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Isolation and Enumeration of *Clostridium botulinum* by Direct Inoculation of Infant Fecal Specimens on Egg Yolk Agar and *Clostridium botulinum* Isolation Media

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Direct plating of stool specimens on selective (*Clostridium botulinum* isolation) and nonselective (egg yolk agar) media was evaluated as an aid in confirming infant botulism. *C. botulinum* was isolated from 13 of 14 culture-positive specimens with *C. botulinum* isolation and from 8 of 14 egg yolk agar. No lipase reaction was seen on plates of 31 culture-negative specimens.

Coproexamination has proven to be a valuable technique in the diagnosis and confirmation of botulism (4, 5, 6). Since circulating botulinal toxin is usually not detected in the sera of infants with botulism, the technique is especially helpful in the diagnosis of infant botulism (5). Coproexamination involves testing stool extracts for toxin by mouse toxin neutralization tests and culturing the feces for *Clostridium botulinum*. At the Centers for Disease Control, the usual method for isolation of *C. botulinum* from fecal samples involves streaking of unheated and heated enrichment cultures (cooked meat glucose starch) on Centers for Disease Control-modified McClung-Toabe egg yolk agar (EYA) medium (3). EYA is differential for lipase-positive organisms, including most types of *C. botulinum*, but also allows the growth of many other species of bacteria found in the fecal flora. Because of the nonselective nature of EYA, it has not been used at the Centers for Disease Control for direct plating of stool specimens.

C. botulinum isolation medium (CBI) was developed by Dezfulian et al. (2) for the selective isolation of *C. botulinum* from mixed fecal flora. The medium is similar to EYA but contains cycloserine, sulfamethoxazole, and trimethoprim. The antimicrobial agents inhibit the majority of microorganisms present in the feces but do not inhibit group I *C. botulinum* (toxin types A, B, and F), which includes the types involved in infant botulism (1). This group of *C. botulinum* grows on CBI and exhibits lipase activity. Preliminary studies with seeded human adult feces showed CBI to be superior to nonselective EYA for the isolation and differentiation of *C. botulinum* from mixed fecal flora in simulated specimens (2). In this study we evaluated CBI with fecal specimens from infants with suspected infant botulism.

(This research was presented in part previously [C. Glasby, Doctor of Public Health dissertation, University of North Carolina-Chapel Hill, 1981].)

Fifty-five specimens from suspected cases of infant botulism were examined culturally for the presence of *C. botulinum* by direct inoculation of specimen suspensions on EYA and CBI plates. All specimens had been examined immediately after arrival at the Centers for Disease Control laboratory by the routine procedures for laboratory confirmation of botulism (5). After the initial tests, a sample of each specimen suspension in gelatin diluent (5) was coded by number and stored at −20°C until use in this study. The suspensions were stored for 3 weeks to 16 months (median of 9 months).

At the time of this study, the samples were removed from the freezer and held at room temperature for 10 to 30 min for thawing. Three 10-fold dilutions of each fecal suspension were made in phosphate-buffered saline, and 0.01 ml of each fecal suspension or dilution thereof was spread evenly over the surface of one plate of each of the two media with a calibrated 10-μl inoculating loop (A/S Nunc, Roskilde, Denmark). After 48 h of anaerobic incubation, the plates were observed for lipase activity and the numbers of lipase-positive colonies were determined if possible. At least two isolated colonies (if accessible on the plate) were picked to identify the organism responsible for the lipase activity by mouse toxicity and neutralization tests and by morphological characteristics. Production of botulinal toxin alone by a lipase-positive organism was considered sufficient for identifying an isolate as *C. botulinum*. Complete characterization of the organisms isolated in the original laboratory examination showed that they were typical of proteolytic strains of *C. botulinum* (1).

