Enzyme-Linked Immunosorbent Assay to Measure Antibodies to Purified Heat-Labile Enterotoxins from Human and Porcine Strains of *Escherichia coli* and to Cholera Toxin: Application in Serodiagnosis and Seroepidemiology

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Serum immunoglobulin G antibodies to purified heat-labile enterotoxin (LT) from human (LTh) and porcine (LTp) *Escherichia coli* strains and cholera enterotoxin (CT) were measured by an enzyme-linked immunosorbent assay. Sera from patients with LTh *E. coli* infection showed a prominent response with LTh, an intermediate response with LTp, and a meager response with CT. Of 47 persons with clinical LTh-producing *E. coli* (herein shortened to LTh *E. coli*) infections, significant rises in antitoxin were detected against LTh in 36 (77%), against LTp in 30 (64%), and against CT in only 13 (28%) patients; seroconversions also occurred in 11 of 14 (79%) patients with subclinical LTh *E. coli* infections. In North Americans with experimental LTh *E. coli* infection, anti-LTh did not remain at high levels for more than 3 months. Persons with cholera manifested antitoxin responses that were similarly potent against all three toxin antigens; in fact, net optical density values were often slightly higher against LTh than against CT. The ratio of CT/LTh ELISA net optical density in convalescent sera proved to be a sensitive means to differentiate LT *E. coli* from cholera infection. All 11 cholera patients tested had CT/LTh ratios of >0.70 , whereas in only 1 of 47 LTh *E. coli* infections did the ratio exceed that value (it was 0.71) ($P < 0.0000000001$). In single serum specimens, a net optical density of ≥ 0.30 against LTh was shown to be a useful cutoff in screening sera for recent LTh *E. coli* or past cholera infection. The CT/LTh ratio was then used to differentiate definitively. Sera from healthy 3- to 5-year-olds and 15- to 19-year-olds in Maryland, Chile, and Bangladesh were tested against LTh and CT. The serological results fit known epidemiological observations. (i) LTh infections are rare in the United States (only 2 of 60 sera had LTh net optical density values of ≥ 0.30). (ii) In contrast, evidence of recent LTh *E. coli* infections was very common in Chilean (69%) and Bangladeshi (57%) 3- to 5-year-olds and not uncommon in 15- to 19-year-olds (38 and 31%, respectively) in those countries. (iii) Only Bangladeshi sera showed serological evidence of cholera infections (CT/LTh ratios of >0.70). The immunoglobulin G enzyme-linked immunosorbent assay measuring antibodies to purified LTh and CT represents a practical and effective tool for the serological study of LTh *E. coli* and cholera diarrheal infections.

Approximately one-half of the enterotoxigenic *Escherichia coli* (ETEC) strains that cause diarrhea in humans produce heat-labile enterotoxin (LTh), alone or in conjunction with heat-stable enterotoxin (ST). LTh is quite immunogenic and stimulates the appearance of neutralizing antitoxin in sera of convalescent patients (6, 7, 10, 20, 27); in contrast, anti-ST is not detectable after natural infection. Heretofore, most LTh serology has involved cumbersome biological assays (1, 6, 10, 16, 20, 25-28, 32). Consequently, there has been much interest over the years in developing more practical assays for LTh antitoxin for purposes of serodiagnosis, large seroepidemiological surveys, and the study of immune response after infection and immunization. It has long been known that LTh is immunologically related to cholera enterotoxin (CT) (3, 4, 9, 11, 13, 14, 22, 30). More recently it has been shown that heat-labile enterotoxins (LT) from human (LTh) and from porcine (LTp) ETEC pathogens

are antigenically related, but distinct, molecules (8, 15, 22, 30).

Previously, we described a microtiter enzyme-linked immunosorbent assay (ELISA) for measurement of serum immunoglobulin G (IgG) antibody to purified CT (24, 33). This assay, which is simple to perform, highly sensitive, and repeatable, correlates closely with neutralizing antitoxin as measured by more cumbersome biological assays. The IgG ELISA was found to be particularly suitable for seroepidemiological studies of cholera (21). Svennerholm et al. (29) utilized crude LTh and purified CT in a GM₁ ganglioside ELISA to measure anti-LTh and anti-CT and compared responses in patients with cholera and LTh ETEC infection. These investigators were able to show differences in the antitoxin response between persons infected with cholera and LTh-producing *E. coli* (herein shortened to LTh *E. coli*).

