enterotoxigenic* C. perfringens *cells were diluted in phosphate buffered saline containing 0.1% Tween 80 and filtered through a 0.45 μm cellulose nitrate filter. The membranes were placed onto the surface of solid Duncan and Strong medium and incubated anaerobically for 4–18 h at 37C. The membranes were sequentially overlaid with* C. perfringens *anti-enterotoxin and an alkaline phosphatase conjugated antibody resulting in a chromogenic reaction that enabled detection and enumeration. The membrane immunostaining technique proved to be sensitive enough to detect as low as 2 CFU/g of enterotoxigenic* C. perfringens.

INTRODUCTION

Clostridium perfringens is a Gram positive, anaerobic bacillus normally found in soil and the intestinal tract of man and animals. The bacterium is frequently

[1]Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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associated with food poisoning in humans acquired through the consumption of contaminated meat and poultry products (Granum 1990; McClane 1992). Bean and Griffin (1990) reported that the organism accounted for 190 of the total 1869 cases of the bacterial foodborne disease outbreaks between 1973 and 1987. The pathological effects associated with *C. perfringens* food poisoning have been linked to an enterotoxin produced during sporulation in the intestinal tract (Duncan *et al.* 1972; Frieben and Duncan 1973; McDonel 1979; Stark and Duncan 1971; Walker 1975).

Ready-to-eat uncured, refrigerated meat products are increasingly common in the U.S. markets. Food poisoning by *C. perfringens* has been associated with such products if the rate and extent of cooling is not sufficient (Bryan 1988), or when products become contaminated after cooking (Bean and Griffin 1990). Juneja *et al.* (1994) reported that the cyclic and static temperature abuse of precooked, refrigerated meat products for a relatively long period allowed *C. perfringens* to grow to potential infective dose levels of at least 5 $\log_{10}$ CFU/g.

The prolonged testing procedures required for traditional detection and identification of enterotoxigenic *C. perfringens* are unsuitable for analysis of food with a limited shelf-life. For example, if a precooked meat product must be held until testing is complete, distribution is delayed, storage fees can increase processing costs, and the final quality and shelf-life of the product may also be affected. Traditional identification of *C. perfringens* is accomplished through the use of selective enrichment followed by biochemical tests and finally confirmation of enterotoxigenic stains by culturing in Duncan and Strong sporulation medium and final serological analysis. A method for *C. perfringens* enterotoxin detection based on a reversed passive latex agglutination test (Oxoid, Columbia, MD) is commercially available. Other reported methods for the detection of enterotoxigenic *C. perfringens* from food samples include enzyme immunoassays (Cudjoe *et al.* 1991; Jackson *et al.* 1986; Stelma *et al.* 1985), gene probes (Van Damme-Jongsten *et al.* 1990) and combinations of serotyping, bacteriocin and plasmid analysis (Mahony *et al.* 1992). These techniques have been applied in detection and identification schemes following the cultivation of the organism, which increases the time required to provide a positive identification. Accordingly, the objective of the present study was to develop a rapid method to detect and enumerate enterotoxigenic *C. perfringens* strains in beef by combining a microcolony technique with an indirect alkaline phosphatase immunostaining method specific for *C. perfringens* enterotoxin A.

MATERIALS AND METHODS

Bacterial Strains

Clostridium perfringens strains NCTC 8238, NCTC 8239, and ATCC 10288 were used for enterotoxin detection. A nonenterotoxigenic *C. perfringens* strain

FD-1, used as a negative control, was kindly provided by Dr. R. Labbe (U. Massachusetts). The potential for enterotoxin production was confirmed by a reversed passive latex agglutination assay (SPET-RPLA) (Oxoid Inc., Columbia, MD). Stock cultures were maintained in cooked-meat medium (Difco Laboratories, Detroit, MI) stored at 4C. Vegetative cell cultures were grown by inoculating 0.1 ml of the stock culture into 10 ml of freshly prepared Fluid Thioglycollate medium (Difco Laboratories, Detroit, MI). The inoculated medium was heat-shocked at 75C for 20 min to induce sporulation, followed by aerobic overnight incubation at 37C. 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) for colony counts.

