

Enumeration of Food-Borne *Clostridium perfringens* in Egg Yolk-Free Tryptose-Sulfite-Cycloserine Agar

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The SFP (Shahidi-Ferguson perfringens), TSC (tryptose-sulfite-cycloserine), EY (egg yolk)-free TSC, and OPSP (oleandomycin-polymyxin-sulfadiazine perfringens) agars have been tested for their suitability to enumerate *Clostridium perfringens* in naturally contaminated foods. Complete recoveries of *C. perfringens* were obtained in each of the four media, but only the TSC and EY-free TSC agars were sufficiently selective to ensure subsequent confirmatory tests without interference from facultative anaerobes. Because of some disadvantages associated with the use of egg yolk, EY-free TSC agar is recommended for enumeration of *C. perfringens* in foods. Several conditions for convenient shipment of foods and *C. perfringens* isolates with minimum loss of viability have been tested. The highest viable counts were preserved when foods were mixed 1:1 (wt/vol) with 20% glycerol and kept in a container with dry ice. Isolated *C. perfringens* strains remained viable for at least 2 weeks at ambient temperatures on blood agar slopes with a 2% agar overlay in screw-cap culture tubes.

The presumptive enumeration of *Clostridium perfringens* is commonly based on the reduction of sulfite and the hydrolysis of lecithin in egg yolk media (10, 11, 22). In a recent publication (14), we listed a number of disadvantages associated with the use of egg yolk and showed that these could be overcome by a modified method. The method involves presumptive enumeration in pour plates with egg yolk (EY)-free agar containing 0.04% D-cycloserine (D-CS), tentatively designated EY-free TSC (tryptose-sulfite-cycloserine) agar, and stab-culturing black colonies in nitrate motility (NM) agar supplemented with glycerol and galactose. All of 71 strains of *C. perfringens* tested were recovered quantitatively in EY-free TSC agar, and all reduced nitrate to nitrite in supplemented NM agar.

The present work was undertaken (i) to determine the applicability of EY-free TSC agar for the enumeration of *C. perfringens* in foods, and (ii) to evaluate conditions for the transport of foods and of *C. perfringens* isolates without undue loss of viability.

MATERIALS AND METHODS

Foods. Foods were purchased at local food service and retail establishments and abused to simulate conditions that have led to food-poisoning outbreaks in the past (2, 5, 23); i.e., they were handled with naturally soiled cutlery and incubated at temperatures conducive to growth of *C. perfringens* (Table 1).

For incubation, the barbecued foods and roasts were wrapped in thin plastic film (saran) and enclosed in aluminum-lined food bags; the meat pie was kept in the original packaging (aluminum tray covered with plastic film); the other products were placed in sidearm flasks and deaerated with a water aspirator for 10 min. After incubation, food samples of 10 to 20 g were homogenized in a Waring blender with 0.1% peptone (9 ml per g of food) for 2 min at high speed. Ten-fold dilutions were made immediately with 0.1% peptone.

Enumeration procedures. The following media were used for presumptive enumeration of *C. perfringens*: SFP (Shahidi-Ferguson perfringens) agar (22), TSC agar (11), EY-free TSC agar (14), and OPSP (oleandomycin-polymyxin-sulfadiazine perfringens) agar (9). They were prepared as described before (14). Volumes of 0.1 ml of diluted sample were spread on the surface of SFP and TSC agars, which were then poured over with 10 ml of cover agar (22); the other two media were used in pour plates with 1.0 ml of diluted sample per plate. All plates were incubated anaerobically at 37 C for 20 h.

For enumeration of *C. perfringens* spores, 10-ml samples of food homogenate at dilution 10^{-1} in screw-cap test tubes (16 by 150 mm) were incubated for 20 min in a water bath at 75 C and cooled immediately in ice water.

