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Research Note

Comparison of Five Anaerobic Incubation Methods for Enumeration of *Clostridium perfringens* from Foods

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ABSTRACT

This study compared the cost, speed, convenience, and sensitivity of five anaerobic systems. Fung's double-tube (FDT) method, the minitube method (MT), the sandwiched microtiter plate (SMP) method, and the Mitsubishi AnaeroPack System were compared with the Brewer anaerobic jar for total anaerobic bacterial counts in foods. Incubation was at 37°C for 4, 8, 12, and 24 h. The results indicated that FDT, MT, SMP, and the Mitsubishi AnaeroPack System were as sensitive as the Brewer anaerobic jar for the detection and enumeration of *Clostridium perfringens* from food products. The FDT, MT, and SMP methods recovered higher numbers of *C. perfringens* compared to the Brewer anaerobic jar ($P < 0.05$) after 12 and 24 h incubation. The Brewer anaerobic jar was the most expensive method.

Cases of foodborne infections and intoxications today are increasing in industrial and developing countries. The Centers for Disease Control and Prevention (CDC) classify foodborne disease outbreaks as bacterial, chemical, parasitic, or viral. The majority of cases of foodborne disease are caused by bacterial agents *(16).* In the U.S., the most common bacterial agents of foodborne disease include *Salmonella, Staphylococcus aureus*, and *Clostridium perfringens*. An estimated 3 to 6 million cases of foodborne disease in the U.S. are attributed to these three organisms *(16).* Therefore, determination of the bacterial load in food products is important to the food industry in ensuring the safety and quality of its products. Most food microbiology laboratories routinely perform aerobic plate counts, yeast and mold counts, coliform counts, and detection of certain pathogens. Because anaerobes are found in poultry and meat products, routine monitoring of anaerobes is needed to assess total microbial contamination in food systems *(6, 7, 11, 14).* In addition, *C. perfringens* continues to be an important foodborne pathogen, and anaerobic conditions are needed to detect and enumerate this organism. A number of methods have been developed to grow and detect anaerobic bacteria. Commonly used anaerobic cultivation systems include the Brewer anaerobic jar *(4),* the Hungate roll tube system *(8, 9),* and the anaerobe glove box *(2, 3, 15).* Other convenient methods developed to detect and enumerate anaerobes include the Lee tube *(12)* and Fung's double-tube (FDT), devised by Fung and Lee *(5).* However, some of these conventional methods are time consuming, require specific equipment and procedures, and are expensive. In an effort to find more convenient and efficient anaerobic procedures, three new simple methods have been developed and/or improved for the recovery of anaerobic microorganisms. These include the minitube (MT), the sandwiched microtiter plate (SMP) method *(10),* and the Mitsubishi AnaeroPack System. The objectives of this study were to compare cost, speed, convenience, and sensitivity of five anaerobic systems. The Brewer anaerobic jar, which is considered the conventional method, was compared with the FDT, MT, SMP, and Mitsubishi AnaeroPack System for the recovery of *C. perfringens* in ground beef, ground turkey breast, and ground pork.

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MATERIALS AND METHODS

Test organisms. *C. perfringens* (ATCC 12915) was obtained from the Food Microbiology Culture Collection at Kansas State University. The purity and characteristics of this strain were determined by Gram morphology, spore stain, and motility test. The test organism was grown in cooked meat medium (Difco, Detroit, Mich.) in test tubes at 37°C for 48 h under anaerobic conditions with a gas pak anaerobic jar. After incubation, cultures were kept under refrigeration (4°C) as stock cultures and transferred every 3 months to ensure viability. Prior to testing, the strain was transferred to fresh cooked meat medium and incubated at 37°C for 48 h.

Medium used. Shahidi–Ferguson–Perfringens (SFP) agar base (Difco) was dissolved in distilled water by heating, autoclaved for 15 min at 121°C, and then tempered (48 to 50°C). A filtered-sterilized solution of D-cycloserine (Sigma Chemical Co., St. Louis, Mo.) was added (400 μg/ml) to SFP agar to make tryptose sulfite cycloserine (TSC) agar. Egg yolk enrichment, kanamycin, and polymyxin B sulfite were not added to the agar base because of the D-cycloserine supplementation.

