JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1984, p. 379–383
0095-1137/84/090379-05$02.00/0

Vol. 20, No. 3

Enzyme-Linked Immunosorbent Assay for Detection of *Clostridium botulinum* Type A and Type B Toxins in Stool Samples of Infants with Botulism

MANOUCHEHR DEZFULIAN,[1]* CHARLES L. HATHEWAY,[2] ROBERT H. YOLKEN,[3] AND JOHN G. BARTLETT[1]

Infectious Disease Division, Department of Medicine,[1] and Eudowood Division of Infectious Diseases, Department of Pediatrics,[3] The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and Division of Bacterial Diseases, Centers for Disease Control, Atlanta, Georgia 30333[2]

Received 30 January 1984/Accepted 4 June 1984

An enzyme-linked immunosorbent assay (ELISA) for *Clostridium botulinum* type A and type B toxins was assessed for diagnostic accuracy in cases of infant botulism. This test was positive in all 22 cases confirmed by the conventional tests, which included the mouse lethality assay and stool culture. Stool specimens from five cases were positive by culture, but the mouse lethality bioassay was either negative or toxicity was judged nonspecific since it could not be neutralized by specific antitoxin. The positive ELISA results in these specimens suggested that this assay may be more reliable, in some cases, than the mouse bioassay. Of the 21 fecal specimens from suspected foodborne cases, 2 contained botulinal toxin demonstrable by the mouse assay and the ELISA. With regard to specificity, 35 fecal specimens from infants and 19 from suspected foodborne cases which were negative in the bioassay for botulinal toxins A and B were also negative in the ELISA. Only two fecal specimens with negative bioassay gave positive ELISA readings, providing a specificity rate of 96%. These results suggest that the ELISA may serve as a useful screening test to detect *C. botulinum* toxin in clinical specimens.

Infant botulism is an enteric disease that results from in vivo production of neurotoxin by *Clostridium botulinum*, which colonizes the intestinal tract of susceptible infants 1 to 9 months old (1). Virtually all cases have been associated with *C. botulinum* type A or type B (4, 8, 11); type F has been implicated in only one case (9). The diagnosis of infant botulism can be established by demonstration of botulinal toxin and *C. botulinum* in feces of an infant exhibiting typical signs and symptoms of the disease (8, 11). The preferred test for toxin detection is the mouse bioassay and toxin neutralization test. However, this assay is generally considered cumbersome and inconvenient, since it requires large numbers of mice and facilities which are not readily available in clinical microbiology laboratories. In addition, mouse bioassay is not suitable for examination of specimens containing other lethal substances which may cause nonspecific death or interfere with neutralization of botulinal toxin (10; M. Dezfulian, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C317, p. 324).

We recently reported the preparation of antibodies against *C. botulinum* toxins A (5) and B (M. Dezfulian and J. G. Bartlett, Diagn. Microbiol. Infect. Dis., in press) in immunologically tolerant animals. By selective suppression of the immunological response of the host to unwanted antigens (by repeated inoculation of infant rabbits with culture filtrates of *C. sporogenes*) and subsequent immunization with botulinum toxoid A or B, we were able to produce the desired antitoxin types without purifying the toxins. Except for cross-reactions between *C. botulinum* toxins A and B, these antitoxins appeared specific. They did not react with culture filtrates of nontoxigenic variants of type B, any other *C. botulinum* type (C, D, E, F, and G), or 18 other *Clostridium* species, including *C. sporogenes*. Using these antitoxins, we developed a sensitive enzyme-linked immunosorbent assay (ELISA) for detection of *C. botulinum* toxins A and B. The present report concerns the application of these assays with stool specimens from infants with suspected botulism and a preliminary investigation of fecal specimens from suspected cases of foodborne botulism.

MATERIALS AND METHODS

Fecal specimens. Seventy-eight fecal specimens that had been examined for diagnostic confirmation of botulism by mouse bioassay at the Centers for Disease Control, Atlanta, Ga., were used for evaluation of the ELISA system. These included 57 specimens from infants and children less than 28 months old, one from a 9-year-old child, 2 from teenagers, and 18 from adults (see Tables 3 and 4). The specimens from persons over 28 months old were from patients suspected of suffering from foodborne botulism. Two additional specimens (JHIB1 and JHIB2) were from cases of infant botulism confirmed by mouse assay at Johns Hopkins Hospital, Baltimore, Md. (13). Laboratory confirmation of the presence of botulinum toxin in the JHIB1 specimen was achieved after dialysis of the fecal extract against phosphate buffer (Dezfulian and Bartlett, in press).

