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Enumeration of Fecal *Clostridium perfringens* Spores in Egg Yolk-Free Tryptose-Sulfite-Cycloserine Agar

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The Shahidi-Ferguson perfringens, tryptose-sulfite-cycloserine (TSC), and egg yolk-free TSC agars have been tested for their suitability to enumerate fecal spores of *Clostridium perfringens*. When these spores comprised at least 20% of the total anaerobe spores, equally accurate counts were obtained in the three media. With lower ratios of *C. perfringens* spores, the most accurate counts were obtained in egg yolk-free TSC agar. The median *C. perfringens* spore count of 60 normal fecal specimens was log 3.4/g. A nonmotile, sulfite- and nitrate-reducing *Clostridium*, not identifiable with any known clostridial species, was isolated from 14 out of 60 fecal specimens. It was not differentiated from *C. perfringens* in the nitrite motility test, but could be distinguished by its inability to liquefy gelatin.

In a preceding communication (11) we recommended a modified method for presumptive enumeration of food-borne *Clostridium perfringens*. The present work was undertaken to determine the applicability of this method for the enumeration of *C. perfringens* spores in feces.

MATERIALS AND METHODS

Fecal samples. The specimens, collected in clean styrofoam cups, were provided by 60 healthy laboratory and office employees.

Enumeration of C. perfringens spores. Fecal samples of 1.5 g were weighed into screw-cap test tubes (16 by 150 mm) and homogenized in 13.5 ml of 0.1% peptone with a Vortex mixer. For spore activation and destruction of the vegetative cells, the tubes were kept in a water bath at 75 C for 20 min, cooled in ice water, and diluted with 0.1% peptone. The enumeration procedures in Shahidi-Ferguson perfringens (SFP), tryptose-sulfite-cycloserine (TSC), and egg yolk (EY)-free TSC agars have been described (10, 11). Heat-resistant spores were enumerated in the same way, except that the 10^{-1} dilutions of fecal homogenates were kept at 100 C for 30 min (7).

Confirmatory tests. Five or ten presumptive *C. perfringens* colonies from each enumeration agar were inoculated into supplemented nitrate motility (NM) agar (10), lactose motility (LM) agar (16), and lactose gelatin (11). For samples J to M (Table 2), the number of isolates was increased to 20. The tubes were incubated at 37 C for 20 to 24 h; liquefaction of gelatin was also recorded after 44 h. Nitrite was determined according to Angelotti et al. (2).

Hemolysis and lecithinase activity. Clostridial isolates were streaked on blood agar base containing 5% of defibrinated human, bovine, sheep, and rabbit blood, and on antibiotic-free SFP agar. Hemolysis and lecithinase activity were recorded after 24 to 48 h of anaerobic incubation at 37 C.

Lysozyme treatment. EY-free TSC agar plates were prepared as described (10, 11), except that each plate contained 0.1 ml of 0.06% lysozyme (Sigma Chemical Co., St. Louis) before the agar was poured. The final lysozyme concentration was approximately 3 μg/ml.

RESULTS

Fifteen fecal samples (A to O) were divided into three groups, depending on which of the nonmotile, nitrite-producing clostridial species had the largest number of spores. In the first group (A to I; Table 1), the spores of *C. perfringens* predominated; in the second group

TABLE 1. *Enumeration of C. perfringens spores from normal feces*

Fecal sample	*C. perfringens* counts (spores/g)		
	SFP	TSC	EY-free TSC
A	4.8×10^5	6.0×10^5	7.7×10^5
B	1.4×10^5	2.8×10^4	1.1×10^5
C	5.2×10^6	5.8×10^6	6.0×10^6
D	1.4×10^6	1.0×10^6	2.2×10^6
E	6.0×10^{4a}	2.7×10^4	3.8×10^4
F	5.8×10^4	6.8×10^4	8.1×10^4
G	1.8×10^5	1.6×10^5	2.1×10^5
H	4.0×10^{5a}	3.2×10^5	2.7×10^5
I	1.0×10^{4a}	1.1×10^{4a}	2.0×10^4

[a] Comprising 20 to 40% of the total spore count.

(J to M; Table 2), these were exceeded by spores of an unidentified species (*Clostridium* sp.). Samples N and O had $<10^2$ spores of either species.

Table 1 shows the *C. perfringens* spore counts for samples A to I in SFP, TSC, and EY-free TSC agars. For each sample, they were of the same order, except for a low count of sample B in TSC agar. Counts in the corresponding media without antibiotics (not shown) were of the same order. With few exceptions (footnote *a*), the *C. perfringens* spores comprised at least 50% of the total black-colony count.

