Rapid Visual Detection of *Escherichia coli* and *Vibrio cholerae* Heat-Labile Enterotoxins by Nitrocellulose Enzyme-Linked Immunosorbent Assay

LOTHAR BEUTIN,^{1*} LIV BODE,¹ THOMAS RICHTER,² GABRIEL PELTRE,³ AND RUDOLF STEPHAN¹

Robert Koch-Institut des Bundesgesundheitsamtes, 1 Berlin 65,¹ and Freie Universität Berlin, Institut für Mikrobiologie, Fachbereich Veterinärmedizin, 1 Berlin 33,² Federal Republic of Germany, and Institut Pasteur, Unité d'Immuno-Allergie, 75724 Paris, France³

Received 18 August 1983/Accepted 17 November 1983

A modification of the enzyme-linked immunosorbent assay for a sensitive and rapid visual detection of heat-labile enterotoxins from *Escherichia coli* and *Vibrio cholerae* is described. Small amounts of bacterial supernatant fluids are bound to nitrocellulose filters which are used as sorbents in the nitrocellulose enzyme-linked immunosorbent assay. The test is based on the immunological similarity between *V. cholerae* and *E. coli* heat-labile enterotoxins. Six isolates of *V. cholerae* and 48 isolates of *E. coli* were examined for heat-labile enterotoxins by the nitrocellulose enzyme-linked immunosorbent assay and the Vero cell bioassay. With some strains, the nitrocellulose enzyme-linked immunosorbent assay was found to be more sensitive for detection of *E. coli* heat-labile enterotoxin than the Vero cell test. A similar result was obtained by endpoint titration of heat-labile enterotoxin-positive *E. coli* H10407 culture fluid in both assays. The sensitivity of the nitrocellulose enzyme-linked immunosorbent assay for the detection of purified cholera toxin was at a total level of 1 ng, which is a good result when compared with other serological assays.

Heat-labile enterotoxins which are produced by *Vibrio cholerae* (CT) and by some pathogenic strains of *Escherichia coli* (LT) can be assayed by various methods.

Previously developed in vivo enterotoxin tests, such as the ileal loop test (3, 17) or the vascular permeability assay (6), proved to be sufficiently sensitive but too laborious for routine application.

In the last decade, a number of different in vitro enterotoxin assays were established which are more suitable for screening examinations of clinical specimens. At present, bioassays with tissue cultures of, e.g., Y-1 cells (20), Chinese hamster ovary cells (10), and Vero cells (26) are most commonly used. It is a disadvantage of these bioassays that they require experience and special tools not always present in standard clinical laboratories.

Alternatively, seroimmunological methods were employed to examine bacterial supernatant fluids for the presence of LT or CT. LT and CT are immunogenic and have been used for the preparation of immunologically cross-reactive antisera in experimental animals (2, 11, 21). A number of serological assays have been described which employ anti-LT or -CT immune serum in precipitation reactions (14), coagglutination tests (1, 13, 15), or complement-mediated, passive-immune hemolysis (8, 23) for enterotoxin screening. Furthermore, very small amounts of LT and CT may be detected by using either the radioimmunoassay (9, 24) or modifications of the enzyme-linked immunosorbent assay (ELISA) (12, 27, 28, 30). In particular, the latter tests are highly sensitive, but until now their application has been limited to well-equipped, high-level laboratories. As a consequence, we developed a modified ELISA by using nitrocellulose as sorbent (NC-ELISA) for the detection of LT and CT in bacterial supernatant fluids. This modification com-

bines the sensitivity of the original ELISA with the advantage that special laboratory equipment and refinements of the test as described for other LT ELISAs (27, 28, 30) are not required. The NC-ELISA is a simple and economical test and can be performed in any small laboratory equipped for bacteriological work.

MATERIALS AND METHODS

Bacteria. Six strains of *V. cholerae* and 48 isolates of *E. coli* were used in this study. The properties of *V. cholerae* strains are summarized in Table 1. All *E. coli* strains except W8, C600, 168-32, and 168-150 were from human clinical isolates. Table 2 presents the characteristics of 16 *E. coli* strains used for documentation of the NC-ELISA in Fig. 1. *E. coli* H10407 and 408 were taken as standard positive controls and the K-12 strains W8 and C600 as standard negative controls for heat-labile enterotoxin.

