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Improved Selective Medium for the Isolation of Lipase-Positive *Clostridium botulinum* from Feces of Human Infants

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Isolation of lipase-positive *Clostridium botulinum* from fecal specimens establishes the diagnosis of infant botulism, contributes to the diagnosis of food-borne botulism, and is most easily accomplished by use of selective media. Modification of an available selective medium, *C. botulinum* inhibitory medium (CBI), enabled more rapid isolation of *C. botulinum*. The modified medium (botulinum selective medium [BSM] contained (per liter) 25 g of dehydrated heart infusion broth, 20 g of agar, 30 ml of egg yolk suspension, 250 mg of cycloserine, 76 mg of sulfamethoxazole, 4 mg of trimethoprim, and 100 IU of thymidine phosphorylase at pH 7.4. The two media were compared by using 15 fresh fecal specimens from infant botulism patients (10 type A and 5 type B) and a *C. botulinum* isolate that had been obtained from an inflant botulism patient and that was mixed into a fresh stool specimen from a healthy human infant. In comparison to CBI, BSM always provided better suppression of the nonbotulinum fecal flora and earlier emergence of lipase-positive colonies. Diagnosis of infant botulism was accomplished sooner with BSM than with CBI because isolation of lipase-positive *C. botulinum* was easier.

Despite recognition of infant botulism almost 9 years ago (12, 16), marked geographical variation in perceived incidence continues to be reported (1, 2). Whether these disparities in apparent incidence result from regional differences in physician awareness and in laboratory diagnostic capability or from actual differences in environmental distribution of *Clostridium botulinum* remains unclear. Improved diagnostic techniques might help resolve this uncertainty.

The diagnosis of infant botulism is established by isolating *C. botulinum* from the feces of an infant with flaccid paralysis (6, 10, 12), characteristically a challenging task because of other competing fecal bacteria. Recently, Dezfulian et al. (4) described an antibiotic-containing plating medium, *C. botulinum* inhibitory medium (CBI), that selected for *C. botulinum*, but the ability of CBI to recover *C. botulinum* directly from the feces of patients with infant botulism was not assessed.

In our experience, three factors have limited the diagnostic usefulness of CBI: (i) occasional overgrowth of *C. botulinum* by competing fecal flora, (ii) the variety and expense of its ingredients, and (iii) the time required to obtain a pure culture isolate of *C. botulinum* (72 h for two sequential streakings). To overcome these limitations, we have through modifications of CBI developed an improved selective plating medium referred to as botulinum selective medium (BSM). BSM has several advantages over CBI: its suppression of the infant fecal flora is superior, it requires fewer ingredients, it costs half as much, and it permits quantitative recovery of *C. botulinum* in 48 h. This report compares BSM and CBI agars for direct recovery of *C. botulinum* from feces of patients with infant botulism.

MATERIALS AND METHODS

Sources of *C. botulinum*. For most medium comparisons, the feces of hospitalized patients acutely ill with infant botulism were used. In addition, an isolate of *C. botulinum* maintained in cooked-meat plus glucose (19) with 0.5% yeast extract added (CMG) (Difco Laboratories, Detroit, Mich.) was obtained from feces of an infant with botulism.

Preparation of media. BSM contained (per liter) 25.0 g of dehydrated heart infusion broth, 20.0 g of Bacto-Agar (Difco), 30 ml of egg yolk suspension (50% egg yolk in saline; Difco), 250.0 mg of cycloserine (U.S. Biochemical, Cleveland, Ohio), 76.0 mg of sulfamethoxazole, and 4.0 mg of trimethoprim (Burroughs Wellcome, Research Triangle Park, N.C.). BSM contained the same concentrations of antibiotics and agar as did CBI (4). However, BSM differed from CBI both in its basal medium (heart infusion versus McClung-Toabe agar medium, respectively) and in its concentration of egg yolk (3 versus 10%, respectively). For purposes of comparison, CBI was also prepared with 30 ml of egg yolk suspension per liter (CBI-3%), in addition to its original formulation with 100 ml of egg yolk suspension per liter (CBI-10%). Also, because the thymidine contained in both CBI and BSM may enable some fecal bacteria to overcome suppression by trimethoprim-sulfamethoxazole (7, 11), 100 IU of thymidine phosphorylase (Burroughs Wellcome) was added (per liter) to both media, even though it was not part of the original formulation of CBI (4).