Meat Preparation

Ground beef obtained from local retail markets was dispersed on plastic trays, covered with aluminum foil, and autoclaved at 121C for 20 min. The fat was drained off aseptically and the meat allowed to cool to room temperature. Individual 20 g portions were aseptically weighed into filter stomacher bags (SFB-0410; Spiral Biotech., Bethesda, MD) and inoculated with 1 ml of the appropriate dilution of *C. perfringens* cell suspension to obtain 2, 3, 4, 5, or 6 $\log_{10}$ CFU/g. Meat samples were also inoculated with 1 ml of 0.1% (w/v) peptone water with no bacterial cells, as a negative control. The bags were manually mixed to ensure even distribution of the organisms in the meat sample.

Detection of *C. perfringens*

Each sample was diluted with 20 ml of filter-sterilized 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). Each meat homogenate (7–10 ml) was transferred to a sterile 15 ml conical screw cap tube (Sarstedt Inc., Princeton, NJ) and centrifuged at 500 rpm for 5 min in a Sorvall GLC-1 centrifuge (DuPont Company, Wilmington, DE). Aliquots of 1 ml from the supernatant fluid were filtered under vacuum through 0.45 μm cellulose nitrate filters (Nalgene Company, Rochester, NY) along with 5 ml of sterile 0.1% (w/v) peptone water to allow for uniform dispersion of the cells onto the filter surface. The membranes were then transferred, with the inoculated side facing up, onto the surface of solid Duncan and Strong (DS) medium. The agar plates were placed in anaerobic jars (BBL GasPack Anaerobic Systems, Beckton Dickinson, Cockeysville, MD) and incubated at 37C. After anaerobic incubation for 4, 6, 8, or 18 h, the mem-

branes were removed from the agar surface and placed into 15 × 100mm Petri plates (Fisher Scientific Co., Malvern, PA). Thereafter, the following steps were done at room temperature with constant agitation on a rotary platform: *C. perfringens* colonies on the membrane surface were fixed in 15 ml of phosphate buffered saline (PBS) containing 1% formalin and incubated for 30 min. The membranes were washed twice for 15 min each time with PBS. Next, the membranes were floated on Tris buffered saline (TBST; 0.010 M Tris-HCl, pH 8.0, 0.150 M NaCl, 0.5% Tween 20) containing 1% bovine serum albumin. Blocking was carried out for 30 min followed by incubation with a 1:1,000 dilution in TBST of a rabbit polyvalent antibody against the enterotoxin A of *Clostridium perfringens* (Calbiochem Corp., San Diego, CA). After 30 min incubation, the membranes were washed 3 times with 10 ml of TBST. Each washing step was performed for 10–15 min. The membranes were then floated on TBST containing goat-anti rabbit antibody conjugated to calf-intestinal alkaline phosphatase at a 1:10,000 dilution (Organon Teknika Corp., West Chester, PA). The membranes were incubated for 30 min, washed 3 times in TBST and then transferred onto the surface of a Whatman filter paper to absorb excess fluid. For color development, a substrate solution containing 33 μl of Nitro Blue Tetrazolium (NBT, 50 mg/ml in 70% dimethylformamide) and 16.5 μl of 5-bromo-4-chloro-3-indoly phosphate (BCIP, 50 mg/ml in dimethylformamide) was prepared in 5 ml of alkaline phosphatase buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.005 M $MgCl_2$). The damp membranes were immersed in the substrate solution and color development was monitored for 15–20 min. The reaction was stopped by transferring the membranes to Tris-EDTA solution (0.02M Tris-HCl, pH 8.0, 0.005M EDTA). The stained membranes were removed and stored in the dark. Two replications were performed for each *C. perfringens* strain.

Bacterial Enumeration

C. perfringens numbers in the meat samples were determined using the membrane-filtration immunostaining (MFI) technique as described in Fig. 1. The stained colonies corresponding to enterotoxin producing *C. perfringens* were counted visually. The cell numbers for the enterotoxin positive and enterotoxin negative strains were also determined by plating onto TSC agar as described before. After homogenization and centrifugation, the meat supernatants used for enumeration by both methods (anaerobic plate count and MFI), were serially diluted in 0.1% (w/v) peptone water. 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 meat supernatant fluid onto TSC agar.