Stock cultures of *V. cholerae* were maintained on nutrient agar slants (pH 7.8) and subcultured monthly. *E. coli* strains were usually kept frozen at -20°C in a dilution of 1.5% Bacto-Peptone (Difco Laboratories, Detroit, Mich.) with an equal amount of sterile 87% glycerol.

Preparation of bacterial culture filtrates. A loopful of bacteria was inoculated into 100-ml culture flasks, each containing 10 ml of 2% Casamino Acids, 0.6% yeast extract (Difco), and salts medium (CYE) as described by Evans et al. (6). Bacterial cultures were grown overnight in a rotary shaker at 250 rpm and 37°C. After overnight growth, the cells were removed from the supernatant by centrifugation for 30 min at 13,000 × g. The culture fluid was subjected to sterile filtration through 0.22-μm disposable filter units (Millex; Millipore Corp., Bedford, Mass.), and aliquots were stored at -20°C.

Preparation of rabbit antiserum against cholera toxin. Purified cholera toxin and cholera toxin (Sigma Chemical Co., St. Louis, Mo.) were used as immunizing antigens.

* Corresponding author.

TABLE 1. *V. cholerae* strains

Strain (biotype)	Serotype	Source	Yr isolated
1360	Ogawa	Human, Bangkok, Thailand	1959
1576	Inaba	Human, Bangkok, Thailand	1959
71	Hikojima	Institut Pasteur, Paris, France	
CH69 (El Tor)	Ogawa	Human, Gaziantep, Turkey	1976
CH57 (El Tor)	Inaba	Human, Portugal	1974
HK51 (El Tor)	Hikojima	Institut Pasteur, Paris, France	

Serum samples were taken from rabbits before immunization and stored at -20°C . For immunization, the rabbits were injected intramuscularly three times within 3 days with 25 μg of cholera toxin in 50 mM Tris–200 mM NaCl–1 mM EDTA (pH 7.5) and emulsified with an equal volume of Freund complete adjuvant (Pel-Freez Biologicals, Rogers, Ark.), followed by three injections of 50 μg of CT in the same buffer with adjuvant at 7-day intervals. Blood was collected 10 days after the last injection and after booster injections. Serum samples were stored in equal portions at -20°C .

Vero cell assay. Vero cells and culture media were purchased from Seromed (Berlin, Federal Republic of Germany). The Vero cell assay was performed as previously described (26) with the following modifications. Cells were grown in minimal essential medium with Earle salts and glutamine supplemented with 5% fetal calf serum and 12.5 μg of kanamycin per ml in a 5% CO_2 atmosphere. Vero cells were maintained by weekly passage in fresh growth medium. For the test, cells were adjusted to $10^5/\text{ml}$ and seeded 3 days before use in 1-ml portions into 24-well tissue culture plates (BD Labware, Oxnard, Calif.). When monolayers had formed, minimal essential medium was replaced by a test medium (a threefold dilution of supplemented minimal essential medium without fetal calf serum in phosphate-buffered saline [PBS] at pH 7.0). After 30 min of preincubation at 37°C , 100 μl of bacterial culture filtrate per well was added. The plates were incubated at 37°C for 24 h. Morphological effects caused by enterotoxin were evaluated with an inverted microscope.

ELISA. The technique originally described by Engvall and Perlmann (5) was subjected to the following modifications. Bacterial supernatant fluid and purified CT were diluted in carbonate buffer (15 mM Na_2CO_3 –35 mM NaHCO_3 [pH 9.6]) before use. One hundred microliters of diluted antigen was added per well in a flat-bottom polystyrene plate (Immulon, 129B; Dynatech Deutschland GmbH, Plochingen, Federal Republic of Germany). The plates were stored for 16 h at 4°C overnight for coating. The next day plates were washed for 10 min in a solution of 0.9% NaCl–0.1% Tween 20. After washing, 100 μl of diluted rabbit anti-CT serum or preimmune serum in PBS containing 0.1% Tween 20 and 1% bovine serum albumin (BSA) (Sigma) was added per well, followed by incubation for 1 h at 37°C .

The serum was removed, and the plates were washed as described above. The plates were further incubated for 1 h at 37°C with 100 μl of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G per well (Tago, Inc., Immuno-diagnostic Reagents, Burlingame, Calif.) diluted 1:1,000 in 50 mM Tris (pH 8.0) containing 0.1% Tween 20 and 1% BSA.

