JOURNAL OF CLINICAL MICROBIOLOGY, July 1983, p. 23–28
0095-1137/83/070023-06$02.00/0

Vol. 18, No. 1

Rapid Test for Identification of Heat-Labile Enterotoxin-Producing *Escherichia coli* Colonies

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Received 22 February 1983/Accepted 1 April 1983

A latex particle agglutination test is described which is suitable for the recognition of heat-labile enterotoxin-producing colonies of *Escherichia coli* immediately after primary culture on a variety of commonly used enteric diagnostic media. The test is simple and economical to perform, and the results are available in minutes.

Strains of *Escherichia coli* which elaborate either the cholera enterotoxin-related heat-labile enterotoxin(s) (LT[s]) or the unrelated heat-stable enterotoxin(s) or both are increasingly being recognized as causative agents of diarrheal disease in humans and in food supply animals (5, 18). Despite the importance of the problem (19), identification of toxin-producing strains can presently only be accomplished in relatively few laboratories in the world. The currently available methods each suffer serious limitations which preclude their widespread application. In each instance, they involve delays in obtaining results such that, although they may be useful epidemiologically, they are of little or no use for directing the therapy of an individual patient. As emerging observations suggest the possibility of specific pharmacological intervention (3, 6, 7, 12, 16) in lieu of, or in addition to, supportive rehydration therapy, the need for more rapid and practical tests for the respective enterotoxins becomes increasingly urgent.

With the exception of the recently introduced gene-specific DNA probes (15), presently available diagnostic tests for the detection of LT-producing strains depend upon the demonstration of enterotoxin activity (in cultured cells or laboratory animal models) or of enterotoxin-specific antigen (in various immunoassays) and involve subculture in appropriate media and conditions for the production of detectable amounts of enterotoxin. The DNA hybridization technique has the significant advantage that it can be applied directly to a stool sample without culture at all, but it requires, at the present state of the art, a radiolabeled, cloned gene probe and several days to develop autoradiograms. The most practical test available is the Biken test, introduced by researchers at the Research Institute for Microbial Diseases (Biken) of Osaka University, Osaka, Japan (8, 9, 11). This test has the very significant advantages of simplicity and economy. It also is extremely reliable and reproducible, having been demonstrated to be so in a multinational, multilaboratory study conducted under the auspices of the World Health Organization (R. G. A. Sutton, Y. Takeda and T. Miwatani (ed.), *Bacterial diarrheal diseases: an international symposium*, in press). However, as it also requires subculture and a development period, its use is restricted to epidemiological surveys and ex post facto diagnoses.

We present here a simple, economical, rapid, and reliable latex particle agglutination test (LPAT) for LT-producing *E. coli* which is directly applicable to the colonies which appear on primary culture on a variety of commonly used enteric diagnostic media. The results are available in minutes.

MATERIALS AND METHODS

***E. coli* strains.** Most of the work was performed with a series of 100 strains which was provided by the Japanese group (8, 9, 11) for the World Health Organization study mentioned above (Sutton, in press). They were sent to us, through the generosity and courtesy of Yoshifumi Takeda and Takeshi Honda (8, 9, 11), as sequentially numbered cultures, 1 through 100, on Dorset medium. After we sent Takeda and Honda our findings, the code was broken by them. Fifty of the strains produced LT, as determined by Takeda and Honda in the Biken test, the CHO cell assay (10), and the passive immune hemolysis test (21). Of these, 21 also produced heat-stable enterotoxin as determined by the suckling mouse assay (20). Of the LT-negative strains, 21 produced heat-stable enterotoxin. The latter two observations are irrelevant to the present study. The strains were subcultured onto MacConkey agar and meat extract agar and stored, in suspension in 20% glycerol in tryptic soy broth (Difco Laboratories), at −70°C.

Biken test. The Biken test was performed in our

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laboratory as described previously (8, 9, 11), with the exception that we used our own goat anti-human LT (H-LT) antiserum (4) at a dilution of 1:10 in lieu of the anti-cholera toxin and other sera previously reported (8, 9, 11).

