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Enumeration and Characterization of *Clostridium perfringens* Spores in the Feces of Food Poisoning Patients and Normal Controls

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ABSTRACT

The fecal spore enumeration method for confirming *Clostridium perfringens* as the cause of food poisoning was evaluated using strains implicated in nine outbreaks in the United States. Confirmed spore counts from 66 stool specimens were made on tryptose-sulfite-cycloserine (TSC) without egg yolk and trypticase soy-sheep blood (TSB) agars. Counts from outbreak stools on TSC agar ranged from 2.0×10^4 to 3.5×10^8 (mean $\geq1.4\times10^6$/g) as compared with $<10^3$ to 5.0×10^5/g (overall mean 9.5×10^3/g) from normal stools. Similar results were obtained with TSB agar. Isolates from seven of the nine outbreaks were nonhemolytic and produced ≥100 ng enterotoxin/ml in spore broth, as measured by an enzyme-linked immunosorbent assay. Spores in stools from six of the outbreaks were heat-resistant and survived heating for 30 to 60 min at 100°C in cooked meat medium. Strains from the three remaining outbreaks were heat-sensitive and survived heating for only 15 min at 100°C. Enterotoxigenic isolates from all but one of the outbreaks were serotyped. In all instances, the predominant strain in specimens from an outbreak was of the same serotype, indicating that it was the causative strain. Reexamination of five specimens from each of three outbreaks after storage at -20°C for 6 months showed only a minimal reduction in the spore counts.

Confirming *Clostridium perfringens* as the cause of food poisoning by its enumeration in stools of food poisoning patients was first proposed by Sutton in 1969 (*16*). Results of that study and related studies, including details of the methods used, were summarized by Sutton et al. in 1971 (*18*). Since then, *C. perfringens* spores in stools usually have been enumerated because total counts and spore counts are similar, and spores are more stable than vegetative cells (*7,9*).

Although workers in the United Kingdom have made many important contributions to research on *C. perfringens* food poisoning, the fecal spore count is one of the least appreciated, perhaps because *C. perfringens* is widely recognized as a normal constituent of human feces (*9,12,15*) and because many investigators have continued to emphasize the examination of incriminated foods for confirming outbreaks (*2,4,11*).

Because of its apparent effectiveness, we decided to evaluate the use of the fecal spore count for investigating food poisoning outbreaks in the United States and to thoroughly characterize a large number of fecal isolates associated with the outbreaks. From December 1981 to November 1984, more than 100 stool specimens were examined from persons who had experienced afebrile, diarrheal illness suspected to be *C. perfringens* food poisoning. A small number of post-symptomatic and control specimens were also studied. These specimens were obtained through the assistance of the Epidemic Intelligence Officers of the Centers for Disease Control (CDC) who were assigned to various state and local health departments. Confirmed *C. perfringens* spore counts were made on two different types of plating media, and representative isolates from each of the positive specimens were characterized.

MATERIALS AND METHODS

Stool specimens

Stool specimens were collected by personnel from the health departments of Colorado, Florida, Illinois, Maryland, Tennessee and Ontario, Canada, during routine investigations of nine food poisoning outbreaks. All specimens were collected within 48 h after onset of illness, except for 12 specimens associated with outbreaks 8 and 9, which were collected after 4 or 5 d. Post-symptomatic specimens were collected 4 wk after recovery from ten persons who had been ill during outbreaks 6 and 7. Thirty-five fecal specimens from healthly adults were used as controls.

Storage of specimens

The specimens were frozen and stored at ca. -20°C and shipped to the Food and Drug Administration (FDA) laboratory, where they were examined immediately or held at -20°C until examination. To determine the effect of long-term frozen storage on spore counts, five stool specimens from each of three

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outbreaks were thawed and examined immediately after receipt, and then quickly refrozen and held at -20°C for 6 months before reexamination.

