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Improvements in the Passive Immune Hemolysis Test for Assaying Enterotoxigenic *Escherichia coli*

A. F. PESTANA DE CASTRO,* MARLENE B. SERAFIM, JOYCE A. GOMES, AND MARIA S. V. GATTI

Department of Microbiology and Immunology, University of Campinas, 13100-Campinas, São Paulo, Brazil

The sensitivity of the passive immune hemolysis test for the detection of heat-labile enterotoxin from *Escherichia coli* was increased when the test was carried out with Veronal-buffered saline plus Ca^{2+} and Mg^{2+} as diluent, instead of phosphate-buffered saline. The performance of the test was further improved by using stationary cultures to which mitomycin C had been added at the end of the lag phase.

In a previous paper (8), it was demonstrated that the passive immune hemolysis (PIH) test carried out with phosphate-buffered saline (PBS) (PIH-PBS test) was not as reliable as the Y-1 adrenal cell assay when used mainly for the detection of enterotoxigenic *Escherichia coli* (ETEC) from porcine origin. Because tissue culture techniques to detect ETEC (1, 4, 9) cannot be adapted by laboratories equipped for serological work, it was decided to investigate some modifications of the PIH-PBS test with the main objectives of increasing its sensitivity and rendering it easier to perform. Thus, the following modifications were studied: (i) use of Veronal-buffered saline (VBS) as diluent; (6, 10) (ii) detection of heat-labile (LT) enterotoxin by the PIH-VBS test in stationary and shaking cultures of ETEC; and (iii) influence of mitomycin C in the production of LT (7) as detected by the PIH-VBS test in stationary cultures.

The source and designations of LT-producing strains of *E. coli* used are listed in Table 1. *E. coli* H-10407 and K-12, kindly provided by D. J. Evans, were included in all tests, respectively, as positive and negative standard controls for LT production as detected by the PIH test (8) and Y-1 adrenal cell assay (1). Stock cultures were maintained on peptone agar slants. Subcultures in brain heart infusion (Difco Laboratories, Detroit, Mich.), to which 15% glycerol had been added after growth, were kept at −70°C.

E. coli LT antitoxin was kindly supplied by D. J. Evans. Cholera antitoxin and anticholeragenoid were generous gifts from Carl E. Miller, Enteric Diseases Program Officer, National Institutes of Health, Bethesda, Md. According to preliminary data (8), cholera antitoxin was diluted either 1:80 or 1:640 for use in the PIH test. Choleragenoid and LT antitoxins were used at 1:80 and 1:60 dilutions, respectively.

For enterotoxin production, cultures from ETEC were inoculated in 125-ml Erlenmeyer flasks containing 10 ml of CAYE medium (3) consisting of 2% Casamino Acids (Difco), 0.6% yeast extract (Difco), 0.25% NaCl, 0.871 K_2PO_4, and 0.1% (vol/vol) trace salts solution (5% $MgSO_4$-0.5% $MnCl_2$-0.5% $FeCl_3$-0.001 N H_2SO_4). The pH of the medium was 8.5. Flasks were incubated at 37°C in a rotary incubator-shaker at 150 rpm for 18 h. For the Y-1 adrenal cell assay (1), the bacterial cells were removed by centrifugation at 12,000 × *g* for 30 min, followed by filtration through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.) These preparations were stored at −70°C for no longer than 15 days, before testing. Unless stated otherwise, cultures for examination by the PIH test were incubated similarly. Exceptions were stationary cultures (10 ml of CAYE medium in 125-ml Erlenmeyer flasks) incubated at 37°C for 24 and 48 h to investigate the effect of shaking and time of incubation on the production of LT enterotoxin as detected by the test.

Whenever the PIH test was used for the de-

TABLE 1. *Sources of ETEC and non-ETEC strains*[a]

Source[b]	Strains	
	ETEC	non-ETEC
Human	5/75, 13D, 60/1, 82/2, 103/3, 150/2, 204/75, 269/7, 2161, F11-2, 40,	3, 55, 22/75
Swine	A, B, 306, 339, 446, 2423, 3406	104, 243, 247, 343
River water	36, 151	

[a] ETEC (LT^+) and non-ETEC (LT^-) as detected by the Y-1 adrenal cell assay.

