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# Automatic and Manual Latex Agglutination Tests for Measurement of Cholera Toxin and Heat-Labile Enterotoxin of *Escherichia coli*

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Automated and manual latex agglutination methods were employed to measure cholera toxin (CT), heat-labile enterotoxin (LT) of *Escherichia coli*, and their subunits A and B. Dow polystyrene latex particles (diameter, 0.22 μm) and polystyrene-chlorostyrene latex particles (diameter, 1 μm) were sensitized by rabbit-specific immunoglobulin for each antigen and used as the reagents of the automated and manual agglutination tests, respectively. Automated agglutination was performed by a nephelometric assay system measuring time-dependent differences of light scattering due to agglutination, and manual latex agglutination was carried out in microtiter plates. As low as 1,000 and 31 pg of CT per ml were estimated by the automated and manual agglutination tests, respectively. Using these methods, the amount of CT and LT was measured in several clinical isolates of *Vibrio cholerae* and *E. coli*. Furthermore, it was discovered that cyclic AMP is not essential for the production of CT by measuring the amount of the toxin in numbers of cyclic AMP-dependent mutants of *V. cholerae* (with the agglutination tests).

The latex agglutination test was first introduced by Singer and Plotz for the measurement of rheumatoid factors (19). The techniques are straightforward, and reagents are stable for a long time; it quickly became popular for measurements of various antigens and antibodies (17, 18). To determine agglutination reactions rapidly, instrumental reading methods, such as scattering spectroscopy, were also devised (1); these enabled us to measure many samples within a very short time.

This paper deals with automatic and manual latex agglutination tests for cholera toxin (CT) and heat-labile enterotoxin (LT) of *Escherichia coli*.

## MATERIALS AND METHODS

**Bacteria.** Three nontoxigenic and seven toxigenic clinical isolates, four cyclic AMP-deficient mutants of *Vibrio cholerae*, and five enterotoxigenic and two nontoxigenic strains of *E. coli* were used (Tables 1 and 2).

**Chemicals.** Purified CT (Choletox) was purchased from Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. Polystyrene-chlorostyrene stable latex particles (particle diameter, 1 μm; specific gravity, 1.14) were manufactured by Takeda Chemical Industry, Osaka, Japan and were a gift from S. Matsuzawa, Department of Forensic Medicine, Juntendo University, Tokyo, Japan. Dow polystyrene latex particle suspension (particle diameter, 0.22 μm) was obtained from the Dow Chemical Co., Indianapolis, Ind.

**Preparation of antisera.** The purified CT was split into subunits A and B by the method of Ohtomo et al. (16). LT was purified from *E. coli* 20S0 (*ent amp*) by the method of Clements and Finkelstein (2–4). Rabbit antisera to CT, subunits A and B, and LT were prepared by the method of Heyningen (12). Antisera were further purified by affinity column chromatography (14).

**Sensitization of latex particles.** A 1% suspension of the stable polystyrene-chlorostyrene particles or 0.1% Dow latex suspension in 0.05 M glycine-NaOH buffer (pH 8.2) containing 0.05 M sodium chloride was mixed with 10 or 40 μg of specific immunoglobulin G (IgG) per mg of latex particles at room temperature for 60 min with gentle shaking, followed by the addition of one drop of 30% bovine serum albumin solution per ml for stabilization and thorough washing with glycine buffer containing 0.05 M sodium chloride and 0.1% bovine serum albumin. The sensitized latex particles were suspended in the same buffer to make a final concentration of 0.5%.

**Automatic agglutination test.** A latex agglutination nephelometric system automatic immunochemistry analyzer (Eiken Chemical Co., Ltd., Tokyo, Japan) was employed for this purpose. A 400-μl amount of a 0.1% suspension of the sensitized latex particles and 80 μl of samples were mechanically mixed in the analyzer and irradiated with a visible light beam (400- to 700-nm wavelength). The scattered light intensity in the forward direction was recorded, starting 35 s after the mixing with 100-s intervals. The change of scattered light in the initial 100 s was calculated automati-