Confirmatory tests. Five or 10 presumptive *C. perfringens* colonies from each enumeration agar per food sample were stab inoculated into supplemented NM agar (14). For additional confirmation, colonies derived from food samples N to T (Table 1) and enumerated in EY-free TSC agar were also inoculated into lactose motility (LM) agar (22) and lactose

TABLE 1. Foods for enumeration of *C. perfringens*

Designation	Food	Incubation	Smell after incubation
A	Barbecued chicken	18 h at 22 C	Wholesome
B	Barbecued chicken	18 h at 30 C	Wholesome
C	Chicken hash	18 h at 30 C	"Off"
D	Beef roast	18 h at 35 C	Putrid
E	Beef in gravy	18 h at 20 C plus 4.5 h at 46 C	Wholesome
F	Chicken chow mein	18 h at 20 C plus 4.5 h at 46 C	Wholesome
G	Meat pie	18 h at 20 C plus 4.5 h at 46 C	Wholesome
H	Meat pie in gravy	18 h at 20 C plus 4.5 h at 46 C	Wholesome
I	Beef in gravy	18 h at 22 C	Wholesome
J	Turkey in gravy	18 h at 30 C	Wholesome
K	Beef in gravy	18 h at 30 C	"Off"
L	Turkey in gravy	18 h at 20 C plus 4.5 h at 46 C	Wholesome
M	Hungarian goulash	18 h at 20 C plus 4.5 h at 46 C	Wholesome
N	Barbecued chicken	18 h at 30 C	"Off"
O	Turkey in gravy	18 h at 30 C	Wholesome
P	Barbecued pork	18 h at 30 C	"Off"
Q	Turkey in gravy	18 h at 20 C plus 4.5 h at 46 C	Putrid
R	Beef roast	18 h at 20 C plus 4.5 h at 46 C	"Off"
S	Beef in gravy	18 h at 20 C plus 4.5 h at 46 C	"Off"
T	Pork in gravy	18 h at 20 C plus 4.5 h at 46 C	Wholesome

gelatin medium. The latter had the composition of LM agar, except that the 0.3% agar (Difco) was replaced with 12% gelatin (Difco). All tubes were incubated at 37 C for 20 h. Nitrite formation was determined by the method of Angelotti et al. (1). Liquefaction of gelatin was also recorded after 44 h.

Hemolysis. Five *C. perfringens* isolates from each of the food samples A to M were selected at random and streaked on sheep blood agar plates (Mogul Diagnostics, Madison, Wis.). Hemolysis was recorded after 24 h of anaerobic incubation at 37 C.

Viability of *C. perfringens* in foods during storage. Food samples were finely chopped, mixed, distributed in amounts of about 10 g into tared 24-ml screw-cap vials, and weighed. The vials were stored at 4 and -18 C, and in a container with dry ice placed in a freezing compartment at -27 C to reduce sublimation of CO₂. Within the dry-ice container, the vials were kept in small boxes with inside temperatures between -55 and -60 C, depending on the amount of dry ice. Some food samples were mixed 1:1 (wt/vol) with 20% glycerol (20) before storage. Viable counts were made in EY-free TSC agar after 0, 1, 2, 3, 4, 5, and 8 weeks of storage.

Viability of *C. perfringens* on blood agar. Sixteen of the *C. perfringens* strains listed previously (14) were grown at 37 C for 20 h in 15 ml of cooked meat medium contained in screw-cap test tubes (20 by 150 mm). Each strain was transferred to 20 replicate blood agar slopes (Difco blood agar base with 5% defibrinated sheep blood) in screw-cap test tubes (16 by 150 mm). These were incubated at 37 C for 20 h. Ten slopes per strain were then poured over and completely covered with 2% agar (Difco) in distilled water. For each strain, groups of five identically treated tubes were stored at 4 C and at ambient temperature (21 to 24 C) for 1 to 8 weeks. After each storage period, single transfers were made from one

tube of each group to cooked meat medium. Growth of *C. perfringens* at 37 C was recorded after 1 to 3 days.

RESULTS

Comparison of SFP, TSC, and EY-free TSC agars. Table 2 shows the *C. perfringens* counts for 13 foods (A to M). The data obtained with each of the three enumeration agars were almost identical. The *C. perfringens* counts for samples B, I, J, and L in SFP agar represent maximum counts because the large numbers of facultative anaerobes interfered in the confirmatory nitrite motility test. To ascertain that the recoveries were complete, we included SFP agar (Table 2) and EY-free TSC agar (not shown) without antibiotics for foods A to H. Although most of these data represented maximum counts, they did not exceed those of the three selective media.

In contrast to experiments with known strains (14), the *C. perfringens* colonies from nearly all the 13 foods produced opaque halos in both egg yolk media (SFP and TSC). Only 25 to 50% of the *C. perfringens* colonies from sample L had no discernible halos in either of the two media. In SFP agar, the egg yolk reaction of *C. perfringens* from sample B was completely masked by the large numbers of egg yolk-positive facultative anaerobes (Table 2).