Enumeration of spores

Fecal specimens weighing 1 g were homogenized in 9 ml of 0.1% peptone in screw-cap tubes on a Vortex mixer. The homogenates were heated in a water bath at 75°C for 20 min and cooled in ice water before diluting and plating. Tenfold dilutions of the homogenates were made in 0.1% peptone, and appropriate dilutions were plated in duplicate on Trypticase soy agar containing 5% defibrinated sheep blood (TSB) (England Laboratories, Beltsville, MD) and tryptose-sulfite-cycloserine (TSC) agar without egg yolk. TSB was inoculated by spreading 0.1 ml of the diluted specimen onto each plate with a sterile glass spreading rod. Volumes of 1 ml were pour plated in TSC agar, as described by Hauschild et al. (*8*). The plates were incubated at 35°C for 20 to 24 h in an anaerobic jar (Oxoid USA, Columbia, MD) and the presumptive *C. perfringens* colonies were counted. Ten presumptive colonies from each plating medium were confirmed by testing in buffered motility nitrate medium and lactose gelatin (*4*). The presumptive counts were adjusted by multiplying by the fraction of isolates that were confirmed as gram-positive, nonmotile rods, positive for nitrate reduction, lactose fermentation and gelatin liquefaction.

Heat resistance

The relative heat resistance of *C. perfringens* spores in stools was determined by the method of Hobbs et al. (*10*). Approximately 1 g of feces was added to each of three tubes of cooked meat medium, and the tubes were immersed in a boiling water bath. After 15, 30 and 60 min, one tube was removed, cooled in ice water and incubated at 35°C for 48 h. Tubes that exhibited growth and gas production were streaked on TSB agar, and representative presumptive colonies were confirmed as *C. perfringens* as described above.

Hemolytic activity

Whenever possible, the hemolytic acitivity of colonies was observed directly on the TSB plates. If the presence of other bacteria prevented this, isolates were transferred to cooked meat medium and streaked onto fresh TSB plates. The hemolytic acitivty of organisms that survived heating for 15 min or longer at 100°C was also assessed on TSB plates.

Enterotoxigenicity of isolates

The ability of isolates to produce *C. perfringens* enterotoxin (CPE) was determined by the double antibody sandwich enzyme-linked immunosorbent assay (ELISA) method (*22*), modified specificially for this purpose by Wimsatt et al. (*23*). Antisera and purified enterotoxin for the ELISA were supplied by John Wimsatt, Division of Microbiology, FDA, Cincinnati, OH. Isolates were cultured in Duncan-Strong (DS) sporulation medium (*1*) or in the AEA spore broth of Tanaguti (*19*), modified by substituting raffinose for starch; the culture supernatant fluid was examined by ELISA. A 0.5-ml volume of culture in cooked meat medium (Difco, Detroit, MI) was inoculated into fluid thioglycollate medium, and the tubes were heated at 75°C for 20 min before incubation to enhance germination and sporulation, as recommended by Uemura et al. (*21*). After 18 h of incubation at 37°C, the resulting culture was transferred into fresh thioglycollate medium and incubated at 37°C for 4 h. A 0.5-ml portion of this culture was then transferred into 15 ml of freshly steamed spore broth and incubated in an anaerobic jar at 37°C for 48 h. Stained smears were prepared from the spore broth and examined microscopically for spores. The supernatant fluids of cultures which had an estimated 20% or more of free spores were assayed for CPE. Supernatant fluids with at least 0.3 ng CPE/ml were retested at dilutions of 1:1, 1:10 and 1:100 to quantitate the enterotoxin within the range of 0.3 to 100 ng/ml. Purified enterotoxin from *C. perfringens* strain NCTC 8239 was used as the standard. Undiluted supernatant fluids that contained <1 ng of CPE/ml were considered to be negative for enterotoxin.

Serotyping

CPE-positive isolates from outbreak stools were serotyped as previously described (*6*) by using a battery of 91 *C. perfringens* antisera prepared at CDC along with the slide agglutination technique of Hobbs et al. (*10*).

Statistical analysis

The spore count data were converted to logarithms for normal probability distribution, and an analysis of variance was performed. A least significant difference test was used to differentiate the means. The statistical methods were those of Snedecor and Cochran (*13*).

RESULTS AND DISCUSSION

Confirmed spore counts

Of the 66 stools from patients in confirmed outbreaks, 56 had *C. perfringens* spore counts between 10^6 and 10^8/g, whereas counts of post-symptomatic and normal control specimens ranged from $<10^3$ to 10^5/g. The plate counts were recorded as the $\log_{10}$/g of stool; the range and means for each group are presented in Table 1. Although the range of counts differed considerably with specimens from all three groups, the mean values for counts on outbreak stools were more than 3 $\log_{10}$ units higher than those for control groups. This was a highly significant difference ($P<0.001$) (Table 2). Average counts also differed statistically with individual specimens within the same group.