All specimens had been tested for toxin by mouse assay and cultured for *C. botulinum* by methods published elsewhere (7, 8). At the time of the original examination, each specimen was suspended in sufficient sterile gelatin diluent (0.2% gelatin, 0.4% Na_2HPO_4, adjusted to pH 6.2 with HCl) so that a satisfactory extract for mouse testing could be obtained after centrifugation. The volume of diluent per gram of specimen varied, depending on the dryness or consistency of the specimen. In some cases, a very dilute suspension was made due to sparse fecal material. In a few others, no diluent was added because the specimen was quite liquid, presumably reflecting collection by enema. A small sample (about 1 ml) of each suspension was placed in a

* Corresponding author.

screw-cap vial, coded, and stored in a freezer (−20°C) at the time of the original laboratory examination. The frozen samples had been thawed on at least one occasion when some of the material was removed for a different study; the remaining material was refrozen.

Six unsuspended fecal samples (no. 81 through 84, JHIB1 and JHIB2) were provided as known positive specimens for developmental work. The remaining 74 specimens were submitted for ELISA tests with no information other than the code numbers.

Organisms. Three isolates of *C. botulinum* type A (strains A28, a10K, and a3K) were obtained from the stock collection of the Centers for Disease Control. Details regarding confirmation of their identity have been described previously (6). The centrifuged supernatant of a 4-day-old bacterial culture was filtered through a 0.45-μm Millipore filter, and the filtrate was used for the detection of toxin by ELISA.

Antitoxin. Type A, type B, and polyvalent (A, B, C, D, E, and F) diagnostic botulinal antitoxins were obtained from the Centers for Disease Control. Antigens to produce these antitoxins in burros were the partially purified toxoid prepared for human immunization (3). Rabbit antitoxins specific for botulinal toxins A and B were prepared by the method described previously (5). The optimal dilutions of rabbit and burro antitoxins used for coating wells for the ELISA were determined by checkerboard titration.

ELISA. The ELISA procedure described by Voller et al. (15) was adapted with some modifications (5, 16). Polyvinyl U-bottomed microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of a 1:5,000 dilution of burro type A, type B, or polyvalent botulinal antitoxins in 0.05 M carbonate buffer (pH 9.6). Blank wells were coated with normal horse serum. The specificity of the test system was not affected by the type of antitoxin used, but for the sake of convenience, wells were usually coated with the polyvalent antitoxin. After overnight incubation at 4°C, the coated plates were either used immediately or stored at 4°C and used within 2 weeks. Before the specimens were tested, the coated plates were washed 5 times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Unless otherwise specified, a 25-μl sample of the fecal extract was added to appropriate wells containing 75 μl of 40% fetal bovine serum (FBS) in PBS-T with 0.05% gelatin (PBS-TG). After an overnight incubation at 4°C, plates were washed as described above, and 100 μl of a 1:500 dilution of rabbit type A or type B botulinal antitoxin in PBS-TG was added. Plates were incubated for 90 min at 37°C and washed 5 times with PBS-T. A 100-μl sample of a 1:1,000 dilution (in PBS-TG) of alkaline phosphatase-conjugated goat antirabbit immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.) was added. After incubation at 37°C for 90 min, the excess conjugate was removed by washing, and 100 μl of a 1-mg/ml solution of *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) in diethanolamine buffer (pH 9.8) was added. Plates were incubated at room temperature for 60 min, and the absorbance was measured at 405 nm in a microplate colorimeter reader (model MR 480; Dynatech Laboratories). The ELISA was performed in duplicate with type A and type B antitoxins when the volume of the fecal specimen was sufficient. In a few cases, the quantity of specimens was such that only one antiserum could be used. Net absorbance was calculated by subtracting the mean absorbance of wells coated with botulinal antitoxin from the mean absorbance of the blank wells. The criterion for a positive assay was an absorbance value exceeding three standard deviations above the mean value of the negative fecal specimens. The diagnostic limits for infant and foodborne cases were determined from absorbance values of negative specimens obtained from infant and foodborne cases, respectively.

