Quantitation of *Clostridium perfringens* in Foods

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Received for publication October 30, 1961

ABSTRACT

ANGELOTTI, ROBERT (U. S. Department of Health, Education, and Welfare, Cincinnati), HERBERT E. HALL, MILTON J. FOTER, AND KEITH H. LEWIS. Quantitation of *Clostridium perfringens* in foods. Appl. Microbiol. **10**:193–199. 1962.—A procedure is described for identifying and enumerating *Clostridium perfringens* in foods by means of a simplified agar plating method, followed by confirmation of black colonies in tubes of motility-nitrate medium and sporulation broth. The test is routinely completed within 48 hr. Under experimental conditions, the procedure has been used to quantitatively recover various levels of *C. perfringens* contamination in a variety of foods and has recovered as few as ten *C. perfringens* per g without interference from food constituents and associated flora. Under practical conditions of field application, the method has been used to investigate five food-poisoning outbreaks, and *C. perfringens* was implicated as the etiological agent in two of these outbreaks.

Clostridium perfringens is recognized as the principal cause of gas gangrene in man and as the specific causal agent of lamb dysentery; lamb, sheep, and newborn calf enterotoxemia; "struck" in sheep; and an acute and fatal disease of very young piglets. However, outside of Great Britain, relatively little attention has been given to the possibility that this organism is also capable of causing gastroenteritis in man.

C. perfringens is one of the principal causes of foodborne illness in the British Isles, and practically all of the outbreaks described in the literature involve meat dishes. Usually the meat was cooked the day before serving and allowed to cool slowly, either in its own juices or separately. Apparently, the two factors of precooking and lack of immediate refrigeration allow the development of the extremely large numbers of *C. perfringens* associated with this type of food poisoning.

English studies (Knox and MacDonald, 1943; Duncan, 1944; Hobbs et al.; 1953; Dische and Elek, 1957) reveal *C. perfringens* food poisoning is caused by atypical type A strains of the organisms. They produce heat-resistant spores, produce relatively small amounts of the α-toxin, and rarely produce any θ-toxin. In the outbreaks reported by McClung (1945) and by Hart, Sherwood, and Wilson (1960) in the United States, the clinical picture resembled that of the English outbreaks, and strains of *C. perfringens*, type A, were isolated.

At the present time, only a few laboratories examine foods incriminated in foodborne disease outbreaks for *C. perfringens* because of the lack of quantitative methods and the difficulties associated with anaerobic cultivation. To facilitate examination of foods for *C. perfringens*, investigations have been in progress in this laboratory on the simplification of methods for identification and enumeration of this organism. A procedure has been developed and is presented below.

MATERIALS AND METHODS

Cultures. Included in this study were members of each of the six recognized types of *C. perfringens* (A through F) obtained from both the American Type Culture Collection and National Collection of Type Cultures (Great Britain). Also included were those strains most frequently encountered in food-poisoning outbreaks in England and designated as Hobbs' strains no. 1 through 11. In addition, 18 strains isolated from three food-poisoning outbreaks in the United States were employed.

Anaerobiosis. Anaerobic incubation of plates was accomplished in Case-Anaero jars[1] under an atmosphere of 90% nitrogen and 10% carbon dioxide.[2] Jars were exhausted by connecting them to a vacuum line sufficient to obtain a reading of from 20 to 26 in. of vacuum on a mercury manometer. After evacuation, they were filled to atmospheric pressure with the gas mixture, evacuated once more to flush out residual oxygen, and again filled with the gas mixture. To minimize airborne contamination, a cotton filter was placed in the connection between the tank of gas and the anaerobic jar. To serve as a check on the maintenance of anaerobiosis, a tube of methylene blue indicator solution (Fildes, 1931) was placed inside each jar before evacuation. Jars so treated were placed in a 37 C incubator and held for 24 hr, after which the plates were removed and counted.

Bacteriological media.

1) Sulfite-polymyxin-sulfadiazine (SPS) agar for enu-

[1] Case Laboratories, Inc., Chicago, Ill. The names of the manufacturers are given for materials solely for the purpose of identification and not as an endorsement thereof by the U. S. Public Health Service.