For additional confirmation of *C. perfringens*, colonies from EY-free TSC agar were also transferred to LM agar and lactose gelatin. Each of the colonies confirmed as *C. perfringens* in supplemented NM agar was found to produce acid and gas and to liquefy gelatin within 20 to 24 h.

The *C. perfringens* colonies from three of the nine samples all had discernible halos after 24 h in the two EY media (SFP and TSC), but of the colonies from the other six samples, only 1 to 80% showed the EY reaction. However, when the overpour agar was omitted, all of the *C. perfringens* colonies produced clearly visible halos on the SFP and TSC agars.

In contrast to samples A to I, the total black-colony counts for samples J to M varied considerably between the three selective media (Table 2). Of these, SFP agar gave the highest counts, which approached those in the corresponding agar without antibiotics.

Between 80 and 100% of all colonies transferred from these two media to supplemented NM agar were nonmotile and positive for nitrite, but growth was slow and the nitrite reactions were faint. For consistent nitrite reactions, the NM agar tubes had to be incubated for about 40 h. Little or no acid and gas was produced in LM agar within 20 h, and gelatin was not liquefied. The isolates differed from *C. perfringens* also in their organic acid spectrum (L. V. Holdeman, personal communication), produced no zone of hemolysis on human, bovine, sheep, or rabbit blood agar, and had no lecithinase activity on SFP agar. So far, attempts to identify these isolates with a known species have failed.

Very few colonies of this species (*Clostridium* sp.) were found in EY-free TSC agar. The slightly higher black-colony counts in complete TSC agar (Table 2) were accounted for by colonies of this species. Similar numbers of *Clostridium* sp. developed in EY-free TSC agar in the presence of lysozyme which caused 1.5- to threefold increases in black-colony counts for samples J to L and a 10-fold increase for sample M. The partial recovery of *Clostridium* sp. in complete TSC agar therefore seems to be due to the EY lysozyme. Effects of lysozyme on germination have been demonstrated for heated spores of other clostridia (3, 6, 15).

For each of the four fecal specimens enumerated in antibiotic-free agar, three specimens in SFP agar, and one specimen in TSC agar, the *C. perfringens* counts could be expressed only in lower and/or upper limits (Table 2). The lower values represent EY-positive colonies which were confirmed as *C. perfringens*. Because none of these plates contained a single *C. perfringens* colony out of 20 EY-negative colonies tested, the differences between the lower and upper limits correspond to 5% of the total counts. In spite of these errors, the *C. perfringens* counts in the three media were of the same order. A relatively low count was obtained for sample K in TSC agar.

Because few data are available regarding *C. perfringens* spore counts in normal stools (1, 12, 14), we used EY-free TSC agar to enumerate these spores in fecal samples from an additional 45 healthy adults. The results were combined with the counts of samples A to O and are summarized in Fig. 1. The median spore count

TABLE 2. *Enumeration of C. perfringens spores from normal feces in which spores of Clostridium sp. were predominant*

Fecal sample	Count	Spores/g			
		SFP	TSC	EY-free TSC	Antibiotic-free SFP
J	*C. perfringens*	$(0.4\text{–}1.4) \times 10^3$	1.0×10^3	0.9×10^3	$(0.4\text{–}2.4) \times 10^3$
	Total black colonies	2.0×10^4	3.2×10^3	1.0×10^3	4.1×10^4
K	*C. perfringens*	$(0.8\text{–}3.0) \times 10^4$	1.1×10^4	2.8×10^4	$(0.8\text{–}4.6) \times 10^4$
	Total black colonies	4.5×10^5	2.5×10^4	2.8×10^4	7.7×10^5
L	*C. perfringens*	5×10^2	3×10^2	3×10^2	$<10^3$
	Total black colonies	9.1×10^3	1.0×10^3	5×10^2	2.2×10^4
M	*C. perfringens*	$(1.0\text{–}4.4) \times 10^3$	$(1.0\text{–}3.0) \times 10^3$	1.1×10^3	$<3 \times 10^4$
	Total black colonies	6.8×10^4	4.1×10^4	2.2×10^3	5.3×10^5

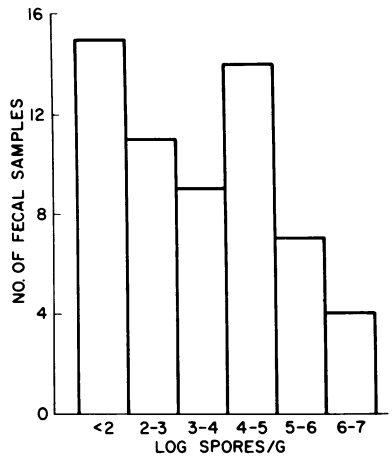

FIG. 1. *Concentrations of C. perfringens spores in the stools of 60 healthy adults.*

per gram was log 3.4 (range: <log 1 to log 6.8). The mean count (log 5.4/g) has little meaning, because it is essentially determined by the few counts over log 6/g. Heat-resistant spores (log 2.3/g) were found in 1 out of 25 specimens tested.

