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New Medium for Rapid Screening and Enumeration of *Clostridium perfringens* in Foods†

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A rapid and sensitive procedure for estimating low numbers of *Clostridium perfringens* has been investigated and compared to methods used currently in the food industry. The new liquid medium, RPM (rapid perfringens medium), was compared with sulfite-polymyxin-sulfadiazine agar and tryptose-sulfite-cycloserine agar in recovery studies with naturally contaminated and with inoculated foods. The medium consists of a mixture of litmus milk and fluid thioglycolate medium fortified with glucose, peptone, gelatin, yeast extract, sodium chloride, and ferrous sulfate. Selectivity is based on an antibiotic system (polymyxin B sulfate and neomycin sulfate) incorporated into the medium, coupled with an incubation temprature of 46 to 48°C for 24 h. Tubes were scored as positive if a stormy fermentation was observed. All tubes demonstrating stormy fermentation were confirmed as containing *C. perfringens*. Of a total of 774 naturally contaminated food samples, 546 samples (71%) were found to contain *C. perfringens* with RPM, whereas only 168 (22%) of the samples were positive using sulfite-polymyxin-sulfadiazine agar. *C. perfringens* was isolated from 71% of 85 other samples using RPM as compared to 14% with tryptose-sulfite-cycloserine agar. Enumeration studies on 14 individual samples using the most probable number technique also demonstrated greater sensitivity with RPM.

Several media are currently available for the selective isolation of *Clostridium perfringens*. Basically, the media are similar in their mode of action in that they rely on the ability of *C. perfringens* ro reduce sulfite to sulfide, thereby producing black colonies. Since many other organisms (i.e., *Enterobacteriaceae*) have the ability to reduce sulfites, a variety of selective agents are incorporated into these media to suppress competing growth. Among the media most commonly employed are SPS (sulfite-polymyxin-sulfadiazine) (2), SFP (Shahidi-Ferguson perfringens) (10), TSC (tryptose-sulfite-cycloserine) (5), and TSN (tryptose-sulfite-neomycin (9).

SPS incorporates two antibiotics to suppress the growth of sulfite-reducing *Enterobacteriaceae* and many other facultative anaerobes, including strains of *Pseudomonas* and *Bacillus*. However, low recoveries of *C. perfringens* in commercially prepared SPS have been reported by L. F. Harris and J. V. Lawrence (Bacteriol. Proc. 70:6, 1970), Hauschild et al. (7), and Harmon et al. (5).

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Marshall et al. (9) developed TSN agar and concluded that it was superior to commercial preparations of SPS. However, it was shown that TSN inhibits a number of strains of *C. perfringens* (5, 6). SFP agar appears to be excellent for the quantitative recovery of *C. perfringens* (10), but lacks the selectivity of the other two media. A large number of facultative anaerobes, including many sulfite-reducing strains, were not suppressed by the antibiotic system in this medium. The TSC medium developed by Harmon et al. (6) substituted 0.04% cycloserine for polymyxin B and kanamycin in the SFP formulation. Cycloserine inhibits the growth of virtually all facultative anaerobes (3). Preliminary work by Harmon et al. (6), with two strains of *C. perfringens*, showed high recovery levels from pure cultures. Hauschild and Hilsheimer (8) modified TSC by eliminating the egg yolk and employing the pour-plate procedure. They demonstrated increased quantitative recoveries by using the above modifications. Harmon (4) subjected TSC agar, with and without added egg yolk, and SPS agar to a collaborative study in 10 different laboratories. The percent recoveries of four *C. perfringens* strains from inoculated roast beef were two to three times higher with the TSC agars than with SPC

agar and, as a result, the TSC agars were adopted for use as official first action by the Association of Official Analytical Chemists (AOAC).

The media described above are primarily designed for the isolation of *C. perfringens* from highly contaminated foods associated with food-poisoning outbreaks where large numbers of the organisms are present. All procedures involve the preparation of plates with agar overlays, anaerobic incubation, and isolation and biochemical identification of isolates. These procedures are excessively laborious and time consuming. Therefore, a need exists for a simple and rapid method that will detect low to moderate numbers of *C. perfringens*. Such a medium would satisfy the needs of the quality control microbiologist at the manufacturing level.

In the present study, a rapid perfringens medium (RPM) consisting of fortified litmus milk broth with selectivity based on the antibiotics polymyxin B sulfate and neomycin sulfate was developed and evaluated. This new medium was compared primarily against SPS agar, which at the time was specified for use in the official first action method for enumeration of *C. perfringens*. Studies have also been included comparing RPM broth to TSC agar because the latter has been found superior to SPC agar and was adopted as a replacement by the AOAC (6).

