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Improved Medium for Enumeration of *Clostridium perfringens*

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An improved selective medium, Tryptose-sulfite-cycloserine (TSC) agar, for the enumeration of *Clostridium perfringens* is described. It consists of the same basal medium as Shahidi-Ferguson-perfringens (SFP) agar, but with 400 μg of D-cycloserine per ml substituted for the kanamycin and polymyxin. Tolerance of *C. perfringens* for D-cycloserine, its production of lecithinase, and its ability to reduce sulfite were used as the basis for development of this medium. Comparisons were made between TSC and SFP agars for the recovery of vegetative cells of *C. perfringens* by using statistical methods. The results showed that TSC allowed virtually complete recovery of most of the *C. perfringens* strains while inhibiting practically all facultative anaerobes tested. SFP agar allowed a slightly higher rate of recovery of *C. perfringens* but was found to be much less selective.

In recent years, several new media as well as new methods have been developed for the enumeration and isolation of *Clostridium perfringens* from foods (1, 4, 8, 9). In the United States, the method of Angelotti et al. (1) which utilizes sulfite-polymyxin-sulfadiazine (SPS) agar as the enumerating medium has been the most widely used of these methods. Marshall et al. (8) modified SPS agar by substituting neomycin sulfate for sulfadiazine and increased the concentration of polymyxin. They recommended incubation of the modified medium, tryptone-sulfite-neomycin (TSN) agar, at 46 C to limit the outgrowth of other sulfite-reducing clostridia, specifically *C. bifermentans*. Shahidi and Ferguson (11) recently developed a new medium, Shahidi-Ferguson-perfringens (SFP) agar, which combines the principle of sulfite reduction with that of the egg yolk reaction (7) for enumeration of *C. perfringens*.

The results of a previous comparative study in our laboratory, using laboratory prepared media, showed that average recovery of vegetative cells of 10 strains of *C. perfringens* was significantly higher with SFP agar than with SPS or TSN agars when 10^6 organisms per g was used as the inoculum level (4*a*). However, all three media permitted the growth of undesirable facultative anaerobes which interfere with the isolation of *C. perfringens*. Others have shown that streptococci, especially the enterococci (group D), *Bacillus cereus*, and *Proteus* species grow well in some of these media (1, 8, 9, 13). Although it has been reported that facultative anaerobes do not interfere with the enumeration of sulfite-reducing clostridia, the isolation of *C. perfringens* is difficult when they are present in large numbers. Since isolation and identification of the organism is a necessary adjunct to the enumeration procedure (1, 11), the importance of the selectivity of a medium is obvious.

Füzi and Csukás (3) reported that blood-agar containing 800 μg of D-cycloserine per ml is a very selective medium for the isolation of *C. perfringens* because it inhibits the growth of many facultative anaerobes. The following report describes a modification of SFP agar by substituting 400 μg of D-cycloserine per ml for the antibiotics recommended by Shahidi and Ferguson and comparison of the modified medium, Tryptose-sulfite-cycloserine (TSC) agar, with SFP agar for recovery of vegetative cells of *C. perfringens* from inoculated chicken broth.

MATERIALS AND METHODS

Cultures. All *C. perfringens* strains used in this investigation were isolated from foods associated with foodborne disease or feces from patients stricken in the outbreaks and are identified in Table 1. The facultative anaerobes and other clostridial species used to evaluate the selectivity of the media were from the stock culture collection of the Food and Drug Administration and are as follows: *Arizona* 5045, *B. cereus*, *B. subtilis* (two strains), *Citrobacter* (2 strains), *C. bifermentans*, *C. botulinum* (types A, B, E, F), *C.*

TABLE 1. *Identification of strains of Clostridium perfringens*

Strain	Serotype[a]	Source of isolate	Supplied by
FD-1	PS 24	Roast beef	Food and Drug Administration
NCTC 8797	Hobbs 1	Stewed steak	National Collection of Type Cultures, London, England
NCTC 8238	Hobbs 2	Boiled beef	
NCTC 8798	Hobbs 9	Meat rissole	
T-65	Hobbs 10	Turkey	D. H. Strong, University of Wisconsin, Madison
S-34	Nontypable[b]	Turkey	H. E. Hall, Food and Drug Administration, Cincinnati Laboratories, Cincinnati, Ohio
80535	Hobbs 10	Beef gravy	A. H. W. Hauschild, Food and Drug Directorate, Ottawa, Ontario, Canada
NCDC 2078	PS 35	Feces of food poisoning patient	V. R. Dowell, Center for Disease Control, Atlanta, Ga.
IU-686	Nontypable	Chicken broth	L. S. McClung, Indiana University, Bloomington
IU-3344	—[c]	Turkey gravy	

[a] Agglutinated by Hobbs or PS antisera.
[b] Nontypable by Hobbs antisera.
[c] No data available.

sordelli, *C. sporogenes*, *Enterobacter aerogenes*, *E. hafniae*, enterococci (20 strains), *Escherichia coli*, *E. freundii*, *Proteus inconstans*, *P. mirabilis*, *P. morganii*, *P. rettgeri*, *Pseudomonas aeruginosa*, *Salmonella senftenberg*, *S. typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus bovis*, *S. durans*, *S. faecalis*, *S. lactis*, and *S. salivarius*.