[2] These gases are obtained commercially as a mixture of purified gases from The Matheson Company, Inc., Joliet, Ill.

merating clostridia in food—The basal medium is prepared separately and contains 1.5% Bacto-Tryptone,[3] 1.0% Bacto-yeast extract, 1.5% Bacto agar, and 0.05% iron citrate. The medium is adjusted to pH 7.0 ± 0.1 and sterilized at 121 C for 15 min. To each liter of sterile medium, the following Seitz-filtered solutions are added: 5.0 ml of a freshly prepared 10% solution of sodium sulfite ($Na_2SO_3 \cdot 7H_2O$), 10.0 ml of a 0.1% solution of polymyxin B sulfate, and 10 ml of a solution of sodium sulfadiazine containing 12 mg per ml.

2) Motility-nitrate medium—Add 0.3% agar to Bacto-nitrate broth and distribute in screw-capped tubes (150 by 15 mm) filled to at least ⅔ of tube capacity. Autoclave for 15 min at 121 C.

3) Sporulation broth—Modified after medium described by Lund (1955-1956). The medium contains 2% Trypticase,[4] 2% vitamin-free Casamino acids,[4] 0.1% sodium thioglycolate. Dispense 15 ml per screw-capped tube (150 by 20 mm). Sterilize at 121 C for 15 min. After autoclaving, the reaction of the medium is pH 6.6. Just before use, add 0.1 ml of a sterile solution of thiamine HCl containing 150 μg per ml. A heavy inoculum is necessary and 2% by volume is adequate. Incubate for 16 to 24 hr in a 37 C water bath.

4) Noyes (*personal communication*, 1960) veal broth for toxin production—Contains 2% Bacto Proteose peptone no. 3, 0.5% NaCl, 0.5% Na_2HPO_4. Adjust to pH 7.3 before autoclaving and dispense 16 ml per screw-capped tube (150 by 20 mm) and add approximately 2 g of freshly ground veal to each. Autoclave at 121 C for 1 hr. Just before using medium, add aseptically 0.4 ml sterile 50% glucose solution per tube.

All other media employed were obtained as commercial products and prepared according to manufacturer's instructions, except when indicated in the text. All tubed media were steamed 10 min just before use to expel dissolved oxygen.

Foods. A variety of frozen foods and raw meats was obtained from local retail markets with no particular selection of brand names.

Preparation of microbial inocula and plate-counting procedures. Inocula for experiments in which yeasts were employed were prepared from 24-hr cultures incubated at room temperature in Fleishmann's Diamalt[5] broth (150 g per liter). Plate counts of yeasts were made in Diamalt agar (1.5% agar added) after 24 hr of incubation at room temperature.

Lactobacilli inocula were prepared from 24-hr-old BBL-Eugon broth[4] cultures incubated at 37 C. Plate counts of the lactobacilli were made in BBL-Eugon agar after 24 hr of incubation at 37 C under anaerobic conditions.

Inocula for experiments in which the remaining aerobic organisms were employed were prepared from 24-hr-old brain heart infusion broth cultures incubated at 37 C. Plate counts of these cultures were made in brain heart infusion agar after 24 hr of incubation at 37 C.

In those experiments requiring clostridia as an inoculum, the suspensions were prepared from either thioglycolate broth or cooked veal broth cultures incubated at 37 C for 24 hr. The ability of various media to recover *C. perfringens* from broth cultures was determined by making comparative plate counts in the following: brain heart infusion agar to which 0.1% sodium thioglycolate was added, Bacto-fluid thioglycolate medium to which 1.5% agar was added, Oxoid-reinforced clostridial agar medium (RCM), sulfite-polymyxin agar (Mossel, 1959), and SPS agar. The plates were incubated anaerobically as previously described.

Results

Sulfite-iron agar (a modified Wilson-Blair medium) has been proposed by several investigators for enumerating clostridia (Cameron, 1938; Thompson, 1939; Lyons and Owen, 1942; Prevot, 1948; Buttiaux et al., 1955). In this medium, clostridia reduce sulfite which is precipitated as iron sulfide, resulting in black colonies. Unfortunately, other organisms may yield the same black colony reaction. The organisms included in this group are salmonellae, *Proteus*, *Escherichia freundii*, *Paracolobactrum*, and certain species of the genera *Erwinia*, *Flavobacterium*, and *Achromobacter*. In addition to the formation of black colonies by these interfering groups, most of the facultative anaerobic bacteria grow well in this medium, and, when present in large numbers in the original sample, outgrow the clostridia and mask or interfere with black-colony formation.