Uncoupled conjugate was removed by washing. As substrate, 100 μl per well of a solution of 1 mg of *p*-nitrophenyl phosphate per ml (Sigma) in 10% diethanolamine–1 mM MgCl_2 (pH 9.8) was added. After 30 min of incubation at 37°C , the reaction was terminated by addition of 50 μl of 1 N NaOH per well. The color reaction was measured photometrically at a 405-nm wavelength with a Multiscan ELISA-reader (Flow Laboratories, Bonn, Federal Republic of Germany). A positive-negative (P/N) value was calculated as a quotient of the optical densities obtained from the reaction of antigen with immune serum and preimmune serum. A P/N value of 2.0 or greater was considered to be positive for the presence of CT or LT (4).

NC-ELISA. Discs (5-mm diameter) were punched from sheets of nitrocellulose membranes (HATF type, pore size 0.45 μm ; Millipore) with a perforator, and each was transferred into a well of a polypropylene microtiter plate (95 F bottom wells; Dynatech). The discs were pushed down to the bottom of the wells with a plastic tip to obtain a good contact between the disc and the plastic plate. The following

TABLE 2. *E. coli* strains

Strain	Serotype	Enterotoxin ^a (reference)	Source
164/36	O148:K ⁻	LT ⁺ , ST ^{++b}	Cholera-like disease, India, 1982 ^c
262/5	O78:K80	LT ⁺ , ST ^{++b}	Infantile diarrhea, Peru, 1978 ^c
205/3	O78:K80	LT ^{++b}	Infantile diarrhea, Peru, 1978 ^c
H10407	O78:H11	LT ⁺ , ST ⁺ (7)	Human ^d
408	O78:H12	LT ⁺ , ST ⁺ (21)	Human ^c
DI8/2	O?:K39	LT ^{++f}	Cholera-like disease, Federal Republic of Germany (FRG), 1981 ^g
C3936	O86:K61	LT ^{++f}	Infantile diarrhea, FRG, 1981 ^g
DR2/2	O15:K ⁻	LT ^{++f}	Cholera-like disease, FRG, 1981 ^g
168-32	O8:K200:H31	LT ^{++h}	Porcine ⁱ
C3949	O128:K67	LT ^{++f}	Infantile diarrhea, FRG, 1982 ^g
C3952	O25:K11	LT ^{++f}	Infantile diarrhea, FRG, 1982 ^g
C600		LT ^{++f}	Laboratory strain collection
W8		LT ^{++f}	W. Suter, Sandoz, Ltd., Basel, Switzerland
89/83	O6	LT ^{++f}	Human urinary tract infection, FRG, 1983 ^g
167/36	O9	LT ⁺ , ST ^{++b}	Cholera-like disease, India, 1982 ^c
168-150	O9:K30:K99	ST ^{++h}	Bovine ⁱ

^a ST, Heat stable.

^b J. Bockemühl, personal communication.

^c J. Bockemühl, Hygienisches Institut Hamburg, FRG.

^d D. J. Evans and D. G. Evans, via T. Chakraborti, Federal Health Office, FRG.

^e B. Sack via B. Rüger, Institut für Mikrobiologie, Erlangen, FRG.

^f This work.

^g Laboratory strain collection.

^h P. A. M. Guinée, personal communication.

ⁱ P. A. M. Guinée, Rijksinstituut voor de Volksgezondheid, Bilthoven, the Netherlands.

test procedure was carried out inside the original microtiter wells, without further transfer of the nitrocellulose as described by Palfrey and Elliott (19). Five-microliter samples of filtered bacterial supernatant fluid or purified CT were spotted on the discs with a micropipette. The loaded discs were air dried overnight at room temperature. After desiccation, the discs were incubated with 200 μ l of 3% BSA in PBS at 45°C for 1 h. This step was necessary to block remaining protein-binding sites on the nitrocellulose membranes. The BSA solution was carefully removed with the aid of a vacuum pump and replaced by an equal volume of washing buffer (0.9% NaCl–0.1% Tween 20) for 10 min at room temperature. After being washed, the discs were incubated for 1 h at 37°C with 100 μ l of rabbit anti-CT or preimmune serum diluted in PBS containing 1% Tween 20. The serum was removed as described above, and the discs were washed with 200 μ l of washing buffer per well. The washing procedure was repeated 10 times at 5-min intervals by addition and removal of washing buffer from the wells. This step was followed by a 1-h incubation at 37°C with 100 μ l of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G per well diluted in 50 mM Tris (pH 8.0) containing 1% Tween 20. For all experiments, conjugate was used in an optimal working dilution of 1:1,000 that was determined by titration. After removal of the unbound conjugate, the discs were washed as described above. The alkaline phosphatase activity was assayed as described by O'Connor and Ashman (18). Equal volumes of freshly prepared solutions of naphthol AS-MX phosphate (0.4 mg/ml) and Fast Red TR salt (6 mg/ml in 200 mM Tris [pH 8.0]–2 mM MgCl₂) (both from Sigma) were mixed and filtered before use. The filter discs were incubated in 100 μ l of this mixture per well for 30 min at room temperature. The color reaction was terminated by washing the discs three times in distilled water. Discs with a positive reaction showed a distinct red spot on the labeled site. The intensity of color corresponded to the amount of nitrocellulose-bound antigen.