TABLE 1. Influence of human stool and FBS on sensitivity of ELISA in detecting botulinal toxin

C. botulinum type A culture filtrate	ELISA values ± SD[a] with type A rabbit antitoxin		
	PBS-TG	Fecal extract	Fecal extract plus FBS
A28	0.541 ± 0.02	0.027 ± 0.006	0.162 ± 0.04
a10K	0.238 ± 0.008	0.002 ± 0.001	0.080 ± 0.004
a3K	0.528 ± 0.114	0.084 ± 0.006	Not done

[a] Net absorbance (three replications) at 405 nm ± standard deviation.

TABLE 2. Comparison of two diluents for detection of botulinal toxins in stool of infants with botulism

Fecal specimen	Toxin type	ELISA values ± SD[a] with homologous antitoxin	
		PBS-TG	FBS
81	A	0.055 ± 0.008	0.149 ± 0.001
84	B	0.092 ± 0.015	0.170 ± 0.001
JHIB1	B	0.123 ± 0.01	0.292 ± 0.02
JHIB2	A	1.167 ± 0.047	1.271 ± 0.189

[a] Net absorbance (three replications) at 405 nm ± standard deviation.

Effects of normal human feces and FBS on sensitivity of ELISA in detecting botulinal toxin. We examined the effects of human feces in our ELISA system by measuring the level of toxin in the presence and absence of normal human fecal extract. The dual effects of fecal extract and FBS on toxin detection were also tested. The extract was the centrifuged supernatant of a 1:4 suspension of a fecal specimen in PBS-TG from a healthy 10-month-old infant. Samples of 25 μl of crude botulinal toxin (culture filtrate) were tested in ELISA after being mixed with 75 μl of the fecal extract, the extract in 40% FBS, or PBS-TG. We also studied the effects of FBS on the sensitivity of ELISA in detecting *C. botulinum* toxin in the stool of infants with a similar method.

RESULTS

Interference of toxin detection in ELISA and its reversal by FBS. The absorbance values of ELISA were drastically reduced when the fecal extract was added to culture filtrates of *C. botulinum* isolates (Table 1). FBS, on the other hand, partially blocked the fecal interference (Table 1). The sensitivity of ELISA in detecting *C. botulinum* toxins in fecal specimens was also improved when 40% FBS was used as a diluent (Table 2).

ELISA compared with mouse bioassay and culture for evidence of botulism. Of 59 fecal specimens from cases of suspected infant botulism, 22 were positive for *C. botulinum* or botulinal toxin A or B by conventional tests. These included 17 specimens that were positive by mouse bioassay for toxins A or B and 5 that were negative in mouse bioassay but positive by culture for *C. botulinum* type A or B. The mouse lethality test in four of these five cases showed nonspecific toxicity, indicating that the specimen was toxic to mice, but there was no neutralization with specific antitoxins. One specimen was culture positive for *C. botulinum* type A and nontoxic to mice at the dilution tested. Two additional specimens were culture positive for *C. botulinum* type F, and one of these was positive in mouse bioassay for the homologous toxin (Table 3). Diagnostic limits for ELISA readings were based on absorbance values exceeding three deviations above the mean value of the negative specimens.