MATERIALS AND METHODS

Cultures. Six strains of *C. perfringens* were obtained from C. L. Duncan, Food Research Institute, The University of Wisconsin, Madison. Cultures were maintained in thioglycolate broth (Baltimore Biological Laboratory) and transferred twice weekly. The stock cultures were stored at −4°C in sterilized skim milk suspensions.

Media. Dehydrated SPS agar (Difco) was prepared according to label instructions and only used on the day of preparation. TSC agar without egg yolk was prepared as described by Harmon (4). The medium was used only on the day of preparation.

The RPM developed in this work consisted of two solutions (A and B) which were prepared independently in double-strength concentrations, autoclaved, cooled, antibiotic fortified, and mixed in sterile screw-capped glass tubes. Solution A consisted of 140 g of litmus milk powder (Difco) in 1 liter of distilled water. This was autoclaved (15 lb/in^2) in flasks for 5 min and cooled, and 150 mg of neomycin sulfate (Calbiochem, B-grade) and 25 mg of polymyxin B sulfate (Calbiochem, B-grade) were added. Solution B contained the following: fluid thioglycolate medium (Difco), 60 g; gelatin, 120 g; peptone, 10 g; glucose, 10 g; K_2HPO_4, 10 g; yeast extract, 6 g; NaCl, 3 g; ferrous sulfate, 1 g; and distilled water, 1 liter. This preparation was boiled gently to dissolve the gelatin, and 5-ml amounts were dispensed in tubes and autoclaved for 5 min. The final medium was prepared by aseptically adding 5 ml of A to each tube of B. The tubes were tightly capped, inverted several times to facilitate mixing, and stored at 4°C.

Isolation and confirmation of *C. perfringens*. For purification of cultures, samples from RPM tubes exhibiting a stormy fermentation were streaked on both Columbia blood agar 5% (Scott Lab 5% sheep blood) and Willis-Hobbs medium (2) and incubated anaerobically at 37°C for 24 to 48 h. The latter medium contains egg yolk and was used also for the detection of lecithinase activity. The plates were visually examined for typical *C. perfringens* colonies. Criteria included lecithinase production, general morphology, and double zone hemolysis. Individual colonies were picked to freshly steamed tubes of fluid thioglycolate medium and incubated at 37°C for 24 h. After incubation, samples from tubes exhibiting heavy frothing (gassing) were examined microscopically by using phase optics and were streaked onto Columbia blood agar (aerobic incubation) to test for contaminants.

Biochemical confirmation tests. Isolates from RPM were confirmed as *C. perfringens* by using the following media: 1% phenol red glucose, 1% phenol red lactose, 1% phenol red sucrose, 1% phenol red mannitol, nitrate broth (Difco), motility agar (Difco), and nutrient gelatin (Difco). The four phenol red sugar broths were prepared (Baltimore Biological Laboratory) and dispensed in 10-ml amounts into screw-capped glass tubes. The other media were prepared according to manufacturer's instructions and dispensed in 10-ml amounts in screw-capped glass tubes. After adding 0.1 ml of 10% filter-sterilized sodium thioglycolate, all tubes of media were steamed 5 to 10 min to drive off residual oxygen. Inoculated tubes were read after incubation at 37°C for 24 h.

An isolate was confirmed as *C. perfringens* if it was an obligate anaerobe (did not grow on the aerobic blood agar plate)—the wet mount revealed asporogenous, nonmotile large rods, usually arranged in pairs, singles, and short chains—and if the biochemical pattern was typical. Classical strains of *C. perfringens* ferment glucose, sucrose, and lactose; do not ferment mannitol; reduce nitrates to nitrites; and liquefy gelatin.

Isolates from SPS agar were confirmed by the nitrate-motility reactions, as determined by the methods of Angelotti et al. (1).

Anaerobiosis and incubation. The Baltimore Biological Laboratory GasPak system was used for incubation of plates under anaerobic conditions. This was not necessary for RPM and the biochemical identification media because thioglycolate and other reducing agents plus the depth of the medium provided sufficient anaerobiosis for the growth of *C. perfringens*. All plating and biochemical confirmatory media were incubated at 37°C for 24 to 48 h. The RPM medium was incubated at 46 to 48°C for 24 h.

Inocula. A 24-h thioglycolate broth culture of *C. perfringens* was used for the food inoculation studies. A direct count using a Petroff-Houser slide chamber was used to determine the approximate population levels. Cultures were serially diluted in 0.1% peptone water to obtain ca. 10^6, 10^4, and 10^2 cells per g of food.