Recently described methods to purify LTh and LTp have made the development of ELISA serologies with these purified reagents feasible (4, 8). In this report we compare the serological antitoxin response, as measured by ELISA against purified LTh, LTp, and CT antigens, in persons with either cholera or ETEC infection. Criteria were developed to

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TABLE 1. IgG ELISA antitoxin response to LTh, LTp and CT antigens in sera from 12 volunteers with diarrhea due to LTh *E. coli*

Volunteer	Serum sample ^a	LTh ^b	LTh ^c	LTp ^b	LTp ^c
4006-2	Pre	0.08	0.06	0.05	0.04
	+10	0.56	0.53	0.06	0.12
	+28	0.38	0.33	0.04	0.06
4006-4	Pre	0.00	0.00	0.00	0.04
	+10	0.00	0.01	0.00	0.01
	+28	0.12	0.09	0.04	0.00
4006-16	Pre	0.10	0.12	0.08	0.08
	+10	0.23	0.16	0.10	0.11
	+28	0.26	0.25	0.10	0.13
4006-21	Pre	0.05	0.06	0.03	0.03
	+10	0.77	0.79	0.29	0.46
	+28	0.63	0.69	0.24	0.38
4006-22	Pre	0.09	0.07	0.03	0.04
	+10	0.39	0.32	0.16	0.18
	+28	0.55	0.59	0.37	0.39
4006-23	Pre	0.09	0.10	0.05	0.08
	+10	0.68	0.71	0.51	0.59
	+28	0.61	0.55	0.44	0.51
4009-2	Pre	0.10	0.08	0.08	0.07
	+10	0.82	0.76	0.21	0.36
	+28	0.90	0.83	0.17	0.34
4009-14	Pre	0.09	0.08	0.01	0.04
	+10	0.10	0.08	0.00	0.04
4009-6	Pre	0.09	0.10	0.05	0.11
	+10	0.31	0.30	0.08	0.20
	+28	0.18	0.22	0.04	0.14
2025-2	Pre	0.09	0.08	0.06	0.07
	+10	0.31	0.25	0.08	0.14
	+28	0.58	0.51	0.24	0.31
2025-5	Pre	0.20	0.21	0.02	0.14
	+10	0.36	0.36	0.03	0.22
	+28	0.64	0.54	0.19	0.37
2025-3	Pre	0.04	0.04	0.00	0.00
	+10	0.02	0.02	0.00	0.00
	+28	0.55	0.63	0.50	0.50

^a Pre, prechallenge sample; +10 and +28, samples taken on days 10 and 28, respectively, after challenge.

^b LT antigens were prepared in the University of Missouri laboratory.

^c LT antigens were produced in the Osaka University laboratory.

apply these serological assays to the serodiagnosis of recent and past LTh infection and to differentiate past cholera from LTh *E. coli* infection.

MATERIALS AND METHODS

Antigens. LTh and LTp were purified to homogeneity as previously described (4, 8, 15, 31). CT purified to the same extent was purchased from Schwarz/Mann, Cambridge, Mass.

Sera. Pre- and postchallenge serum specimens were available from volunteers who participated as controls in experimental challenge studies involving *Vibrio cholerae* and LTh *E. coli* (18). Sera were also obtained before and after challenge from 29 volunteers who developed diarrhea due to *E. coli* that do not elaborate LTh, including strains that produce only ST or classical serotype enteropathogenic *E. coli* (that elaborate neither LTh nor ST) (17, 19); these sera comprised a negative control population.

Single serum specimens were available from groups of approximately 20 to 30 healthy 3 to 5 year olds and 15 to 19 year olds in Maryland, Chile, and Bangladesh.

ELISA. Goat anti-human IgG antiserum conjugated to alkaline phosphatase was obtained from Kirkegaard Co.,

Gaithersburg, Md. The specificity of the conjugate was verified by testing against purified IgG, IgM, and IgA in an ELISA as previously described (33). The working dilution of the conjugate was determined in a cholera IgG ELISA with known positive and negative sera.

Checkerboard titrations of positive and negative sera were carried out to determine the optimal concentrations of antigen and to select a single dilution of serum that could be used in the assay. Concentrations of LTh, LTp, and CT tested included 0.1, 1.0, 2.0, 5.0, and 10.0 µg/ml. Serum was serially diluted twofold (1:25 to 1:800) in washing buffer as previously described (33).