Plating media. SFP agar (11) was prepared according to the directions of the authors (*personal communication*). The basal medium consisted of 1.5% Tryptose (Difco), 0.5% soytone (Difco), 0.5% yeast extract, 0.1% sodium bisulfite (meta), 0.1% ferric ammonium citrate (NF Brown Pearls, Mallinckrodt Chemical Works), and 2.0% agar. Three milliliters of a 0.1% solution of polymyxin B sulfate and 10 ml of a 0.12% solution of kanamycin sulfate were added per liter of basal medium; the medium was adjusted to *p*H 7.6 and sterilized for 10 min at 121 C.

SFP agar without antibiotics served as the noninhibitory control medium. This medium was prepared in the manner previously described with the polymyxin B sulfate and kanamycin sulfate solutions omitted.

The same basal medium used for preparing SFP agar (antibiotics omitted) was used for TSC agar. To each liter of medium, adjusted to *p*H 7.6 and sterilized for 10 min at 121 C, 40 ml of a 1.0% filter-sterilized solution of D-cycloserine (Seromycin, Lilly) was added to give a final concentration of 400 μg per ml. All media were cooled to 50 C, and 40 ml of a sterile 50% egg yolk emulsion was added per 500 ml with the exception of that utilized to overlay the plates. The media were dispensed in standard petri dishes, air dried at room temperature for 24 hr, and stored at 4 C.

Preparation of cultures and inoculation of chicken broth. Vegetative cells of the 10 strains of *C. perfringens* were grown in cooked meat medium (CMM) for 18 to 24 hr at 35 C. After incubation, the cultures were diluted in 0.1% peptone water (5) and added to chicken broth to give a final concentration of approximately 10^6 organisms per g of food.

Tolerance of C. perfringens for D-cycloserine. The tolerance of vegetative cells of 10 strains of *C. perfringens* for D-cycloserine was determined by plating CMM cultures on SFP basal medium containing 200, 400, 600, and 800 μg of D-cycloserine per ml. The SFP basal medium served as a noninhibitory control medium.

Enumeration. Twenty-five grams of chicken broth, inoculated with vegetative cells of *C. perfringens*, was homogenized with 225 ml of 0.1% peptone water for 1 min at 8,000 rev/min in a Waring Blendor and diluted serially in 0.1% peptone water. A 0.1-ml amount of each of the appropriate dilutions was surface-plated in duplicate on all test media. After the agar had dried, the plates were overlaid with an additional 10 ml of the respective media without egg yolk. All plates were incubated under a nitrogen atmosphere in an upright position for 24 hr at 35 C in Anaero jars (Case Laboratories, Chicago, Ill.). Plates containing between 30 and 300 colonies were counted, and five colonies were picked for confirmation in motility-nitrate medium (1).

Selectivity. Cultures of the facultative anaerobes were grown in Brain Heart Infusion (BHI) broth for 18 to 24 hr at 35 C. Spore stocks of clostridia other than *C. perfringens* were produced in CMM or in Trypticase-peptone-glucose (TPG) broth by the procedures described by Solomon et al. (12). The cultures or spore stocks were diluted in 0.1% peptone water, and 0.06 ml of an appropriate dilution containing approximately 10^5 vegetative cells or spores was placed on the surface of each medium by using the replicating device and method described by Evans et al. (Bacteriol. Proc., p. 56, 1964). SFP agar without antibiotics served as a control for the evaluation. All plates were incubated for 24 hr at 35 C under a nitrogen atmosphere.

Statistical analysis. The data obtained from the enumeration experiments were analyzed by statistical methods. A factorial design was set up for the experiments. All three sets of data were analyzed for 3 main effects (i.e., media, strain, and experimental runs). Each set was the result of an experimental design in which it was expected that a 10% difference between the average recovery of *C. perfringens* with the two media giving the high and low results would be detected as significant nine times in 10 when it occurred. The probability that the null hypothesis (i.e., that the recovery in the media is equal) would be rejected when true was set at $\alpha = 0.01$. Counts were transformed to logarithms to assume and use the normal probability distribution. An analysis of variance was performed on the logarithmic counts. The calculations and assumptions for the three way design with interactions was given by Ostle (10). Duncan's multiple-range test (2) was also performed to determine which means differed from one another.