In both TSC agars (with or without egg yolk), the nonspecific counts (total counts minus confirmed *C. perfringens* counts) were either below the *C. perfringens* counts or were of the same order. In contrast, the nonspecific counts for

TABLE 2. Enumeration of *C. perfringens* from various foods in SFP, TSC, and EY-free TSC agars

Food	Organism	Count (cells/g)			
		SFP	TSC	EY-free TSC	Antibiotic-free SFP
A	<i>C. perfringens</i>	5.5×10^5	7.8×10^5	10.8×10^5	7.0×10^5
	Nonspecific	$<10^5$	$<10^5$	$<10^5$	$<10^5$
B	<i>C. perfringens</i>	1.4×10^4	1.3×10^4	1.3×10^4	1.9×10^4
	Nonspecific	10^7-10^8 ^a	10^3-10^4	10^3-10^4	10^7-10^8 ^b
C	<i>C. perfringens</i>	7.4×10^7	6.3×10^7	8.8×10^7	8.3×10^7
	Nonspecific	$<10^5$	$<10^5$	$<10^5$	10^6-10^7
D	<i>C. perfringens</i>	1.2×10^8	1.2×10^7	1.2×10^8	1.1×10^8
	Nonspecific	$<10^5$	$<10^5$	$<10^5$	10^6-10^7
E	<i>C. perfringens</i>	4.7×10^8	5.4×10^8	5.4×10^8	5.2×10^8
	Nonspecific	$<10^4$	$<10^4$	$<10^4$	$<10^4$
F	<i>C. perfringens</i>	3.0×10^7	1.4×10^7	3.2×10^7	4.0×10^7
	Nonspecific	$<10^4$	$<10^4$	$<10^4$	10^7-10^8
G	<i>C. perfringens</i>	7.3×10^8	7.4×10^8	8.3×10^8	7.5×10^8
	Nonspecific	$<10^4$	$<10^4$	$<10^4$	$<10^4$
H	<i>C. perfringens</i>	1.0×10^8	1.2×10^8	1.5×10^8	1.4×10^8
	Nonspecific	10^8-10^9	$<10^5$	$<10^5$	10^8-10^9
I	<i>C. perfringens</i>	1.8×10^4	1.0×10^4	1.4×10^4	ND ^c
	Nonspecific	10^7-10^8	$<10^3$	$<10^3$	
J	<i>C. perfringens</i>	1.8×10^7	1.9×10^7	2.6×10^7	ND
	Nonspecific	10^8-10^9	10^7-10^8	10^7-10^8	
K	<i>C. perfringens</i>	6.8×10^7	5.0×10^7	6.2×10^7	ND
	Nonspecific	10^8-10^9	$<10^5$	$<10^5$	
L	<i>C. perfringens</i>	3.2×10^8	2.1×10^8	4.2×10^8	ND
	Nonspecific	10^7-10^8 ^d	$<10^5$	$<10^5$	
M	<i>C. perfringens</i>	3.4×10^5	3.5×10^5	4.3×10^5	ND
	Nonspecific	$<10^3$	$<10^3$	$<10^3$	

^a 2.4×10^6 produced opaque halos.

^b 3.5×10^6 produced opaque halos.

^c ND, Not determined.

^d 3.0×10^6 produced opaque halos.

some foods in SFP agar exceeded the *C. perfringens* counts by several logs. For samples B and H, the nonspecific counts were essentially as high in SFP agar as in the same agar without antibiotics (Table 2).

Since we counted on possible inhibition of *C. perfringens* in media with 400 μ g of D-CS/ml, we also included EY-free TSC agar with 300 μ g of D-CS/ml in each experiment (not shown). At both D-CS concentrations, the *C. perfringens* and nonspecific counts were essentially the same, except for sample L where the nonspecific black-colony count was significantly higher at 300 μ g of D-CS/ml.

Hemolysis. After enumeration in EY-free TSC agar and confirmation as *C. perfringens* by the nitrite motility test, five isolates per food were characterized further by hemolysis on sheep blood agar. Within each of 9 out of 13 groups, the 5 isolates all showed identical hemolytic patterns: they were all beta-hemolytic in 6 groups and partially hemolytic in 3 groups. Within each of these groups, the nitrite reac-

tions were of similar intensities, but they varied between different groups. The isolates from the remaining 4 groups each showed two distinct hemolytic patterns.

Viability of *C. perfringens* during storage.

