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Microtiter Enzyme-Linked Immunosorbent Assay for Immunoglobulin G Cholera Antitoxin in Humans: Method and Correlation with Rabbit Skin Vascular Permeability Factor Technique

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A microtiter enzyme-linked immunosorbent assay (ELISA) to measure immunoglobulin G cholera antitoxin in human serum has been developed. The ELISA employs commercially available reagents, including cholera enterotoxin and goat anti-human immunoglobulin G. It is specific, sensitive, and reproducible and requires as little as 5 μl of serum. ELISA, moreover, permits quantitative determination of cholera antitoxin at a single serum dilution of 1:200. A total of 162 pre- and postchallenge sera from 49 volunteers who ingested *Vibrio cholerae* classical biotype, and 165 sera from 43 volunteers who ingested *V. cholerae* El Tor biotype, were tested for cholera antitoxin by ELISA and by the rabbit skin vascular permeability factor assay. The correlation between the two assays was statistically significant ($P < 0.001$). ELISA for immunoglobulin G cholera antitoxin thus provides a valuable in vitro correlate of in vivo toxin-neutralizing capacity. Microtiter ELISA permits duplicate evaluation of at least 14 sera per 96-well plate including blanks and controls, is readily adapted to use in field studies, and therefore is particularly well suited to seroepidemiological surveys.

The diarrhea-producing enterotoxin of *Vibrio cholerae* is immunogenic, and the majority of individuals with clinical or subclinical *V. cholerae* infection develop significant rises in circulating antitoxin (1, 2, 10, 17, 19). Current assays for cholera antitoxin include the rabbit skin vascular permeability factor (PF) assay (5, 16), Y-1 adrenal cell assay (7, 16), rabbit ileal loop assay (6, 19), radioimmunoassay (11, 13), and passive hemagglutination test (10, 14). Each suffers from one or more of the following drawbacks: not readily adaptable to large numbers of specimens; expensive; requires sophisticated tissue culture capability or radioisotopes; does not measure specific immunoglobulin class; requires relatively large volumes of serum.

The microtiter enzyme-linked immunosorbent assay (ELISA), currently used for measurement of antibodies to a wide range of antigens (9, 20), offers a method that could overcome all the aforementioned drawbacks of cholera antitoxin serology.

Macro-ELISA techniques for measurement of cholera antitoxin have been reported (4, 15). Herein we report the development of a microtiter ELISA for measurement of cholera antitoxin in humans.

MATERIALS AND METHODS

Anti-human IgG conjugate. Goat antiserum against human immunoglobulin G (IgG) (Meloy Co., Springfield, Va.) was precipitated with Na_2SO_4 to isolate the immunoglobulin fraction, which was then coupled to alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, Mo.) by the method of Voller et al. (20).

The washing buffer, which served as diluent for both sera and conjugate, consisted of phosphate-buffered saline (pH 7.2) with 0.05% polyoxyethylene-(20) sorbitan monolaurate (Tween 20; Fisher Chemical Co., Pittsburgh, Pa.), and 1% heat-inactivated fetal calf serum.

Conjugate working dilution. The sensitivity and specificity of the conjugate were determined in a checkerboard fashion. Ten-fold increasing concentrations, from 1 to 1,000 ng of human IgG (purified by chromatography) per ml in sodium carbonate coating buffer (pH 9.6), were adsorbed onto wells of polyvinyl chloride microtiter plates (Cooke, Division of Dynatech Laboratories, Alexandria, Va.) overnight at 4°C. Samples (100 μl) of twofold dilutions (1:100 to 1:3,200) of conjugate were added to wells coated with purified IgG as well as to blank wells similarly treated with washing buffer. The plates were incubated for 60 min at 37°C, and the wells were washed three times (3 min each) with buffer. Samples (100 μl) of *p*-nitrophenyl phosphate substrate (1 mg/ml) (Sigma 104, Phospha-

tase Substrate Tablets) were applied to the wells for reaction with the enzyme. After 30 min of incubation at 37°C, the reaction was arrested with 3 M NaOH (25 μl per well). The optical density (OD) in each well was read at 400-nm wavelength using an ELISA microtiter colorimeter (3). The OD of each blank well without immunoglobulin was subtracted from the corresponding well with immunoglobulin to give the net OD. The lowest concentration of conjugate that gave a net OD of approximately 0.10 (i.e., just visible to the naked eye) when reacted with the lowest concentration of IgG was selected as the working dilution of conjugate. To evaluate its specificity, the IgG conjugate at working dilution, usually 1:200, was reacted against 10-fold graded doses (from 1 to 1,000 ng/ml) of purified IgM and IgA.