TABLE 3. Infant fecal specimens examined for evidence of botulism

Specimen code no.	Age (mo)	Dilution of fecal suspension	Original laboratory results			
			Toxin	Culture	ELISA[a]	
					A	B
1	25				0.002	
2	28	1:4	NS[b]		0.001	
4	3	1:6	B	B	0.147	
5	5	1:5			0.000	
6	10	1:9		A	0.067	
8	5	1:2			0.001	
9	25	1:3			0.000	
10	2	1:5			0.048	
13	3	1:4	B	B	0.140	
15[c]	7	1:3			0.367	
16[c]	7	1:2.5			0.000	
17[c]	2	1:3.5			0.000	
18[c]	2	1:2.5			0.000	
19[c]	2	1:2.5			<0.000	
20	9	1:3.5	NS		<0.000	0.079
21	6	1:4	NS		<0.000	0.001
22	9	1:3			0.000	0.001
23	12	1:11			0.000	0.022
24	12	1:6			0.000	<0.000
25	3	1:3.5			<0.000	0.000
26	4	1:6	B	B	0.111	0.241
27	2	1:4	B	B	0.096	0.146
28	1	1:11		F	<0.000	
29	1	1:5	F	F	<0.000	<0.000
30	4	1:5	B	B	0.586	0.881
31[d]	2	1:1.5	B	B	0.842	0.922
32	2	1:6			0.000	0.000
33	2	1:3			0.000	0.000
34[d]	5	1:1	B	B	0.125	0.243
35	(duplicate of 34)		B	B	0.067	0.145
37	4		A	A	0.339	0.067
38[c]	1	1:2			0.000	0.000
39	5	1:11	B	B	0.031	0.145
40	4	1:4	NS	A	0.200	0.066
41	5	1:4	B	B	0.050	0.110
43	8	1:4			0.000	0.000
44	8	1:2			0.033	0.000
45	8	1:4			0.000	0.000
53	4	1:4			<0.000	0.000
57	1	1:11			0.000	0.003
58	2	1:2			0.000	0.000
59		1:20(est)			0.000	0.000
60	27	1:2			0.000	0.000
62	5	1:3	NS[e]	A	0.540	0.473
63	(duplicate of 62)		NS[e]	A	0.474	0.704
64	1	1:3.5	NS		0.000	0.000
68	13	1:2.5			0.029	<0.000
71	2	1:11			0.001	<0.000
73	3	1:4	A	A	0.449	0.457
74	10	1:2			0.000	0.174
76	10	1:4			0.000	0.000
78	1	1:16			0.015	0.000
79[d]	1	1:1	A	A	0.241	0.242
80[c]					0.000	0.000
81	8	1:1	A	A	0.210	0.138
82[d]	1	1:4	B	B	0.585	
84	3	1:4	NS	B	0.170	
JHIB1	4	1:4	B	B	0.179	0.292
JHIB2	1	1:4	A	A	1.271	

[a] Net absorbance >0.051 (for type A) and >0.083 (for type B) was considered positive.
[b] NS, Nonspecific toxicity.
[c] Specimens from cases of sudden infant death syndrome.
[d] Specimen possibly obtained by enema.
[e] Nonspecific toxicity, but evidence of botulinal toxin because of signs of botulism in mice.

The limits were 0.051 for type A and 0.083 for type B. By these criteria all 22 infant stool specimens that were positive by conventional tests for *C. botulinum* or botulinal toxin A or B were positive by ELISA. With nine specimens containing type A toxin, the mean ELISA reading was 0.421 ± 0.353 (± 1 standard deviation) with type A antitoxin and 0.307 ± 0.243 with type B antitoxin. With 13 type B specimens, these values were 0.240 ± 0.256 and 0.347 ± 0.320 with type A and type B antitoxins, respectively. Despite a significant cross-reactivity between the type A and type B antitoxins, higher absorbance readings were usually produced with the homologous antiserum. Of 35 specimens that were negative with conventional tests, 33 were negative in the ELISA and 2 were positive. In addition, specimens from the two type F cases were both negative in the ELISA (Table 3) with antitoxins for types A and B.

The 21 fecal specimens from suspected cases of foodborne botulism gave essentially zero ELISA readings with the exception of two (no. 42 and 83) which were the only toxin-positive specimens by mouse assay (Table 4). For toxin-negative specimens, the average ELISA readings were −0.002 ± 0.006 for type A and −0.010 ± 0.020 for type B. In this case, absorbance values above 0.016 for type A and above 0.050 for type B were considered positive.

A comparison between the ELISA and combined testing by mouse assay and culture for all the fecal specimens is summarized in Table 5. This shows that 24 of 25 specimens that were positive by conventional tests for *C. botulinum* or botulinal toxin A or B were positive by ELISA. Of 55 specimens that were negative by conventional tests, 53 were negative by ELISA. Thus, sensitivity and specificity rates were both 96%.

DISCUSSION

Infant botulism was initially described in 1976 (11, 12), has been recognized with increasing frequency, and now outranks foodborne cases as the most common form of botulism in the United States. Infant botulism may account for some cases of sudden infant death syndrome or "crib death" (2). However, this is regarded as one end of a spectrum of disease in which many victims may have rather trivial symptoms, leading some authorities to conclude that the condition may be greatly underdiagnosed.