Mossel et al. (1956) and Mossel (1959), reported on an iron-sulfite agar medium containing 0.05% sodium sulfite and 10 ppm polymyxin B sulfate, which yielded quantitative recovery of pure cultures of several species of clostridia in Miller-Prickett tubes. However, when mixed cultures were used, growth of enterococci, staphylococci, and aerobic bacilli occurred and black-colony formation by *Proteus*, *Salmonella*, and certain *Paracolobactrum* was observed.

An improvement of this medium was sought to yield quantitative recovery of clostridia from foods, as well as broth cultures, and to restrict black-colony formation to clostridia alone. Because of the inconvenience associated with isolating colonies from Miller-Prickett tube cultures, the development of a method employing conventional plate count techniques also was desirable. Galton, Lowery and Hardy (1954), reported on the successful addition of sulfadiazine to suppress growth of *Proteus*, *Pseudomonas*, and coliform organisms on brilliant green agar used as a counting medium for salmonellae. Because many of the sulfite-reducing bacteria comprising normal food flora are members of the *Enterobacteriaceae*, the addition of sulfadiazine to Mosel's sulfite-polymyxin agar appeared ap-

[3] Difco Laboratories, Inc., Detroit, Mich.

[4] Baltimore Biological Laboratory, Inc., Baltimore, Md.

[5] Fleishmann's Dry Diamalt, Dry Malt Syrup, Standard Brands, Inc., New York, N. Y.

propriate. Preliminary experiments, in which salmonellae, *Proteus*, *Pseudomonas*, and coliform organisms were plated in sulfite-polymyxin agar containing various concentrations of sodium sulfadiazine, revealed that the addition of 0.12 mg per ml was necessary to inhibit members of the *Enterobacteriaceae*. To establish that this concentration was not inhibitory to *C. perfringens*, comparative plate counts of broth cultures of 20 strains of this organism were made in the agar media previously described. (See last paragraph of Materials and Methods.) The results of these comparative counts are shown in Table 1, in which the percentage recovery of *C. perfringens* in the various media is compared to that of SPS agar taken as 100%. These data revealed that *C. perfringens* formed black colonies in SPS agar and that counts comparable to those obtained in sulfite-polymyxin agar (Mossel, 1959) were recorded. Additionally, in most instances, higher plate count values were obtained than those found in brain heart infusion, thioglycolate, and RCM agars.

Having established that the addition of 0.12 mg per ml of sulfadiazine to the basal medium did not affect adversely the quantitative recovery of *C. perfringens*, a number of experiments were conducted to determine whether the sulfadiazine would inhibit growth of staphylococci, enterococci, and aerobic bacilli, and, at the same time, eliminate black-colony formation by sulfite-reducing organisms other than *C. perfringens*. Comparative counts of broth cultures, containing between several million and 18 billion viable cells per ml, were made in suitable agar media and SPS agar of species of both sulfite-reducing and non-sulfite-reducing bacteria. Of the sulfite-reducing organisms tested (Table 2), only the clostridial species formed black colonies, and they were morphologically indistinguishable from the type of black colony produced by *C. perfringens*. Growth of *Paracolobactrum intermedium* was observed; however, black colonies did not develop. No growth of *Paracolobactrum arizonae* occurred. *Paracolobactrum aerogenoides* and *Paracolobactrum coliforme* were not tested because of their failure to produce black colonies in the basal medium lacking sulfadiazine (Mossel, 1959). Of the non-sulfite-reducing organisms tested (Table 3), the enterococci, *Streptococcus lactis*, *Bacillus cereus*, *Serratia marcescens*, and yeasts were only slightly inhibited. Growth of these organisms appeared as pinpoint white colonies. The coli-aerogenes organisms also grew as pinpoint white colonies; however, they were severely inhibited, and counts were obtainable only from those plates receiving 1.0 ml of undiluted broth culture.

Though growth of various clostridial species occurred in this medium with the formation of black colonies, the data indicated that black-colony formation was restricted to clostridia and that inhibition of many of the groups of organisms encountered as food flora was possible.