Sera. Sera were collected from 49 volunteers who were challenged with the classical biotype of *V. cholerae*, and from 43 volunteers who were challenged with the El Tor biotype of *V. cholerae*, as a part of studies designed to assess the protective value of cholera vaccines. All challenge studies were carried out under quarantine in the Isolation Ward of the Center for Vaccine Development (16, 17). Sera were obtained before and 10, 21, and 28 days after challenge. Each serum sample was tested for antitoxin by ELISA at the Center for Vaccine Development and by rabbit skin PF assay at Downstate Medical Center.

A negative control serum standard was obtained by pooling prechallenge sera from six volunteers. This pooled serum contained no detectable cholera antitoxin by either the PF (5, 16) or Y-1 adrenal cell methods (7, 16).

A positive control serum (Chol. 2005-3-28) for use as a standard was obtained from a volunteer convalescent from induced clinical cholera (17). This serum contained 11 antitoxin units per ml and 40 antitoxin units per ml, as measured by the PF and adrenal cell techniques, respectively.

ELISA technique. (i) Concentration of antigen. Polyvinyl plates with U-bottom wells were coated with 100 μl of varying concentrations (1, 5, 10, 20, and 40 μg) of purified cholera enterotoxin (lot DZ 3100; Schwarz/Mann Co., Orangeburg, N.Y.) in phosphate-buffered saline (pH 7.2) and incubated at 4°C overnight. The plates were washed three times (3 min each) with washing buffer, after which 100-μl samples of twofold serial dilutions of the positive and negative control sera were added. After 60 min at 37°C the wells were washed as above, conjugate was added, and the plates were further incubated for 60 min at 37°C, followed by another wash. Substrate was added as described above, and the net ODs were determined. In this manner, an antigen concentration of 10 μg/ml was found to best meet the criteria recommended by Voller et al. (20) and was used exclusively thereafter.

(ii) Test procedures. The test wells of U-bottom polyvinyl microtiter plates were coated with 100 μl of purified cholera toxin (10 μg/ml in phosphate-buffered saline) and incubated overnight at 4°C; each test well had a corresponding blank well coated with washing buffer. After the plates were washed as previously described, 100 μl of two-fold serial dilutions of unknown or control sera was added to the appropriate wells and incubated for 60 min at 37°C. The triple wash was repeated, after which 100 μl of the working dilution of conjugate was added to all wells and incubated for 1 h at 37°C. The washing procedure was repeated, and 100 μl of *p*-nitrophenyl phosphate substrate (1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added to each well and incubated for 30 min at 37°C. The reaction was stopped by adding 25 μl of 3 M NaOH to each well. The color intensity was read at 400 nm wavelength as described above, and net OD values were calculated.

(iii) Reproducibility of assay. To examine the reproducibility of the assay, twofold serial dilutions (1:100 to 1:3,200) of the positive control serum were assayed in quintuplicate, twice daily, on 5 separate days. The coefficient of variation was estimated from the mean and standard deviation of the net OD of each dilution.

(iv) Determination of serum screening dilution. During the experiments designed to investigate the reproducibility of ELISA, it was apparent that there existed a linear relationship between net OD and serum dilution, suggesting that antibody could be quantitated by testing sera at a single dilution. To confirm this impression, we performed ELISA on five serially diluted (1:100 to 1:1,600) postchallenge sera, in all of which PF and Y-1 adrenal cell assays had demonstrated the presence of cholera antitoxin.

To select the most suitable dilution with minimal nonspecific reactivity for screening unknown sera, we tested twofold serial dilutions (1:25 to 1:400) of the pooled negative control serum in quintuplicate, twice daily, for 5 days.