All medium components except egg yolk, the three antibiotics, and thymidine phosphorylase were mixed, adjusted to pH 7.4 with NaOH, autoclaved at 121°C for 20 min and cooled to 55°C in a water bath, at which point the five initially omitted ingredients were added.

Stock solutions of the antibiotics were prepared separately in advance by dissolving cycloserine (25 mg/ml) in distilled water and sulfamethoxazole (15 mg/ml) in 0.1 N NaOH. The trimethoprim (50 mg) was first dissolved in 5 ml of 0.05 N HCl at 55°C, and then distilled water was added to make a final volume of 50 ml (1-mg/ml final concentration). The filter-sterilized stock solutions were stored at 4°C before addition to CBI or BSM. After the media were poured, the plates were immediately entered into a Coy anaerobic cham-

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TABLE 1. Recovery of pure *C. botulinum* vegetative cell suspensions on two selective media

C. botulinum isolate	Type	CFU/ml on:	
		BSM-3%	CBI-3%
1	A	1.2×10^6	6.0×10^5
2	A	1.0×10^4	9.5×10^3
3	B	1.5×10^6	1.1×10^6

ber (Coy Laboratories, Ann Arbor, Mich.), dried with lids in place for approximately 36 h, and then stored in the chamber in polyethylene bags at ambient temperature (25°C) for at least 2 days, but not longer than 14 days, before use. When BSM and CBI were compared, all media for a given experiment were prepared from common stock ingredients as appropriate, poured, and put into the anaerobic chamber at the same time.

Pure culture suspensions. Vegetative cell suspensions of pure cultures were grown in 2.5% heart infusion broth (Difco) with 0.5% dextrose or in Trypticase (BBL Microbiology Systems)-peptone-glucose-yeast extract without trypsin (TPGY broth) (8) for 10 to 12 h in the anaerobic chamber and checked for absence of spores by phase-contrast microscopy. In the chamber, a series of 10-fold dilutions was made in peptone-saline (PS) solution (0.086 M NaCl, 1.0% Bacto-Peptone [pH 7.2]). From this series, 0.1-ml samples of appropriate dilutions were spread inoculated on two or three plates of each experimental medium to be compared. As a reference point, it was known from experience that a *C. botulinum* suspension with barely visible turbidity has about 10^6 CFU/ml. Dilutions of the series were also heated at 75°C for 15 min and plated on BSM to determine the proportion of spores in the cell suspension.

Patient feces. After collection and refrigerated transport to the laboratory (maximum interval, 60 h), a sample of feces was passed into the anaerobic chamber, suspended 1:10, and serially diluted 10-fold in PS. To ensure that a countable number (30 to 300) of lipase-positive colonies would be obtained, 0.1-ml samples from the 1:10 through 1:100,000 dilutions were spread plated on the experimental media. The suspensions were also heated at 75°C for 15 min and again plated on BSM to determine the concentration of *C. botulinum* spores in the patients' feces.

Seeded stools. A fresh fecal specimen from a healthy, mostly breast-fed infant 6 months of age was kept at 3 to 10°C and within 6 h was passed into the anaerobic chamber for immediate seeding with *C. botulinum*. In the chamber a 12-h pure culture of *C. botulinum* actively growing in TPGY broth was diluted in PS to a concentration of approximately 10^6 cells per ml (estimated visually by turbidity as before). An appropriate volume of this dilution was added to a weighed portion of the feces to yield a 1:10 dilution (wt/vol, feces/suspension), and the resulting mixture was then thoroughly homogenized. The homogenate was serially diluted in PS in 10-fold decrements, and 0.1-ml samples were plated on experimental media as described above to yield countable numbers of colonies.

