J. Milk Food Technol. Vol. 39, No. 2, Pages 107-110 (February, 1976)

Estimating Population Levels of *Clostridium perfringens* in Foods Based on Alpha-toxin

STANLEY M. HARMON and DONALD A. KAUTTER

Division of Microbiology
Food and Drug Administration, Washington, D.C. 20204

(Received for publication August 4, 1975)

ABSTRACT

A method for estimating population levels of *Clostridium perfringens* in foods based on the titer of *a*-toxin in food extracts was evaluated. Twenty-two samples of food associated with 15 food poisoning outbreaks and 12 ready-to-serve foods inoculated with approximately 100 *C. perfringens* spores/g and incubated to simulate improper holding conditions were examined. Alpha-toxin was present in extracts from most of the foods which could be correlated with the viable population as determined by plate counts in sulfite-polymyxin-sulfadiazine (SPS) agar. The amount of *a*-toxin present could be correlated with previous growth of *C. perfringens* in food regardless of whether the organisms were viable when the examination was made. This is important because storage and shipment of the food specimens for brief periods at low temperature results in a decrease of 2 to 4 log cycles in the viable plate count in SPS agar, but does not affect the accuracy of estimates of the population level based on the presence of *a*-toxin.

In many countries, including the United States, *Clostridium perfringens* is now widely recognized as one of the principal causes of food poisoning of bacterial origin (*3, 6, 10*). The illness occurs upon ingestion of foods containing large populations of viable vegetative cells of *C. perfringens* and the subsequent production of an enterotoxin in the intestine (*9, 11*). The rapid loss of viability of this organism, which occurs when food samples associated with food poisoning outbreaks are frozen for storage or shipment to the laboratory, has led to much difficulty in firmly establishing *C. perfringens* as the cause of many outbreaks. A decrease of 2 to 4 log cycles in viable plate counts of this organism is not uncommon when food samples are stored at low temperature for only a few days (*2, 7*). Therefore, cultural methods used for quantitation of this organism are often inadequate for an accurate determination of population levels in outbreak foods.

A method for estimating population levels of *C. perfringens* in food, which utilizes the hemolytic and lecithinase activities of *a*-toxin (phospholipase C) produced by this organism as an index of growth, was developed earlier in our laboratory (*7, 8*). The purpose of this study was to further evaluate the utility of the *a*-toxin method for the examination of a large variety of different foods associated with foodborne disease outbreaks. It should be understood that *a*-toxin and the enterotoxin produced by *C. perfringens* type A are different toxins. The enterotoxin which is responsible for gastroenteritis in outbreaks of food poisoning due to *C. perfringens* is produced only after ingestion of the contaminated food and is not usually present in the incriminated food.

MATERIALS AND METHODS

Examination of outbreak foods

A variety of foods associated with 15 food poisoning outbreaks which were attributed to *C. perfringens* were examined. All of the foods were supplied by laboratories of various state and municipal health departments who were conducting the investigation of the different food poisoning outbreaks. The incriminated food samples were collected from the cooperating laboratories by personnel from the District Laboratories of the Food and Drug Administration or the Center for Disease Control in Atlanta, Ga., and shipped frozen with dry ice to our laboratory. A few samples were sent directly to us by the cooperating laboratories.

The samples were stored frozen and examined within 18 to 24 h for viable *C. perfringens* by plate counts in sulfite-polymyxin-sulfadiazine (SPS) agar and for the presence of *a*-toxin by the method described previously (*7*). Of the various foods examined, eight consisted of roast beef, three of beef juice, three of turkey, two of beef hash, two of beef gravy, and one each of sliced ham, pork barbeque, chicken salad, and clam sauce. Most of the foods tested had been shown to contain a large viable population of *C. perfringens* by the laboratory that initially investigated the outbreak.

Examination of inoculated foods

The 12 ready-to-serve foods used for the temperature abuse experiments were all obtained from local food service establishments. The various foods tested included: roast beef, ground beef, meat loaf, beef hash, turkey, beef gravy-mix, chicken salad, sliced pork, and crabmeat. Between 100 and 200 g of the food were placed in sterile plastic bags and either inoculated directly with *C. perfringens* spores or immersed in a water bath for 2 h at 50 C to reduce oxygen levels in the food before inoculation. Each food was inoculated with approximately 100 spores/g of one of the strains studied. As much air as possible was evacuated from the bags, which were heat sealed and incubated at 35 C.