(v) Test of unknown samples. ELISA was performed on pre- and postchallenge sera, diluted 1:200 in washing buffer, from 49 volunteers who ingested classical *V. cholerae* and from 43 who ingested *V. cholerae* El Tor. Scatter diagrams were constructed of net OD against rabbit skin PF antitoxin units per ml. The least squares linear regression and correlation coefficient (*r*) were derived from these data by using a programmable calculator.

RESULTS

Sensitivity and specificity of the anti-IgG conjugate. The alkaline phosphatase-conjugated anti-human IgG was found to be both sensitive and specific in detection of purified IgG. As seen in Fig. 1, a 1:200 dilution of the conjugate gave high net OD values against IgG but not against IgM or IgA. The conjugate allowed detection of IgG at concentrations of 15 ng/ml and higher.

Reproducibility of ELISA. Twofold serial dilutions (1:100 to 1:3,200) of the positive control serum tested in quintuplicate, twice daily, on 5 days revealed little variation (Table 1).

Use of serum at a single dilution. Examination of five serially diluted postchallenge sera of known cholera toxin-neutralizing capacity disclosed a linear relationship between serum di-

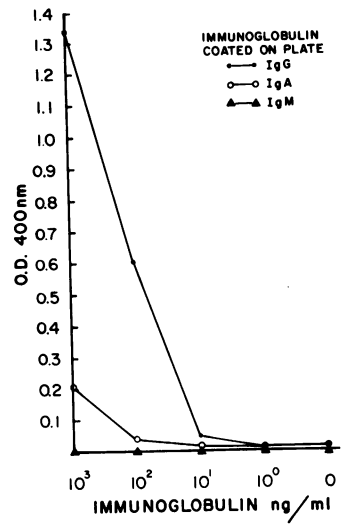

FIG. 1. *Sensitivity and specificity of anti-human IgG conjugate at working dilution 1:200.*

TABLE 1. *Reproducibility of ELISA for IgG cholera antitoxin*[a]

Reciprocal dilution	Mean net OD	Standard deviation	Coefficient of variation (%)
100	0.87	0.084	9.7
200	0.70	0.088	12.6
400	0.50	0.06	12.2
800	0.33	0.044	13.3
1,600	0.21	0.029	13.8
3,200	0.12	0.019	15.9

[a] Results of the positive control serum (Chol. 2005-3-28) tested in quintuplicate, twice daily, for 5 days.

lution and net OD (Fig. 2). It was concluded, therefore, that sera could be screened at a single dilution, provided that nonspecific reactivity could be minimized. Serial dilution of the negative control serum indicated that at serum concentrations greater than 1:200, net OD was elevated significantly above the background value obtained with the washing buffer blank (Table 2). Accordingly, 1:200 was selected as the lowest dilution that yielded no significant nonspecific reactivity.

Correlation between ELISA and rabbit skin PF assay. In 162 serum samples from 49 volunteers who ingested classical *V. cholerae*, levels of cholera toxin-binding IgG measured by ELISA and expressed as net OD correlated closely with toxin-neutralizing antibody determined by rabbit skin PF assay ($r = 0.86$, $P < 0.001$) (Fig. 3). From the regression equation, it was possible to calculate the net OD value, equivalent to 1 antitoxin unit per ml, which could be used as a cutoff between positive and

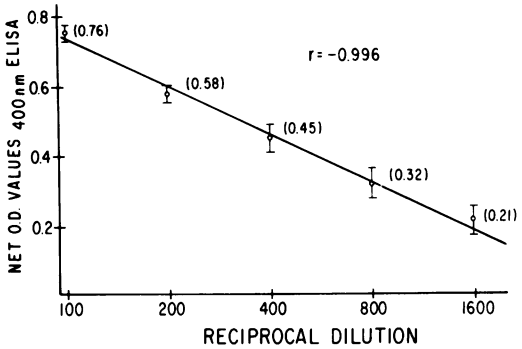

FIG. 2. *Relationship between ELISA values (net OD) and serum dilution. Each point represents the mean ± standard error of the mean of five sera with cholera toxin-neutralizing capacity by rabbit skin PF and Y-1 adrenal cell methods. The regression equation is* $y = -0.45 \log_{10} x + 1.63$, *and the correlation coefficient, r, is −0.996.*