Comparisons of CBI and BSM. The two media were compared for their ability to recover *C. botulinum* from pure cultures and from feces, and in the latter situation, to simultaneously suppress the normal fecal flora. Inoculated plates were inspected after 18, 24, 36, and 48 h of incubation (35°C) in the anaerobic chamber for emergence of lipase-positive colonies and for possible overgrowth by the competing fecal flora. Numbers of *C. botulinum* were determined by counting the lipase-positive colonies that appeared on BSM or CBI. Representative lipase-positive colonies were regularly inoculated into CMG and confirmed to be *C. botulinum* by their production of botulinum toxin, which was identified by the toxin neutralization test in mice (5).

Controls. Growth of *C. botulinum* was expected to be optimal in an antibiotic-free liquid medium. For this reason a comparison was made between the recovery of organisms determined by the fluid medium three-tube most-probable-number (MPN) technique (8), and the recovery was determined by the number of colonies on the antibiotic-containing CBI and BSM solid agar plates. In the anaerobic chamber, suspensions containing *C. botulinum* were serially diluted 10-fold in PS. Samples (0.1 ml) were spread inoculated on BSM and CBI and also inoculated into tubes of liquid CMG which had been held in the anaerobic chamber at least 7 days before inoculation. All cultures were then incubated at 35°C in the anaerobic chamber, the plates for 2 days and the MPN tubes for 5 days. At the MPN endpoints, turbid CMG cultures were checked for the presence of lipase-positive organisms by plating the cultures on BSM and for the presence of type-specific botulinal toxin by injecting culture supernatant fluids into mice (5). To verify the lack of growth of *C. botulinum* in the nonturbid CMG tubes, their supernatant fluids were also injected into mice.

RESULTS

Recovery of *C. botulinum*. Whether the inocula were pure cultures of *C. botulinum* or feces containing this organism, both BSM and CBI provided virtually equivalent recovery of lipase-positive CFU (Tables 1 and 2). However, discrete lipase-positive colonies usually appeared on BSM at least 12 h earlier than on CBI. Colonies of *C. botulinum* on these egg yolk-containing media (BSM, CBI, and the basal medium without antibiotics) had a characteristic appearance and typical lipase zones around the colonies.

TABLE 2. Recovery of *C. botulinum* from infant feces on two selective media

Stool source[a]	No. of days post-onset[b]	Type	% Spores	CFU/g on:		
				BSM-3%	CBI-3%	CBI-10%
N-1		B	<0.01	8.2×10^5	5.0×10^5	8.0×10^5
P-1	10	A	0.96	5.0×10^6	NC[c]	5.0×10^6
P-2	19	A	NT[c]	2.2×10^7	1.9×10^7	2.3×10^7
P-2	20	A	0.03	3.6×10^7	3.0×10^7	2.8×10^7
P-3	24	A	0.034	7.3×10^7	6.0×10^7	5.5×10^7
P-3	32	A	0.0002	1.0×10^8	1.0×10^8	1.0×10^8
P-4	6	B	0.09	1.1×10^8	NC	1.1×10^8
P-5	16	B	100.0[d]	3.1×10^5	2.0×10^5	1.5×10^5
P-6	8	B	0.045	5.5×10^5	5.8×10^5	7.0×10^5
P-6	15	B	0.001	8.8×10^6	9.1×10^6	9.8×10^6
P-7	24	A	<0.5	1.8×10^4	8.2×10^4	NT
P-8	8	A	<0.01	1.7×10^6	2.3×10^6	NT
P-9	6	A	2.2	2.6×10^5	6.6×10^5	NT
P-10	16	A	5.0	1.2×10^5	1.1×10^5	NT
P-11	13	A	14.0	1.0×10^6	1.0×10^6	NT
P-12	24	B	12.0	3.1×10^6	1.7×10^6	NT

[a] N-1 is a suspension of feces from a normal infant that was seeded with a pure culture of an infant botulism patient isolate. P indicates infant botulism patient feces; each number designates a separate patient.