Some counts from the outbreak and control groups overlapped, i.e., the confirmed spore count of one specimen from each of four outbreaks and two specimens from an additional outbreak were $<10^6$/g ($\log_{10}$ values of 4.30 to 5.48). The effect on the spore count means was minimal, however, because the median count of the four specimens from each outbreak with the lowest counts was always $>10^6$/g. Counts of only one of the post-symptomatic and two normal control specimens were within the same population range as those of the six subnormal outbreak specimens ($\log_{10}$ 4.11 to 5.70). Because this represents a worst case situation, it seems unlikely that results with the fecal spore enumeration method would be misinterpreted when as few as three or four specimens are examined or when isolates are characterized. The *C. perfringens* spore counts for ten persons affected in outbreaks 6 and 7 were extremely high at first, but returned to normal within 30 d (Table 1). Although *C. perfringens* spore counts have been shown to vary

TABLE 1. Clostridium perfringens *spores in the feces of food poisoning patients, and post-symptomatic and normal controls counted on two plating media.*

Outbreak	No. of specimens	Spores (log_{10})/g of stools			
		TSC[a]		TSB[b]	
		Range	Mean	Range	Mean
1	7	5.30-7.04	6.37	5.32-7.08	6.33
2	4	4.43-6.90	6.16	4.36-6.90	6.13
3	8	5.30-7.95	6.79	5.20-7.90	6.66
4	19	6.49-8.30	7.81	6.48-8.36	7.62
5	2	6.70-6.90	6.68	6.71-7.00	6.86
6	8	7.18-8.54	7.70	7.11-8.95	7.99
7	6	6.48-8.61	7.59	6.94-9.08	7.98
8[c]	7	4.43-6.92	6.16	4.75-6.82	6.12
9[c]	5	4.30-8.24	6.40	4.18-8.15	6.38
Total	66	4.30-8.61	7.08	4.18-9.08	7.15
Post-symptomatic controls[d]	10	<3.00-3.95	3.28	<3.00-4.11	3.28
Normal controls	25	<3.00-5.70	3.62	<3.00-5.35	3.61

[a]TSC agar without egg yolk (pour plated).
[b]Trypticase soy sheep blood agar.
[c]Specimens were collected 4 or 5 d after onset of illness.
[d]Submitted by patients 30 d after illness.

TABLE 2. *Analysis of variance summary of log_{10}* Clostridium perfringens *spore counts.*

Source of variation	Degrees of freedom	Mean squares	Significance level
Specimen group	2	235.95	P<0.001[a]
Patient (specimen group)	8	7.23	P<0.001[a]
Sample (patient group)	80	1.06	P<0.001[a]
Medium	1	0.09	P>0.05
Specimen group × medium	2	0.04	P>0.10
Medium × patient (group)	8	0.22	P<0.001[a]

[a]Statistically significant difference in the values compared.

considerably in stools of normal persons (*9,12,15*), our data suggest that they are usually quite low among healthy adults in the United States.

Effect of media

TSB agar without antibiotics was used in this study instead of the neomycin horse blood agar recommended by Hobbs et al. (*10*) because it gave slightly higher counts and was more readily available. We had also found in previous work (unpublished) that addition of neomycin to TSB was of limited value because so few competing bacteria were present in heat-treated specimens, except in normal stools.

An analysis of variance showed no statistically significant differences in the rates of recovery with TSC and TSB agars (Table 2). However, TSB was more useful because in most instances food poisoning strains were nonhemolytic and thus could be readily distinguished from the nonenterotoxigenic hemolytic strains normally found in human feces. In addition, TSB was more convenient because prepared plates could be stored up to 2 wk at 4°C before use.

Effect of frozen storage

As the study progressed, the importance of determining the effect of prolonged frozen storage on spore counts became clear. Stools associated with three of the first four outbreaks had been stored frozen at -20°C for 3 to 6 wk before they were submitted to our laboratory. Although the spore counts were still quite high (Table 1), we were not sure that these counts accurately reflected the situation at the time the stools were collected. Therefore, we determined the ability of spores to remain viable during long-term frozen storage with specimens from three subsequent outbreaks. Initial counts were made on TSB agar as soon as the specimens were received, and five representative specimens from each outbreak were quickly refrozen and stored at -20°C for 6 months before reexamination. In most instances, confirmed spore counts before and after storage were almost the same (Table 3).