TABLE 2. *ELISA values of a negative control serum standard*

Reciprocal serum dilution	Mean[a]	Standard deviation	t[b]	P
25	0.34	0.06		
			5.2	<0.01
50	0.20	0.06		
			2.6	<0.02
100	0.14	0.04		
			2.8	<0.02
200	0.09	0.04		
			1.3	>0.1
400	0.07	0.03		
			1.7	>0.1
Washing buffer blank	0.05	0.02		

[a] Mean net OD of 10 quintuplicate determinations.
[b] Student's t test.

negative sera. Thus, a net OD of 0.23 may be considered equivalent to 1 antitoxin unit per ml (Fig. 3). The validity of this relationship was confirmed in yet another way by performing ELISA on 40 randomly selected sera with no demonstrable cholera antitoxin in either the rabbit skin PF or adrenal cell assays. The net OD obtained with these sera was 0.11 ± 0.05 (mean ± standard deviation). Therefore, a net OD of 0.23, equivalent to 1 antitoxin unit per ml, is also approximately equal to the mean net OD plus 2.5 standard deviations of the 40 sera from the negative control population.

Correlation between ELISA values and antitoxin units was also demonstrated with 165 sera from 43 volunteers before and after ingestion of *V. cholerae* El Tor ($r = 0.83$, $P < 0.001$) (Fig. 4A). At net OD values greater than 0.9, however, this relationship was no longer demonstrable (r

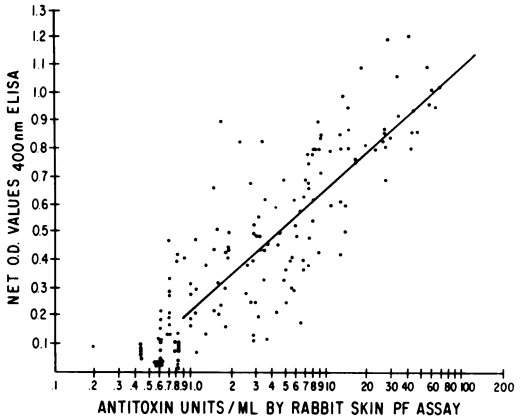

FIG. 3. *Relation between ELISA value (net OD) and antitoxin units measured by rabbit skin PF assay in 162 sera from 49 volunteers before and after ingestion of classical V. cholerae. The regression equation is* $y = 0.437 \log_{10} x + 0.23$, *and the correlation coefficient, r, is 0.86.*

= 0.24, $P > 0.1$). Examination of the high-titer sera (OD > 0.9) diluted 1:800 in washing buffer restored linearity to the relationship between OD and PF antitoxin units ($r = 0.64$, $P < 0.001$) (Fig. 4B).

DISCUSSION

Measurement of cholera antitoxin is useful in serologically detecting clinical and subclinical cases of cholera, in epidemiological investigations, and in studies of immunity to *V. cholerae* and its toxin and toxoids. The rabbit ileal loop (6, 19), Y-1 adrenal cell (7, 16), and rabbit skin techniques (5, 16) measure cholera toxin-neutralizing antibody in serum. As currently performed, they require larger volumes of sera than does ELISA and are less sensitive, although it has been shown that the PF assay can be rendered approximately 10 times more sensitive by reducing the toxin test dose (J. P. Craig, unpublished data). The passive hemagglutination test (10, 14) and radioimmunoassay (11, 13) for cholera antitoxin resemble ELISA in that they measure binding antibody. Passive hemagglutination is simple and inexpensive, but it cannot be used to identify immunoglobulin class, and it is less sensitive than the other assays in detecting low levels of antitoxin. Radioimmunoassay is highly sensitive and specific, but requires a scintillation counter and special safety precautions to handle isotopes. Microtiter ELISA overcomes many of the above-mentioned drawbacks. ELISA is inexpensive, highly sensitive and specific, and technically simple; it measures immunoglobulin class, and requires only minute quantities (5 μl) of serum. ELISA permits duplicate evaluation