Foods were examined periodically during incubation for evidence of the growth of *C. perfringens*. When large populations of *C. perfringens* were present in foods, as indicated by gas production in bags, or by direct microscopic examination, a portion of each sample was examined for viable *C. perfringens* by plate counts in SPS agar and for *a*-toxin by the method previously described. The remainder of each food was frozen and stored for 3 weeks at -20 C and reexamined for viable *C. perfringens* and for *a*-toxin.

Spore stocks

All of the *C. perfringens* strains used in these experiments were isolated from food or feces associated with foodborne disease outbreaks. Strains FD-1, FD-2, and FD-26 were isolated in our laboratory. Strains CDC 1861 and CDC 2078 were obtained from V. R. Dowell, Center for Disease Control, Atlanta, Ga. Strains A-86 and S-34 were obtained from H. E. Hall, U.S. Public Health Service, Cincinnati, Ohio. Strains NCTC 8797, NCTC 8238, NCTC 8798, and NCTC 10240 were supplied by the National Collection of Type Cultures, London, England.

The spore stocks used for inoculation of the ready-to-serve foods were grown in either Ellner's medium (5) or in Duncan-Strong spore broth (4). Fifteen-milliliter volumes of each spore broth were inoculated with 2 ml of an 18-h fluid thioglycollate culture of each strain and incubated at 35 C for 24 to 48 h. The spores were harvested by centrifugation, resuspended in sterile water, and stored at 4 C.

Extraction and quantitation of a-toxin

The procedures for extracting and quantitating *a*-toxin in foods were described previously (7). These procedures were followed throughout the present study except that the supernatant extract was filtered with Whatman No. 1 filter paper before Seitz filtration. Removal of fat and particulate material by filtration with paper greatly facilitated filter sterilization of the extract. Extracts from turkey samples and the chicken salad sample also required treatment with ethyl ether to remove lipids before filter-sterilization of the extract, but this step was not necessary for any of the other foods. Details of this procedure were presented in a previous publication (7).

Viable counts

A 25-g portion of food was blended with 225 ml of 0.1% peptone water, diluted serially in the same diluent, and plated in laboratory-prepared SPS agar (1). After solidification, this agar was overlaid with an additional 5 ml of SPS agar and incubated for 24 h in a Case Anaero-jar (Case Laboratories Inc., Chicago, Ill.) under an atmosphere of nitrogen or in Gas Pak jars (BBL, Cockeysville, Md.). Black colonies were counted, and 10 isolates were picked to motility-nitrate medium for confirmation as *C. perfringens*.

Correlation of viable count with a-toxin

The relationship between viable plate counts in SPS agar and the amount of *a*-toxin produced in a limited number of foods has been established (7). Data obtained with six strains of *C. perfringens* isolated from outbreak foods (chicken broth) were chosen as the basis for correlating population levels with the *a*-toxin titer. The *C. perfringens* strains used for the determinations in chicken broth included three strains which produce heat-sensitive spores (FD-1, CDC 1861, and S-34) and three strains with heat-resistant spores (NCTC 8238, NCTC 8797, and NCTC 8798). Two-hundred grams of chicken broth were heated to expel oxygen and inoculated with approximately 5×10^5 washed cells/ml of the test strain and incubated at 35 C. After 2 h of incubation, samples were removed at 30-min intervals and tested for *a*-toxin and plate counted. Alpha-toxin titers were determined in the hemolysin indicator plate and the lecithovitellin (LV) test as previously described (7). The geometric mean value of the viable counts of the six strains was determined and correlated with the corresponding *a*-toxin titer for each population level.

RESULTS

Correlation of viable count with a-toxin

The relationship between viable plate counts of *C. perfringens* in SPS agar and the amount of *a*-toxin produced in a limited number of foods has been established (7). Since experimental results obtained with roast beef and chicken broth were similar, data from experiments with six representative "food poisoning" strains of *C. perfringens* in chicken broth were chosen as the basis for correlating population levels with the amount of *a*-toxin present in foods. For easy reference in interpreting the results of the present report, these data are presented again in Table 1.

TABLE 1. *Correlation between the viable count of* Clostridium perfringens *and the amount of a-toxin detected in filtrates of chicken broth*

Viable count/g[a]	Alpha toxin titer: Hemolysin indicator plate	Alpha toxin titer: Lecithovitellin test
1.2	Undiluted	No reaction
2.5	1:2[b]	Undiluted
6.5	1:4	1:2[c]
9.5	1:8	1:4
25	1:16	1:8
55	1:32	1:16
80	1:64	1:32
150	1:128	1:128
210	1:256	1:256

[a]Average of viable counts obtained with six strains. Values to be multiplied by 10^6.
[b]Dilution of extract producing a 1-mm zone of hemolysis.
[c]Highest positive dilution.