Table 3 shows the loss of viable *C. perfringens* in a number of foods after 1 week of storage at different temperatures. At 4 C, the log reduction varied from <0.2 (no detectable loss) to >3 . Comparable losses occurred at -18 C. In dry ice, the losses were considerably lower.

Preliminary experiments indicated that the losses could be reduced further by storing the foods in 10% glycerol. This is confirmed by the results in Table 4, which show that the smallest loss in viable counts occurred when the foods were mixed 1:1 (wt/vol) with 20% glycerol and kept in dry ice. The log decreases in viable counts, plotted against storage time from 0 to 8 weeks, approached straight lines.

We also tested the suitability of the common practice of shipping isolated *C. perfringens* strains on blood agar slopes. Table 5 shows that

TABLE 3. Loss of viable *C. perfringens* in foods stored at different temperatures

Food	Log ₁₀ reduction in counts after 1 week		
	4 C ^a	-18 C	-55 to -60 C ^b
A	>3.0	ND ^c	ND
B	2.6	ND	ND
C	<0.2 ^d	ND	ND
E	3.4	ND	ND
F	<0.2	ND	ND
G	3.0	1.9	0.3
H	0.4	0.6	<0.2
J	<0.2	1.0	<0.2
K	0.8	0.4	<0.2
L	2.8	2.3	0.9

^a Storage temperature.^b Stored in a dry-ice container.^c ND, Not determined.^d <0.2, No detectable loss.TABLE 4. Loss of viable *C. perfringens* in foods stored in the absence or presence of glycerol

Food	Log ₁₀ reduction in counts after 4 weeks			
	4 C ^a		-55 to -60 C	
	No ^b	Yes ^c	No	Yes
N	3.0	0.6	<0.2	<0.2
O	ND ^d	ND	2.6	1.1
P	1.7	1.0	<0.2	<0.2
Q	1.9	0.8	0.4	0.4
R	1.8	1.3	0.6	<0.2
S	4.8	1.6	0.5	<0.2
T	2.0	1.6	0.6	0.6

^a Storage temperature.^b Glycerol not added.^c Glycerol added.^d ND, Not determined.TABLE 5. Viability of *C. perfringens* on blood agar slopes with or without agar overlay^a

Slope	No. of viable strains				
	1 ^b	2	3	5	8
Slopes without overlay	16	14	12	8	7
Slopes with overlay	16	16	15	15	12

^a Storage temperature was 21 to 24 C; 16 strains were tested.^b Storage time in weeks.

the loss of viable strains at ambient temperatures could be delayed by an overlay of 2% agar. No strains were lost during 8 weeks of storage at 4 C, with or without agar overlay.

Comparison of OPSP and EY-free TSC

agars. To evaluate the suitability of the recently described OPSP agar (9) for enumeration of *C. perfringens* in foods, this medium was included in our viability studies. Table 6 compares the enumeration data obtained in EY-free TSC and OPSP agars for six foods that had been stored for 4 weeks in 10% glycerol at 4 C. The *C. perfringens* counts were essentially the same in the two agars, but the nonspecific counts were consistently higher in OPSP agar. Essentially the same results were obtained with foods stored at 4 C without glycerol (not shown).

Confirmatory tests. Most of the black colonies derived from foods A to T and transferred to supplemented NM agar were confirmed as *C. perfringens* (1). However, plates of food samples J and L had black colonies that differed in appearance from the "typical" *C. perfringens* colonies; they could be counted and transferred separately and were all motile and negative for nitrite.

Isolates derived from foods N to T were also tested in LM agar and lactose gelatin. All of 63 isolates identified as *C. perfringens* by the nitrite motility test fermented lactose rapidly and liquefied gelatin.

Spores. The tests for *C. perfringens* spores in food samples N to T were all negative (<10 spores/g). Clostridial spores (4×10^3 /g) were encountered in sample N, but these were all motile and did not produce nitrite.

