APPLIED MICROBIOLOGY, May 1971, p. 922-927

Vol. 21, No. 5
Printed in U.S.A.

Comparison of Media for the Enumeration of *Clostridium perfringens*

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Received for publication 13 January 1971

For the enumeration of viable vegetative cells and spores of *Clostridium perfringens*, noncommercial (laboratory prepared) sulfite-polymyxin-sulfadiazine (SPS) agar, tryptone-sulfite-neomycin (TSN) agar, and Shahidi-Ferguson-perfringens (SFP) agar were statistically compared to SPS agar without antibiotics. The selectivities of these four media were also evaluated on the basis of their ability to inhibit the growth of pure cultures of a variety of other organisms. The average recovery of vegetative cells of 10 strains of *C. perfringens* with SFP agar was not significantly higher than with SPS agar with 10^4 organisms per g, but with 10^6 organisms per g it yielded significantly higher recoveries than SPS agar. TSN agar yielded significantly lower recoveries at both inoculum levels. SFP agar gave significantly higher recoveries of spores than SPS and TSN agars. Average plate counts of spores in SFP agar were 75% as high as in SPS agar without antibiotics, but only 45% of the spores grew in SPS agar and 25% in TSN agar. TSN agar was the most selective of the three media, but the selectivity of SPS agar approached that of TSN agar under the test conditions. SFP agar, which was the least selective of the media, allowed growth to some extent of nearly all of the facultative anaerobes tested.

In recent years, several new media as well as new methods have been developed for the enumeration and isolation of *Clostridium perfringens* from foods. In 1956, Mossel et al. (9) proposed a sulfite agar for enumerating sulfite-reducing clostridia in foods. Later he described a sulfite-iron medium containing polymyxin B (8) which selectively suppressed the growth of most *Enterobacteriaceae*.

Based on Mossel's medium, Angelotti et al. (1) developed a sulfite-polymyxin-sulfadiazine (SPS) agar and recommended its use for enumerating *C. perfringens* in food. This medium has been widely used in the United States and elsewhere (4, 12, 13). Marshall et al. (7) modified SPS agar by substituting neomycin sulfate for sulfadiazine and increasing the concentration of polymyxin. They recommended incubation of the modified medium, tryptone-sulfite-neomycin (TSN) agar, at 46 C to limit the out-growth of other sulfite-reducing clostridia, specifically *Clostridium bifermentans*. Shahidi and Ferguson (Bacteriol. Proc., p. 1, 1969) recently developed a new medium, Shahidi-Ferguson-perfringens (SFP) agar, which combines the principle of sulfite reduction with that of the egg yolk reaction for enumeration of *C. perfringens* (6).

Marshall et al. (7) and Shahidi and Ferguson compared their media with commercial SPS agar and concluded that their media were equal to or superior to SPS agar. However, Hauschild et al. (5) and Harris and Lawrence (Bacteriol. Proc., p. 6, 1970) have reported that commercial preparations of SPS agar are unsatisfactory for quantitative recovery of *C. perfringens*. Failures in quantitative recovery with commercial lots of SPS and TSN agars have also been experienced in this laboratory.

The purpose of this study was to compare the enumeration of *C. perfringens* vegetative cells and spores on SPS, TSN, and SFP agars with SPS agar from which the antibiotics were omitted and to evaluate the selectivity of these agars. Analysis of variance techniques were used to compare the mean recoveries.

MATERIALS AND METHODS

Strains. All *C. perfringens* strains used in this investigation were isolated from foods associated with foodborne disease or from feces of 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 Food and Drug Administration stock culture

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
FD-2	Nontypable[b]	Chicken broth	Food and Drug Administration
NCTC 8238	Hobbs 2	Boiled beef	National Collection of Type Cultures, London, England
NCTC 8798	Hobbs 9	Meat rissole	
T-65	Hobbs 10	Turkey	D. H. Strong, University of Wisconsin, Madison
S-34	Nontypable	Turkey	H. E. Hall, Food and Drug Administration, Cincinnati Laboratories, Cincinnati, Ohio
80535	—[c]	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.

collection and are as follows: *Escherichia coli*, *E. freundii*, *Enterobacter hafniae*, *E. aerogenes*, *Proteus mirabilis*, *P. inconstans*, *P. morganii*, *P. rettgeri*, *Salmonella typhimurium*, *S. senftenberg*, *Arizona* 5045, *Citrobacter* (two strains), *Serratia marcescens*, *Bacillus subtilis*, *B. cereus*, *Staphylococcus aureus*, *Streptococcus faecalis*, *S. durans*, *S. lactis*, *S. bovis*, *S. salivarius*, *Enterococci* (20 strains), *Clostridium botulinum* (types A and B), *C. sporogenes*, *C. bifermentans*, *C. novyi*, and *C. tetani*.

