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Rapid GM1-Enzyme-Linked Immunosorbent Assay with Visual Reading for Identification of *Escherichia coli* Heat-Labile Enterotoxin

ANN-MARI SVENNERHOLM* AND GUDRUN WIKLUND

Department of Medical Microbiology, University of Göteborg, S-413 46 Göteborg, Sweden

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A modified, quicker and simpler GM1-enzyme-linked immunosorbent assay procedure for detection of *Escherichia coli* heat-labile enterotoxin (LT) has been developed with the intent that it should be useful in less well-equipped laboratories. The method, which makes use of stable reagents including commercially available horseradish peroxidase immunoglobulin conjugate, can detect LT in overnight cultures within 1 working day (8 h), and the tests can be read with the naked eye. This GM1-horseradish peroxidase-enzyme-linked immunosorbent assay shows excellent quantitative and qualitative correlation with the conventional GM1-enzyme-linked immunosorbent assay. When 100 human *E. coli* strains were analyzed blindly and in parallel by the two methods, LT production was identified in 50 out of 50 LT-positive strains and in 0 out of 50 LT-negative strains by either method.

Enterotoxin-producing *Escherichia coli* are a common cause of watery diarrhea in all age groups (14). These bacteria may produce either or both of a heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST) (14). During the last decade, a number of toxicological as well as immunological tests for the demonstration of LT have been reported. These tests have depended either on animal (4, 12) or tissue culture (5, 8) facilities or on radioactive (7) or spectrophotometric (15, 16) registration, making them unsuitable for use in less well-equipped laboratories or in the field; the recently reported Biken immunoprecipitation test avoids these difficulties, but it is time-consuming (11).

The ideal method for demonstration of LT production in the field should be accurate, simple, and rapid. It should also be based on stable, readily available, and inexpensive reagents and be possible to read without any complicated equipment. Recently, we developed a sensitive and accurate immunological method for detection of LT-producing *E. coli*, the GM1-ganglioside enzyme-linked immunosorbent assay (GM1-ELISA) (2, 15). This method was based on the binding of LT to solid-phase-coupled GM1, utilizing the known affinity of this toxin for the cholera toxin receptor ganglioside, GM1 (9). The original GM1-ELISA has now been modified to permit detection of LT within one working day (8 h), the use of stable reagents including commercially available horseradish peroxidase (HRP)-immunoglobulin conjugate, and reading of the test with the naked eye.

MATERIALS AND METHODS

Bacterial strains. Thirteen *E. coli* strains that had been isolated from humans with diarrhea by B. Stoll, International Centre for Diarrheal Disease Research, Dacca, Bangladesh, and that had been shown to produce LT by means of the Chinese hamster ovary cell test (8) and the original GM1-ELISA assay (15) were studied. In addition, *E. coli* strains H10407 (LT/ST; obtained from D. Evans, University of Texas, Houston), 286-C2 (LT only; obtained from D. Robertson, University of Kansas, Lawrence, Kans.), and 64124 and 64052 (ST only; isolated by M. H. Merson in Dacca) were studied. Eight *E. coli* urinary isolates of different serotypes obtained from the strain collection of K. Lincoln, University of Göteborg, and five fecal *E. coli* strains that did not produce LT (Chinese hamster ovary cell negative) were used as negative controls. Finally, 100 *E. coli* strains with different enterotoxin profiles (50 representing LT only or LT/ST and 50 ST only or non-enterotoxin-producing strains), kindly provided by Y. Takeda, Osaka University, Osaka, Japan, were used. These 100 strains were distributed under a World Health Organization program by R. Sutton, Diarrhoeal Disease Control Programme, Geneva, Switzerland, to different laboratories for analysis of LT production; the toxin production of these strains was not revealed to the different laboratories until they had reported their results. All strains were kept on agar slants at room temperature until used.

Cultivation of bacteria. The day before testing, the bacteria were inoculated from the agar slants into 100-ml flasks containing 30 ml of a Casamino Acids-yeast extract medium (6). When not otherwise stated, the bacteria were incubated at 37°C for 16 to 18 h with shaking (200 rpm), and then polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) was added to

the cultures to a final concentration of 2,000 IU/ml, and the cultures were incubated for another 45 min. In some experiments, lincomycin (Sigma) was added to the cultures at a final concentration of 10 to 90 μg/ml. The cultures were then centrifuged at 3,000 × *g* for 5 min, and the supernatants were analyzed for LT activity.