LPAT. LPAT involves three phases: (i) the preparation of sensitized latex, (ii) the preparation of the colony supernatant, and (iii) the actual test. In the actual test, 10 μl of the supernatant and 5 μl of the sensitized latex are mixed in a well of a Boerner slide (Scientific Products, Inc.) or other appropriate container (e.g., a ring slide [Scientific Products]). The mixture is gently shaken for 3 min and then observed for agglutination by using transmitted oblique illumination. A hand lens or stereoscope is useful for interpreting weak reactions. A positive reaction is any evidence of agglutination of the latex particles, in comparison with a control, which can be observed with the naked eye, a hand lens, or a stereoscope (×10 magnification). In practice, it is convenient to record results as +++ (very strong agglutination), ++ (strong agglutination clearly visible with the naked eye), + (weak reaction visible with the naked eye but clearer with magnification), and − (no detectable agglutination).

Sensitization of latex. The specific anti-LT serum, or immunoaffinity-purified anti-LT antibody, prepared as described previously (B. A. Marchlewicz and R. A. Finkelstein, Diag. Microbiol. Infect. Dis., in press), is diluted to its optimal level (see below) in glycine-buffered saline solution (7.505 g of glycine and 5.85 g of NaCl in distilled water to 1 liter, adjusted to pH 8.2 with 6 N NaOH). In a typical preparation, 10 μl of goat anti-H-LT is added to 190 μl of glycine-buffered saline and mixed thoroughly. An equal volume of a suitable latex preparation is added to this preparation (see below) and also mixed thoroughly. The sensitized latex is incubated in a water bath at 37°C for 2 h and shaken at 15-min intervals. After this, 600 μl of 0.1% bovine serum albumin (Sigma Chemical Co.) in glycine-buffered saline is added. The mixture can then be used after 30 min. In our studies, the sensitized latex has been stable for 2 weeks at room temperature or at 4°C. The sensitized latex can also be preserved by lyophilization.

Preparation of colony supernatant. Colonies to be tested (a variety of primary isolation media are suitable [see below]) are emulsified, in an Eppendorf-type microcentrifuge tube, in 30 μl of polymyxin B-Tris-NaCl buffer prepared as follows. Polymyxin B sulfate (Aerosporin; Burroughs Wellcome Co.) sterile powder is suspended in distilled water to a concentration of 100,000 U/ml; 0.5 ml of that solution is added to 2 ml of 0.1875 M Tris–0.9% NaCl (pH 6.6) to yield a final solution of 20,000 U of polymyxin B per ml in 0.15 M Tris–0.12 M NaCl. Increasing the concentration of polymyxin B above 20,000 U/ml does not increase the sensitivity of the test. The bacterial suspension is incubated in polymyxin B-Tris-NaCl buffer at 37°C in a water bath for 5 to 30 min (a matter of convenience, depending on how many different colonies are to be tested) and centrifuged for 5 min in a tabletop Brinkmann Eppendorf Centrifuge 3200 (or its equivalent).

Purified H-LT. H-LT was purified, as described previously (4), from *E. coli* MM294 pWD600, a transformed K-12 derivative which contains multiple copies of the LT gene(s) of the Ent plasmid from *E. coli* H74-114 (4). It was kindly and generously provided by Walter Dallas (2). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis and in Ouchterlony tests, it behaved identically with LT previously isolated from strain H74-114 (4), and it was equally as active in the Y-1 adrenal cell assay (4).

Comparison of sensitivity of LPAT with the Biken test. To compare the relative sensitivities of LPAT and the Biken test, the Biken test was performed as described above, with the exception that a filter paper disk saturated with various levels of H-LT (20 μl) was substituted for the bacterial colony, and the center well was filled with 20 μl of various dilutions of antiserum or purified antibody. Purified H-LT was also titrated in LPAT with optimal levels of antiserum or purified antibody, and the minimal detectable levels of H-LT in both tests were compared. It is recognized that this is not strictly comparable to the actual Biken test in which the bacterial colonies may constantly be liberating some enterotoxin and then release more after treatment with polymyxin B. Thus, the results may only be a rough approximation.