These results show the relationship between the average plate count in SPS agar and the corresponding *a*-toxin titer produced in chicken broth as measured in hemolysin indicator plates and the LV test. Data in Table 1 were used throughout the study for estimating population levels in the various outbreak foods and in the inoculated ready-to-serve foods.

Viable counts and population estimates with outbreak foods

A total of 22 food samples associated with 15 food poisoning outbreaks were examined. The viable plate counts of *C. perfringens* determined before and after

TABLE 2. *Comparison of viable count with an estimation of the previous growth based on the quantity of a-toxin detected in foods associated with foodborne disease outbreaks*

Food	Viable count per g: Before freezing[a]	Viable count per g: After frozen storage[b]	Alpha toxin titer in hemolysin indicator plate	Estimated population/g based on *a*-toxin titer
Beef prime ribs[c]	1.0×10^7	1.2×10^6	1:8	9.5×10^6
Roast beef[c]	5.5×10^6	5.0×10^4	1:4	6.5×10^6
Roast beef[c]	1.4×10^3	1.0×10^2	1:2	2.5×10^6
Roast beef	ND[d]	2.1×10^4[d]	0	$< 10^6$
Roast beef	ND	3.0×10^4	0	$< 10^6$
Barbecue beef hash[c]	6.3×10^7	1.5×10^3	1:64	8.0×10^7
Beef hash[c]	9.0×10^4	10	1:2	2.5×10^6
Beef juice	3.0×10^7	2.7×10^5	1:16	2.5×10^7
Beef juice	1.2×10^7	-7.0×10^4	1:8	9.5×10^6
Beef gravy	3.5×10^7	2.3×10^5	1:16	2.5×10^7
Ham	3.0×10^7	4.7×10^3	1:32	5.5×10^7
Barbecue pork	1.0×10^7	3.0×10^2	1:4	6.5×10^6
Chicken salad	4.0×10^5	5.0×10^2	conc. × 2	5.0×10^5
Turkey	ND	1.0×10^2	undiluted	1.2×10^6
Turkey salad	5.3×10^6	2.1×10^3	undiluted	1.2×10^6
Clam sauce	5.0×10^5	5.0×10^2	undiluted	1.2×10^6

[a]Data supplied by the laboratory conducting investigations of the outbreak.
[b]Data obtained in FDA laboratory after frozen shipment of the food.
[c]Previously published data (7).
[d]No data available.

frozen shipment and storage and estimated population levels based on the quantity of *a*-toxin present which were obtained with 16 representative outbreak foods are presented in Table 2. Data obtained with some of the outbreak foods has been reported previously (*7*), but are included in Table 2 because they were more typical of the outbreak foods examined than those for which no data are presented. Similar results were obtained with the remaining six food samples examined. Plate counts with these foods ranged from 1.4×10^3 to 3.5×10^7/g before they were frozen for shipment to our laboratory. Alpha-toxin was present in 14 of these foods which could be quantitated and utilized for estimating the population level of *C. perfringens*.

Population estimates were based on the titer of *a*-toxin in hemolysin indicator plates and *a*-toxin present identified by neutralization with *C. perfringens* alpha antitoxin. Identity of *a*-toxin in food extracts was also considered to be more firmly established if lecithinase activity of extracts in the LV test was prevented by addition of *a*-antitoxin. There was very good agreement between the plate counts obtained in other laboratories before frozen shipment of the samples and the estimated population level based on *a*-toxin with most of the outbreak foods. The estimated population levels ranged from $<10^6$/g to 5.5×10^7/g and most instances were within 1 $\log_{10}$ of the plate count reported by the original investigators. However, after frozen shipment of the food samples, plate counts in SPS agar were reduced substantially and therefore were unreliable for determining population levels because of the loss of viability of *C. perfringens* in the incriminated foods. A decrease of 2 to 4 log cycles in the plate count in SPS agar usually occurred during storage and frozen shipment to our laboratory.

With two samples, the estimated population level based on *a*-toxin was substantially higher than the original plate counts indicating that the viable population may have declined before the plate counts were made. Alpha-toxin was not detected in any of the foods which had a population level less than 5.0×10^5/g.