RESULTS

Neutralization of LT in *E. coli* culture fluid by rabbit anti-CT serum. Rabbit preimmune and anti-CT sera were tested for neutralization of LT from culture fluid of *E. coli* H10407. Serial twofold dilutions of serum in PBS (100 μ l) were incubated for 1 h at room temperature with an equal volume of sterile, filtered H10407 supernatant. The mixtures were then added to Vero cells. The toxic effect of LT from the supernatant was inhibited by high concentrations of (preimmune) serum in a maximal dilution of 1:80. Such inhibitory effects of high serum concentration have been reported for LT cell bioassays (26). Dilutions of anti-CT serum from the same animal neutralized LT with an endpoint of 1:640.

Effect of serum concentration on the sensitivity of NC-ELISA. Rabbit preimmune and anti-CT sera were tested in dilutions from 1:50 to 1:2,000. When low serum dilutions were used (1:50 to 1:200), unspecific reactions occurred with some bacterial culture filtrates but not with purified CT in NC-ELISA and ELISA. This effect was absent when higher serum dilutions were tested. A 1,000-fold dilution of serum was determined as optimal for reliable detection of LT and CT from bacterial supernatant fluids for both assays. Higher serum dilutions (1:2,000) resulted in a weaker response with some *E. coli* culture filtrates (mostly poor LT producers). On the other hand, the test sensitivity for detection of purified CT was not enhanced when serum concentrations higher than 1:1,000 were used.

Sensitivity of the NC-ELISA for the detection of LT and CT. A total of 1 ng of purified CT per filter disc was clearly detectable by visual reading of the NC-ELISA. The amount of 0.1 ng per filter was too small to allow a distinct detection, whatever concentration of serum was used (Fig. 1). Similarly, when tested by ELISA, 1 ng (10 ng/ml) of CT resulted in a slightly positive P/N ratio (>2.0), whereas 0.1 ng (1 ng/ml) of CT was below the detectable level (Table 3). With both assays, a quantitative correlation between increasing amounts of bound CT and color intensity (absorbance rate) was found.

The sensitivity of the NC-ELISA for detection of purified CT (a total of 1, 5, and 10 ng) was not markedly decreased by the presence of other protein (BSA) up to a concentration of 25 mg/ml in the sample added to the filters. Higher concentrations of BSA prevented clear detection of bound CT.

The sensitivity of the NC-ELISA for the detection of LT and CT in bacterial supernatant fluids was tested. The degree of color intensity on the nitrocellulose test filters varied strongly and specifically between different tested supernatant fluids from independent strains or species. This finding correlated with the different strength of the toxic effect caused by several tested LT⁺ or CT⁺ supernatant fluids on Vero cells. These variations observed in both assays appeared to be dependent on the quantities of LT or CT present in the culture fluid. Quantitative differences between some representative low and strong enterotoxin-producing strains were also measured by ELISA (Table 4).

In contrast to the NC-ELISA, only strong and nonenterotoxin producing bacteria could be clearly discerned by this method. The variations of absorbance rates which were obtained from repeated experiments with the same antigen samples were too high to distinguish reliably between poor (strains 168-32 and DI8/2) and non-LT-producing (strains W8 and 89/83 and CYE control) *E. coli* (Table 4). In contrast, these strains were clearly differentiated by the NC-ELISA (Fig. 1).