TABLE 1. *Recovery of Clostridium perfringens in various agar media*

Origin of cultures	Strain designation	Inoculum per ml (3×10^6 to 350×10^6 recovered in:*		
		Thioglycolate brain heart infusion agar	Thioglycolate agar	Oxoid reinforced clostridial medium
		%	%	%
Hobbs' type A British food poisoning	2	55	164	121
	6	115	104	90
	7	74	75	85
	8	118	90	130
	9	76	84	95
National Collection of Type Cultures (Great Britain)	Type B	65	94	91
	Type C	58	77	74
	Type D	49	84	76
	Type E	55	50	29
	Type F	179	50	38
American Type Culture Collection	Type A	46	78	57
	Type B	109	—	—
	Type C	29	—	—
	Type D	105	80	68
Isolated from food poisoning outbreaks in U. S.	B2	82	93	93
	B3	87	89	68
	B4	56	23	80
	B5	52	87	105
	B7	81	96	112
	MW	97	83	114

* Percentage recovery compared with that of SPS agar taken as 100%.

TABLE 2. *Comparative growth response of various sulfite-reducing bacteria in noninhibitory plating medium and SPS agar*

Species	No. of organisms per ml of 24-hr broth cultures determined by plating in:	
	Brain heart infusion agar	SPS agar
Salmonella enteritidis	4.1×10^8	0*
Salmonella gallinarum	18.8×10^8	0
Salmonella typhimurium	13.0×10^8	0
Salmonella choleraesuis	12.0×10^8	0
Proteus vulgaris	14.0×10^8	0
Proteus mirabilis	16.0×10^8	0
Proteus morganii	17.9×10^8	0
Proteus rettgeri	12.7×10^8	0
Escherichia freundii	5.8×10^8	0
Paracolobactrum intermedium	12.2×10^8	23.8×10^7†
Paracolobactrum arizonae	5.0×10^7	0
Erwinia nimipressuralis	24.8×10^8	0
Flavobacterium suaveolens	15.8×10^8	0
Archromobacter parvulus	7.6×10^7	0
Alcaligenes viscolactis	2.2×10^6	0
Clostridium bifermentans	8.6×10^7‡	4.7×10^7
Clostridium sporogenes	7.8×10^7	5.0×10^7
Clostridium tetani	5.3×10^8	4.6×10^8
Clostridium putrefaciens	1.4×10^7	2.9×10^7

* No growth from 1.0 ml of undiluted broth suspension.

† Did not form black colonies.

‡ Number per milliliter of broth culture determined by plating in thioglycolate-brain heart infusion agar and incubating anaerobically.

Because it is recognized that food constituents may affect the differential or selective properties of a medium (Galton et al., 1954; Silliker and Taylor, 1958; North, 1961), further tests were performed to determine the effects of various foods upon the reliability of SPS medium. The foods employed were frozen tuna, poultry, and beef pot pies, because of the diversity of ingredients available in a single composite form. Two 50-g portions of each of the frozen pies were removed and placed in separate, sterile Waring Blendor cups. One portion of each pie was homogenized in 450 ml of sterile phosphate dilution water (APHA, 1960) for 2 min at slow speed, and additional 10-fold dilutions were plated in plate count agar and SPS agar to determine the number of organisms per g present initially. To the second 50-g portion of each pie, 1.0 ml of a 24-hr broth culture of *C. perfringens* was added to yield an inoculum of approximately 5 to 30 $\times$ 10^5 organisms per g. Sterile dilution water was added to yield a 1 to 10 dilution, and the inoculated food homogenized as above. Additional 10-fold dilutions were plated in SPS agar. The results presented in Table 4 are typical of those obtained in these studies. Plate counts of the frozen foods before artificial contamination with *C. perfringens* revealed them to contain relatively few organisms as measured by aerobic or anaerobic incubation at 37 C. Black colonies were not observed on the SPS agar plates, although stained preparations of colonies revealed a number of morphological types to be present. Similar plates of the same foods with added *C. perfringens* developed black colonies, and the number per g coincided very closely with the number of *C. perfringens* added. These data indicated food constituents did not interfere with black colony formation by *C. perfringens* or with the ability of the medium to yield quantitative recovery of this organism.