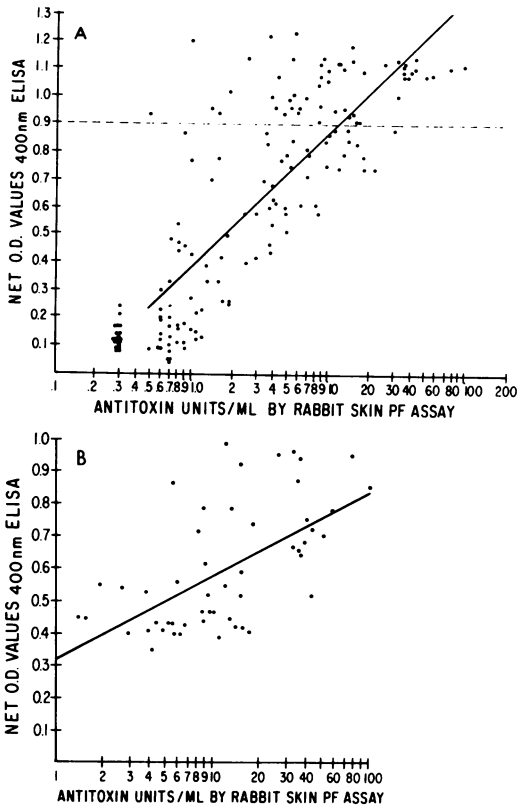

FIG. 4. *(A) Relation between ELISA value (net OD) and antitoxin units measured by rabbit skin PF assay in 165 sera diluted 1:200 from 43 volunteers before and after ingestion of V. cholerae El Tor. The regression equation is* $y = 0.485 \log_{10} x + 0.39$, *and the correlation coefficient, r, is 0.83. (B) Relation between ELISA value (net OD) and antitoxin units in 53 sera diluted 1:800 with high-titer ELISA antibody (net OD > 0.9, from Fig. 4a). The regression equation is* $y = 0.259 \log_{10} x + 0.325$, *and the correlation coefficient, r, is 0.64.*

of at least 14 sera per 96-well microtiter plate and, therefore, is particularly well suited for assay of large numbers of specimens. Although the ELISA described in this paper calls for the use of a spectrophotometer, naked-eye readings could provide data suitable for epidemiological surveys. Thus, microtiter ELISA could be undertaken in field laboratories.

An additional advantage of our ELISA method is the ability to quantitate antitoxin by testing serum at a single dilution. As a consequence, requirements for reagents, plates, and technicians' time are minimized. Use of a single dilution of serum, however, precludes reliable quantification of antitoxin when present in high levels. Accordingly, sera that yield net OD values greater than 0.9, when tested at 1:200 dilu-

tion, should be reexamined at 1:800 dilution.

The antitoxin assayed in ELISA was IgG because animal studies have shown that cholera toxin-neutralizing activity in serum is virtually confined to this immunoglobulin class (15); although IgM antibodies bind toxin, they do not neutralize its biological activity (15). Therefore, the demonstration of a close correlation between antitoxin levels measured by ELISA and those determined by rabbit skin PF was not unexpected. ELISA cannot reveal the in vivo neutralizing capacity of a serum. Nevertheless, since neutralizing activity is associated largely with IgG, ELISA provides an excellent overall in vitro correlate of toxin-neutralizing activity, even though an occasional serum may have binding antibody of high avidity lacking potent neutralizing activity.

Assays that measure binding or neutralizing antibody to cholera toxin also detect cross-reacting antibodies to *Escherichia coli* heat-labile enterotoxin (8, 11, 18), which is immunologically related to cholera enterotoxin (12). The ELISA method described herein shares this property. Thus, persons challenged with *E. coli* that produce heat-labile enterotoxin had significant antitoxin rises by ELISA with cholera toxin as the test antigen (Levine et al., unpublished data).

In summary, the ELISA method for measurement of cholera antitoxin has distinct advantages over previous assays. As ELISA methodology in general becomes more widespread, laboratories currently measuring cholera antitoxin by more cumbersome or less sensitive methods should find it practical to switch to ELISA.

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