FIG. 1. NC-ELISA. Bacterial supernatant fluids and purified CT were incubated with rabbit anti-CT serum (rows 1, 4, 7, and 10), preimmune serum (rows 2, 5, 8, and 11), and PBS–1% Tween 20 (rows 3, 6, 9, and 12). Supernatant fluids are as follows: Rows A1 through F1: *V. cholerae* 1360, 71, CH57, 1576, HK51, and CH96. Row G1: CYE medium. Row H1: PBS. Rows A4 through H4: *E. coli* 164/36, 262/5, 205/3, H10407, 408, DI8/2, C3936, and DR2/2. Rows A7 through H7: *E. coli* 168-32, C3949, C3952, C600, W8, 89/83, 167/36, and 168-150. Rows A10 through H10: 50, 20, 10, 7.5, 5, 2.5, 1, and 0.1 total ng, respectively, of purified CT.

TABLE 3. Sensitivity of ELISA for detection of purified CT

CT (ng)	P/N	Absorbance at 405 nm
10.0	>12	>2.000
7.5	10.2	1.696
5.0	7.4	1.221
2.5	6.0	0.985
1.0	2.4	0.403
0.1	1.6	0.263

Comparison of the NC-ELISA with the Vero cell assay for the detection of LT and CT. Six strains of *V. cholerae* and 48 *E. coli* isolates were tested for their enterotoxigenicity with both assays. The results obtained were tested blindly and compared afterwards. For the NC-ELISA the results referring to six *V. cholerae* (Table 1) and 16 *E. coli* (Table 2) strains are documented in Fig. 1. Some of the *E. coli* strains have already been described as LT⁺ (Table 2). These and strains found to be LT⁺ in this study by the Vero cell assay were all positive when tested by the NC-ELISA. Supernatants of *V. cholerae* strains exhibited a stronger effect on Vero cells (>90% of cells affected) and a stronger color reaction in the NC-ELISA than LT⁺ *E. coli* (Fig. 1).

A disagreement between both tests was obtained for *E. coli* 167-36 (see Fig. 1) and two other *E. coli* strains which were negative in the Vero cell test but weakly positive for LT by the NC-ELISA. These three strains have been reported to cause morphological alterations of Chinese hamster ovary cells and to be LT⁺ when tested by GM1 ELISA (J. Bockemühl, personal communication).

The other *E. coli* strains which are examined for LT in this study and are not listed in Table 2 are independent isolates of O serogroups 1, 2, 3, 4, 25, 26, 55, 78, 111, 114, 126, and 128. These strains were LT⁻ in both the NC-ELISA and the Vero cell assay.

The partial discrepancy which was found between the Vero bioassay and the NC-ELISA was probably due to their different sensitivity levels for LT detection. This was tested by endpoint determination with serial twofold dilutions of H10407 culture fluid in both assays. The highest dilution inducing morphological effect on Vero cells was 16-fold, whereas in the NC-ELISA, LT was still detectable in a 1:64 dilution of the culture supernatant.

DISCUSSION

In this study, the use of a modified ELISA with nitrocellulose as a solid phase for the rapid detection of LT and CT in bacterial culture fluid is described. The results reported indicate that the NC-ELISA possesses sufficient specificity and a better sensitivity for detection of LT as compared with the Vero cell assay. By the NC-ELISA, LT⁻ strains of *E. coli* were clearly discerned from isolates which produced LT in relatively high and low amounts. In contrast to other methods (8, 14, 28), it was not necessary to prepare polymyxin extracts of bacteria for LT detection.

The binding capacity of nitrocellulose favorably compared with that of polystyrene ELISA plates. Similar observations have been made by others, especially for the case in which high concentrations of additional protein other than antigen were present in the sample (19). Nitrocellulose is known to have excellent binding characteristics for DNA and protein (25, 29). For ¹²⁵I-labeled immunoglobulin G, a binding capacity of 80 to 100 µg of protein per cm² was reported (16). The ability of polystyrene to adsorb proteins is much more

limited, even when an excess of protein is used for coating (22). Furthermore, the effective binding capacity of polystyrene shows a certain variation between different batches of plastic material and even between different lots from the same batch. In our study, this phenomenon was observed when bacterial supernatant fluids were screened for the presence of LT by repeated ELISA tests (Table 4). As a consequence, Svennerholm and Holmgren (27) developed the GM1 ELISA, in which the ganglioside GM1 is used to enhance the specific binding of LT to GM1-coated plastic material. This technique, which is very specific, requires at least partial purification of *E. coli* LT for antiserum preparation and purified GM1 ganglioside, which is a relatively expensive substance.