In view of the ability of certain aerobic organisms to grow fairly well in SPS agar (Table 3), more rigorous test conditions were required to note the effect of high concentrations of these organisms in foods on quantitative recovery of *C. perfringens*. Accordingly, high, medium, and low concentrations of *C. perfringens* were mixed with relatively high concentrations of enterococci, *Bacillus* sp., and

TABLE 3. *Comparative growth response of various non-sulfite-reducing microorganisms in noninhibitory plating media and SPS agar*

Species	No. of organisms per ml of 24-hr broth cultures determined by plating in:	
	Brain heart infusion agar	SPS agar
Streptococcus faecalis	10.1×10^7	8.0×10^7
Streptococcus zymogenes	9.9×10^8	6.4×10^7
Streptococcus liquefaciens	2.7×10^8	1.0×10^8
Streptococcus durans	6.3×10^8	2.6×10^8
Streptococcus salivarius	3.5×10^8	0*
Streptococcus bovis	1.4×10^8	0
Streptococcus mitis	7.1×10^7	0
Streptococcus lactis	8.9×10^8	2.8×10^8
Streptococcus pyogenes	11.3×10^7	0
Staphylococcus aureus (196E)	2.4×10^8	0
S. aureus (Ms149)	4.1×10^7	0
S. aureus (80/81)	2.9×10^8	0
Serratia marcescens	8.4×10^8	4.9×10^8
Shigella sonnei	6.4×10^8	0
Shigella flexneri	8.0×10^7	0
Aerobacter aerogenes	9.3×10^8	26
A. aerogenes (K17)	7.8×10^8	129
Escherichia coli	11.0×10^8	17
E. coli (11229)	8.8×10^8	25
E. coli (K3)	11.3×10^8	15
Alcaligenes faecalis	1.3×10^8	0
Pseudomonas aeruginosa	3.5×10^8	0
Bacillus subtilis	1.1×10^7	0
Bacillus cereus	2.0×10^7	1.1×10^7
Lactobacillus casei	4.1×10^7†	0
Lactobacillus bulgaricus	4.4×10^7	0
Yeasts:		
Candida lipolytica	2.1×10^7‡	2.0×10^6
Pichia fermentens	3.2×10^7	1.4×10^7
Hansenula subpelliculosa	5.7×10^6	1.7×10^6

* No growth from 1.0 ml of undiluted broth suspension.

† Number of lactobacilli per ml of broth culture determined by plating in Eugon agar.

‡ Number of yeasts per ml of broth culture determined by plating in Diamalt agar.

TABLE 4. *Effect of various food constituents on selectivity of SPS agar and quantitative recovery of Clostridium perfringens*

Pot pies	Organism per g before inoculation		*C. perfringens* per g after inoculation		
	Aerobic count*	Anaerobic count†	Culture used	Added per g $\times$ 10^5	Recovered per g $\times$ 10^5
Tuna	185	30	Hobbs no. 7	28	14
Beef	520	0	Hobbs no. 8	5.4	6.6
Turkey	600	50	B_1	19	18
Chicken	1,080	590	B_2	14	12

* Morphological types: gram-positive and negative bacilli, streptococci, micrococci. (Growth on plate count agar.)

† Morphological types: gram-positive bacilli, streptococci. No black colonies. (Growth on SPS agar.)

TABLE 5. *Recovery of Clostridium perfringens from foods in the presence of other organisms*

Food	No. of miscellaneous organisms added per g*	No. of clostridia added per g†	No. of clostridia recovered per g†
	Enterococci		
Chow mein	3,250	15,500	15,000
	3,250	16	10
	Bacilli		
Chicken a la king	20,625	3,125	3,300
	20,625	31	30
	Serratia marcescens		
Tuna pot pie	27,000	8,900	7,800
	27,000	890	820

* Plate counts made with plate count agar.

† Plate counts made with SPS agar.

S. marcescens in various foods, employing procedures similar to those described in the previous experiment. In every instance, quantitative recovery of *C. perfringens* was obtained as determined by the number of black colonies developed in SPS agar (Table 5). In some trials, a 1,500:1 ratio of aerobic species to *C. perfringens* existed but did not affect quantitative recovery of the anaerobe.

The results of the experiments outlined above indicated that the formation of black colonies in SPS agar after 24 hr of anaerobic incubation at 37 C provided presumptive evidence for the presence of clostridial species. However, the problem of differentiating among black colonies produced by *C. perfringens* and other sulfite-reducing clostridial species was acute and reduced the effectiveness of the medium as a differential test for *C. perfringens*.

In reviewing *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957), it was determined that, of the 93 recognized species of clostridia, only 13 are nonmotile in addition to *C. perfringens*. Of these nonmotile species, all but one may be differentiated from *C. perfringens* on the basis of their inability to form black colonies in SPS agar or produce nitrite from nitrate. The exception is *Clostridium filiforme*, an extremely rare organism, apparently having been isolated but once (Debono, 1912), and for which the ability to produce nitrite and hydrogen sulfide has not been recorded.