For 10 out of the 45 additional fecal samples, at least 1 of the 10 colonies transferred from EY-free TSC agar to the confirmatory media showed the characteristics of *Clostridium* sp.: nonmotility and nitrite formation, production of little or no gas and acid within 20 h in LM agar, and no liquefaction of gelatin. In addition, these isolates had no hemolytic or lecithinase activities. For more accurate counts of *Clostridium* sp. spores, the 10 fecal samples were subsequently plated in EY-free TSC agar without the antibiotic. The counts varied from 8×10^2 to 2×10^5; in six of the samples, they exceeded the *C. perfringens* counts by 1 to 2 logs.

DISCUSSION

Each of the three media tested appeared to be suitable for enumeration of *C. perfringens* spores in feces, provided that these spores predominated (Table 1). Some counts in TSC agar were lower than in SFP and EY-free TSC agars; this may have been due to a combination of high oxygen tension during surface plating and the cycloserine in the medium (10).

The recoveries of *Clostridium* sp. in SFP agar were essentially as high as in antibiotic-free agar (Table 2); they were lower in TSC agar and approached 0% in EY-free TSC agar. Taking into account the other disadvantages of EY-containing media (10), it is apparent that EY-free TSC agar is superior to the two other media for enumeration of fecal *C. perfringens* spores. The same conclusion was drawn for enumeration of food-borne *C. perfringens*. In this work, the OPSP agar (8) was not tested because previous work showed that it suppressed colony formation of some *C. perfringens* strains (10) and was not sufficiently inhibitory to facultative anaerobes (11).

Spores of *Clostridium* sp. were encountered in 14 out of 60 normal fecal samples. Because 45 of the samples were plated only in EY-free TSC agar, which is inhibitory to *Clostridium* sp., the actual number of specimens with spores of this species was probably even higher. This high incidence of *Clostridium* sp. in normal stools and our inability to identify it with a recognized clostridial species are difficult to reconcile. Similarly, Debono (5) stated that *C. filiforme*, which has several characteristics in common with *Clostridium* sp., was "commonly met with in faeces," yet it has apparently not been isolated since 1912. In collaboration with L. V. Holdeman, a detailed description of our isolates will be published elsewhere.

For several days after an episode of *C. perfringens* food poisoning, the total *C. perfringens* counts (no heat activation) as well as the spore counts (after heat activation) were significantly higher in the stools of recovering patients than in normal stools (12, 19). In investigations of food-poisoning outbreaks, both these counts might be used as criteria for implicating *C. perfringens* as the causative organism. The spore counts are generally preferred because most of the potentially interfering intestinal microflora is killed during heat activation of the spores, and total counts are ill-defined; because the spores constitute a significant proportion of the total *C. perfringens* (vegetative cells plus spores), but vary in dormancy, the "total count" is usually incomplete and may even be lower than the spore count (14, 19). In addition, the spore counts remain virtually unchanged during storage of fecal specimens for at least 1 week at +4, −18, or −16 C (unpublished data).

The median *C. perfringens* spore count of log 3.4/g of normal feces is in agreement with reports from the United Kingdom (14, 19) which indicate median concentrations of about log 3/g. However, spore concentrations of up to log 9/g with a median of about log 5/g of normal feces have been reported from Japan (1).

In feces collected within a few days after *C. perfringens* food poisoning, the log spore concentrations per g ranged from 6.2 to 7.8 (12) and from 5.6 to 7.2 (19). The latter data are median counts and correspond to total counts of log 6.6/g to log 7.5/g (19). It is obvious that only

median fecal spore counts, rather than a few individual counts, are meaningful criteria for implicating *C. perfringens* as the cause of food poisoning.

The low incidence of heat-resistant *C. perfringens* spores in normal feces is consistent with reports from the United States (7), the United Kingdom (4, 13) and Australia (17, 18), whereas Japanese workers have detected heat-resistent spores in over 50% of normal stools (1).

This work has shown that strains of *Clostridium* sp. are not completely inhibited in EY-free TSC agar and that confirmation of presumptive *C. perfringens* colonies in supplemented NM agar is not adequate. As discussed previously (11), none of the clostridia that might be falsely identified as *C. perfringens* in the nitrite motility test liquefied gelatin within a week in lactose gelatin (11), whereas *C. perfringens* caused liquefaction within 40 h, usually within 24 h. We propose, therefore, to confirm *C. perfringens* by simultaneous transfers of presumptive colonies to supplemented nitrate motility agar and to lactose gelatin.

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