Preparation of cultures and inoculation of food samples. 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 and added to canned roast beef with gravy to give a final concentration of approximately 10^4 or 10^6 organisms per g of food. Spores of eight strains were grown in Duncan-Strong (DS) sporulation broth by the method described by the authors (2). Spore stocks in DS broth were diluted in 0.1% peptone water, heat-shocked for 15 min at 75 C, and added to dehydrated beef stroganoff or dehydrated chicken soup to a final concentration of 10,000 to 20,000 spores per g.

Plating media. The four plating media selected for comparison were prepared as follows.

(i) SPS agar of Angelotti et al. (1). The basal medium was prepared separately and consisted of 1.5% tryptone (Difco), 1% yeast extract, 1.5% agar, and 0.05% iron citrate. The medium was adjusted to *p*H 7.0 and sterilized at 121 C for 15 min. To each liter of sterile medium, the following filter-sterilized (0.45 μm Nalgene filter, Sybron Corp., Rochester, N.Y.) solutions were added: 5.0 ml of 5% sodium sulfite (anhydrous), 10 ml of 0.1% polymyxin B sulfate, and 10 ml of 1.2% sodium sulfadiazine.

(ii) SPS agar without antibiotics. This medium was prepared in the manner previously described with the polymyxin B sulfate and sodium sulfadiazine solutions omitted. It was utilized as the control medium because it has been shown to give recovery of *C. perfringens* equal to that obtained in other nonselective media (8, 12).

(iii) TSN agar of Marshall et al. (7). The same basal medium used for preparing SPS agar was used for TSN agar. To each liter of sterile medium, the following filter-sterilized solutions were added: 10 ml of 5% sodium sulfite (anhydrous), 20 ml of 0.1% polymyxin B sulfate, and 10 ml of 0.5% neomycin sulfate.

(iv) SFP agar was prepared according to the directions of the authors (*personal communication*). It 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, N.Y.), 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; the medium was adjusted to *p*H 7.6 and sterilized for 10 min at 121 C. The medium was cooled to 50 C, and 40 ml of a sterile 50% egg yolk emulsion was added per 500 ml. The medium was dispensed in standard petri dishes, air-dried at room temperature for 24 hr, and stored at 4 C.

Enumeration. Twenty-five grams of food, inoculated with either vegetative cells or spores of *C. perfringens*, was homogenized with 225 ml of 0.1% peptone water (5) for 1 min at 8,000 rev/min in a Waring Blendor and diluted serially in 0.1% peptone water. A 1-ml amount of each of the appropriate dilutions was pour-plated in duplicate in all test media except SFP, on which 0.1-ml samples were surface-plated. After the agar had solidified, all plates with the exception of the SFP agar were overlaid with an additional 5 ml of the respective media. The SFP agar was overlaid with 10 ml of additional medium without egg yolk. All plates were incubated in an upright position for 24 hr at 35 C in Anaero-Jars (Case Laboratories, Chicago, Ill.) under a nitrogen atmosphere. Plates containing between 30 and 300

TABLE 2. *Per cent recovery of vegetative cells of Clostridium perfringens from roast beef in SFP, SPS, and TSN agars compared with SPS agar without antibiotics*[a]

Strain	Recovery (%) in various media at inoculum level of					
	SFP agar[b]		SPS agar		TSN agar	
	10^4[c]	10^6	10^4	10^6	10^4	10^6
FD-1	109	101	100	99	100	97
FD-2	97	103	102	104	91	103
NCTC 8238	119	93	105	99	97	81
NCTC 8798	106	104	101	106	105	106
T-65	107	99	95	97	92	81
S-34	102	101	99	100	100	96
80535	103	126	99	89	85	78
NCDC 2078	113	102	99	89	102	92
IU-686	101	100	101	91	101	67
IU-3344	92	100	94	99	90	102
Overall avg	106	103	100	97	96	90

[a] Average of three trials. Plate count obtained in SPS agar without antibiotics is equal to 100%.