[b] Strains from humans were isolated by L. R. Trabulsi, São Paulo, Brazil, except for strain F11-2, which was supplied by R. L. Guerrant, Charlottesville, Va., and strains 3 and 55 (LT^- ST^+) (ST, heat-stable), serotype 0128 ac:H21, isolated by us. Strains from swine were isolated by us. Strains from river water were isolated by L. R. Trabulsi.

TABLE 2. *Results of the PIH test with ETEC and non-ETEC strains from several sources, using VBS or PBS as diluent*[a]

Source	PBS plus antiserum at the following dilution:				VBS plus antiserum at the following dilution:			
	Cholera		Cholerage-noid	LT	Cholera		Cholerage-noid	LT
	1:80	1:640	1:80	1:60	1:80	1:640	1:80	1:60
ETEC								
Human	8/11 (0.62)	5/11 (0.33)	5/11 (0.35)	5/11 (0.25)	11/11 (0.97)	11/11 (0.85)	10/11 (0.81)	10/11 (0.67)
Swine	1/7 (0.11)	0/7 (0.02)	0/7 (0.01)	0/7 (0.01)	7/7 (0.77)	6/7 (0.37)	5/7 (0.30)	1/78 (0.09)
River water	1/2 (0.39)	1/2 (0.46)	1/2 (0.41)	1/2 (0.35)	2/2 (1.00)	2/2 (0.85)	2/2 (0.85)	2/2 (0.67)
Subtotal	10/20 (0.39)	6/20 (0.27)	6/20 (0.25)	6/20 (0.20)	20/20 (0.91)	19/20 (0.69)	17/20 (0.65)	13/20 (0.47)
non-ETEC	0/7 (0.02)	0/7 (0.02)	0/7 (0.01)	0/7 (0.02)	0/7 (0.03)	0/7 (0.02)	0/7 (0.02)	0/7 (0.02)

[a] ETEC and non-ETEC as detected by the Y-1 adrenal cell assay. Data are reported as the number of strains positive in the PIH test over the total number of strains examined from each source; numbers within parentheses represent the arithmetic mean of the A_{420} values observed in the PIH test for the strains studied.

TABLE 3. *Effect of mitomycin C on LT production in stationary cultures in CAYE medium incubated at 37°C for 24 h, as assayed by the PIH-VBS test*

Enterotoxin prepn	Titer[a] in the following *E. coli* strain:													
	F11-2		40		204/75		306		A		H-10407[b]		K-12[c]	
	w/h[d]	w/c[e]	w/h	w/c	w/h	w/c	w/h	w/c	w/h	w/c	w/h	w/c	w/h	w/c
Neat[f]	1.11	1.11	0.96	1.13	1.12	1.12	0.65	0.92	0.62	0.13	1.12	1.12	—	—
1:2	1.11	1.11	0.88	1.13	0.86	1.12	0.30	0.82	0.50	0.91	0.90	0.98	—	—
1:4	1.04	1.11	0.69	1.13	0.62	1.12	—	0.64	0.21	0.53	0.78	0.86	—	—
1:8	0.86	1.04	0.40	0.86	0.34	1.02	—	0.22	—	0.27	0.40	0.62	—	—
1:16	0.44	0.64	—	0.67	—	0.84	—	—	—	—		0.44	—	—
1:32	—[g]	0.46	—	0.42	—	0.61	—	—	—	—		0.28	—	—
1:64	—	0.28	—	0.20	—	0.35	—	—	—	—			—	—
1:128	—	—	—	—		0.23	—	—	—	—			—	—
1:256	—	—	—	—	—	—	—	—	—	—			—	—
Ratio of w/c to w/h titers	4		8		16		4		2		4		—	

[a] Reported as A_{420} values. Cholera antitoxin diluted 1:80 was used in the test.
[b] Standard positive control.
[c] Standard negative control.
[d] No mitomycin C added for enterotoxin production.
[e] Addition of mitomycin C (0.5 μg/ml) at the lag phase.
[f] Dilutions of polymyxin B-release enterotoxin preparations.
[g] —, Negative, or absorbance values less than 0.16 at 420 nm.

tection of LT, this enterotoxin was extracted from CAYE broth cultures by the polymyxin B-release technique (3). Briefly, 2.2 mg of polymyxin B (Aerosporin, Burroughs Wellcome Co., Research Triangle Park, N.C.), diluted in 1 ml of 0.04 M PBS, (pH 6.7), was added to 10 ml of colibacilli cultures in CAYE medium. Shaking cultures were reincubated at 37°C in a rotary shaker at 150 rpm for an additional 15 min. For stationary cultures, flasks were shaken to allow better contact between the drug and bacterial cells. Supernatants, obtained from either shaking or stationary cultures were recovered by centrifugation at 16,000 × *g* for 30 min and then stored at −70°C.