Recovery of *C. perfringens* from inoculated foods. Fresh ground beef (80% lean), fresh ground turkey breast (20% fat), and fresh ground pork (20% fat) were purchased from a local com-

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mercial store. All products were kept under refrigerated conditions (4°C) until usage. One hundred-gram samples of each meat were placed aseptically into individual stomacher bags. Three different samples were used for each experiment. The samples were inoculated with 1 ml of *C. perfringens* to make initial inocula of about 6.0 to 7.0 log CFU/g, mixed with gloved hands for 2 min, and incubated anaerobically at 37°C for 24 h in an anaerobic jar. After incubation, 11 g of sample were transferred aseptically to a new stomacher bag and mixed with 99 ml sterile fluid thioglycollate solution. This 1:10 dilution sample was stomached for 2 min. The anaerobe viable cell counts from the diluted samples were evaluated by the five methods.

Cultivation units: Brewer anaerobic jar. Diluted food suspensions (1 ml) were placed into test tubes with 9 ml of sterile fluid thioglycollate solution to make dilutions (from 10^{-2} to 10^{-8}). TSC agar then was poured into duplicate agar plates containing 1.0 or 0.1 ml of sample (dilutions 10^{-3} – 10^{-8}). A BBL (BBL Microbiology Systems, Cockeysville, Md.) gas pack was opened, and 10 ml of water was added to allow generation of hydrogen and carbon dioxide. The envelope was placed in the jar along with a methylene blue indicator strip (BBL) to monitor the anaerobic condition within the jar. The plates were inverted and placed into the anaerobic jar, the lid was sealed tightly, and the jar was incubated at 37°C. After 4, 8, 12, and 24 h, black colonies were counted.

FDT method. This system consisted of a smaller glass test tube (16 by 150 mm, Kimex) inserted into a larger glass test tube (25 by 150 mm, Kimex). Shahidi–Ferguson–Perfringens agar (24.5 ml) was poured into the larger test tubes that were autoclaved. The smaller test tubes were wrapped in aluminum foil and autoclaved separately. After autoclaving, D-cycloserine (400 μg/ml) was added to the tempered agar (48 to 50°C) to make TSC agar. Aliquots of diluted food suspensions (1 ml) were introduced quickly to the melted agar in the larger duplicate tubes. The screw cap was replaced, and the tube was inverted four times for even dispersal. The cap was removed, and the smaller test tube then was inserted aseptically into the larger tube with flamed-sterilized forceps. The inner tube squeezed the agar between the two tubes to form a thin agar layer and create an anaerobic environment. The cap was replaced before incubation. The tubes were incubated at 37°C. After 4, 8, 12, and 24 h, black colonies were counted.

MT method. One milliliter of tempered TSC agar containing 0.1 ml of diluted food sample was aspirated into sterile 1.2-ml duplicate pipets. The agar solidified, creating an anaerobic environment within the pipet, and the ends of the pipet were sealed with sterilized parafilm. These tubes were incubated at 37°C. After 4, 8, 12, and 24 h, black colonies were counted.

SMP method. This anaerobic system consisted of two microtiter plates placed together in a sandwich formation. The plates were sterilized by UV light for 30 min. The bottom plate was filled with 100 μl of TSC agar and 10 μl of diluted food sample, with dilutions of 10^{-3} to 10^{-8}. The top microtiter plate was placed into the bottom plate, creating an anaerobic environment. The rows in the microtiter plate consisted of eight wells representing eight replicates of each sample. The sandwiched microtiter plates were incubated at 37°C, and black colonies were counted after 4, 8, 12, and 24 h.

Mitsubishi AnaeroPack System. Tempered TSC agar was poured into duplicate agar plates containing 1 ml or 0.1 ml of sample (dilutions 10^{-3} to 10^{-8}). The agar and sample were dispersed evenly within the plate and allowed to solidify. The plates were inverted and placed into the AnaeroPack System (2.5-liter jar). A sealed AnaeroPack sachet was opened to allow generation of carbon dioxide and absorption of oxygen and placed inside the jar, along with an indicator to monitor the anaerobic conditions inside. Then the jar was sealed. Additions of water to the pouch or a catalyst to the system were not needed. The AnaeroPack System was incubated at 37°C, and black colonies were counted after 4, 8, 12, and 24 h.