TABLE 1. Bacterial strains

| Species | Strain | Place | Yr isolated | Source |
|---|---|---|---|---|
| *V. cholerae* biotype *eltor* | BR1074 | Sao Paulo, Brazil | 1978 | River |
| | BR2633 | Lio, Brazil | 1978 | River |
| | JR290 | Yoshikawa, Japan | 1979 | River |
| | LO23 | Louisiana, U.S.A. | 1978 | Patient |
| | K20 | Philippines | 1971 | Patient |
| | H23446 | Bangladesh | 1970 | Patient |
| | S249 | Bangladesh | 1961 | Patient |
| *V. cholerae* biotype *cholerae* | CC4476 | Calcutta, India | 1941 | Patient |
| | CC4560 | Calcutta, India | 1941 | Patient |
| | 569B | India | 1940? | Patient |
| Cyclic AMP-deficient mutants of *V. cholerae* | IK12, *cya* | Tokyo, Japan | 1978 | *cya* mutant of Ikenohata strain (21) |
| | IK23, *cya* | Tokyo, Japan | 1978 | Same as above |
| *V. cholerae* biotype *eltor* | E2511, *cya* | Philippines | 1964 | *cya* mutant of E2 strain (22) |
| *V. cholerae* biotype *cholerae* | B42, *cya* | India | 1940 | *cya* mutant of 569B |

cally. The intensity of agglutination was expressed as ΔLSE (an increase of light-scattering energy per 100 s). From the standard curve of CT or LT, the toxin concentration in culture filtrates was estimated.

**Manual agglutination test.** Microtiter plates with U-type bottoms were used. Twenty-five microliters of a 0.05% suspension of the sensitized latex particles was poured into each well, followed by the addition of the same volume of samples which were serially diluted with glycine-NaOH buffer (pH 8.2). The plates were sealed with plastic tape and left at room temperature overnight until the reaction was read at 18 h macroscopically. The positive reaction appeared as an irregular ring formation of agglutinated latex particles, whereas the negative reaction appeared as a smooth contoured sedimentation of particles.

TABLE 2. Bacterial strains

| Species | Strain | Isolated by: |
|---|---|---|
| Enterotoxigenic *E. coli* | H10407 | D. J. Evans, Jr., and D. G. Evans[a] |
| | 339t5 | R. A. Finkelstein et al. (8) |
| | TD218C1 | R. B. Sack[a] |
| | B16-4 | R. L. Guerrant et al. (11) |
| | 20S0, *ent amp* | T. Yokota et al. |
| Nontoxigenic *E. coli* | K-12 20S0 | Stock in our laboratory |
| | CSH-2 | Stock in our laboratory |

[a] Kindly provided by M. Ohashi, Department of Microbiology, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan.

**Preparation of culture filtrates of *V. cholerae* and *E. coli*.** Purified test strains of *V. cholerae* and *E. coli* were inoculated into 10 and 5 ml, respectively, of liquid medium consisting of 30 g of peptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), 5 g of sodium chloride, and 1,000 ml of distilled water adjusted to pH 7.2. The microbes were cultured at 37°C overnight with a Monod-type shaker (Taiyo Bussan Co., Tokyo, Japan) for *V. cholerae* and a reciprocal shaker for *E. coli*. For cyclic AMP-deficient mutants of *V. cholerae*, the medium was supplemented with and without 0.5 mM cyclic AMP. The culture filtrates were obtained by refrigerated centrifugation at 10,000 × *g* for 30 min followed by passage through a membrane filter (0.3 μm; Millipore Corp., Bedford, Mass.).

**Preparation of sonicated cell-free extracts.** The cell-free extracts were prepared as the supernatant of the cell suspension sonicated at 10 kc four times at 15 s and 0°C.

**Bioassay of CT and LT with CHO-K1 cells.** The activities of the enterotoxins in culture fluids or cell-free extracts were assayed by the tissue culture method with Chinese hamster ovary cells, CHO-K1 (10).

## RESULTS

**Optimum conditions for the manual latex agglutination test.** Choletox (Chemo-Sero Therapeutic Research Institute; lot no. BC14) was used as the standard CT. The lyophilized toxin was dissolved in the original volume of distilled water and kept at 4°C. Before use, the toxin was diluted with the medium employed for the cultivation of test microbes adjusted to pH 6.0 to

TABLE 3. Influence of the concentration of the latex particles on the sensitivity of the manual latex agglutination test

| Concn of latex particles (%) | Minimum amt of CT determined (pg/ml) |
|---|---|
| 0.1 | 1,000 |
| 0.05 | 31 |
| 0.025 | 2 |
| 0.0125 | 0.5 |

make a final concentration of 1 μg/ml. The use of a weakly acidic medium was critical to get a stable working reference. If an alkaline medium was employed, the endpoint of the reference test was unsatisfactory. The minimum detection limit of CT was 31 pg/ml at pH 6.0, 31 to 63 pg/ml at pH 7.0, and 250 pg/ml at pH 8.0.