Once it was determined that toxin concentrations of 1.0 µg/ml and serum dilutions of 1:50 gave optimal results, all further assays utilized those conditions. Wells of polystyrene microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 µl of purified CT, LTh, or LTp (1.0 µg/ml) in carbonate buffer (pH 9.6) and incubated for 1 h at 37°C. Each well coated with antigen had a corresponding blank well coated with buffer, but no antigen.

After washing three times (3 min each) with washing buffer, 100 µl of 5% heat-inactivated fetal calf serum diluted in phosphate-buffered saline (pH 7.2) was added for 1 h at 37°C. The assay was completed as previously reported (33). Net optical density (OD) refers to the resultant value when the OD of the blank well without antigen is subtracted from its corresponding well with antigen (other than the initial antigen coating step, the paired wells were otherwise identically treated). Net OD is a direct measure of the concentration of specific antitoxin (21, 24, 33).

GM₁ ELISA. Antitoxin to LTh and CT in sera from 10 patients with *E. coli* infection and 10 patients with cholera was also measured by the GM₁ ganglioside technique of Svennerholm et al. (29) with a filtered supernatant from LT *E. coli* 408-3 as a source of crude LTh. GM₁, a receptor for both LTh and CT (12), was obtained from Supelco, Bellefonte, Pa. After coating of the microtiter plates with GM₁, either crude LTh (0.1 mg/ml), purified LTh (0.1 µg/ml), or purified CT was added, after which the ELISA was completed (29).

Y-1 adrenal cell neutralization. Selected sera were assayed for the presence of neutralizing antitoxin by the Y-1 adrenal cell method as previously described (20).

RESULTS

Comparability of two LTh and two LTp antigens. Pre- and postchallenge sera from 12 volunteers experimentally infected with LTh *E. coli* were tested against LTh and LTp purified in two different laboratories (Missouri and Osaka) as well as against CT. The two LTh and two LTp preparations gave essentially identical results (Table 1). Because of the virtual equality of the purified LT preparations from the two laboratories, in all further work we used the Missouri LTh and the Osaka LTp as antigens.

Determination of the value denoting a significant rise in antitoxin. Sera obtained before and 10, 21, and 28 days after challenge from 29 persons who developed diarrhea due to *E. coli* that do not produce LTh, i.e., from persons representing a negative control population, were tested against LTh, LTp, and CT to determine the inherent variability of the ELISA. Against LTh, the mean difference in net OD between the prechallenge and any of the postchallenge specimens for the 29 individuals in the negative control population was 0.06 with a standard deviation (SD) of ±0.03. For LTp the mean difference in net OD between the specimens was 0.04 ± 0.03, whereas against CT the mean ± SD was

0.04 ± 0.04 . Based on these measurements denoting minimal innate variability in the assay, a rise in net OD of 0.20 or greater of a postchallenge specimen over the prechallenge baseline was considered significant. For each antigen this represents a value equal to or surpassing the mean plus 4 SD. No persons in the negative control population had rises in net OD ≥ 0.20 against any of the antigens.

Positive control population. Pre- and postchallenge sera from 47 volunteers with diarrhea due to LTh *E. coli* were run against LTh, LTp, and CT (Table 2). Significant rises (postchallenge net OD of 0.20 or more) were detected in 77% of persons against LTh, but in only 64 and 28% when LTp and CT, respectively, were used as antigens.

The kinetics and magnitude of the antitoxic response of this group of 47 persons with LTh *E. coli* disease are shown in Fig. 1. Although the mean net OD of the group increased by day +10, the peak was not reached until day +21; the day +28 value was similar to that on day +21.

Pre- and postchallenge sera were also tested from 14 volunteers who had positive coprocultures, but who did not develop diarrhea after challenge with LTh-producing *E. coli* (Table 2). Significant rises in serum IgG ELISA antitoxin were detected in 11 (79%) with LTh, 9 (64%) with LTp, and 2 (14%) with CT as antigen. The magnitude of the antibody response to LTh in these 14 persons with subclinical infection (mean net OD on day +28 of 0.53) was similar to that of the group with clinically apparent infection (mean net OD on day +28 of 0.56).