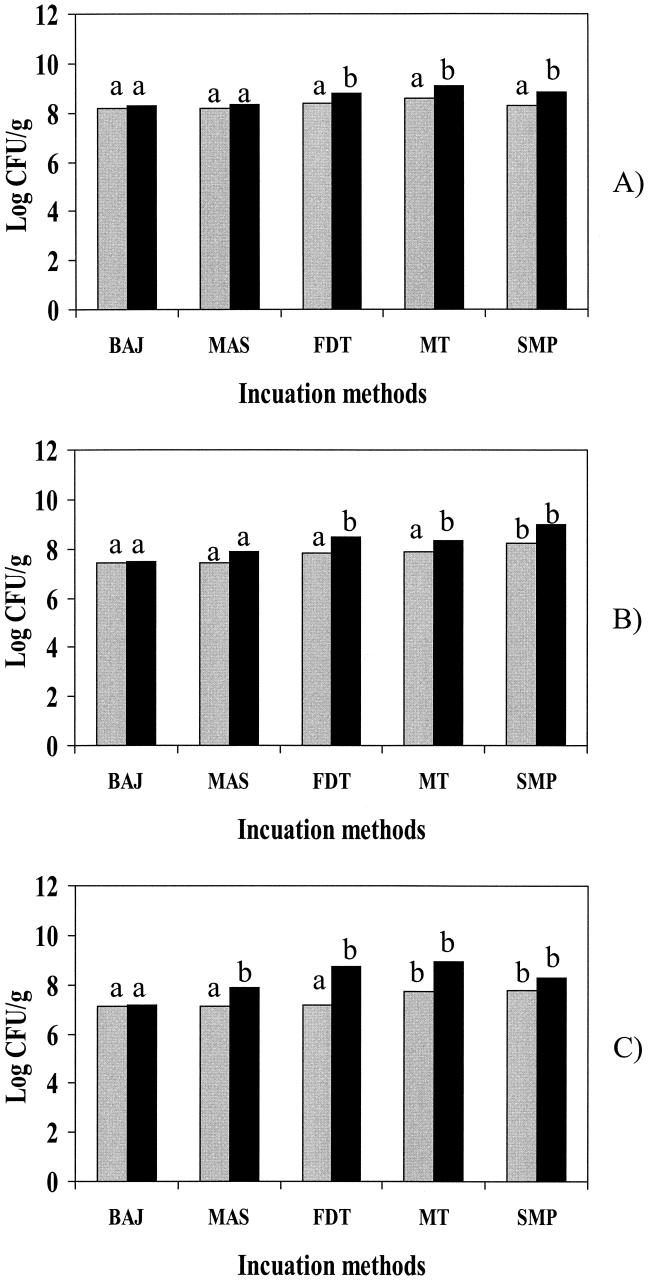

FIGURE 1. *The recovery rates of five methods (Brewer anaerobic jar [BAJ], Mitsubishi AnaeroPack System [MAS], Fung's double-tube [FDT], minitube [MT], and sandwiched microtiter plate [SMP]) for enumeration of* C. perfringens *from ground beef (A), ground turkey (B), and ground pork (C) after 12 h (□) and 24 h (■) incubation. Data with the same superscript are not significantly different (*$P > 0.05$*).*

Cost analysis. Time and total cost of each system were calculated from material, media, and labor costs. A stopwatch was used to record time needed for stomaching of sample, dilution,

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TABLE 1. *Total cost analysis of five anaerobic systems (per viable cell count[a])*

Systems	Cost ($) per sample (cost per viable cell count[a])		
	Material and media cost	Labor cost	Total cost
Brewer anaerobic jar[b]	3.82 (22.92)	0.77 (4.63)	4.59 (27.55)
AnaeroPack System[b]	3.81 (22.86)	0.73 (4.38)	4.54 (27.24)
Double-tube[b]	0.93 (5.58)	0.65 (3.88)	1.58 (9.46)
Minitube	0.71 (4.26)	0.56 (3.38)	1.27 (7.64)
SMP	0.37 (2.22)	0.42 (2.50)	0.79 (4.72)

[a] Six units (plates, tubes, wells, pipets) per viable cell count.

[b] Does not include initial cost of equipment. The cost of the Brewer anaerobic jar (2.5 liters) was $367.40/each. The Mitsubishi AnaeroPack System has two different-sized jars. The pack-rectangular jar (2.5 liters) was $55.00/jar and the pack-rectangular (5.5 liters) was $70.00/jar. For Fung's double-tube method, the Kimax tubes (16 × 150 mm) were $39.50/package of 72 and Kimax tubes (25 × 150 mm) were $83.80/package of 72 (approximate costs as of 1 May 1998).

inoculation, and mixing of sample with agar, if required. These recorded times were used in calculating cost of labor. Labor was calculated at an average cost of $15.00/h. Time involved in plate counts was not included in the calculation.