Estimation by LPAT of the amount of H-LT released by bacterial colonies. An attempt to approximate the amount of H-LT released by bacterial colonies was made in the following manner. Growth of five different LT-producing strains (selected at random) on the various enteric media, with and without lincomycin (90 μg/ml; Sigma) (8, 9, 11, 14), was suspended to a constant turbidity (approximately 1.5 optical density [OD] units at 640 nm as determined in a Gilford model 250 spectrophotometer) in polymyxin B-Tris-NaCl buffer and processed for LPAT as usual. [Clindamycin (The Upjohn Co., Kalamazoo, Mich.; kindly provided by Robert Yancey), a semisynthetic antibiotic produced by a (7*S*)-chloro substitution of the (7*R*)-hydroxyl group of the parent compound lincomycin, can also be used]. Twofold serial dilutions of each supernatant were tested in LPAT, and the endpoint, i.e., the highest dilution giving a positive reaction, was determined. These results were compared with the smallest amount of purified H-LT which gave a positive reaction. Additionally, the turbidity of suspensions of individual colonies which were picked from the different media was determined. The amount of LT found in an individual colony was then calculated by relating the average colony OD to 1.5. As there was considerable variation in the amount of LT detected with the five strains on any individual medium, for the sake of this approximation, the results were averaged. This, too, should be regarded as a crude approximation at best.

Culture media. The culture media used included MacConkey agar (Difco), eosin methylene blue (EMB) agar (BBL Microbiology Systems), Hektoen agar (BBL), deoxycholate-citrate agar (Oxoid Ltd.), deoxycholate agar (Difco), and XLD agar (BBL), each prepared according to the specifications of the manufacturer, with or without lincomycin (90 μg/ml; Sigma).

RESULTS

Biken test. In our initial screening of the battery of 100 strains by the Biken test with our goat anti-H-LT, 46 of 50 LT-producing strains were detected, and 1 of 50 LT-negative strains was considered to be a doubtful positive. The

test was repeated after the code was broken, and the results were in complete conformance with those obtained by the Japanese workers. The four false-negative reactions in the first assay were due to improper spacing of the colonies and wells, whereas the doubtful false-positive reaction was due to a nonspecific haze around the growth of that strain. It is evident that our goat anti-H-LT is suitable for use in the Biken test; this may also be regarded as independent confirmation of the reliability of that test.

Sensitization of latex. To determine the optimal concentration of antibody for sensitization of latex (Table 1), latex particles were sensitized with various dilutions of anti-H-LT antiserum or immunoaffinity-purified (Marchlewicz and Finkelstein, in press) anti-H-LT antibody. As might be expected, the results indicated that there was an optimal level of antibody for sensitization. Each serum, therefore, must be pretested before use in the test. Interestingly, immunopurified antibody exhibited a broader range of activity than the crude antiserum from which it was derived. Apparently, irrelevant serum proteins may occupy sites on the latex particles and interfere with the binding of specific antibody. In this representative assay, at a dilution of 1:20, the whole serum detected as little as 80 ng of H-LT in 10 μl. The purified antibody was more sensitive, and at a dilution of 1:20 (0.16 μg of antibody protein per ml), it detected 0.32 ng of H-LT in 10 μl. Unless specifically noted, purified antibody (1:20) was used in all subsequent experiments.

Comparison of sensitivity of LPAT with the Biken test. To evaluate the relative sensitivities of the two tests, the Biken test was performed as described above, with the exception that a filter paper disk saturated with various concentrations of purified H-LT (20 μl) was substituted for the bacterial colony, and the center well was filled with 20 μl of various dilutions of antiserum or purified antibody. The results (Table 2) indicated that the Biken test, with whole serum diluted 1:20 (the most sensitive level), could detect levels of H-LT as low as 130 μg/ml (or 2.6 μg/20-μl sample). With purified antibody it could detect a level of 32 μg/ml (0.6 μg/20-μl sample). In comparison, LPAT could detect a level of 8 μg/ml (with whole serum) or 0.032 μg/ml (with purified antibody). Thus, according to these results, LPAT is 16 to 1,000 times more sensitive than the Biken test, depending on whether whole serum or purified antibody is used. Interestingly, purified antibody was not proportionately more active than whole antiserum in the Biken test, although it was in LPAT.