Viable counts and population estimates in inoculated foods

In addition to the outbreak foods, samples of 12 ready-to-serve foods which were inoculated with 100 *C. perfringens* spores/g and incubated to simulate improper storage conditions were examined. The viable plate counts in SPS agar and estimated population levels based on the titers of *a*-toxin found in extracts of the inoculated foods are shown in Table 3. Immediately following incubation, a large population of *C. perfringens* was present in all of the foods tested. Viable counts in SPS agar ranged from 3.0×10^6/g in roast beef inoculated with strain NCTC 8798 to 2.2×10^8/g in beef hash inoculated with strain FD-1. Detectable *a*-toxin was also present in all of the foods which could be quantitated and correlated with the viable plate counts in SPS agar with the exception of roast beef inoculated with strain NCTC 8798. Storage of the inoculated foods for 3 weeks at -20 C resulted in a substantial decrease in the viable plate counts in SPS agar as shown in Table 3. The viable counts decreased from 4.5×10^7/g to 3.4×10^3/g during frozen storage of a sample of roast beef inoculated with strain FD-1. A similar result was obtained with many of the other foods.

The *a*-toxin titer present in extracts from the various foods remained unchanged during the 3-week storage period. Therefore, only the results obtained after frozen storage are presented. The estimated population levels based on the titer of *a*-toxin present in food extracts were virtually identical with plate counts obtained in SPS agar with six of the inoculated foods. However, the estimated population levels obtained with foods inoculated with strains NCTC 8238, NCTC 10240, and FD-26, which produce less *a*-toxin, were lower than the viable plate count by a factor of 2.5 to 16 times. One sample of roast beef inoculated with strain NCTC 8798 was negative for *a*-toxin but had a plate count indicating a population of 3.0×10^6/g.

DISCUSSION

Results obtained with most of the outbreak foods and

TABLE 3. *Comparison of viable count with an estimation of growth based on the quantity of a-toxin detected in a variety of foods inoculated with spores of* Clostridium perfringens[a]

Food	Strain	Viable count per g		Alpha-toxin titer in hemolysin indicator plate[b]	Estimated population/g based on the *a*-toxin titer
		Before freezing	After 3 wk of frozen storage		
Roast beef	FD-1	4.5×10^7	3.4×10^3	1:64	8.0×10^7
Roast beef	NCTC 8798	3.0×10^6	1.6×10^4	0	$< 10^6$
Meat loaf	NCTC 8797	9.0×10^7	9.3×10^4	1:256	2.1×10^8
Beef hash	FD-1	2.2×10^8	9.1×10^6	1:128	1.5×10^8
Ground beef	FD-2	1.9×10^8	6.9×10^4	1:256	2.1×10^8
Ground beef	CDC 1861	1.7×10^8	1.1×10^5	1:128	1.5×10^8
Ground beef	NCTC 8238	1.6×10^7	2.1×10^5	1:2	2.5×10^6
Turkey	A 86	2.8×10^7	7.2×10^3	1:16	2.5×10^7
Beef gravy mix	NCTC 10240	4.0×10^7	5.5×10^4	1:2	2.5×10^6
Chicken salad	FD-26	2.3×10^7	8.7×10^3	1:8	9.5×10^6
Sliced pork	CDC 2078	1.9×10^8	4.4×10^5	1:256	2.1×10^8
Crab meat	S-34	3.0×10^7	2.8×10^4	1:32	5.5×10^7

[a]Foods were inoculated with approximately 100 spores per gram and incubated for 18-48 h at 35 C.
[b]Alpha-toxin titers were determined after 3 weeks of storage at −20 C.

inoculated ready-to-serve foods showed that there was a reasonably good correlation between the viable population of *C. perfringens* and the estimated population level based on the titer of *a*-toxin found in the food extracts. These results support our previous findings that in most cases detectable *a*-toxin is present in extracts from foods containing populations of *C. perfringens* greater than 10^6/g which can be utilized to estimate population levels when the organisms themselves are no longer viable. Data obtained with a variety of outbreak foods naturally contaminated with *C. perfringens* and with food inoculated with spores and incubated to simulate improper holding of foods, indicate that population estimates based on the titer of *a*-toxin in food extracts are usually quite reliable.