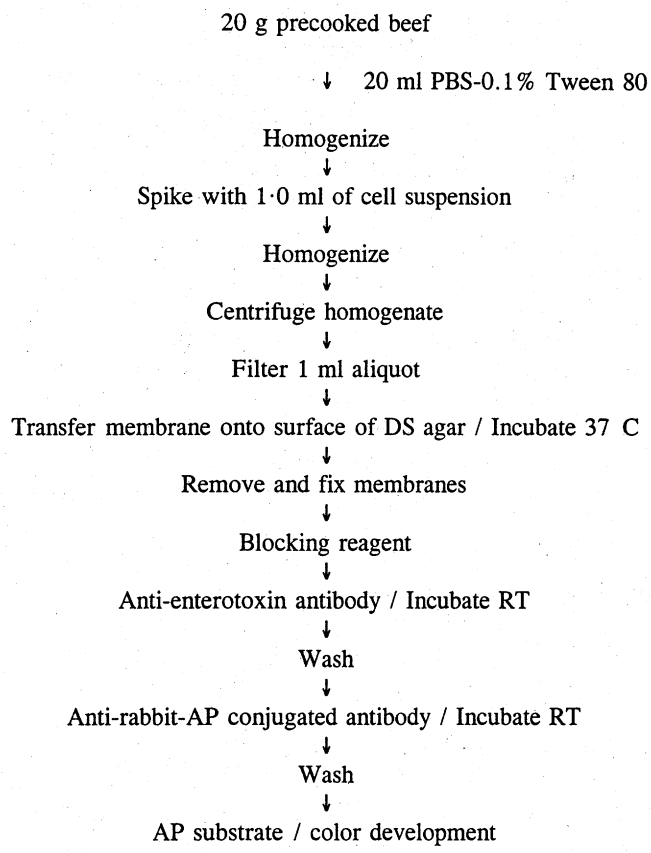

FIG. 1. MEMBRANE FILTRATION IMMUNOSTAINING PROCEDURE FOR DETECTION OF ENTEROTOXIN-PRODUCING *C. PERFRINGENS* FROM SPIKED BEEF SAMPLES

RESULTS

The membrane filtration-immunostaining procedure was developed for direct identification and enumeration of enterotoxin producing *C. perfringens* from precooked meat samples. The procedure, outlined in Fig. 1, was designed for direct testing of meat specimens without the need for enrichments or sample extraction steps.

Membranes incubated for as short as 6 h provided a discernable staining for the three enterotoxin producing *C. perfringens* strains, while shorter incubation resulted in faint staining. The anti-enterotoxin stained colonies appeared dark blue with decreasing intensity farther from the center (Fig. 2). The negative controls, which included uninoculated beef samples and the enterotoxin negative FD-1 strain, showed no reaction as reflected by the absence of stained colonies (Fig. 2b, 2d and 2e).

The alkaline phosphatase staining method demonstrated adequate resolution to provide a quantitative assessment of the presence of enterotoxin-producing *C. perfringens* strains. Table 1 shows a representative example of the bacterial colony counts for the enterotoxigenic *C. perfringens* strains from the spiked meat samples using the conventional plate count method and the membrane-filtration immunostaining (MFI) procedure developed in this study. The MFI procedure proved to be sensitive enough to detect as low as 2 CFU/g. Samples enumerated by anaerobic plate count showed lower counts compared to the MFI procedure with all *C. perfringens* strains (Table 1). Samples containing $\geqslant 3.5$ $\log_{10}$ CFU/g resulted in too many stained colonies to enumerate by the MFI technique. To facilitate enumeration these meat supernatants were serially diluted to obtain cell numbers within a reasonable density for staining and enumeration by the MFI technique. Serial dilutions of meat samples containing $\geqslant 3$ $\log_{10}$ followed by enumeration by the MFI technique and anaerobic plate count resulted in similar counts (data not shown).

Data obtained by conventional plate count and the MFI procedure (Table 1) were compared by paired t-test (Snedecor and Cochran 1980). For statistical purposes, the bacterial counts ''not detectable'' (Table 1) by anaerobic plate count were considered as zero for the paired t-test computations. The analysis showed that the MFI procedure resulted in significant ($P < 0.05$) higher counts than the plate count method.