This is a very important advantage of the fecal spore enumeration method for confirming outbreaks. Often *C. perfringens* cannot be confirmed as the causative agent because the implicated food is not available for examination, or viable counts are too low to give a clear-cut answer (*5*). Our results indicate that stool specimens collected within 3 d after the onset of illness can be stored frozen and examined at a convenient time or shipped to a reference laboratory for confirmation of food poisoning outbreaks caused by *C. perfringens*.

TABLE 3. *Effect of 6 months storage at -20°C on viability of* Clostridium perfringens *spores in stools of food poisoning patients.*

Outbreak	Stool No.	No. ($\times 10^6$) of *C. perfringens* spores/g of stool[a]	
		Before storage	After storage
4	1	122	110
	2	189	166
	3	70	43
	4	39	44
	5	87	73
7	1	97	77
	2	40	33
	3	48	31
	4	240	240
	5	74	78
8	1	1.7	1.2
	2	3.0	3.8
	3	13	9.3
	4	1.2	2.8
	5	2.5	2.1

[a]Determined on TSB agar.

Characteristics of isolates

Hemolytic activity on TSB agar, relative heat resistance of spores in the stool, and the ability of isolates to produce CPE in modified AEA or DS spore broth were determined (Table 4). Serotypes of representative isolates from each of the nine outbreaks are summarized and the characteristics of isolates from control specimens are included (Table 4).

Hemolytic activity. Whenever possible, the hemolytic activity of strains present in the stools was determined directly on the TSB plates. In seven of the nine outbreaks, the predominant strain was of the nonhemolytic type frequently implicated in food poisoning in the United Kingdom (*10*) (Table 4). The strains implicated in outbreaks 2 and 3 were hemolytic and similar to the hemolytic, heat-sensitive strains described by Sutton and Hobbs (*17*). Hemolytic colonies were also noted occasionally on TSB plates with specimens in which the nonhemolytic type predominated; however, they did not produce detectable amounts of CPE. This suggests that they were part of the patient's normal flora or were a concomitant strain that had little if anything to do with the food poisoning. Only two of the 25 normal controls and none of the 10 post-symptomatic controls contained nonhemolytic strains, and neither of these produced detectable amounts of CPE.

Heat resistance of spores. The predominant strain present in stools of food poisoning patients from four outbreaks were nonhemolytic and heat-resistant, surviving

TABLE 4. *Characteristics of* Clostridium perfringens *isolates from feces of food poisoning patients, and post-symptomatic and normal controls.*

Outbreak	No. of isolates tested	Hemolytic colonies[a] (%)	Heat resistance of spores at 100°C (min)[b]	Serotype(s)	Enterotoxin production[c]
1	70	3	>60	Hobbs 1	ND[d]
2	18	100	30	Hobbs 5	+
3	35	100	15	PS-65	+
4	68	8	>60	PS 66, 88[e]	+
5	20	0	30	Hobbs 10	+
6	80	0	30-60	PS 66, 58	+
7	90	0	>60	PS 66, 58	+
8	69	5	15	ND	+
9	28	0	15	Hobbs 5,6 (13)[f]	+
Post-symptomatic controls	20	100	<15	ND	−
Normal controls	38	94	<15	ND	−

[a]On TSB agar.
[b]As determined by the method of Hobbs et al. (*11*).
[c]+, >100 ng CPE/ml; −, <1 ng CPE/ml.
[d]ND, not determined.
[e]Agglutinated with more than one antiserum.
[f]( ), weak reaction.

heating for more than 1 h at 100°C (Table 4). The implicated strains in two additional outbreaks might also be classified as heat resistant because they survived heating at 100°C for 30 min (although not for 1 h) and agglutinated with antisera prepared against one of Hobb's heat-resistant types. Two of the nonhemolytic CPE-positive strains (from outbreaks 8 and 9) and one hemolytic strain from outbreak 3 survived heating for only 15 min at 100°C. These strains would definitely be classified as heat sensitive by the criteria of Hobbs et al. (*10*). None of the strains present in the normal or follow-up specimens survived heating for 15 min at 100°C, perhaps because of the low populations of spores present, but more likely because they were the heat-sensitive, hemolytic type usually found in normal stools.