[b] SFP, Shahidi-Ferguson-perfringens; SPS, sulfite-polymixin-sulfadiazine; TSN, tryptone-sulfite-neomycin.

[c] Inoculum level of vegetative cells added per gram of roast beef.

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. (11). The cultures or spore stocks were diluted in 0.1% peptone water and one drop of an appropriate dilution containing approximately 10^6 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). SPS agar without antibiotics served as a control for the evaluation. All plates were incubated for 24 hr at 35 C under a nitrogen atmosphere and examined for growth.

Statistical analysis. A factorial design was set up for the experiments. All three sets of data were analyzed for three main effects (i.e., media, strain, and experimental runs). The data from each series of experiments were analyzed separately. Each series was the result of an experimental design in which it was expected that a 10% difference between the average recovery of *C. perfringens* in the two media with 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 as $\alpha = 0.01$. 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 were given by Ostle (10). Duncan's multiple range test (3) was also performed to determine which means differed from one another.

RESULTS

Recovery of vegetative cells. The recovery of vegetative cells of *C. perfringens* from roast beef is presented in Table 2. To determine whether the inoculum level would affect recovery, two series of three experiments each were performed by using 10^4 and 10^6 organisms per g of roast beef. The highest overall recovery was obtained in SFP agar, which gave 106 and 103% recoveries at respective levels of 10^4 and 10^6 organisms per g, compared with SPS agar without antibiotics. SPS agar gave slightly lower recoveries with averages of 100 and 97% at respective levels of 10^4 and 10^6 organisms per g. The lowest recovery was obtained with TSN agar, which gave an average recovery of 96% at the 10^4 organisms per g level and 90% at 10^6 per g level. The recovery of vegetative cells in the three test media when compared with SPS agar without antibiotics differed significantly from strain to strain, and different strains gave different relative recovery rates (interaction between media and strain) for the three media at the inoculum level of 10^6 organisms per g. For example, the recovery for strain IU-686 was shown to be 100, 91, and 67%, respectively, in SFP, SPS, and TSN agars, whereas the recovery of strain FD-2 was 103, 104, and 103%, respectively, for the three test media. Thus, TSN agar gave satisfactory recovery for some strains,

TABLE 3. *Per cent recovery of Clostridium perfringens spores from dehydrated foods in SFP, SPS, and TSN agars compared with SPS agar without antibiotics*[a]

Strain	Recovery from beef stroganoff in various media[b] (%)			Recovery from chicken soup in various media[b] (%)		
	SFP agar	SPS agar	TSN agar	SFP agar	SPS agar	TSN agar
FD-1	63	31	12	38	28	9
FD-2	54	18	9	47	38	14
NCTC 8238	77	37	14	73	55	27
NCTC 8798	220	88	61	224	100	75
S-34	52	24	13	53	27	18
NCDC 2078	106	81	62	117	75	48
IU-686	56	48	31	60	69	32
IU-3344	78	74	48	100	71	65
Overall avg	78	44	24	72	50	28

[a] Average of two trials. Plate count obtained in SPS agar without antibiotics is equal to 100%.

[b] *See* footnote *b*, Table 2.

TABLE 4. *Summary of analysis of variance in the recovery of vegetative cells of Clostridium perfringens from roast beef*

Source of variation	Analysis of variance at inoculum level of			
	10^4 organisms/g		10^6 organisms/g	
	Degrees of freedom (DF)	Result of F test	Degrees of freedom (DF)	Result of F test
(A) Media	3	6.18[a]	3	17.24[a]
(B) Strain	9	120.16[a]	9	54.49[a]
(C) Experiments	1	0.36	2	5.45[a]
Interaction AB	27	0.99	27	3.73[a]
Interaction AC	3	0.71	6	3.10[a]
Interaction BC	9	39.70[a]	18	68.30[a]
Interaction ABC	27	1.39	0.54	2.02[a]
Experimental error	80	0.00152[b]	120	0.00228
Per cent coefficient of variation		9		11

[a] Significant at $\alpha = 0.01$.
[b] Experimental error in log units.

but the overall recovery for this agar was the lowest.