RESULTS AND DISCUSSION

A preliminary study was undertaken with

pure cultures of *C. perfringens* to determine whether litmus milk media could be modified to enhance the onset of stormy fermentation. *C. perfringens* and other anaerobes require reduced conditions, such as found in iron litmus milk (litmus milk containing iron filings or nails). Under optimal conditions, iron litmus milk requires 48 to 96 h to exhibit a stormy fermentation. It was thought that with the addition of various media supplements, the growth of the organism could be greatly enhanced, thereby shortening the incubation time. The addition of 0.5% peptone significantly shortened the time to 24 h. However, autoclaving the peptone and milk together resulted in coagulation, and the sterilized medium had a white clot with a brown supernatant fluid. Subsequent experiments demonstrated a further enhancement of the growth response when the litmus milk-peptone medium was supplemented with other materials such as glucose, fluid thioglycolate, ferrous sulfate, and gelatin. The gelatin prevented clotting and allowed the medium to be autoclaved for 5 min and subsequently used at elevated incubation temperatures. After these improvements an effort was made to introduce selectivity into the supplemented milk medium. It was found that a combination of high-temperature incubation and antibiotics produced a highly selective environment which favored the growth of *C. perfringens* over most organisms. Addition of two antibiotics, neomycin sulfate (75 μg/ml) and polymyxin B sulfate (12.5 μg/ml), coupled with an incubation temperature of 46 to 48°C, afforded selectivity without reducing the recovery levels or interfering with the stormy fermentation reaction of the six strains examined. The composition of the rapid detection medium as finally developed is listed in the methods section.

A number of experiments were performed to investigate the utility of the new medium. The six strains of *C. perfringens* (24-h cultures) were diluted to three levels (10^2, 10^4, and 10^6) with 0.1% peptone water. Ten grams of blended raw chicken liver was mixed with 0.1 ml of each dilution in a plastic bag and incubated for 24 h at 37°C to afford growth of the inocula as well as any natural contaminants present. In Table 1 the recovery of inoculated *C. perfringens* is compared by using the RPM and SPS methods. In every instance the recovery level was higher in the RPM series.

In another experiment dealing with laboratory-contaminated foods, three classes of foods (raw meats, soups, and gravy) were inoculated with ca. 10^3 cells of *C. perfringens*. The six strains were inoculated independently into plastic bags containing the specific food product, which were sealed and incubated as described above. The products were then blended and serially diluted in 0.1% peptone water, and the dilutions (10^2, 10^3, and 10^4) were examined comparatively for the presence of *C. perfringens* by using both methods. Table 2 shows that *C. perfringens* was easier to detect by using the new medium. Although both Tables 1 and 2 show significantly better recovery with RPM, the differences are not great compared to those found with SPS. In both experiments, actively growing, vegetative cells were used as the inocula and the products favored their increased proliferation.

Another series of experiments was undertaken to see whether the new medium was more useful for detection of *C. perfringens* in naturally contaminated food products. A wide variety of prod-

TABLE 1. *Approximate numbers of C. perfringens cells recovered from chicken liver for each of the six strains tested*

Strain	Level of inoculation (cells/g)	Recovery level/g	
		SPS[a]	RPM[b]
FD-5	10^2	1.7×10^2	6.4×10^2
FD-5	10^4	2.5×10^4	3.5×10^4
FD-5	10^6	2.7×10^4	2.9×10^5
H-9	10^2	1.6×10^3	2.0×10^3
H-9	10^4	5.5×10^5	4.3×10^6
H-9	10^6	1.4×10^4	4.3×10^5
H-4	10^2	2.2×10^2	9.3×10^2
H-4	10^4	1.5×10^2	4.3×10^3
H-4	10^6	3.0×10^2	3.5×10^5
H-13	10^2	3.6×10^3	9.5×10^3
H-13	10^4	2.5×10^3	2.7×10^4
H-13	10^6	4.3×10^2	4.4×10^5
A	10^2	2.8×10^3	3.4×10^4
A	10^4	2.5×10^5	4.2×10^5
A	10^6	3.9×10^5	7.5×10^5
T-65	10^2	3.2×10^3	2.9×10^4
T-65	10^4	4.1×10^4	6.4×10^4
T-65	10^6	3.4×10^4	5.3×10^5

[a] Numbers determined by counts on pour plates incubated anaerobically.

[b] Numbers determined by the three-tube, most-probable-number procedure.

TABLE 2. *Comparison of SPS and RPM for detection of C. perfringens in inoculated foods*

Food inoculated[a]	No. of samples examined	No. of positive samples	
		SPS	RPM
Chicken liver	18	8	15
Chicken gizzards	12	12	12
Pork sausage	12	12	12
Chicken kidneys	12	8	12
Chicken necks	12	12	12
Beef broth (soup)	18	9	12
Chicken broth (soup)	18	11	12
Beef gravy	12	12	12

[a] Ca. 10^3 cells per g.

ucts were tested, including spices, dried soups, dried gravy products, animal by-products, vegetable extracts, and other miscellaneous food products. One sample of soil was also examined. Table 3 gives a complete listing of the products and the resultant recovery levels by using the two media. For each sample, SPS and RPM were inoculated at the 1/10 dilution level (10 g of product to 90 ml of sterile 0.1% peptone water diluent). The results clearly indicate an increased recovery and sensitivity level with RPM. By using RPM, we isolated *C. perfringens* from 71% (546/774) of the samples tested, whereas only 22% (168/774) of the samples were positive with SPS. In a subsequent experiment (Table 4), RPM was compared to TSC medium with similar results. *C. perfringens* was isolated from 71% of the 85 samples with RPM and isolated from only 14% with TSC.