Reference enterotoxins. Crude *E. coli* LT was prepared as previously reported (15), and pure LT was prepared as described by Clements and Finkelstein (3), with some modifications (10). Purified cholera toxin was purchased from Sigma.

Ganglioside. Ganglioside GM1 (purchased from Supelco Inc., Supelco Park, Bellefonte, Pa. or from Seromed, Munich, West Germany) was evaporated to dryness and kept in portions at +4°C or at room temperature until used. For control purposes, highly purified GM1, kindly provided by L. Svennerholm, University of Göteborg, was used.

Antisera. Antisera to *E. coli* LT were produced in adult New Zealand white rabbits given four to five subcutaneous injections with 1 ml of crude *E. coli* LT or two to three subcutaneous immunizations with 20 μg of purified LT in each at 2- to 3-week intervals. The initial two immunizations were given in Freund complete adjuvant, and the bleedings were performed 1 to 2 weeks after the last injections. The sera prepared were freeze-dried in portions and stored at 4°C or room temperature until dissolved and used.

GM1-ELISA procedures. Microtiter ELISA plates (Dynatech Laboratories, Sussex, England) were coated with GM1 by incubating the plates with 0.1 ml of 1.5 μM GM1 diluted in phosphate-buffered saline (PBS; 0.05 M phosphate, 0.15 M NaCl, pH 7.2) at 37°C for 6 h; the coated plates could be kept at 4°C for up to 1 month before use. After washing the plates twice in PBS, the remaining binding sites were blocked by incubating the plates with a 1% bovine serum albumin (BSA)-PBS solution at 37°C for 30 min (200 μl/well). The subsequent procedures for analyzing LT production by means of the new GM1-HRP-ELISA and the original GM1-alkaline phosphatase (AP)-ELISA (15) are given in Table 1.

The enzyme-substrate reaction in the GM1-AP-ELISA was read spectrophotometrically with a Titertek Multiskan (Flow Laboratories Ef-lab, Helsinki, Finland) at 405 nm, and in the GM1-HRP-ELISA it was read both visually and spectrophotometrically at 450 nm. In the GM1-AP-ELISA, readings with an absorbance of ≥0.3 above the background were regarded as positive (LT containing) (15); in the GM1-HRP-ELISA, a brown color that differed significantly from that of medium controls as determined by visual readings by two persons independently, an absorbance of ≥0.1 above the background, or both were considered as positive. The toxin titer was determined as the interpolated dilution of the test sample giving an absorbance of 0.3 (GM1-AP-ELISA) or 0.1 (GM1-HRP-ELISA) above the background when letting the bound enzyme conjugate react with its substrate for 100 or 20 min, respectively.

RESULTS

Culture conditions. The production of LT by strains 286-C2 and H10407 under different culture conditions was tested by means of the GM1-AP-ELISA and the GM1-HRP-ELISA. Cultivation with shaking was superior to incubation without shaking, and growth in flasks was better than cultivation in 5-ml tubes. Addition of lincomycin, polymyxin B sulfate, or both to the initial culture (11) resulted in a marked depression both of the bacterial growth and of the LT production. Addition of polymyxin B to cultures which had grown overnight and continued incubation with the drug for 45 min resulted in release of up to threefold higher LT concentrations than in nontreated cultures; addition of lincomycin (10 to 90 μg/ml) to the stationary culture had no detectable effect on the release of LT.

Experimental conditions. Similar LT titers were obtained when highly purified GM1 from L. Svennerholm or any of the two commercially available GM1 preparations was used to coat the polystyrene wells. Incubation with anti-LT antibodies for 4 h and with the enzyme conjugate for 18 h permitted detection of three- to fivefold lower toxin concentrations than incubations with the antiserum for 1 h and the conjugate for 2 h. Antisera to highly purified LT and to crude LT gave comparable results when they were used diluted to the same anti-LT titer. The sensitivity of the assays increased with increasing anti-LT concentrations; in most of the experiments, the anti-LT sera were used in a final dilution of 1:200, corresponding to 1,000 times the GM1-ELISA titer (15) against LT. When the standard procedures described in Table 1 and antiserum against purified LT were used, GM1-HRP-ELISA detected LT in concentrations down to ~10 ng/ml and cholera toxin in concentrations down to about 15 to 20 ng/ml; the corresponding values for the GM1-AP-ELISA were 4 and 8 ng/ml, respectively.