In the mitomycin C (Calbiochem, La Jolla, Calif.) experiments, the inhibitor (0.5 μg/ml of medium) was added to stationary cultures at the end of the lag phase, that is, after 3 h of incubation at 37°C. For the extraction of LT by the polymyxin B-release technique, the procedure followed was that described above for stationary cultures.

Enterotoxin testing by the Y-1 adrenal cell assay was carried out with filtered (0.45-μm membrane filter, Millipore Corp.) supernatants from CAYE broth cultures. The PIH test was performed as recommended by Evans and Evans

(2) but was slightly modified (8). Readings were made spectrophotometrically (Coleman Junior spectrometer, model 6) at 420 nm. Those results which, after correction for nonimmune hemolysis, gave absorbance values at 420 nm (A_{420}) which were lower than 0.16 (less than 30 μg of hemoglobin released) were considered negative. Conversely higher A_{420} values were recorded as positive.

The results of the PIH test, carried out with ETEC and non-ETEC, and the respective arithmetic means of the A_{420} values, according to the source of the strains, are shown in Table 2. Regardless of the origin of the colibacilli studied, these values were always higher for the PIH-VBS test. A better sensitivity achieved in the PIH-VBS test did not render this assay less specific, since non-ETEC continued to give A_{420} values lower than 0.16, usually between 0.05 and 0.12.

Experiments carried out with strains 204/75, F11-2, 40, 60/1, 13D, 2423, B, and 36 demonstrated that the amount of enterotoxin as detected by the PIH-VBS test was equivalent in shaking and stationary cultures. Similarly, no significant increase was obtained in the A_{420} values of the PIH-VBS test carried out with strains 204/75, F11-2, 40, and 36, with incubation without shaking for 48 h (data not shown).

The results of the PIH-VBS test with polymyxin B-release extracts from five mitomycin-C-treated stationary cultures are presented in Table 3. The LT activity of these extracts as detected by the PIH-VBS test was higher than that observed in the untreated cultures, with titers increasing from 2- to 16-fold. On the other hand, extracts from *E. coli* K-12 treated similarly remained negative, with A_{420} readings lower than 0.16.

The increase in the sensitivity of the PIH-VBS test for the detection of LT cannot be taken as an unexpected result, since the effect of Ca^{2+} and Mg^{2+} in the activation of C1 and C2 components of the guinea pig complement as well as the use of VBS diluent have been known for a long time (5, 10). We call attention to the fact that there was total agreement between the adrenal cell assay and the PIH-VBS test when cholera antitoxin diluted to 1:80 was used in the latter. On the other hand, cholera antitoxin (1:640) and anticholeragenoid (1:80) yielded lower A_{420} values. This was even more noticeable when LT antitoxin (1:60) was used in the PIH-VBS test for the detection of LT produced by porcine ETEC strains; that is, six of seven strains gave negative results either in the PIH-PBS or PIH-VBS test.

These findings are unclear and deserve further investigation, but it is possible that porcine strains produce either less LT or that this enterotoxin is not suitably extracted by the polymyxin B treatment.

Based upon our results, the following modifications are suggested to render the PIH test adaptable to any laboratory likely to be concerned with LT assay: (i) use of VBS plus Ca^{2+} and Mg^{2+} in the PIH test, as diluent, instead of PBS; (ii) production of LT in stationary cultures by using low volume-of-medium-to-volume-of-flask ratios; and (iii) addition of mitomycin C (0.5 μg/ml of medium) at the end of the lag phase of growth in stationary cultures.

Undoubtedly, these modifications will make the test easier to be carried out by any laboratory not equipped for tissue culture techniques but for serological work. Although quantitative studies have not been performed between tissue culture assays and the PIH test, with the suggested modifications, we believe that the resulting increase in the sensitivity of the latter makes this test as reliable as the Y-1 adrenal cell assay.

Since the PIH test measures an antigen-antibody reaction, it is conceivable that if strains of ETEC produce an inactive LT, false-positive results may be obtained. Although this condition may be rare among ETEC isolated from clinical cases of diarrheal disease (2), we recommend that this possibility be taken into consideration when using the PIH test for diagnostic purposes.

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