These results indicate that the heat resistance of strains implicated in food poisoning in the United States varies considerably, as has been noted by other workers (*3*). A heat-sensitive strain was the only type found in the feces of patients from three outbreaks. The food implicated in all three of these outbreaks was roast beef, which often receives only minimal cooking before being served.

Serotyping. Representative CPE-positive isolates from outbreak specimens were serotyped by slide agglutination (Table 4). Isolates from only one of the outbreaks were nontypable with the 91 antisera available. In most instances, the serotype(s) present had been implicated previously in food poisoning in the United States (*6*). Isolates from outbreak 3 were of a serotype not encountered in previous incidents; those from outbreaks 4, 6, 7 and 9 agglutinated with more than one antiserum and thus appeared to belong to a group of strains that are antigenically complex, as reported by Hatheway et al. (*6*). The strain implicated in outbreak 4 agglutinated with antisera to types 66 and 88. Strains of this type have been implicated in food poisoning in the United States on three previous occasions (*6*). Isolates from outbreak 4, which occurred in a Florida state hospital in 1982, were serologically related to those from outbreaks 6 and 7, which took place in a state correctional facility in Florida within an 8-d period in 1984 and involved the same inmate population (*20*). Our serotyping results suggest that both outbreaks 6 and 7 may have been caused by the same strain. Whether the implicated strain persisted in the kitchen environment and caused the second outbreak or merely remained at high levels in feces of the food poisoning patients during their convalescence could not be determined from the available information. Some isolates from outbreak 9 agglutinated with as many as three antisera. The cultures were of the rough type and this may have affected the results obtained. Although we were able to serotype the isolates from all but one of the nine outbreaks, we concluded that routine serotyping of isolates as a means of confirming outbreaks may not be practical because of the work involved and the fact that complete serotyping can only be done in a few reference laboratories throughout the world.

Enterotoxin production. The predominant strains from all nine outbreaks produced CPE (Table 4). All of the normal hemolytic, heat-sensitive strains from the control and post-symptomatic specimens were negative for CPE by the ELISA. The few hemolytic isolates from specimens from outbreaks in which nonhemolytic strains predominated were also negative. All of the strains implicated in the outbreaks produced an amount of CPE $\geq$100 ng/ml, whereas strains from the normal control groups produced $<$1 ng/ml. The biological activity of the enterotoxin was not determined; however, we believe that a positive ELISA reaction is a reliable indication that the strains are able to produce CPE, because only those strains that were definitely associated with food poisoning were positive.

CONCLUSIONS

Our results support the conclusions of Sutton et al. (*18*) and others that enumeration of *C. perfringens* in the feces of food poisoning patients is a reliable means of confirming this organism as the cause of food poisoning outbreaks. Further, our results show that the determination may be confined to the enumeration of spores that survive heating for 20 min at 75°C, as recommended by Hauschild et al. (*9*). *C. perfringens* spore counts were greater with outbreak stools than with normal stools (Table 1); in addition, the predominant strains found in outbreak stools frequently differed from those present in normal stools by their inability to produce beta-hemolysis on TSB, the often greater heat resistance of their spores, and their ability to produce large amounts of CPE.

Characterization and enumeration of the strains present in the feces of food poisoning patients along with a screening technique for CPE, such as the ELISA procedure of Wimsatt et al. (*23*), would validate the involvement of specific *C. perfringens* strains and offer a more consistent picture of the role of other strains in food poisoning. The recent development of simpler methods of enumeration, such as the iron milk method of St. John et al. (*14*), and the likelihood that reagents for sensitive serological assays for CPE, such as ELISA, will soon be available commercially should enable state and municipal health departments, which have the primary responsibility for investigating outbreaks, to perform these tests on a routine basis. In the meantime, use of the fecal spore count with either TSB or TSC agar is recommended. This count, which requires only the most basic anaerobic culture apparatus, can be performed in most public health laboratories and appears to be more reliable than either serotyping or culturing of suspect foods for confirming outbreaks for two reasons: (a) some *C. perfringens* strains are not typable with presently available antisera (*6*) and (b) the viability of the organism declines rapidly in leftover foods stored at low temperatures (*5*).

Because food poisoning strains often differ from the heat-sensitive, hemolytic type A strains normally found in human feces, the hemolytic activity and relative heat-resistance of spores present in outbreak stools should also

be determined. This information would be helpful in interpreting results obtained with stool specimens from particular outbreaks and in defining the role of different *C. perfringens* biotypes in the etiology of foodborne disease.

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