Statistical analysis. The experiment was replicated three times. All data were expressed as $\log_{10}$ CFU/g. The experimental design used was a randomized black design. Using the SAS system *(13)*, results were compared by analysis of variance (ANOVA) to determine the statistical significance of observed differences ($P > 0.05$)

RESULTS AND DISCUSSION

Figure 1A represents data for *C. perfringens* growth in ground beef after 12 and 24 h incubation. After 12 h incubation, no significant difference occurred in the recovery of *C. perfringens* with the five methods ($P > 0.05$). After 24 h, the Brewer anaerobic jar and Mitsubishi AnaeroPack System recovered *C. perfringens* without significant difference ($P > 0.05$), whereas FDT, MT, and SMP recovered higher numbers of *C. perfringens* compared to the two other methods ($P < 0.05$). Kang and Fung *(10)* also reported that no significant difference occurred in the recovery of *C. sporogenes* with FDT, MT, and SMP from ground beef. Figure 1B shows the recovery rate of *C. perfringens* inoculated in ground turkey with five methods. After 12 h incubation, only the SMP method recovered higher numbers of *C. perfringens* compared to the four other methods ($P < 0.05$). However, after 24 h incubation, the FDT, MT, and SMP methods recovered higher numbers of *C. perfringens* compared to the two other methods ($P < 0.05$). These results from inoculated ground turkey were similar to those from ground beef. In the case of ground pork, the MT and SMP methods recovered higher numbers of *C. perfringens* compared to the other three methods after 12 h incubation ($P < 0.05$) (Fig. 1C). After 24 h incubation, only the Brewer anaerobic jar recovered lower numbers of *C. perfringens* compared to the other four methods ($P < 0.05$). In the case of 4- and 8-h incubations, the recovery rates of *C. perfringens* by the five methods were not significantly different regardless of samples (data not shown). Overall, the SMP method recovered the highest counts of *C. perfringens* after 12 h incubation. After 24 h of incubation, FDT, MT, and SMP recovered higher numbers of *C. perfringens* compared to the other two methods. The results indicated that the FDT, MT, and SMP methods probably provided more anaerobic conditions, such that clostridial colonies started to develop faster than in the two chamber systems. Therefore, these three methods might be used for a more rapid detection of *Clostridium.* The Brewer anaerobic jar method has been the most widely known conventional method for many years. However, it does have disadvantages. The jar is not always reliable in producing an anaerobic environment, the procedure requires many steps, and the jar also has a large space requirement. The Mitsubishi AnaeroPack System is similar to the Brewer anaerobic jar, but provides many advantages. (i) This system is less expensive than other jars and chambers. (ii) It does not require a catalyst or addition of water to the AnaeroPack sachet. (iii) The system is easy to store. (iv) The containers are stackable. (v) The system is more reliable and provides a more airtight environment than the Brewer anaerobic jar. (vi) The handle-like closure on the container is easy to use compared to the hand-tightened screw closure on the GasPak system. The FDT method is easy to use. The tubes can be used more than once, and they are easy to clean *(5).* Previous studies using this method have shown higher anaerobic counts than with the Brewer anaerobic jar *(1).* The FDT method can be used for food products that have either a high level or very low level of contamination. However, this system does require several steps of setup, and all tubes need to be sterilized before use. The MT method is similar to the FDT method, however, it is more practical and requires less time *(10).* This method is simple to operate, and the pipets are sterile and ready to use. The MT method requires small amounts of inoculum and is better for foods with a high level of contamination. The advantages of this method include: (i) low cost, (ii) speed, (iii) ease of use, and (iv) less use of agar. The SMP method is also easy to operate and is practical. The SMP method also requires small amounts of inoculum and is better for foods with a high level of contamination *(10).* Advantages of the SMP method include: (i) low cost, (ii) speed, (iii) ease of use, and (iv) less use of agar. If food was contaminated with high numbers of *C. perfringens,* then the MT and SMP methods are reliable to enumerate the numbers of *C. perfringens.* However, if food contained

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low levels of *C. perfringens* (ca. 1.0 to 3.0 log CFU/g or ml), the Brewer anaerobic jar, Mitsubishi AnaeroPack System, and FDT methods are reliable for enumeration of *C. perfringens.* The costs per test and total cost analyses of the five anaerobic systems are given in Table 1. The Brewer anaerobic jar ($4.59/sample) was found to be the most expensive method, followed by the Mitsubishi AnaeroPack System ($4.54/sample), the FDT method ($1.58/sample), the MT method ($1.27/sample), and the SMP method ($0.79/sample). Note that the Brewer anaerobic jar, Mitsubishi AnaeroPack System, and FDT also require initial costs of equipment.

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