Evaluation of LPAT with bacterial colonies on different media. Table 3 summarizes results found when LPAT was used to detect LT-

TABLE 1. Titration of antibody for latex sensitization

Type of antibody	Dilution of antibody	Reaction[a] with an H-LT concn (μg/ml) of:												
		525	263	131	66	33	16.5	8.25	4.1	2.0	1.0	0.5	0.25	0.125	0.06	0.03	0.015
Goat anti-H-LT antiserum	0	+++	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−	−
	1:5	−	−	−	++	++	−	−	−	−	−	−	−	−	−	−	−
	1:10	−	−	−	+	+++	++	−	−	−	−	−	−	−	−	−	−
	1:20	−	−	−	−	−	++	++	−	−	−	−	−	−	−	−	−
Immunopurified goat anti-H-LT antibody[b]	0	−	+	++	+++	++	++	−	−	−	−	−	−	−	−	−	−
	1:5	−	−	−	−	+	++	+++	+++	++	+	−	−	−	−	−	−
	1:10	−	−	−	−	−	−	++	+++	+++	+++	++	+	−	−	−	−
	1:20	−	−	−	−	−	−	++	+++	+++	+++	+++	++	++	+	−	−

[a] +++, Very strong agglutination; ++, strong agglutination clearly visible with the naked eye; +, weak reaction visible with the naked eye but clearer with magnification; −, no detectable agglutination.

[b] 3.2 mg of protein per ml.

TABLE 2. Comparison of sensitivities of LPAT and the Biken test

Type of antibody	Test	Minimal amt of H-LT detectable (μg/10 μl)	LPAT/Biken ratio of sensitivity
Whole serum	Biken	1.3	16
	LPAT	0.08	
Purified	Biken	0.32	1,000
	LPAT	0.0003	

producing colonies on several different commonly used enteric diagnostic culture media with and without lincomycin.

The results indicate that a variety of culture media are suitable for LPAT, although XLD agar appears to reduce the number of positive strains detected. The addition of lincomycin (see Table 4) to the various culture media increased the levels of toxin antigen produced, but it was not required for the recognition of LT-producing colonies in LPAT. In XLD agar, it actually reduced the number of strains which were positive.

Approximation of the amount of LT per colony. As described above, the amount of LT extracted from single colonies on the various media, both with and without lincomycin, was estimated in LPAT (Table 4). The amounts varied from 0.03 to 1.3 ng/10 μl. MacConkey agar and EMB agar gave the highest values. Lincomycin increased the recovery of LT by four to fivefold. Although the lowest average value obtained appears to fall at the limit of sensitivity of the test (Table 1), it should be recognized that there are also day-to-day variations in quantitation and the results in Table 1 are representative only.

Comparison of different sources of latex. All of the above data were obtained using latex from Difco (lot 519984) or latex from Polysciences, Inc. (lot 11907). Examination of latex from various sources revealed (Table 5) that different brands and sizes of latex particles vary in their suitability for this test. The best results were obtained with particle sizes stated to range from approximately 0.8 to 1.25 μm, although not all lots of that particle size are suitable.

TABLE 3. Evaluation of LPAT[a] with 100 *E. coli* strains on different culture media, with and without lincomycin

Medium	Lincomycin[b]	No. of strains	
		LPAT$^+$/ Biken test$^+$	LPAT$^-$/ Biken test$^-$
MacConkey	−	50/50	50/50
	+	50/50	50/50
EMB	−	49/50	51/50
	+	50/50	50/50
Deoxycholate-citrate	−	50/50	50/50
	+	50/50	50/50
Deoxycholate	−	49/50	51/50
	+	50/50	50/50
Hektoen	−	50/50	50/50
	+	50/50	50/50
XLD	−	47/50	53/50
	+	41/50	59/50

[a] With immunopurified antibody (1:20).
[b] Without (−) or with (+) lincomycin (90 μg/ml) in the culture medium.

DISCUSSION

A new LPAT has been described which is suitable for the detection of LT-producing colonies of *E. coli* on a variety of commonly used selective and differential primary isolation culture media.

The following are essential to the test: high-quality specific antiserum or, preferably, affinity-purified specific anti-H-LT antibodies; a suitable latex preparation; prior titration of the antibody preparation to determine its optimal level; inclusion of polymyxin B in a Tris buffer of appropriate pH in the cocktail in which the bacterial colony is suspended; a tabletop microcentrifuge and other readily available laboratory supplies. The sensitized latex preparation is relatively stable at room temperature or under refrigeration, and it can be lyophilized for longer-term preservation. One milliliter of sensitized latex is sufficient for 200 tests, and the results are available in minutes.