In view of this information, it was possible to develop a differential test for identifying *C. perfringens*. A single tube test was developed which utilizes the characteristics of nonmotile growth and production of nitrite from nitrate as follows. A portion of black colony, developed in SPS agar, is stab inoculated into a tube of Bacto-nitrate broth containing 0.3% added agar. The inoculated tube is incubated in a 37 C water bath until visible growth appears (12 to 24 hr, depending on strain). Motile organisms produce diffuse turbidity throughout the tube, whereas nonmotile organisms form a discrete line of growth along the stab. The addition of 0.3% agar eliminates lines of growth at right angles to the stab, as sometimes occurs with nonmotile organisms in media containing less agar. Nonmotile cultures are tested for nitrite production in the same tube, as soon as growth is easily visible, by the addition of about 5 drops each of sulfanilic acid and dimethyl-α-naphthylamine reagent solutions. The development of a pink color denotes the presence of nitrites and is considered a positive test for nitrate reduction.

It appeared desirable also to demonstrate spore production by cultures isolated from SPS agar plates. Therefore, the ability of the test strains to sporulate in a number of sporulation broths was determined (Ellner, 1956; Zoha-Sadoff, 1958; Sames, 1961, *personal communication*). The modification of Lund's medium (1955-1956), described above, generally yielded the highest sporulation rate. Spore production may be determined by inoculating a por-

TABLE 6. *Examination of foods involved in outbreaks of gastroenteritis for Clostridium perfringens employing SPS agar, motility-nitrate medium, and sporulation broth*

Outbreak	Probable etiological agent of outbreak	Foods examined	Total count per g (aerobic)	Predominant aerobic flora	No. of black colonies/g developed in SPS agar	Characteristics of black colonies developed in SPS agar			*Clostridium* isolated
						NO_2 production	Motile	Spore production	
A	*C. perfringens*	Roast beef	100,000	Enterococci	20,000	+	−	+	*C. perfringens*
		Green beens	2,700	Enterococci	0				
		Potatoes	90,000	Enterococci	0				
		Fruit cup	3,200	Enterococci	0				
		Apple pie	60	Staphylococci	0				
		Blue cheese dressing	3,800	Enterococci	0				
B	*C. perfringens*	Chicken salad	40,500	Coliform organisms	560,000	+	−	+	*C. perfringens*
		Mayonnaise	No growth						
		Pickles	No growth						
C	Unknown	Three cans of dried beef	23,500,000 to 68,000,000	*Bacillus stearothermophilus*	2 to 10	+	−	+	*C. perfringens*
D	Unknown	Nine ham sandwiches	41,000,000 to 129,000,000	Staphylococci*	0				
E	Unknown	Shrimp salad	610,000	Coliform organisms	2,800	−	+	+	*C. bifermentans*

* All isolates tested were coagulase negative.

tion of a black colony into a tube of thioglycolate broth and incubating in a 37 C water bath for 4 hr. The 4-hr thioglycolate culture is used as an inoculum into sporulation broth. In the latter, the majority of *C. perfringens* strains tested to date produces sufficient numbers of spores after 12 to 24 hr of incubation to be easily observed by spore stain or by testing for survivors after heating an aliquot of sporulation broth for 10 min at 80 C and inoculating the aliquot into fluid thioglycolate broth incubated at 37 C for 24 hr. Growth consisting of gram-positive rods indicates the existence of spores in the original heated aliquot.

Since the development of this method, few opportunities have arisen to evaluate the ability of the procedure to identify and enumerate *C. perfringens* in food-poisoning outbreaks. However, to date, food samples from five separate outbreaks of gastroenteritis have been examined (Table 6). *C. perfringens* was isolated and enumerated in foods from three of these outbreaks, and the large concentration recovered from foods in two of the outbreaks helped establish the etiological agent as *C. perfringens*. In one instance (outbreak C), a very low level of from 2 to 10 *C. perfringens* per g was recovered from a canned dried beef product containing several million *Bacillus stearothermophilus* per g. In outbreak E, large numbers of sulfite-reducing clostridia were observed on SPS agar plates; however, subsequent testing in motility-nitrate medium quickly revealed they were not *C. perfringens*. Additional tests identified these organisms as *Clostridium bifermentans*. However, this instance re-emphasized the need to test black colonies in motility-nitrate medium before assuming them to be *C. perfringens*. It also highlighted an inherent disadvantage of the medium and one which requires recognition in order that results be properly interpreted. Should *C. perfringens* be present in food as a mixture with other sulfite-reducing clostridia, dilution plates of SPS agar will contain black colonies of both groups. To establish the concentration of *C. perfringens* per g, a large number of black colonies necessarily will have to be tested in nitrate-motility medium to establish the ratio of *C. perfringens* to the remaining clostridial species. This procedure may be compared to that employed in determining the number of coagulase-positive staphylococci in a food, wherein one picks a representative number of colonies for determining coagulase production.