Toxin detection in stool by mouse assay is currently the method of choice for confirmation of infant botulism (8, 11). The ELISA described here offers an alternative test which, unlike mouse assay, can easily be carried out in routine clinical laboratories. However, because of the rarity of botulism, performance of this assay in such laboratories may not be justified. On the basis of the data presented, the ELISA appeared to be highly sensitive and reasonably specific for the detection of type A and type B botulinal toxins in fecal specimens. It was found positive in all specimens from infant botulism cases that were confirmed for *C. botulinum* toxin A or B by conventional tests. This included five fecal specimens which showed positive cultures but were either nontoxic in mice or showed toxicity that was judged nonspecific since it could not be neutralized by specific antitoxin. In an additional specimen, toxin neutralization was achieved after dialysis of the fecal extract. All of these specimens were positive by ELISA, indicating that this method may actually be more reliable, in some cases, than the conventional mouse assay. Two of the fecal specimens from suspected foodborne cases contained botulinal toxin demonstrable by the mouse assay, and both were positive by the ELISA. As an indication of the specificity of our method, all but two fecal specimens from suspected infants and foodborne cases that were negative in the bioassay for type A and type B botulinal toxin were also negative in the ELISA. The positive ELISA for two bioassay-negative specimens might reflect the presence of a "naturally formed" nonlethal toxoid or a nonspecific reaction.

Inclusion of FBS in fecal diluent was shown to improve the sensitivity of our in vitro system. This finding is in accordance with a recent report (14) that many fecal specimens contain interfering substances which reduce the sensitivity of ELISA for toxin A of *C. difficile* and some viral antigens. The interference is apparently caused by desorption of the immunoreactants from solid-phase surfaces and is partially reversed by FBS as well as by specific and nonspecific protease inhibitors (14).

To achieve the highest sensitivity with limited amounts of the available specimens, we included an overnight incubation of the specimens at 4°C and an additional 4 to 5 h at 37°C for performing the subsequent steps. Insufficient quantities of stool did not allow testing of other alternative incubation schedules. Nevertheless, by using precoated plates and a shorter period for incubation of the specimens, one may be able to complete the assay within 6 h.

TABLE 4. Fecal specimens from noninfants examined for evidence of foodborne botulism

Specimen code no.	Age (yr)	Dilution of fecal suspension	Original laboratory results			
			Toxin	Culture	ELISA[a]	
					A	B
42	53	1:2	A		0.037	0.000
46	38	1:3			0.000	0.000
47	23	1:1.5			0.000	0.000
48	9	1:2			0.000	0.000
49	61	1:3	NS[b]	E	0.000	<0.000
50	61	1:3	NS	E	0.000	0.000
51	71	1:3		A	0.000	0.000
52	35	1:2			0.000	0.000
54[c]	23	1:1			0.000	<0.000
55[c]	27	1:1			0.000	<0.000
56	73	1:2.5	NS		<0.000	0.001
61	16	1:3	NS		0.000	<0.000
65	40	1:3			0.001	<0.000
66	38	1:2			0.000	<0.000
67	14				0.001	0.000
69	72	1:10			<0.000	0.000
70	72	1:2			<0.000	0.001
72	21				0.000	0.000
75[c]	57	1:4			0.000	0.001
77	79				<0.000	0.000
83	34	1:4	B	B	0.059	0.099

[a] Net absorbance >0.016 (for type A) and >0.050 (for type B) was considered positive.
[b] NS, Nonspecific toxicity.
[c] Liquid fecal specimen.

TABLE 5. ELISA compared with mouse bioassay and culture for detection of type A and type B botulinal toxins in stool

ELISA	Combined mouse assay and culture	
	Positive	Negative
Positive	24[a]	2
Negative	1[b]	53

[a] This includes 19 specimens positive by mouse bioassay and 5 culture-positive specimens which, with direct testing of stool by mouse assay, showed nonspecific toxicity (4 specimens) or no toxicity (1 specimen).
[b] This specimen was culture positive but showed no toxicity in mice.

Although our assay was also able to detect *C. botulinum* antigen in specimens obtained from two adults with foodborne disease, the number of positive specimens was insufficient to provide definitive conclusions concerning the reliability of the ELISA test for this type of specimen. The efficiency of the assay system for the detection of *C. botulinum* in foodborne cases, in food and in other environmental specimens, should be determined in additional studies.