TABLE 3. *Comparison of SPS and RPM for detection of C. perfringens in naturally contaminated foods*

Product	No. of samples examined	No. of positive samples	
		SPS	RPM
Marjoram	116	22	87
Oregano	100	16	81
Paprika	61	11	57
Rosemary leaves	46	4	34
Thyme	43	14	43
Whole black pepper	41	5	29
Caraway seeds	25	7	19
Sesame seeds	20	0	10
Celery seeds	20	7	17
Whole cinnamon	17	0	5
Whole ginger	11	0	4
Savory leaves	10	0	8
Sage	9	2	9
Whole nutmeg	5	0	0
Dried stroganoff mix	28	3	16
Dried beef stew mix	11	0	4
Dried vegetable leaves	10	0	3
Dried soups	6	0	1
Dried vegetable powders	6	0	1
Dried gravy base	3	3	3
Spice mix	2	0	1
Tomato powder	10	0	0
Carmine red dye	26	0	1
Chili peppers	8	0	4
Hops	8	0	5
Corn meal	6	0	3
Flour	3	0	0
Mushroom powder	3	0	0
Dried coconut	2	0	0
Dry dog food	45	45	45
Chicken feed	27	22	27
Anchovy paste	20	0	11
Liver powder	15	5	12
Turtle meat	10	1	5
Soil	1	1	1

TABLE 4. *Comparison of TSC and RPM for detection of C. perfringens in naturally contaminated foods*

Product	No. of samples examined	No. of positive samples	
		TSC	RPM
Liver powder	17	4	14
Oregano	24	6	20
Thyme	17	2	11
Marjoram	8	0	8
Sage/stuffing mix	9	0	8
Soup and gravy mixes (dehydrated)	10	0	1

In every category, with the exception of the dried dog food and the dried gravy base, RPM was superior. This was especially evident with spices. The vast majority of the food products tested were expected to contain high levels of naturally contaminating endogenous flora. Most of the spices had spore counts greater than 10^6/g. It was also assumed that the contaminating microflora was primarily spore formers due to the desiccated nature of the products and the methods associated with their manufacture.

Apparently RPM stimulates the germination of *C. perfringens* spores. This could result from a combination of several factors, including the fortified composition of the medium and the high temperature of incubation.

In another experiment RPM and SPS were compared for their ability to enumerate *C. perfringens* levels in various food products and soil (Table 5). Fourteen samples were tested, and in each instance RPM yielded higher recovery levels than SPS pour plates.

The greater sensitivity and selectivity of RPM was emphasized by the fact that even though the food products contained low to moderate levels of *C. perfringens*, RPM consistently detected higher numbers. In each case where RPM was recorded as positive (i.e., showed stormy fermentation), isolates were confirmed biochemically as *C. perfringens*. Two minor problems were noted with the RPM methodology. First, the initial preparation of the medium is somewhat laborious. However, the medium can be made up in large batches and stored at 4°C for an indefinite period. No decline in selectivity or sensitivity was noted in media stored up to 3 months. Second, due to the high glucose and peptone content of the media, a rapid reduction in pH is noted during stormy fermentation. The final pH of the broth after stormy fermentation is between 4.0 and 4.5. Usually within 30 to 60 h, the organisms have been greatly reduced in

TABLE 5. *Comparison of SPS and RPM for enumeration of C. perfringens in naturally contaminated foods and soil*

Product	No./g	
	SPS[a]	RPM[b]
Paprika	ND[c]	43
Paprika	ND	150
Paprika	ND	460
Paprika	ND	43
Paprika	ND	43
Paprika	ND	2.1
Paprika	ND	240
Black pepper	ND	23
Celery seed	ND	93
Celery seed	36	93
Oregano	ND	23
Oregano	ND	4,600
Chicken feed	20	460
Soil	370	11,000

[a] Numbers determined by counts on pour plates incubated anaerobically.

[b] Numbers determined by the three-tube, most-probable-number procedure.

[c] ND, Not detected at 1-g level.

numbers, and direct plating may not reveal viable cells. However, if a sample is transferred to fresh broth or streaked on agar plates within 18 to 24 h after initial inoculation, viable *C. perfringens* are readily recovered.

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