[b] Number of days after onset of infant botulism at which feces were collected.

[c] NC, Not countable because lipase zones were confluent; NT, not tested.

[d] Specimen obtained by sterile water enema.

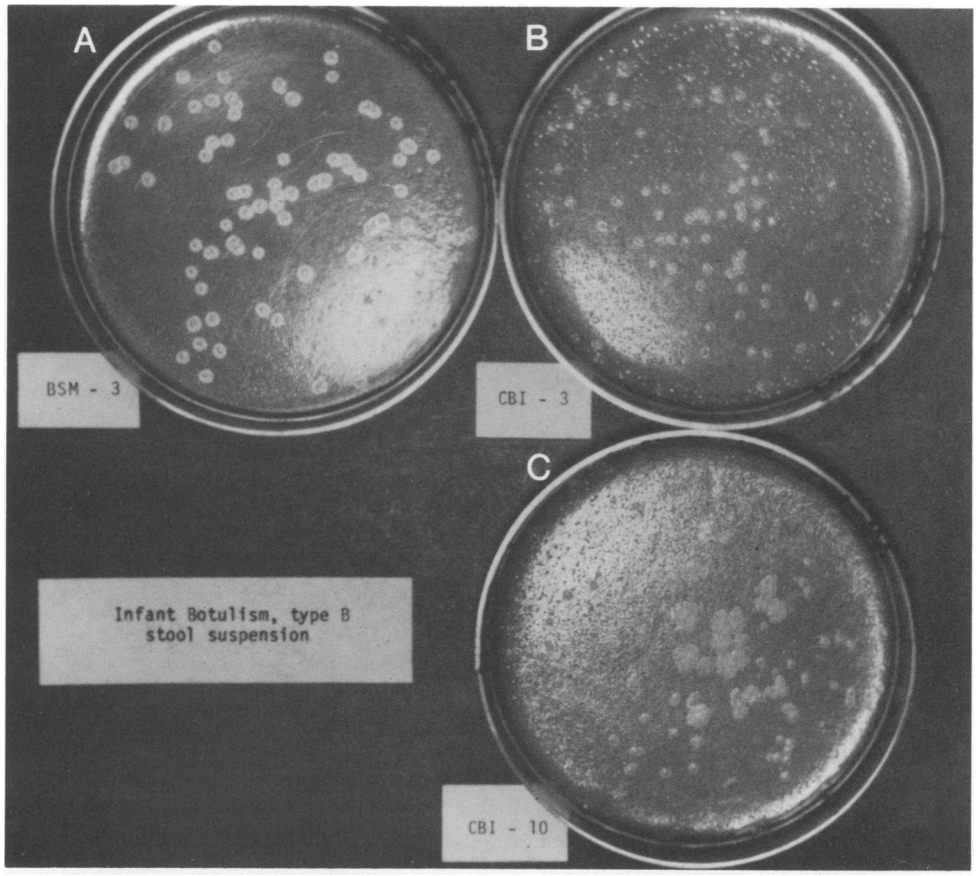

FIG. 1. Comparison of BSM (A) and CBI (B and C) for isolation of lipase-positive *C. botulinum*. Both plates were spread inoculated with 0.1 ml of a stool suspension from an infant with botulism. Note improved suppression of the competing normal fecal flora by BSM (A) and merged borders of lipase zones on CBI-10% (C).

Suppression of nonbotulinum fecal flora. When tested either with feces of infants with botulism or with feces of healthy infants seeded with *C. botulinum*, BSM always provided better suppression of the extraneous fecal flora than did CBI (Fig. 1). Nearly complete suppression of the fecal flora on BSM always enabled easy counting and picking of lipase-positive colonies. Breakthrough colonies of the nonbotulinum fecal flora were fewer and smaller on BSM than on CBI. However, to minimize undesirable spreading of colonies and of lipase zones on either selective medium, it was necessary to incubate the plates under low humidity. Thymidine phosphorylase, when included in BSM, always improved suppression of the normal fecal flora, whereas its addition to CBI did not materially improve suppression.