Infant botulism remains an important cause of morbidity and mortality in the newborn. However, the disease is sufficiently rare in most areas so that the ELISA seems unnecessary for most laboratories, although it does appear appropriate for reference laboratories and those serving large pediatric populations. More widespread availability of this and other sensitive assays for the detection and identification of *C. botulinum* toxins, including type F, in biological samples might facilitate efficient diagnosis and study of this infection. Of particular importance would be the determination of the contribution of this disease to infant mortality among children living in environments where *C. botulinum* is highly prevalent.

ACKNOWLEDGMENT

This work was supported by an institutional research grant from the John J. Hopkins Fund.

LITERATURE CITED

1. **Arnon, S.** 1980. Infant botulism. Annu. Rev. Med. **31**:541–560.
2. **Arnon, S. S., T. F. Midura, K. Damus, R. M. Wood, and J. Chin.** 1978. Intestinal infection and toxin production by *Clostridium botulinum* as one cause of sudden infant death syndrome. Lancet **i**:1273–1277.
3. **Cardella M. A.** 1964. Botulinum toxoids, p. 113–129. *In* K. H. Lewis and K. Cassel (ed.), Public Health Service publication no. 999-FP-1. Public Health Service, Cincinnati, Ohio.
4. **Center for Disease Control.** 1981. Botulism—United States, 1979–80. Morbid. Mortal. Weekly Rep. **30**:121–123.
5. **Dezfulian, M., and J. G. Bartlett.** 1984. Detection of *Clostridium botulinum* type A toxin by enzyme-linked immunosorbent assay with antibodies produced in immunologically tolerant animals. J. Clin. Microbiol. **19**:645–648.
6. **Dezfulian, M., and V. R. Dowell, Jr.** 1980. Cultural and physiological characteristics and antimicrobial susceptibility of *Clostridium botulinum* isolates from foodborne and infant botulism cases. J. Clin. Microbiol. **11**:604–609.
7. **Dowell, V. R., Jr., L. M. McCroskey, C. L. Hatheway, G. L. Lombard, J. M. Hughes, and M. H. Merson.** 1977. Coproexamination for botulinal toxin and *Clostridium botulinum*. J. Am. Med. Assoc. **238**:1829–1832.
8. **Hatheway, C. L.** 1979. Laboratory procedures for cases of suspected infant botulism. Rev. Infect. Dis. **1**:647–651.
9. **Hoffman, R. E., B. J. Pincomb, M. R. Skeels, and M. J. Burkhart.** 1982. Type F infant botulism. Am. J. Dis. Child. **136**:270–271.
10. **Horwitz, M. A., C.L. Hatheway, and V. R. Dowell, Jr.** 1976. Laboratory diagnosis of botulism complicated by pyridostigmine treatment of the patient—a method for selectively removing interfering substances from clinical specimens. Am. J. Clin. Pathol. **66**:737–742.
11. **Midura, T. F., and S. S. Arnon.** 1976. Infant botulism: identification of *Clostridium botulinum* and its toxin in feces. Lancet **ii**:934–936.
12. **Pickett, J., B. Berg, E. Chaplin, and M. Brunstetter-Shafer.** 1976. Syndrome of botulism in infancy: clinical and electrophysiologic study. N. Engl. J. Med. **295**:770–772.
13. **Rubin, L. G., M. Dezfulian, and R. H. Yolken.** 1982. Serum antibody response to *Clostridium botulinum* toxin in infant botulism. J. Clin. Microbiol. **16**:770–771.
14. **Viscidi, R. V., R. B. Laughon, M. Hanvanich, R. H. Yolken, and J. G. Bartlett.** 1984. Improved enzyme immunoassays for the detection of antigens in fecal specimens. J. Immunol. Methods **67**:129–143.
15. **Voller, A., D. Bidwell, and A. Bartlett.** 1980. Enzyme-linked immunosorbent assay, p. 359–371. *In* N. Rose, and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
16. **Yolken, R. H., P. J. Stopa, and C. C. Harris.** 1980. Enzyme immunoassay for the detection of rotavirus antigen and antibody, p. 692–699. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.