DISCUSSION

A rapid method for detection and enumeration of enterotoxigenic *Clostridium perfringens* requires specificity, sensitivity, and simplicity, performed all within a reasonable time frame and at an affordable cost. Compared with techniques such as microcolony immunofluorescence (Insalata *et al.* 1972; Geopfert *et al.* 1970), membrane colony hybridization using labelled oligonucleotide probes (Van Damme-Jongsten *et al.* 1990), and other more sophisticated molecular biology techniques (Mahony *et al.* 1992; Saito *et al.* 1992), the membrane filtration-alkaline phosphatase immunostaining method for enterotoxigenic *C. perfringens* provides an alternative procedure without the requirement for specialized equipment and/or

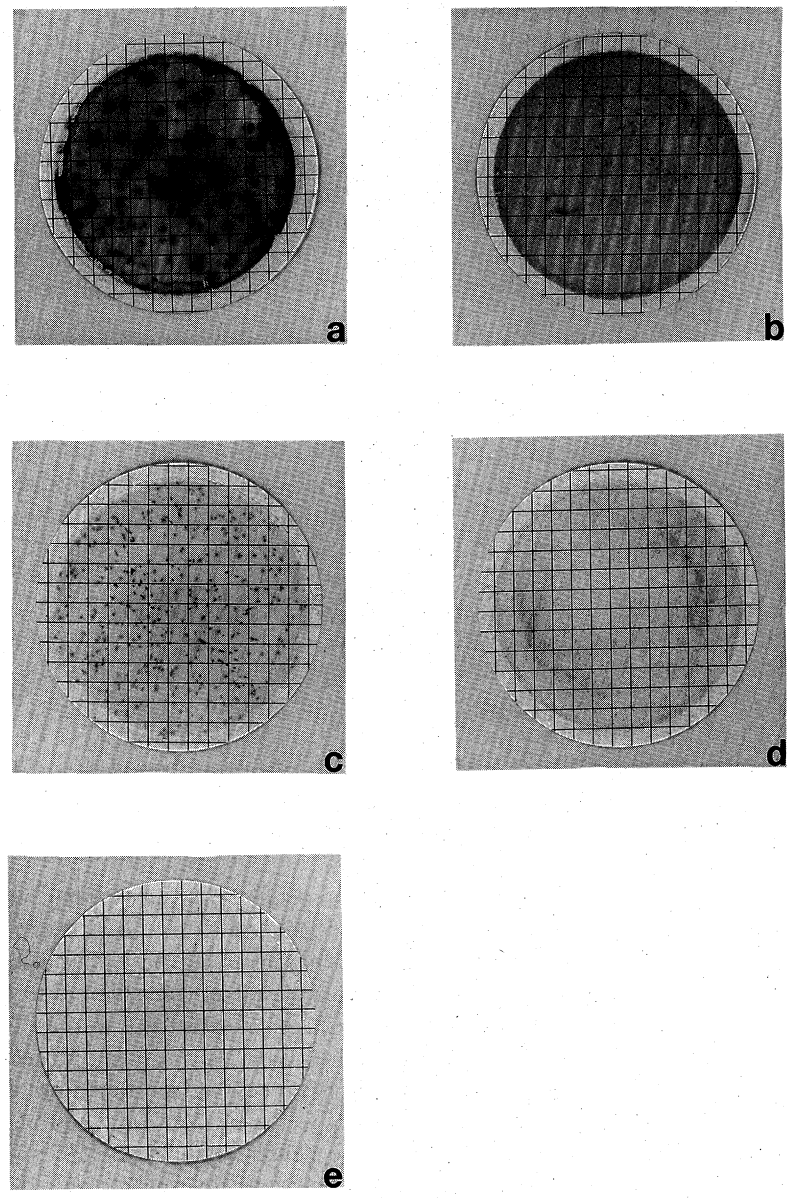

FIG. 2. CELLULOSE NITRATE MEMBRANES STAINED WITH THE *CLOSTRIDIUM PERFRINGENS* ANTI-ENTEROTOXIN

Membranes a and b: Anti-enterotoxin antibody 1:1,000, Alkaline phosphatase conjugated antibody 1:5,000. (a) Enterotoxigenic *C. perfringens* strain NCTC 8239, (b) Enterotoxin negative *C. perfringens* FD-1 strain (10^2 CFU/g determined by anaerobic plate count). Membranes c and d: Anti-enterotoxin antibody 1 :1,000, Alkaline phosphatase conjugated antibody 1:10,000. (c) Enterotoxigenic *C. perfringens* strain NCTC 8239, (d) Enterotoxin negative *C. perfringens* FD-1 strain (10^2 CFU/g determined by anaerobic plate count). (e) Negative control with no cells.