We found *p*-phenylenediamine (PPD) dissolved in acid citrate buffer (0.1 M $Na_3C_6H_5O_7$, pH 4.5) to be the best color indicator for the HRP-H_2O_2 (enzyme-substrate) reaction. PPD in neutral or alkaline citrate buffer or acid 0.1 M phosphate, carbonate, or Tris buffer all resulted in lower specific absorbance, higher background, or both. *o*-Phenylenediamine (1) as well as 5-aminosalicylic acid (13) were also tried as chromogens but were found to be less suitable than PPD for visual reading.

Enterotoxin determinations. The content in overnight cultures of 15 known LT-producing and 15 non-LT-producing *E. coli* strains was determined in parallel with the GM1-AP-ELISA and the GM1-HRP-ELISA. The samples were analyzed both undiluted and in various dilutions in PBS-Tween containing 1% BSA, with the two methods performed as specified in Table 1. All the cultures of the strains previously shown to produce LT had a significant enterotoxin titer as determined with the GM1-AP-ELISA and also

TABLE 1. Comparison of modified GM1-HRP-ELISA procedures with original GM1-AP-ELISA procedures[a]

Step in procedure	Time involved	
	GM1-HRP-ELISA	GM1-AP-ELISA
1. Incubate test sample undiluted in GM1-coated ELISA microtiter plates blocked with 1% BSA-PBS solution	1 h	2 h
2. Wash three times with PBS-0.05% Tween		
3. Incubate with rabbit anti-LT serum diluted in PBS-Tween-0.5% BSA	2 h	4 h
4. Wash 3 times with PBS-Tween		
5. Incubate with anti-rabbit immunoglobulin conjugate diluted in PBS-Tween-BSA	2 h[b]	18 h[c]
6. Wash 3 times with PBS-Tween		
7. Add enzyme substrate and read after	20 min[d]	100 min[e]

[a] All incubations are with 0.1 ml of the reagent per well with the plate kept in a humid chamber at room temperature.

[b] Anti-rabbit immunoglobulins, HRP conjugated (Dakopatts, Copenhagen, Denmark) and diluted 1:300.

[c] Anti-rabbit immunoglobulin G, AP conjugated (15) and diluted 1:250.

[d] H_2O_2 at a final concentration of 0.01% and PPD (1 mg/ml) diluted in citrate buffer (0.1 M $Na_3O_6H_5O_7$, pH 4.5).

[e] Nitrophenylphosphate (1 mg/ml) diluted in ethanolamine buffer (1 M ethanolamine, 0.001 M $MgCl_2$; pH 9.8).

gave a visible brownish color or an absorbance value of ≥0.1 in the GM1-HRP-ELISA (Fig. 1). In no instance with either method did the urinary or previously demonstrated LT-negative fecal strains give any significant reaction. The LT titers determined with the two tests showed a highly significant correlation as determined by regression analysis (r = 0.91) (Fig. 1); the toxin titers registered with the GM1-HRP-ELISA were, however, about twofold lower as a mean.

The qualitative results obtained when the 100 strains with different enterotoxin profiles were tested blindly by the two techniques were compared. The overall agreement between the two tests was 100%. Thus, all of the 50 *E. coli* strains that were positive with the GM1-AP-ELISA, i.e., that gave an absorbance at 400 nm ≥0.3 above background, were also positive by visual reading with the GM1-HRP-ELISA when reacting the enzymes with their substrates for 100 and 20 min, respectively; the remaining 50 strains were negative by both tests.

DISCUSSION

A modified GM1-ELISA that should be suitable for use in less well-equipped laboratories, for instance in developing countries, has been developed. The method shows excellent agreement with the results obtained with the previous GM1-AP-ELISA. The somewhat lower sensitivity obtained with the new GM1-HRP-ELISA could be compensated for by increasing the incubation time with conjugate to 6 to 10 h. However, in no instance was the sensitivity of the GM1-HRP-ELISA too low to detect LT in cultures of any of the 65 strains with known LT-producing capacity, although some strains with low-level enterotoxin-producing capacity were included.