In contrast to the GM1 ELISA, we found rabbit anti-CT immune serum sufficiently cross-reactive with *E. coli* LT of human and animal origin to be used for the NC-ELISA. The use of antiserum to crude or partially purified LT may cause unspecific reactions in the test due to the presence of antibodies to *E. coli* proteins other than LT.

The sensitivity of the NC-ELISA for the detection of LT and CT is good as compared with other serological assays. Moreover, in contrast to other serological tests, which often require biochemical purification of specific antibodies, preparation of purified LT, or, as in the case with the double-sandwich-antibody ELISA (30), two immune sera from different animal species, the NC-ELISA is much more simple to perform. Coagglutination tests require highly purified anti-CT or -LT immunoglobulins, which are needed for sensitization of bacteria or inorganic material (1, 13, 15). The passive hemolysis assay (8, 23) requires sensitization of erythrocytes with purified enterotoxin and has the disadvantage of not being applicable to hemolysin-producing strains of *E. coli*.

Especially for clinical laboratories with low-standard equipment, the NC-ELISA represents a sensitive qualitative method for the rapid diagnosis of enterotoxigenic *E. coli*. All components of the system, except rabbit anti-CT serum, which is simple to prepare, are commercially available at moderate costs and economical in use. No specialized staff and special equipment are needed to carry out the test. Because of its properties, the NC-ELISA is suitable for

TABLE 4. Sensitivity of ELISA for detection of LT and CT in culture fluid of *E. coli* and *V. cholerae* strains

Species and strain ^a	Absorbance at 405 nm (A ₄₀₅) ^b	P/N ^c obtained with:	
		A ₄₀₅ - SD	A ₄₀₅ + SD
<i>V. cholerae</i> 1360	1.805 ± 0.072	8.5	9.2
<i>E. coli</i>			
H10407	1.384 ± 0.124	6.8	8.2
C3936	0.917 ± 0.092	4.0	4.9
DI8/2	1.374 ± 0.234	3.7	5.3
168-32	0.873 ± 0.152	3.6	5.2
89/83	0.678 ± 0.085	2.9	3.7
W8	0.464 ± 0.082	1.7	2.4
CYE ^d	0.557 ± 0.129	1.8	2.8

^a Bacterial supernatant fluids and CYE medium were diluted 1:10 in carbonate buffer for coating.

^b Mean ± standard deviation (n = 22).

^c Maximal and minimal P/N ratios were obtained by addition or subtraction of the standard deviation to the arithmetic mean of A₄₀₅.

^d CYE medium was used as control.

clinical application, particularly in developing countries where diarrhea still constitutes a severe medical problem.

ACKNOWLEDGMENTS

This study was supported by the Federal Ministry for Youth, Family Affairs and Health.

The authors are grateful to Ida and Frits Ørskov for serotyping of some *E. coli* strains, to Sonja Zimmermann for excellent technical help, and to Mark Achtman and Manfred Frank for critical discussions.