Recovery of spores. The average per cent recovery of the spores of eight strains of *C. perfringens* obtained from dehydrated beef stroganoff and dehydrated chicken soup in two trials in each substrate with the four agars is presented in Table 3. The recovery of spores in the three test media when compared with SPS agar without antibiotics differed significantly from strain to strain. The highest overall per cent recovery of spores was obtained in SFP agar with an average of 78% from beef stroganoff and 72% from chicken soup, respectively, compared to SPS agar without antibiotics. Plate counts in SPS agar were consistently lower with an average of 44% recovery from beef stroganoff and 50% from chicken soup. The lowest recovery was obtained in TSN agar with an average of only 24% from beef stroganoff and 28% from chicken soup. Uninoculated portions of the dehydrated foods were negative for *C. perfringens*.

Statistical analysis. The results of analyses of variance in the recovery of vegetative cells are presented in Table 4. The F test was performed to examine each factor and interaction for significance. For example, the hypothesis that recoveries are equal for all four media was tested by computing variation among media. The F ratio was determined by dividing this variation by the experimental error. The value of variation among media was found to be 6.18 times the experimental error at the 10^4 organisms per g inoculum level. The F ratios were compared to the critical values tabulated in Ostle (10) for the degrees of freedom listed as in Table 4. The critical value for F with 3 and 80 degrees of freedom is 4.04 at $\alpha = 0.01$. Since F is 6.18 > 4.04, the recoveries observed for the media were considered to differ significantly at the $\alpha = 0.01$ level. The other tests of significance performed in this manner yielded similar results. Results from Duncan's test indicated that SFP and SPS agars and SPS agar without antibiotics did not differ significantly in their recovery of vegetative cells of *C. perfringens* at the 10^4 organism per g level, but SFP gave a significantly higher recovery than SPS and TSN agars at the 10^6 organisms per g level.

All main effects (media, strain, and trials) as well as their interactions were significant for the recovery of spores from dehydrated foods. The estimate of replicate experimental error was 0.00380 or per cent coefficient of variation equal to 14.5. This is only slightly higher than the 9 and 11% observed for the recovery of vegetative cells.

Selectivity. The growth of various facultative anaerobes and other *Clostridium* species on SFP, SPS, and TSN agars at 35 C is presented in Table 5. TSN agar was the most selective of the three media studied although SPS agar showed partial inhibition of many strains; thus, its selectivity approached that of TSN. However, SFP was the least selective, completely inhibiting very few strains and partially inhibiting a smaller number than either of the other two media. SPS agar did not inhibit the growth of *S. marcescens*, *B. cereus*, *S. faecalis*, *S. durans*, *S. lactis*, or the enterococci or clostridial species, with the exception of *C. novyi* which was only partially inhibited. In addition, this agar permitted some growth of *S. senftenberg*, *S. aureus*, and *S.*

TABLE 5. *Growth response of various faculative anaerobes and other Clostridium species in SFP, SPS, and TSN agars*[a]

Organisms	Response on various media[b]		
	SFP agar	SPS agar	TSN agar
Escherichia coli	(+)	−	−
E. freundii	(+)	−	−
Enterobacter hafniae	(+)	−	−
E. aerogenes	(+)	−	−
Proteus mirabilis	+	−	+
P. inconstans	(+)	−	(+)
P. morganii	(+)	−	+
P. rettgeri	+	−	+
Salmonella typhimurium	−	−	−
S. senftenberg	−	(+)	−
Arizona 5045	(+)	−	−
Citrobacter (2 strains)	(+)	−	−
Serratia marcescens	+	+	+
Bacillus subtilis	−	−	−
B. cereus	+	+	−
Staphylococcus aureus	+	(+)	−
Streptococcus faecalis	+	+	(+)
S. durans	+	+	+
S. lactis	+	+	+
S. bovis	+	−	−
S. salivarius	+	(+)	−
Enterococci (20 strains)	+	+	+
Clostridium botulinum, (types A and B)	+	+	+
C. sporogenes	+	+	+
C. bifermentans	+	+	+
C. novyi	+	(+)	+
C. tetani	+	+	+

[a] All organisms grew in SPS without antibiotics. Symbols: + = no inhibition of growth, (+) = partial inhibition of growth, − = complete inhibition of growth.