At variance with the observation by Honda et al. (11), we did not find any increased release of LT from a number of LT-producing strains when adding lincomycin of various concentrations to

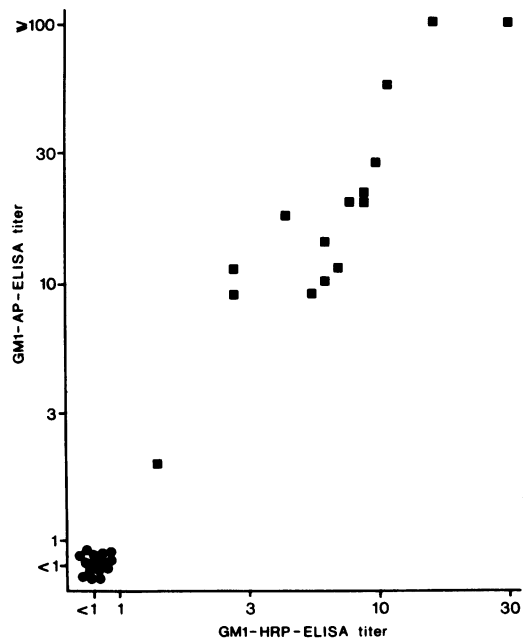

FIG. 1. Correlation between LT titers determined by the GM1-HRP-ELISA and GM1-AP-ELISA methods (15) for overnight cultures of 30 *E. coli* strains. Symbols: ■, LT-positive strains (13 fecal strains, H10407 and 286-C2); ●, LT-negative strains (2 ST-only strains, 8 urinary isolates and 5 fecal strains). Regression analysis gives a correlate of r = 0.91, $P < 0.01$.

the culture medium. Instead, lincomycin at a relatively low concentration (45 μg/ml) had a marked depressive effect on the growth when added to the liquid medium. Addition of polymyxin B, on the other hand, which has been found to cause release of intracellular toxin (3, 6), resulted in significantly higher LT concentrations in the test culture medium of most of the strains tested. Therefore, as a standard procedure, we recommend addition of polymyxin B to overnight cultures for 30 to 45 min; incubation with the drug for up to 2 h resulted in even higher toxin concentrations (up to twofold higher) in the medium than those obtained with the shorter incubation time.

The specificity of the GM1-HRP-ELISA was high. Thus, the 15 known LT-negative *E. coli* strains, including the ST-positive ones, were all negative, and none of the strains from Y. Takeda with reported LT negativity was positive with the GM1-HRP-ELISA. Furthermore, all the 15 known LT-positive strains were positive with the test, and all of the 50 World Health Organization-distributed strains, which after completed testing were reported to be of the LT only or LT/ST type, were positive with the GM1-HRP-ELISA.

At variance with many other immunological methods for LT detection (11, 15, 16), the GM1-HRP-ELISA could be performed within 1 working day (8 h). However, isolation of the test strain from feces and overnight culture for LT production will still delay the diagnosis for several days. One possibility for obtaining the result quicker might be to perform the toxin culture directly on the fecal specimen without isolation of individual bacterial strains for the testing, but this approach would not ordinarily enable differentiation between *Vibrio cholerae* and LT-producing *E. coli* as the cause of a positive test. However, recently developed monoclonal antibodies have been prepared which recognize species-specific, i.e., non-cross-reactive, determinants on LT and cholera toxin, respectively (L. Lindholm, J. Holmgren, M. Wikström, U. Karlsson, K. Andersson, and N. Lycke, submitted for publication), and which might overcome this problem, enabling species-specific enterotoxin detection both in total fecal cultures and directly in stool specimens.

An advantage of the GM1-HRP-ELISA over the GM1-AP-ELISA methods is that all reagents could be stored in dry form, ensuring transport and stability. Moreover, except for the rabbit anti-LT serum, reagents of sufficient quality are presently commercially available. Suitable anti-LT sera may be obtained by repeatedly immunizing rabbits with concentrated culture filtrates of LT-producing *E. coli* strains. However, use of anti-cholera toxin sera is not advisable, since the sensitivity of the test will be about fivefold lower than when using anti-LT antibodies (15).

When using PPD in acid citrate buffer as chromogen, the HRP enzyme-substrate reaction was possible to read with the naked eye. Two persons independently came to exactly the same results when analyzing the 100 strains from the World Health Organization, which were unknown with regard to their LT property at the time of analysis. In all instances, strains that were read as positive with the naked eye gave an absorbance value of ≥0.1 above background in the GM1-HRP-ELISA. Furthermore, in no instance was a reaction that had an absorbance of <0.1 read as positive. Use of *o*-phenylenediamine or aminosalicylic acid as chromogens (1, 13) resulted in color reactions that gave less clear-cut differences from the low-level background staining that might be seen with LT-negative samples.

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