False-negative results were obtained occasionally with this method due to insufficient *a*-toxin production by a few strains. This finding was anticipated from our experimental results which showed that 10 of 34 strains of the *C. perfringens* isolated from outbreak foods produced less than average amounts of *a*-toxin and that 2 of the 34 strains tested were poor *a*-toxin producers (*7*). However, we considered this shortcoming to be outweighed by the potential usefulness of the method. As the data in Tables 2 and 3 show, the estimated population levels based on the titer of *a*-toxin in food extract were much more indicative of the actual *C. perfringens* population than were viable plate counts made after storage and shipment of the foods at low temperature. Since this is the usual practice in handling food specimens for bacteriological examination, plate count determination of *C. perfringens* populations in foods shipped or stored at low temperature are likely to be highly inaccurate.

The type of food associated with the different outbreaks appears to have had little effect on population estimates based on the presence of *a*-toxin. Results obtained with the outbreak foods suggest that whenever conditions were suitable for growth of *C. perfringens* *a*-toxin was usually produced. It appears that the quantity of *a*-toxin detected in food extracts depended more on toxin-producing ability of the strains present than any other factor. In a few cases when population levels were near the lower limits for production of detectable *a*-toxin, no toxin was found in the food extracts.

The stability of *a*-toxin in foods stored for prolonged periods at 4 C or below and the relative ease with which it can be extracted and quantitated provide an additional means for estimating large populations of *C. perfringens* in foods. The population estimates, while not strictly quantitative, should be valuable for identifying foods responsible for *C. perfringens* food poisoning outbreaks and determining the hazard presented by the development of large populations of this organism in foods.

ACKNOWLEDGMENTS

We thank the following individuals and organizations for supplying the outbreak foods used in this investigation: Syed A. Shahidi, Joseph Zitter, and Charles Reissberg, New York City Health Department; Edwin Werner, Sulfolk County New York Health Department; Ronald Gleen, Alabama State Health Department; H. J. Webb, South Carolina State Board of Health; Tom McKinley, Georgia State Health Department; Louise Brown, Illinois Department of Public Health; Gentry Moffitt, Tennessee State Health Department; and the State Health Departments of Colorado, Maryland and Washington.

We appreciate the assistance of V. R. Dowell, Eugene Gangerosa, and Mike Merson of the Center for Disease Control, Atlanta, Ga. and the following personnel of the District Offices of the Food and Drug Administration in procuring the outbreak food samples: John Feldman and Daniel Sitko, New York; Kent Smith, Atlanta; Doyle Smith, Chicago.

REFERENCES

1. Angelotti, R., H. E. Hall, M. J. Foter, and K. H. Lewis. 1962. Quantitation of *Clostridium perfringens* in foods. Appl. Microbiol. 10:193-199.
2. Canada, J. C., D. H. Strong, and L. G. Scott. 1964. Response of *Clostridium perfringens* spores and vegetative cells to temperature variation. Appl. Microbiol. 12:273-276.
3. Center for Disease Control. 1973. Foodborne disease outbreaks; Annual summary for 1972.
4. Duncan, C. L., and D. H. Strong. 1968. Improved medium for sporulation of *Costridium perfringens*. Appl. Microbiol. 16:82-89.
5. Ellner, P. D. 1956. A medium promoting rapid quantitative sporulation in *Clostridium perfringens*. J. Bacteriol. 71:495-496.
6. Hall, H. E. 1969. Current developments in detection of microorganisms in foods—*Clostridium perfringens*. J. Milk Food Technol. 32:426-430.
7. Harmon, S. M., and D. A. Kautter. 1970. Method for estimating the presence of *Clostridium perfringens* in food. Appl. Microbiol. 20:913-918.
8. Harmon, S. M., and D. A. Kautter. 1974. Collaborative study of the alpha-toxin method for estimating population levels of *Clostridium perfringens* in food. J. Assoc. Offic. Anal. Chem. 57:91-94.
9. Hauschild, A. H. W., L. Niilo, and W. J. Dorward. 1971. The role of enterotoxin in *Clostridium perfringens* type A enteritis. Can. J. Microbiol. 17:987-991.
10. Hobbs, B. C., M. E. Smith, C. L. Oakley, G. H. Warrak, and J. C. Cruickshank. 1953. *Clostridium welchii* food poisoning. J. Hyg. 51:75-101.
11. Strong, D. H., C. L. Duncan, and G. Perna. 1971. *Clostridium perfringens* type A food poisoning. II. Response of the rabbit ileum as an indication of enteropathogenicity of strains of *Clostridium perfringens* in human beings. Infec. Immun. 3:171-178.