[b] *See* footnote *b*, Table 2.

salivarius. TSN agar failed to inhibit the growth of three of four *Proteus* species, *S. marcescens*, *S. durans*, *S. lactis*, the enterococci, and the *Clostridium* species tested, but it partially inhibited *P. inconstans* and *S. faecalis*. The only organisms which SFP agar completely inhibited were *S. typhimurium*, *S. senftenberg*, and *B. subtilis*, but there was some suppression of growth of *Escherichia* and *Enterobacter* species, *P. inconstans*, *P. morganii*, *Arizona* 5045, and two strains of *Citrobacter*.

DISCUSSION

The results obtained with vegetative cells of *C. perfringens* indicate that SFP agar is slightly superior to the other two media for enumerating this organism in the test system employed. Plate counts in SPS agar were 5% lower on the average than in SFP agar whereas those in TSN agar averaged 9% lower. From a practical point of view, these differences in the recovery of vegetative cells are too small to recommend SFP agar over SPS agar. They are about the same as the reduction effected by the addition of antibiotics to the basal medium used for SPS and TSN agars (Table 2). On the other hand, the counts obtained with spores of *C. perfringens* in SPS and TSN agars were significantly lower than counts obtained on the same materials in SFP agar. An average recovery of 75% from dehydrated beef stroganoff and chicken soup was obtained by using SFP agar but only 47% with SPS agar and 26% with TSN agar. The greater recovery of spores of strain NCTC 8798 in SFP agar (Table 3) may have been due to a higher rate of germination of this strain in egg yolk-containing media, an effect observed with *C. botulinum* type E (*unpublished data*).

The lower per cent recovery of vegetative cells of many strains in the selective media with an inoculum of 10^6 per g as compared to 10^4 (Table 2) suggests that greater dilution of the food homogenate decreased the capability of some cells to grow in these media. However, the strains tested exhibited considerable variation in their growth in the three media. For example, the recovery of strain FD-1 was virtually 100% in all three media when either 10^4 or 10^6 organisms per g were used, whereas the recoveries of strains IU-686 and 80535 in TSN agar, when 10^6 organisms per g were used, were only 68 and 78%, respectively; but the recoveries were 100% or greater in SFP agar and about 90% in SPS agar (Table 2). TSN agar may grow vegetative cells of some strains as well as SFP and SPS agars, but out of the number of strains tested 40% yielded less than 90% of the colonies obtained on SPS agar without antibiotics. It is possible that incubation of TSN agar at 46 C as recommended by Marshall et al. (7) would have resulted in higher recovery with some strains, but preliminary trials indicated that recovery decreased at this temperature. Incubation at 46 C would have increased the selectivity of TSN agar, but we have found that streptococci when present in large numbers in food grow at this temperature. All recoveries on SFP agar were higher than 90% and only two on SPS agar were below 90% (both 89%).

The difference in recovery of spores was greater than that observed with vegetative cells. Again, the interaction between media and strain was significant, but SFP agar would generally seem to be the most suitable medium. SFP agar gave better recovery of spores with all strains except IU-686 in chicken soup (Table 3).

An important consideration of the media devised, to date, 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 or an egg yolk reaction due to lecithinase production, or both. Though this study was not specifically designed to evaluate the three 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 the criteria described by the original investigators (1, 7; Shahidi and Ferguson, Bacteriol. Proc., p. 1, 1969). 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 important. It has been commonly observed in our laboratories that overgrowth by other microorganisms interferes with the isolation of pure cultures from typical colonies. A medium, therefore, that suppresses outgrowth of concomitant microflora but permits quantitative outgrowth of *C. perfringens* as easily differentiated colonies is ideal. None of the media evaluated in this study met this ideal. The data collected in this study indicate that the choice of medium may depend upon its intended use. Where selectivity is of primary importance as with heavily contaminated foods, SPS or TSN agar might be preferred, whereas SFP agar would be the medium of choice for lightly contaminated foods.

We are currently evaluating the utility of the three media for the enumeration of this organism in foods associated with foodborne disease outbreaks.

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