The optimum amount of specific IgG and the optimum pH for the sensitization of latex particles was determined to be 10 to 40 μg per mg of latex at pH 8.2. Poor sensitization was observed when the IgG was added to latex at a pH lower than 6.2. For stabilization, the source of bovine serum albumin was important. Among some products tested, the product of Armour Pharmaceutical Co., Chicago, Ill. gave the best result. The manual latex agglutination test with the use of the product of Armour was shown to detect 31 pg of CT per ml, whereas with the use of others, it was shown to detect 4 or 8 ng of CT per ml.

When the density of the sensitized latex particles was decreased, the sensitivity increased conversely (Table 3). However, since it was difficult to read the endpoint at low densities, a 0.05% suspension was selected for routine work. Stable results were obtained when the reaction was carried out at room temperature rather than 37°C.

In contrast to the acidic medium used for the preparation of the standard working solution of the toxin, the optimum pH for the further dilution of the standard toxin and for reaction mixtures was found to be 8.2 (0.05 M glycine–NaOH buffer).

Based upon the results that we obtained, the manual latex agglutination test for CT was routinely carried out as follows. Twofold serial dilutions of the working standard toxin solution prepared with medium (pH 6.0) were placed in wells of microtiter plates with glycine-NaOH buffer (pH 8.2; 25 μl each). The same volume of the sensitized latex particles suspended in the same buffer was added to each well followed by thorough mixing, and the endpoint of the reaction was read macroscopically after 18 h at room temperature. Concentrations as low as 31 pg of CT per ml could be estimated by this method.

**Automatic latex agglutination test.** A rapid measurement of CT was possible by the automatic latex agglutination test with a nephelometric assay system (Fig. 1). Since an autosampler and autochanger were connected to the system, an accurate time difference of light-scattering energies in each tube was determined (Fig. 2). The method, however, was rather insensitive when compared with the manual method. The lowest toxin concentration which can be measured by the automatic method was found to be about 1 ng/ml.

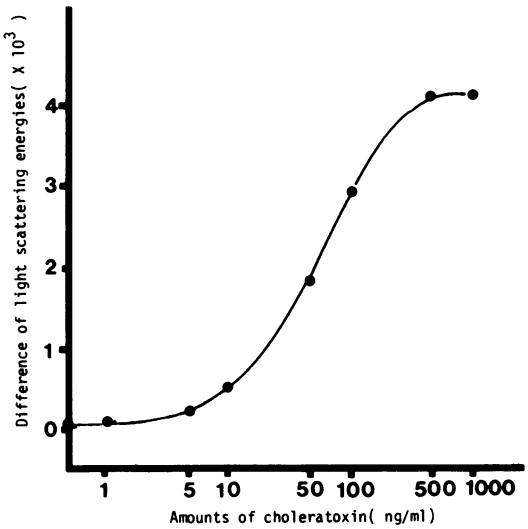


FIG. 1. Standard curve for CT quantitation by automatic latex agglutination.

**Estimation of amounts of CT in culture filtrates of *V. cholerae* strains.** The amount of CT in

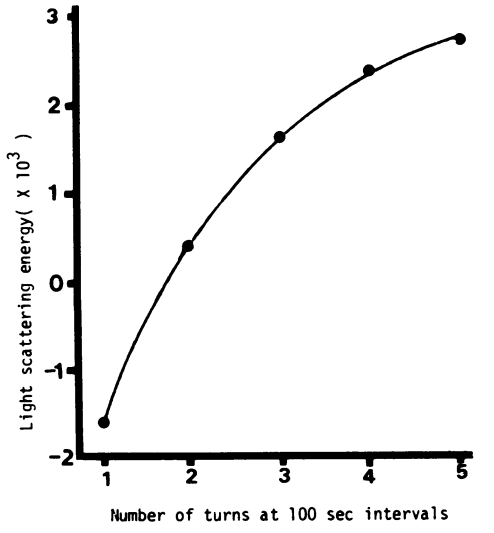


FIG. 2. Increase of agglutination after mixing 100 ng of CT and latex particles per ml.