It was previously shown (4, 18) that the trimethoprim, sulfamethoxazole, and cycloserine in CBI do not reduce quantitative recovery of *C. botulinum*. This observation was checked and confirmed by comparing BSM and CBI plates containing these antibiotics to BSM and CBI plates from which these antibiotics had been omitted. Recovery of either pure spore or vegetative cell cultures of *C. botulinum* on these antibiotic-free plates was quantitatively equivalent to recovery on the antibiotic-containing BSM and CBI plates.

In three experiments that compared the counts of *C. botulinum* on antibiotic-containing BSM and CBI plates, recovery of *C. botulinum* in CMG broth was also determined by the MPN technique (8). In all three experiments, the *C. botulinum* counts obtained by plating on either BSM or CBI fell within the 95% confidence interval of the recovery in liquid CMG determined by the MPN technique (Table 3).

TABLE 3. Recovery of *C. botulinum* on BSM and CBI compared with MPN estimation

Patient	Type	Source[a]	% Spores	MPN	MPN 95% confidence interval	CFU/g (%[b])	
						BSM	CBI
P-11	A	p	14	1.9×10^6	0.3×10^6–7.6×10^6	1.0×10^6 (56)	1.0×10^6 (56)
P-12	B	p	12	9.3×10^6	1.5×10^6–38.0×10^6	3.1×10^6 (34)	1.7×10^6 (18)
P-13	A	ss	100	6.6×10^{5c}	1.4×10^5–23.0×10^{5c}	1.9×10^{5c} (30)	NT[d]

[a] p, Patient stool; ss, spore suspension.
[b] Compared with MPN.
[c] Per milliliter of pure culture spore suspension.
[d] NT, Not tested.

DISCUSSION

Use of BSM permits quantitative recovery of lipase-positive colonies from feces of infants with botulism. BSM performs this function more rapidly, with greater suppression of the competing fecal flora and at lesser expense than does CBI. Present costs of ingredients per liter (excluding the antibiotics common to both media) are $8.16 for BSM and $16.12 for CBI. Also, BSM may be useful in diagnosing food-borne botulism (6) and in clinical (14, 15, 20) and animal (13, 17) studies of infant botulism.

BSM selected and differentiated *C. botulinum* from the infant fecal flora without quantitative reduction in its recovery (Table 2), and on BSM the lipase zones were well-defined and discrete. In contrast, on CBI the lipase zones from some stool suspensions were so large and diffuse that enumeration of colonies was very difficult or impossible (Fig. 1). BSM can be used for toxin types A and B. BSM may also be useful for the other lipase-positive toxin types of *C. botulinum* (C, D, E, and F), although these were not tested in this study. BSM would not be expected to be helpful in isolating *C. botulinum* type G because it is lipase negative (3). Because other anaerobes also produce lipase, definitive confirmation of presumptive *C. botulinum* isolates on BSM requires demonstration of their production of botulinum toxin by the toxin neutralization test in mice (5, 9).

BSM was formulated with 3% egg yolk suspension because this percentage was more economical and in previous work was found to provide satisfactory definition of lipase-positive zones (20). In contrast, CBI as originally described contained 10% egg yolk suspension (4). A comparison of CBI which contained either 3 or 10% egg yolk suspension determined that the 10% egg yolk-containing CBI usually yielded lipase zones that were less diffuse and more easily seen. Both the 3 and 10% egg yolk concentrations in CBI yielded equivalent recovery of *C. botulinum* from feces.

The presence of thymidine phosphorylase in BSM improved the suppression of the nonbotulinum infant fecal flora. To ensure comparability of experimental conditions, thymidine phosphorylase was also added to CBI, but its inclusion in CBI did not improve suppression of the nonbotulinum fecal flora, which in every instance was less than the suppression achieved with BSM. In summary, BSM, when used together with the Gram stain and fecal toxin testing, may aid in the rapid diagnosis of infant botulism.

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