Of the 55 fecal specimens obtained from the infants examined, 14 contained viable *C. botulinum* isolates at the time of this study, as determined by recovery from enrichment cultures. Thirteen of these culture-positive specimens yielded lipase-positive colonies on CBI plates, and *C. botulinum* was isolated in each case by picking an isolated colony. Twelve of the specimens showed observable lipase-positive colonies on EYA, and in only eight instances could isolated colonies be picked. All of the areas in which there was lipase activity on EYA plates from four of the specimens were heavily overgrown with lipase-negative organisms. One specimen (no. 3680; see Table 1) did not yield lipase-positive growth on directly plated EYA or CBI, although *C. botulinum* grew out in enrichment cultures and was isolated as a lipase-positive organism when these cultures were streaked on CBI and EYA. Two other specimens (nos. 2775 and 3301; see Table 1), which were culture positive when originally examined, were negative for lipase activity on both media and, at the time of this study, were also negative on enrichment culture. Every specimen in this study which yielded lipase-positive bacterial growth when directly inoculated onto either EYA or CBI also yielded isolates of *C. botulinum* from the CBI plate. All lipase-pos-

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TABLE 1. Isolation and enumeration of *C. botulinum* by direct inoculation of stools from infant botulism patients initially found positive by enrichment culture

Specimen no.	Medium	No. of lipase$^+$ CFU/g of feces (log_{10})[a]	Toxin type	Isolation at time of study	Directly streaked plates	
					C. botulinum isolation	Dilution yielding isolated colonies (log_{10})
2761	EYA	5.48	B	Yes	Yes	2
	CBI	5.30			Yes	1
2775	EYA		A	No	No	
	CBI				No	
2776	EYA	5.48	A	Yes	Yes	0
	CBI	5.30			Yes	0
2782	EYA	5.32	B	Yes	Yes	3
	CBI	5.80			Yes	1
2978	EYA	4.23	B	Yes	No	
	CBI	4.20			Yes	1
2980	EYA	6.86	B	Yes	Yes	3
	CBI	6.90			Yes	3
3014	EYA	5.18	B	Yes	Yes	3
	CBI	4.98			Yes	3
3021	EYA	2.18	B	Yes	No	
	CBI	2.65			Yes	0
3258	EYA	3.48	B	Yes	Yes	1
	CBI	3.43			Yes	1
3280	EYA		A	Yes	No	
	CBI	3.08			Yes	0
3300	EYA	4.00	B	Yes	No	
	CBI	4.20			Yes	0
3301	EYA		A	No	No	
	CBI				No	
3302	EYA	3.90	B	Yes	No	
	CBI	4.15			Yes	0
3517	EYA	5.56	A	Yes	Yes	3
	CBI	5.70			Yes	3
3675	EYA	6.18	A	Yes	Yes	3
	CBI	6.00			Yes	3
3680	EYA		A	Yes	No	
	CBI				No	

[a] Lipase$^+$, Lipase-positive.

itive organisms which could be isolated after direct streaking of suspensions or streaking of enrichment cultures on either EYA or CBI produced botulinal toxin type A or type B. No lipase activity was seen on any of the plates from specimens which were not culture positive at the time of this study.

Table 1 shows the results obtained with all of the specimens found positive on original examination. Lipase-positive colony counts, when plates were countable, were comparable for the two media. On the EYA plates, many, and sometimes all, of the lipase-positive colonies were overgrown with lipase-negative organisms (see Fig. 1). The results showed that, although *C. botulinum* could be isolated and numerically estimated from direct inoculation of EYA, some specimens gave positive results only on CBI plates (Table 1). Although colony counts of organisms other than *C. botulinum* were sometimes nearly as high on CBI as on EYA, colonies of such organisms on CBI were usually very small and did not occupy much space, leaving many colonies of *C. butulinum* readily accessible for isolation. Figure 1 shows pictures of the colonies found on CBI and EYA.