TABLE 4. Amounts of CT in culture fluids of various strains of *V. cholerae* measured by automatic and manual latex agglutination tests and by the tissue culture method with CHO-K1 cells

| Strain | Amt of CT measured by: | | |
|---|---|---|---|
| | Automatic method (ng/ml) | Manual method (ng/ml) | CHO-K1 method (ng/ml) |
| BR1074 | <1 | <0.01 | <0.01[a] |
| BR2633 | <1 | <0.01 | <0.01 |
| JR290 | <1 | <0.01 | <0.01 |
| LO23 | 6.4 | 1.0 | ?[b] |
| K20 | 50.4 | 1.0[c] | 10 |
| H23446 | 112.6 | 63.0 | 100 |
| S249 | 19.5 | 1.0[c] | 100 |
| CC4476 | 13.0 | 2.0 | 10 |
| CC4560 | 6.9 | 47.0 | 10 |
| 569B | >400 | 250.0 | 1,000 |

[a] Approximate toxin concentration.
[b] Bioassay was impossible because of the cytotoxicity of the culture fluid.
[c] Approximate toxin concentration, as the endpoints were unclear.

culture filtrates of 10 strains of *V. cholerae* isolated in different years and places was measured by the manual and automatic latex agglutination tests and by the tissue culture method with CHO-K1 cells (Table 4). All three strains of *V. cholerae* isolated from rivers were confirmed to be nontoxigenic by the three different methods, whereas seven clinical isolates of the same microbe were recognized as toxin producers even though the amounts varied from one strain to another.

Although some discrepancies were found among the amounts of CT measured by the three methods, all methods were in agreement regarding the toxigenicity of each strain.

The amounts of LT in culture filtrates of five toxigenic strains of *E. coli* are shown in Table 5. The lowest concentration of toxin that could be measured by the manual and automatic methods was almost the same as that of CT. It seemed that the disruption of the cells was essential to get large enough amounts of LT for determination by the latex agglutination tests. In this regard, treatment with polymixin B gave the same good results as sonic disruption (data not shown).

**Amounts of CT produced by cyclic AMP-deficient mutants of *V. cholerae*.** Amounts of CT and subunits A and B produced by four cyclic AMP-deficient mutants of *V. cholerae* are shown in Table 6. Although the excretion of CT, especially that of subunit B, was stimulated in the presence of exogenous cyclic AMP in some mutants, it was concluded that cyclic AMP is not an essential factor for the production of cholera toxin.

## DISCUSSION

Accurate measurement of the amount of enterotoxins produced by *V. cholerae* or enterotoxigenic *E. coli* is frequently required. Although conventional bioassay methods, such as the ileal loop test (6) and the vascular permeability test (5), are complex and time consuming, it is almost impossible to estimate accurately CT or LT in 100 strains by the tissue culture methods with CHO-K1 cells (10) or Y-1 adrenal cells (7), even though these methods are sensitive enough.

For this reason, many immunochemical techniques have been applied for the estimation of enterotoxins, such as the modified Elek test (13), the reverse passive hemagglutination test (14), passive hemolysis (20), the radioimmunoassay (RIA) (9), and the enzyme-linked immunosorbent assay (ELISA) (23).

The modified Elek test is very useful for the detection of the enterotoxigenic *E. coli*, but unsuitable for the quantitation of the enterotoxins.

TABLE 5. Amounts of LT in culture fluids and sonicated cell-free extracts of enterotoxigenic *E. coli*[a]

| Strain | Amt of LT (ng/ml) | | | | | |
|---|---|---|---|---|---|---|
| | In culture fluids determined by: | | | In sonicated cell-free extracts determined by: | | |
| | ALG | MLG | CHO | ALG | MLG | CHO |
| 20SO | <5 | 0 | 0[b] | <5 | 0 | 0[b] |
| CSH-2 | <5 | 0 | 0 | <5 | 0 | 0 |
| 20S0, *ent amp* | <5 | 4 | 1 | 601 | 2,000 | 100 |
| 339t5 | <5 | 2 | 1 | 291 | 250 | 100 |
| B16-4 | 5 | 2 | 0.1 | 155 | 125 | 10 |
| H10407 | <5 | 8 | 1 | 35 | 31 | 10 |
| TD218Cl | 17 | 32 | 10 | 538 | 1,000 | 100 |

[a] Measured by the automatic latex agglutination test (ALG), the manual latex agglutination test (MLG), and the CHO-K1 cell culture method (CHO).
[b] Approximate toxin concentration. Purified LT was used as the standard.