The test was evaluated with a series of 100 well-characterized *E. coli* strains, of which 50 were previously established to produce LT. The 50 LT-producing strains all gave positive reactions when cultured on MacConkey, deoxycholate-citrate, and Hektoen media. One LT-producing strain was missed on EMB agar and on deoxycholate agar, and three were negative on XLD agar. No false-positive reactions were obtained with the 50 negative strains grown on the six commonly used primary isolation media, with or without lincomycin.

LPAT was used to obtain an approximation of the amount of H-LT liberated by *E. coli* colonies, prepared as we have described above. In media without lincomycin, the range was from 0.03 to 0.24 ng/10 μl. Lincomycin increased the yield by four to fivefold.

LPAT may be regarded as one of a class of

TABLE 4. Estimation of LT released in LPAT by individual colonies on different culture media

Medium	Lincomycin[a]	titer in LPAT[b]	H-LT (ng/10 μl)[c]	Avg OD per colony	H-LT per colony (ng/10 μl)[d]
MacConkey	−	40	12.0 (0.3–48)[e]	0.031	0.24
	+	216	64.8 (6.0–192)		1.3
EMB	−	42	12.6 (0.3–48)	0.028	0.24
	+	172.5	52 (3.0–192)		0.99
Deoxycholate citrate	−	11	3.3 (0.3–6.0)	0.014	0.03
	+	64	19.2 (6.0–48)		0.17
Deoxycholate	−	19	5.7 (1.5–12)	0.020	0.08
	+	96	33.2 (6.0–96)		0.40
Hektoen	−	10	3.0 (0.3–6.0)	0.071	0.14
	+	52.5	15.8 (3.0–48)		0.74

[a] −, Without lincomycin; +, with lincomycin.
[b] Average of values determined for five strains suspended at an OD 640 nm of 1.5.
[c] Calculated by relation to Table 1; minimal level detectable, ≅30 ng/ml × titer.
[d] Calculated by multiplying H-LT (nanograms per 10 μl) by average colony OD divided by 1.5.
[e] Numbers in parentheses indicate the range.

diagnostic or serological tests which depend on agglutination of antibody-sensitized particles in the presence of antigen. A particle agglutination test for LT, employing anti-cholera toxin-sensitized staphylococci, has been described previously (1). However, that assay required preparation of a second-generation culture supernatant and a subsequent overnight incubation period; it was not directly applicable to bacterial colonies, and no subsequent reports of its usefulness have appeared (to our knowledge). We have found (unpublished data) that formalinized staphylococci sensitized with rabbit antiserum can be used with colony supernatants, prepared as we described above. However, our goat anti-H-LT was not suitable for use in the staphylococcal coagglutination test, possibly because goat immunoglobulin does not bind well to staphylococcal protein A (17).

TABLE 5. Suitability of various latex preparations for use in LPAT[a]

Latex type (source)	Size of particle (μm)	Minimal amt of LT detectable (μg/ml)
Bacto-Latex (Difco; lot 519984)	0.81	≤0.5
Bacto-Latex (Difco; lot 709672)	0.81	2.0
Polysciences (lot 11907)	1.24	≤0.5
Polysciences (lot 25527—red)	1.1	≤0.5
Polysciences (lot 24417—yellow)	1.14	≤0.5
Polysciences (lot 31215)	0.32	—[b]
Polysciences (lot 24550—blue)	1.19	—
Sigma (lot 21 F-02581)	1.091	2.0
Sigma (lot 32 F-05151)	0.797	4.1
Sigma (lot 28 C-0051)	0.60	—

[a] Latex particles were "sensitized" in the usual manner with immunopurified anti-H-LT (1:20) and then used to titrate purified H-LT as described in Table 1.
[b] —, No agglutination at any level of H-LT.

While this manuscript was in preparation, Ito et al. (13) described automated and manual latex agglutination techniques for the detection of cholera toxin and LT. However, the manual technique, as reported, depended on subculture for the preparation of culture filtrates and an additional overnight incubation period before reading of the results. The automated technique, which required additional sophisticated instrumentation, was stated to be less sensitive.

We conclude that the LPAT described will be a useful technique for the rapid identification of LT-producing colonies which can readily be performed in laboratories in developing, as well as developed, countries.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI-16776 to R.A.F. from the National Institute of Allergy and Infectious Diseases.

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