In addition to comparing CBI and EYA, we found that direct plating of stool suspensions on EYA or CBI can be used to advantage for investigating suspected cases of infant botulism. Isolation has routinely been done by streaking enrichment cultures on plating media. Isolation from a directly inoculated plate saves the 4 days of incubation time necessary for enrichment cultures. Also, these results show that in infant botulism cases *C. botulinum* in most specimens

remains viable and recoverable even after extended periods of storage. On the other hand, frozen storage may have enhanced the recovery of sporeforming organisms since many vegetative cells may not have survived. Further experience with direct plating of fresh specimens is needed.

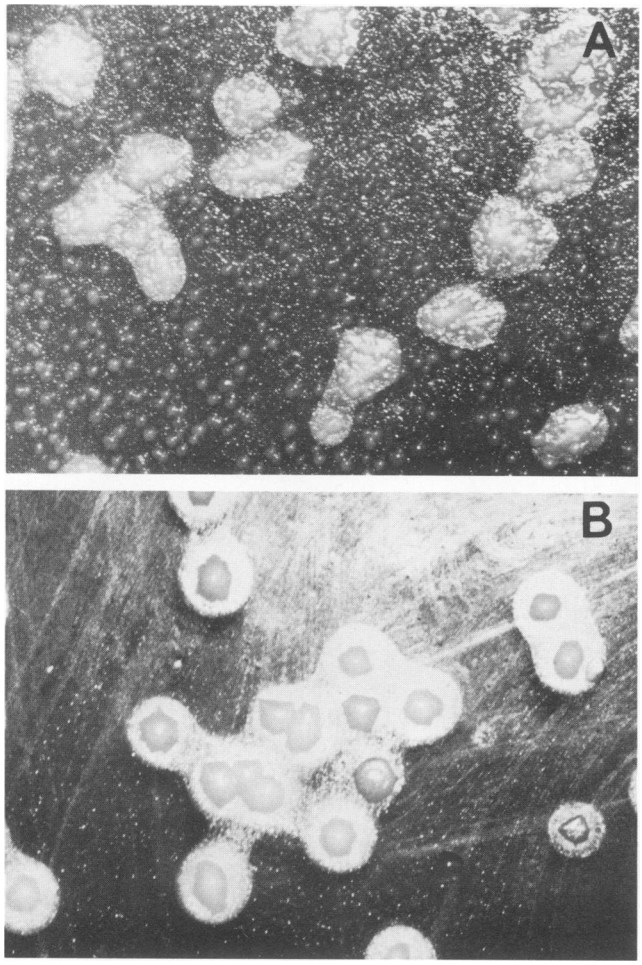

FIG. 1. Colonies (specimen no. 2782) found on plates of EYA (A) and CBI (B) after 48 h of anaerobic incubation at 35°C; 10 μl of a 1:10 dilution of fecal suspension was spread evenly over the surfaces of the plates. The original suspension was a 1:3 dilution of the specimen.

In this study, direct plating of dilutions was done to determine the efficiency of inhibition of organisms other than *C. botulinum* by the antimicrobial agents in CBI. For five of the specimens *C. botulinum* could be isolated only from CBI, and for two of the specimens it was isolated at a lower dilution on CBI. For six of the specimens *C. botulinum* was isolated from the same dilution on both media. Plating of dilutions also allowed us to estimate the number of *C. botulinum* in the specimens by counting the number of lipase-positive colonies, whether isolated or overgrown. The numbers were generally comparable for both media. Since the specimens had been stored, the numbers were probably considerably lower than when the specimens were obtained from the infants. Nevertheless, the numbers shown in Table 1 were probably the result of multiplication in the gut rather than mere passage of ingested bacteria or spores. This evidence would not be provided by isolation of the organism from enrichment cultures. In practice, one would not have to make dilutions. Direct streaking for isolation would probably have been just as successful for quick recovery of the organism and would give an indication whether lipase-positive organisms were numerous or rare. The results of this study indicate that direct streaking of infant botulism specimens on a nonselective medium (EYA) can also often be helpful in providing confirmatory evidence and isolation, even though fecal specimens contain a high load of normal flora. The success rate, however, will be higher with CBI.

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