LITERATURE CITED

- Brill, B. M., B. L. Wasilauskas, and S. H. Richardson. 1979. Adaptation of the staphylococcal coagglutination technique for detection of heat-labile enterotoxin of *Escherichia coli*. *J. Clin. Microbiol.* **9**:49-55.
- Clements, J. D., and R. A. Finkelstein. 1978. Immunological cross-reactivity between a heat-labile enterotoxin(s) of *Escherichia coli* and subunits of *Vibrio cholerae* enterotoxin. *Infect. Immun.* **21**:1036-1039.
- De, S. N., and D. N. Chatterje. 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *J. Pathol. Bacteriol.* **66**:559-562.
- deSavigny, D., and A. Voller. 1980. The communication of ELISA data from laboratory to clinician. *J. Immunoassay* **1**:105-128.
- Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.* **109**:129-135.
- Evans, D. G., D. J. Evans, Jr., and S. L. Gorbach. 1973. Identification of enterotoxigenic *Escherichia coli* and serum antitoxin activity by the vascular permeability factor assay. *Infect. Immun.* **8**:731-735.
- Evans, D. G., and D. J. Evans. 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. *Infect. Immun.* **21**:638-647.
- Evans, D. J., Jr., and D. G. Evans. 1977. Inhibition of immune hemolysis: serological assay for the heat-labile enterotoxin of *Escherichia coli*. *J. Clin. Microbiol.* **5**:100-105.
- Greenberg, H. B., D. A. Sack, W. Rodriguez, R. B. Sack, R. G. Wyatt, A. R. Kalica, R. L. Horswood, R. M. Chanock, and A. Z. Kapikian. 1977. Microtiter solid-phase radioimmunoassay for detection of *Escherichia coli* heat-labile enterotoxin. *Infect. Immun.* **17**:541-545.
- Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. *Infect. Immun.* **10**:320-327.
- Gyles, C. L. 1974. Immunological study of the heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. *Infect. Immun.* **9**:564-570.
- Holmgren, J., and A.-M. Svennerholm. 1973. Enzyme-linked immunosorbent assays for cholera serology. *Infect. Immun.* **7**:759-763.
- Honda, T., R. Samakoses, C. Sornchai, Y. Takeda, and T. Miwatani. 1983. Detection by a staphylococcal coagglutination test of heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* **17**:592-595.
- Honda, T., S. Taga, Y. Takeda, and T. Miwatani. 1981. Modified Elek test for detection of heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* **13**:1-5.
- Ito, T., S. Kuwahara, and T. Yokota. 1983. Automatic and manual latex agglutination tests for measurement of cholera toxin and heat-labile enterotoxin of *Escherichia coli*. *J. Clin. Microbiol.* **17**:7-12.
- Millipore Corporation. 1982. Laboratory products catalogue. A guide to membrane separation technology. Protein assays and DNA hybridization. Millipore Corp., Bedford, Mass.
- Moon, H. W., S. C. Whipp, G. W. Engstrom, and A. L. Baetz. 1970. Response of the rabbit ileal loop to cell-free products from *Escherichia coli* enteropathogenic for swine. *J. Infect. Dis.* **121**:182-187.
- O'Connor, C. G., and L. K. Ashman. 1982. Application of the nitrocellulose transfer technique and alkaline phosphatase conjugated anti-immunoglobulin for determination of the specificity of monoclonal antibodies to protein mixtures. *J. Immunol. Methods* **54**:267-271.
- Palfrey, R. G. E., and B. E. Elliott. 1982. An enzyme-linked immunosorbent assay (ELISA) for detergent solubilized Ia glycoproteins using nitrocellulose membrane discs. *J. Immunol. Methods* **52**:395-408.
- Sack, D. A., and R. B. Sack. 1975. Test for enterotoxigenic *Escherichia coli* using Y1 adrenal cells in miniculture. *Infect. Immun.* **11**:334-336.
- Sack, R. B., and J. L. Froehlich. 1977. Antigenic similarity of heat-labile enterotoxins from diverse strains of *Escherichia coli*. *J. Clin. Microbiol.* **5**:570-572.
- Salonen, E.-M., and A. Vaheri. 1979. Immobilization of viral and mycoplasma antigens and of immunoglobulins on polystyrene surface for immunoassays. *J. Immunol. Methods* **30**:209-218.
- Serafim, M. B., A. F. P. deCastro, M. B. Leonardo, and A. R. Monteiro. 1981. Single radial immune hemolysis test for detection of *Escherichia coli* thermolabile enterotoxin. *J. Clin. Microbiol.* **14**:473-478.
- Shah, D. B., P. E. Kauffman, B. K. Boutin, and C. H. Johnson. 1982. Detection of heat-labile-enterotoxin-producing colonies of *Escherichia coli* and *Vibrio cholerae* by solid-phase sandwich radioimmunoassays. *J. Clin. Microbiol.* **16**:504-508.
- Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
- Speirs, J. I., S. Stavric, and J. Konowalchuk. 1977. Assay of *Escherichia coli* heat-labile enterotoxin with Vero cells. *Infect. Immun.* **16**:617-622.
- Svennerholm, A.-M., and J. Holmgren. 1978. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM₁-ELISA) procedure. *Curr. Microbiol.* **1**:19-23.
- Svennerholm, A.-M., and G. Wiklund. 1983. Rapid GM₁-enzyme-linked immunosorbent assay with visual reading for identification of *Escherichia coli* heat-labile enterotoxin. *J. Clin. Microbiol.* **17**:596-600.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**:4350-4354.
- Yolken, R. H., H. B. Greenberg, M. H. Merson, R. B. Sack, and A. Z. Kapikian. 1977. Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. *J. Clin. Microbiol.* **6**:439-444.