RESULTS

Tolerance of C. perfringens for D-cycloserine. The data for tolerance of vegetative cells of 10 *C. perfringens* strains to D-cycloserine in SFP basal medium are presented in Table 2. Two strains associated with food poisoning tolerated 800 μg of D-cycloserine per ml without a significant reduction in the plate count. However, considerably lower counts were obtained with the other eight strains. The heat-resistant strains described by Hobbs et al. (6) were adversely affected by this concentration. Therefore, 400 μg of D-cycloserine per ml was chosen as the final concentration in the preparation of TSC agar since it permitted good recovery of most of the strains.

Recovery of vegetative cells on TSC and SFP agars. The recovery of vegetative cells of *C. perfringens* from inoculated chicken broth is presented in Table 3. Three trials were conducted with the two selective agars, and the results were analyzed by statistical methods. The per cent recovery was computed for each strain and compared with the geometric mean values for SFP basal medium as 100%. The overall average recovery with the 10 strains for three trials on TSC agar was 90%, and on SFP agar it was 95% compared to the basal medium. The overall difference in recovery of 5%, although small, was found to be significant at the $\alpha = 0.01$ level. Low recoveries with strains T-65 and IU-686 on TSC agar accounted for the large variation noted in the analysis of variance. All strains save FD-1, however, exhibited a slight sensitivity to the antibiotics in both media. This can be observed by comparing the per cent recovery in these media with that obtained in the noninhibitory SFP basal medium.

TABLE 2. *Effect of concentration of* D-*cycloserine on per cent recovery of vegetative cells of Clostridium perfringens*[a]

Strain	Per cent recovery at various concn of D-cycloserine in SFP agar without antibiotics			
	200 μg/ml	400 μg/ml	600 μg/ml	800 μg/ml
FD-1	99	99	88	46
NCTC 8797	90	96	61	8
NCTC 8238	99	99	80	22
NCTC 8798	99	93	93	6
T-65	101	75	51	55
S-34	98	95	101	100
80535	81	86	62	3
NCDC 2078	99	101	92	34
IU-686	97	85	66	9
IU-3344	96	104	86	79

[a] Plate counts obtained in SFP agar without antibiotics are equal to 100%.

TABLE 3. *Per cent recovery of vegetative cells of Clostridium perfringens from chicken broth on TSC and SFP agars compared with SFP without antibiotics*[a]

Strain	Per cent recovery on	
	TSC agar	SFP agar
FD-1	95	101
NCTC 8797	97	96
NCTC 8238	93	97
NCTC 8798	92	91
T-65	79	89
S-34	97	99
80535	94	93
NCDC 2078	97	99
IU-686	76	95
IU-3344	89	93
Over all avg	90	95

[a] Average of three trials. Plate counts in SFP agar without antibiotics are equal to 100%.

Selectivity. The growth of various facultative anaerobes and other *Clostridium* species on TSC agar containing 200 and 400 μg of D-cycloserine per ml and SFP agar is presented in Table 4. TSC agar containing 400 μg of D-cycloserine per ml was the most selective medium studied, whereas SFP agar was the least. *S. marcescens* and *S. lactis* were the only facultative anaerobes which grew on TSC agar containing 400 μg of D-cycloserine per ml. In addition, this agar permitted the growth of the other sulfite-reducing *Clostridium* species tested with the exception of *C. sordelli*, which was completely inhibited, and

TABLE 4. *Growth response of various facultative anaerobes and other Clostridium species in TSC and SFP agars*[a]

Organisms	Growth on media[b]		
	TSC agar		SFP agar
	Concn of D-cycloserine (μg/ml)		
	400	200	
Arizona 5045	−	−	(+)
Bacillus cereus	−	−	+
B. subtilis (2 strains)	−	(+)	−
Citrobacter (2 strains)	−	−	(+)
Clostridium bifermentans	(+)	+	+
C. botulinum (types A, B, E, and F)	+	+	+
C. sordelli	−	(+)	+
C. sporogenes	+	+	+
Enterobacter aerogenes	−	(+)	(+)
E. hafniae	−	(+)	(+)
Enterococci (20 strains)	−	−	+
Escherichia coli	−	−	(+)
E. freundii	−	−	(+)
Proteus inconstans	−	(+)	(+)
P. mirabilis	−	(+)	+
P. morganii	−	+	(+)
P. rettgeri	−	(+)	+
Pseudomonas aeruginosa	−	(+)	+
Salmonella senftenberg	−	−	−
S. typhimurium	−	−	−
Serratia marcescens	+	+	+
Staphylococcus aureus	−	−	+
Streptococcus bovis	−	(+)	+
S. durans	−	−	+
S. faecalis	−	−	+
S. lactis	(+)	(+)	+
S. salivarius	−	−	+

[a] All organisms grew on SFP without antibiotics.