TABLE 6. Amounts of CT measured by the manual latex agglutination test in culture fluids and cell-free extracts of *V. cholerae* grown in the presence or absence of cyclic AMP

| Strain | Presence of 0.5 mM cyclic AMP | Amt (ng/ml) of: | | | | | |
|---|---|---|---|---|---|---|---|
| | | Holotoxin | | Subunit A | | Subunit B | |
| | | I.C.[a] | E.C.[b] | I.C. | E.C. | I.C. | E.C. |
| IK, *cya*$^+$ | − | 32.0 | <0.1 | 16.5 | <0.1 | 32.0 | <0.1 |
| IK, *cya* | − | 2.0 | <0.1 | 1.0 | <0.1 | <0.1 | <0.1 |
| | + | 2.0 | 0.13 | 2.0 | <0.1 | <0.1 | 0.13 |
| IK23, *cya* | − | 16.0 | 2.0 | 8.0 | 0.5 | 8.0 | 4.0 |
| | + | 32.0 | 1.0 | 8.0 | 1.0 | 16.0 | 8.0 |
| E2, *cya*$^+$ | − | 2,000.0 | 4.0 | 1,024.0 | 4.0 | 2,000.0 | 32.0 |
| E2511, *cya* | − | 512.0 | 1.0 | 128.0 | 4.0 | 512.0 | 2.0 |
| | + | 512.0 | 2.0 | 64.0 | 8.0 | 512.0 | 8.0 |
| 569B, *cya*$^+$ | − | 100.0 | 100.0 | 50.0 | 100.0 | 50.0 | 100.0 |
| B42, *cya* | − | 400.0 | 200.0 | 100.0 | 100.0 | 50.0 | 100.0 |
| | + | 400.0 | 400.0 | 200.0 | 100.0 | 100.0 | 200.0 |

[a] I.C., Intracellular toxin. A supernatant of sonically disrupted cells equivalent to 1 ml of culture fluid was used.

[b] E.C., Extracellular toxin. A culture supernatant was used.

The reverse passive hemagglutination test or passive hemolysis test frequently suffers from nonspecific reactions. The RIA is highly specific and sensitive, but the requirement for a radioactive reagent and an expensive detection system make the RIA impractical for many centers. ELISA has been preferred recently. The specificity and sensitivity of ELISA are almost the same as those of the RIA. Moreover, it is free from restrictive legislation, and the reagents used have a long shelf life.

The automatic latex agglutination test is capable of estimating concentrations of CT as low as 1 ng/ml. Although the method is rather insensitive compared with the RIA and the ELISA, which can measure 0.1 pg of CT per ml, it is more rapid and simple and routinely sensitive enough to estimate CT or LT in culture fluids of toxigenic strains of *V. cholerae* and *E. coli*. The concentration of CT or LT can be determined in 100 samples within 1 h by the automatic method, so that the method may be a useful tool for a large-scale epidemiological survey.

The manual latex agglutination test is less expensive and more sensitive than the automatic method, although overnight reaction is required. Since the reagents for the latex agglutination tests are stable for at least 6 months and do not show any nonspecific reactions, it is more convenient than the reverse passive hemagglutination test or passive hemolysis. This method does not require any special equipment and techniques and has been shown to be useful as a routine clinical assay.

The application of the latex agglutination test to the culture filtrates of *V. cholerae* and *E. coli* is shown in Tables 4 and 5. The amounts of LT in sonicated cell-free extracts of enterotoxigenic *E. coli* estimated by three methods were in agreement. Although some discrepancies were found among the amounts of CT measured by the three methods, all methods were in agreement regarding the toxigenicity of each strain.

It was surprising to find that cyclic AMP is not an essential factor for toxin production in *V. cholerae* of both biotypes. We have previously reported that cyclic AMP-deficient mutants of *V. cholerae* E2 required cyclic AMP for the production of toxin as measured by the skin permeability test (15). This conclusion might have resulted from the absence of an immunochemical method for measuring small amounts of CT.

Automatic and manual latex agglutination tests for the measurement of CT and LT may find various uses in the study of *V. cholerae* and enterotoxigenic *E. coli*.

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