TABLE 1.
CLOSTRIDIUM PERFRINGENS CELL COUNTS IN PRECOOKED GROUND BEEF OBTAINED BY PLATING ON TSC AGAR AND BY MFI TECHNIQUE[1]

	TSC agar[2]	MFI-stain[3]
NCTC 8239	3.5	TNTC[4]
	2.4	2.6
	1.2	1.5
	ND[5]	0.3
NCTC 8238	2.4	2.6
	1.4	1.5
	ND[5]	0.5
ATCC 10288	3.6	TNTC[4]
	2.4	2.5
	0.7	1.2

[1]Data shown as mean of two replications and expressed as $\log_{10}$ CFU/g
[2]Tryptose-sulfite-cycloserine agar
[3]Membrane filtration immunostaining after 6 h incubation
[4]Too numerous to count
[5]Nondetectable by anaerobic plate count

complicated protocols. Incubation in Duncan and Strong sporulation medium combined with anaerobic conditions favored the recovery and enterotoxin synthesis by *C. perfringens*.

The assay is quantitative since the staining is specific for the clostridial enterotoxin A and the stained colonies can also be enumerated. While one of the limitations of the conventional plate count is that the minimum number of bacteria that can be enumerated is 10 CFU/g and the agar plates require incubation for at least 18 h, the technique reported here is sensitive enough to detect as low as 2 CFU/g of meat samples within 6 h of incubation followed by 3½ h staining procedure. The total detection time (about 10 h) from the processing and sampling of the meat of the final staining is comparable with gene-probe (Van Damme-Jongsten *et al.* 1990) or polymerase chain reaction procedures (Saito *et al.* 1992) where time consuming enrichments or sample extraction steps are usually required. The MFI procedure also has the added advantage of providing evidence for the presence of the enterotoxigenic *C. perfringens* in the analyzed sample, whereas the nucleic acid detection techniques (Van Damme-Jongsten *et al.* 1990; Saito *et al.* 1992) provide evidence for the presence of the gene but not for its product. Compared with the commercial latex agglutination test (Harmon and Kautter 1986), the MFI procedure not only detects the enterotoxigenic *C. perfringens*, but it also provides an estimate of cell numbers in the food sample analyzed.

Incubation periods of less than 6 h did not provide consistent results. The anti-enterotoxin stained colonies appeared light in staining intensity, suggesting that low amounts of toxin were present. These observations support previous findings that enterotoxin synthesis begins 2 h after inoculation in Duncan and Strong sporulation medium (Granum 1990). After incubation for 18 h, the stained membranes showed larger size halos surrounding a more intense stained colony.

One limitation of the MFI technique was the inability to specifically detect the clostridial enterotoxin in raw beef samples. Membranes showed nonspecific staining, presumably due to antibody binding caused by lipids present in the sample. Presently, studies are underway to overcome the nonspecific background staining in raw beef. For the experiments where cooked meat samples were analyzed, the assay conditions were optimized to minimize nonspecific background staining by assessing the efficacy of different blocking solutions and higher dilutions of the primary and secondary antibodies. A 1:1,000 dilution in the anti-enterotoxin antibody and a 1:5,000 or 1:10,000 dilution for the alkaline phosphatase conjugated secondary antibody resulted in a reduced membrane background as illustrated in Fig. 2.

In conclusion, the membrane filtration immunostaining technique demonstrated its effectiveness in providing a quantitative assessment of the presence of enterotoxigenic *C. perfringens* in cooked meat products. The study reported here indicates the suitability of the MFI technique for analysis of cooked meat samples. While providing a higher sensitivity level than the plate count method for the enumeration of enterotoxigenic *C. perfringens*, the membrane immunostaining technique proved to be faster than conventional cultivation and biochemical characterization of the organism.

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