[b] + = No inhibition of growth, (+) = partial inhibition of growth, and − = complete inhibition of growth.

C. bifermentans, which was partially inhibited. TSC agar containing 200 μg of D-cycloserine per ml did not inhibit the growth of *P. morganii*, *S. marcescens*, or any clostridial species with the exception of *C. sordelli*, which was only partially inhibited. This medium permitted a limited amount of growth of the *Enterobacter* species, three of four *Proteus* species, *P. aeruginosa*, *B. subtilis*, *S. lactis*, and *S. bovis*. A similar inhibition pattern was observed with TSC agar containing 300 μg of D-cycloserine per ml. The only organisms which SFP completely inhibited were *S. typhimurium*, *S. senftenberg*, and *B. subtilis*. There was some suppression of growth of *Escherichia* and *Enterobacter* species, *P. inconstans*, *P. morganii*, *Arizona* 5045, and two strains of *Citrobacter*.

Of particular significance is the fact that the group D streptococci, which are frequently present in large numbers in foods associated with *C. perfringens* outbreaks, were completely inhibited in TSC agar but grew well on SFP agar.

DISCUSSION

It was observed in a recent comparative study in our laboratory (4*a*) and confirmed in this study that SFP agar was not sufficiently selective to limit the growth of facultative anaerobes such as the enterococci which interfere with the isolation of *C. perfringens*. This also occurs with all of the sulfite agars recommended, to date, for the enumeration of *C. perfringens* (1, 8, 9, 11).

The overall recovery rate of vegetative cells of *C. perfringens* on SFP agar was 5% higher than on TSC agar. This difference from a practical standpoint is rather small and was primarily due to the sensitivity of two strains (T-65 and IU-686) to D-cycloserine. Tolerance tests showed these two strains and strain 80535 to be somewhat sensitive to 400 μg per ml, whereas recovery of the other seven strains was greater than 90%. The recovery of these seven strains and strain 80535 was approximately the same on both agars. Since TSC agar is the more selective of the two media while permitting quantitative recovery with the majority of the food poisoning strains tested, it would be the preferred medium. Reduction in the concentration of D-cycloserine to 200 μg per ml would permit higher counts with the less tolerant *C. perfringens* strains (Table 2) but would markedly impair the selectivity of the medium. At least 10 species of facultative anaerobes tested grew to some extent in TSC agar containing 200 μg of D-cycloserine per ml (Table 4). Increasing the concentration of D-cyloserine to 300 μg per ml did not substantially increase selectivity of the medium.

An important consideration in the formulation of a medium for the enumeration of *C. perfringens* is the development of colonies which are easily differentiated from those developed by concomitant microflora. Differentiation is dependent upon blackening of the colony due to sulfite reduction, an egg yolk reaction due to lecithinase production, or both. Though this study was not specifically designed to evaluate the media for their ability to yield typical *C. perfringens* colonies

from naturally contaminated foods containing a mixed microflora, in all cases the *C. perfringens* colonies that did develop met these criteria. Since enumeration is dependent not only upon the development of typical colonies but also upon isolation and confirmation of typical colonies as *C. perfringens*, the ability of a medium to suppress outgrowth of concomitant microflora is extremely important. It has been commonly observed in our laboratories that overgrowth by concomitant microflora interferes in the isolation of pure cultures from typical colonies. TSC agar is highly selective and at the same time permits quantitative recovery with the majority of food poisoning strains tested.

Experience in our laboratory with foods involved in *C. perfringens* outbreaks indicates that other sulfite-reducing clostridia are rarely present in large numbers in these foods. Nevertheless, because TSC agar permits the growth of certain other sulfite-reducing clostridia, confirmation of the identity of organisms which produce black colonies in the medium is still necessary. The spores of organisms such as *C. sporogenes* and *C. botulinum* require at least 48 hr of incubation at 35 C in TSC agar to obtain appreciable growth. It seems unlikely, therefore, that they would be detected within 24 hr. *C. bifermentans* was the only other *Clostridium* species tested which grew well on TSC agar after 24 hr of incubation at 35 C.

Since the development of this medium, there have been few opportunities to evaluate its application in enumerating *C. perfringens* in food-poisoning outbreaks. However, a few food samples from outbreaks of gastroenteritis have been examined employing TSC agar as well as two other agar media used for enumeration of this organism. The results, to date, confirm the utility of TSC agar in the examination of foods associated with *C. perfringens* foodborne disease outbreaks.

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