DISCUSSION

In each of the four enumeration agars (SFP, TSC, EY-free TSC, and OPSP), we obtained quantitative recoveries of *C. perfringens* for

TABLE 6. Enumeration of *C. perfringens* from various foods in EY-free TSC and OPSP agars^a

Food	Organism	Count (cells/g)	
		EY-free TSC	OPSP
N	<i>C. perfringens</i>	5.6×10^6	5.7×10^6
	Nonspecific	10^7-10^8	$>10^8$
P	<i>C. perfringens</i>	2.6×10^2	2.8×10^2
	Nonspecific	10^6-10^7	$>10^7$
Q	<i>C. perfringens</i>	3.8×10^7	3.4×10^7
	Nonspecific	$<10^6$	$>10^6$
R	<i>C. perfringens</i>	9.0×10^7	8.2×10^7
	Nonspecific	$<10^6$	$>10^6$
S	<i>C. perfringens</i>	6.4×10^6	6.1×10^6
	Nonspecific	$<10^6$	$>10^6$
T	<i>C. perfringens</i>	2.8×10^4	1.5×10^4
	Nonspecific	10^4-10^5	$>10^6$

^a Foods listed in Tables 1 and 4, enumerated after 4 weeks of storage in 10% glycerol at 4 C.

each of the foods tested. Previous results with known *C. perfringens* strains were similar, except that the recoveries of some strains in OPSP agar were low (14). The facultative anaerobes were kept to relatively low numbers in both TSC agars but were much less inhibited in the SFP and OPSP agars. We previously listed a number of disadvantages associated with egg yolk agars for enumeration of *C. perfringens*. Since both the specific and nonspecific counts were the same for the two TSC agars, it appears that of the four media tested, the EY-free TSC agar is the most suitable one for enumeration of *C. perfringens* in foods.

In 9 out of 13 foods, each of 5 *C. perfringens* isolates had the same hemolytic pattern. Although we did not determine the serotypes, this finding is consistent with reports of food-poisoning outbreaks (8, 16, 23) in which usually only one serotype of *C. perfringens* could be isolated from each incriminated food. Foods A and B (Table 1) were halves of the same chicken, yet the five isolates from A were all beta-hemolytic, whereas those from B were partially hemolytic. This suggests that, of the factors determining which of the contaminating *C. perfringens* strains becomes predominant in a given food, the temperature is of particular importance.

Duncan et al. (6) demonstrated that the synthesis of enterotoxin is associated with spore formation. Spores and enterotoxin, along with large numbers of vegetative cells, have been demonstrated in certain cooked meats (4, 12, 18). For some of these we have no information regarding their organoleptic qualities (18), but the others had offensive smells (12; F. M. Dework, personal communication). In contrast, cooked meats that are likely to be consumed seem to have either no or very few spores of *C. perfringens* (12, 15). Spores were also absent in contaminated foods tested in this work, although large numbers of *C. perfringens* cells were formed and some products were no longer palatable. These data are in accord with the concept (13) that the enterotoxin responsible for *C. perfringens* type A food poisoning is produced in vivo and that incriminated foods do not have to be assayed for the toxin in routine investigations of food-poisoning outbreaks.

Investigators of *C. perfringens* outbreaks are often faced with the question of how to transport and store incriminated food samples and *C. perfringens* isolates, both without undue loss of viability (7). This work suggests that food samples may be suitably transported in mixture 1:1 (wt/vol) with 20% glycerol in a dry-ice container and stored in the same way or in a freezer at -60 C. Isolated strains may be trans-

ported at ambient temperatures on blood agar slopes with a 2% agar overlay in screw-cap test tubes.

As stated by Angelotti et al. (1), the only clostridial species listed in *Bergey's Manual* (7th ed., 1957) that is nonmotile and produces sulfide and nitrite is *C. perfringens*, with the possible exception of *C. filiforme*, which was isolated only in 1912 (3) and is no longer viable in its isolated form. However, other clostridia with these three characteristics have been described: the unofficial species designated *C. barati*, *C. perenne* (17), *Inflabilis barati*, *I. indolicus*, and *I. lacustris* (21), and recent isolates from pheasant intestine (19). Some of these are likely to be identical. We recently isolated a clostridium with the same three properties from fecal specimens (manuscript to be published) that is distinctly different from *C. perfringens* and the clostridia listed above. These, as well as our fecal isolate and *C. filiforme*, may all be distinguished from *C. perfringens* by their inability to liquefy gelatin. According to Prévot (21), *I. indolicus* and *I. lacustris* may liquefy gelatin after prolonged incubation, but we found no liquefaction in lactose gelatin after 1 week of incubation with strains received from his laboratory, whereas *C. perfringens* strains liquefied gelatin consistently within 40 h, usually within 24 h, in this medium. In this work, all our nonmotile, sulfide, and nitrite-producing food isolates that were tested for liquefaction of gelatin were reconfirmed as *C. perfringens*.

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