It should be recognized also that a situation may arise in which *C. perfringens* represents only a minor portion of the total sulfite-reducing clostridial contamination. Under such a circumstance, the highest dilution yielding black colonies on plates may be beyond the concentration range of *C. perfringens* present originally, and the latter will remain undetected.

Discussion

C. perfringens has been recognized in Great Britain for a number of years as a common cause of food poisoning, and weakly toxigenic strains of type A are usually involved as contaminants of meat dishes. With the exception of the report of McClung (1945), *C. perfringens* generally has been overlooked in this country. Epidemiological reports of gastroenteritis associated with *C. perfringens* contamination of food were received for the first time by the U. S Public Health Service in 1959 (Dauer and Davids, 1959). The experience of the British reveals this type of food poisoning to be more prevalent than the available American statistics indicate. The 1959 "Summary of Disease Outbreaks" (Dauer and Davids, 1959) reports 75 outbreaks of foodborne disease, affecting more than 1,200 persons, in which poultry or other meat was the vehicle of infection, but for which no etiological agent was identified. The July 21, 1961, issue of *Mortality and Morbidity Weekly Reports* (1961) presents a summarization of 25 reported food-poisoning outbreaks of unknown etiology which occurred in the first 5 months of 1961 and affected over 513 individuals. A meat product was invariably involved and associated with establishments preparing food on a mass basis. The usual incubation period was 6 to 24 hr, and the epidemiological characteristics described for many of the outbreaks were those typical of *C. perfringens* gastroenteritis. Undoubtedly, some of these outbreaks were of *C. perfringens* etiology, but went unrecognized because of the basic limitation of methodology for routine detection and identification of anaerobic bacteria.

A simplified method is presented which permits quantitation of *C. perfringens* in food. A critical evaluation of the techniques in laboratories concerned with performing bacteriological examination of foods is required to establish the utility and accuracy of the procedure under practical conditions of application. In any event, the technique has the advantage of permitting quantitation of *C. perfringens* in foods, rather than merely indicating the presence of the organism, as do currently available methods. Pre-enrichment is eliminated, due to the inhibition of most of the food microflora associated with *C. perfringens*, and because the primary plating medium will recover quantitatively extremely low concentrations of the organism. In addition, the method of obtaining anaerobiosis eliminates the necessity for employing combustible gasses or the disorder attendant with providing anaerobic conditions by chemical or biological procedures. The use of motility-nitrate medium provides a rapid one-step procedure for screening black colonies and differentiating *C. perfringens* from other sulfite-reducing clostridia.

The technique also has associated with it certain disadvantages which require recognition to permit proper interpretation of results. The major area of concern is that SPS agar supports growth and black-colony formation not only by *C. perfringens*, but also by other sulfite-reducing clostridia. This necessitates the picking of several such colonies from each countable plate to insure that a representative sampling is obtained. Each colony must then be tested in motility-nitrate medium to obtain the

ratio of *C. perfringens* to other clostridia. In the event that several foods are under examination simultaneously, this may be a laborious procedure. Secondly, in order to quantitate, one necessarily selects for testing in motility-nitrate medium the highest dilution plate containing between 30 and 300 black colonies. Should *C. perfringens* represent a minor portion of the total sulfite-reducing clostridial contamination, it may remain undetected because of the dilution factor.

Data collected on *C. perfringens* food-poisoning incidents which occurred in Great Britain and the United States indicate that, in most instances, the predominant flora of the incriminated food was *C. perfringens*, that it was present in high concentrations, and that the presence simultaneously of other *Clostridium* species is rarely, if ever, encountered. Under such circumstances, the identification and enumeration of *C. perfringens* may be easily achieved by the procedure described in this paper. However, for the reasons discussed above, caution must be used when applying the method to enumerate *C. perfringens* in foods not involved in food-poisoning outbreaks.

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