For each of the 36 persons with clinical ETEC infection who manifested a significant rise in antitoxin to LTh, as well as for the 11 persons with subclinical infection who exhibited significant rises, the CT/LTh ratio was computed. This ratio represents the net OD of the serum specimen tested against CT over the net OD of the specimen tested against LTh. Among the 36 clinical infections associated with significant LTh seroconversions, in only one instance was the CT/LTh ratio greater than 0.70 for the peak postchallenge serum specimen (0.71). In only four instances was the CT/LTh ratio greater than 0.60, and in only nine persons was the CT/LTh ratio greater than 0.50. Similarly, among the 11 persons with subclinical infection who manifested significant rises in IgG ELISA antitoxin, none had CT/LTh ratios greater than 0.50 in the postchallenge sera.

Cholera patients. Pre- and postchallenge sera from nine volunteers who developed clinical cholera while serving as controls in vaccine efficacy studies were tested against all three toxin antigens. Significant rises in antitoxin were shown by all nine persons against all three antigens. Surprisingly, in only two persons was the rise greater against CT than against LTh or LTp; in the other seven persons a slightly greater net OD was recorded against LTh than against CT. The magnitude of the antitoxin response of these nine cholera patients against the three antigens is compared in Table 3. Although the net OD was greater against LTh

FIG. 1. IgG antitoxin measured by ELISA against purified LTh, LTp, and CT in serum from 47 patients with diarrhea due to LTh *E. coli*.

than against CT in seven of these nine persons with cholera diarrhea, the absolute differences were small. Thus, the CT/LTh ratio exceeded 0.70 in all instances and, in fact, surpassed 0.85 in 9 of 11 persons. This provides a clear-cut contrast to the results observed in persons with LTh *E. coli* infection (in whom the CT/LTh ratio exceeded 0.70 in only 1 of 47 infections). This difference, 11 of 11 versus 1 of 47, is highly significant ($P < 0.000000001$).

"Significant" and "diagnostic" levels of antitoxin. For use in seroepidemiological surveys when only a single serum specimen is available for testing, it was necessary to identify a minimal net OD value denoting the presence of LTh antitoxin. To accomplish this, sera were tested from a presumed negative control population consisting of 10 young United States (U.S.) children (3 to 5 years of age). These 10 sera were negative when tested for neutralizing LTh antitoxin in the Y-1 adrenal cell neutralization assay; (one should also note that diarrhea due to LTh *E. coli* in young children is rare in the U.S.). The net OD of one child's serum was 0.11; the rest were <0.05 . A pool was made of equal amounts of these 10 sera to give rise to a pooled negative control serum standard for use in seroepidemiological studies. This pooled negative control was tested against LTh on 10 separate occasions and gave net OD values of 0.00 to 0.06 (mean $\pm$ SD, 0.03 ± 0.02). Thus a net OD of ≥ 0.10 represents a value surpassing the mean plus 3 SD of 10 runs of the pooled negative control serum. Therefore, in seroepidemiological studies any serum yielding a net OD of ≥ 0.10 was considered to contain LTh antitoxin.

Experience with serological studies in cholera had shown that a more stringent criterion was necessary for serodiagnosis of recent clinical infection, as opposed to just demonstrating evidence of prior antigenic contact based on

TABLE 2. Rises in serum IgG-ELISA antitoxin against LTh, LTp, and CT antigens in pre- and postchallenge sera from 47 persons with diarrhea and 14 persons with subclinical infection due to LT *E. coli*^a

Group	LTh	LTp	CT
Clinical infections	36/47 (77)	30/47 (64)	13/47 (28)
Subclinical infections	11/14 (79)	9/14 (64)	2/14 (14)

^a Data are presented as the number with significant rises/number of persons tested. Percentages are given within parentheses.

TABLE 3. Magnitude and kinetics of the IgG ELISA antitoxin response to CT, LTh and LTp in pre- and postchallenge sera from nine volunteers with diarrhea due to *V. cholerae*^a

Antigen	Mean net OD $\pm$ SEM on day postchallenge:		
	0	21	28
CT	0.04 ± 0.01	0.71 ± 0.09	0.77 ± 0.08
LTp	0.08 ± 0.03	0.82 ± 0.07	0.83 ± 0.08
LTh	0.11 ± 0.03	0.94 ± 0.05	0.94 ± 0.06

^a The mean CT/LTh ratio was 0.76 on day 21 and 0.87 on day 28.

TABLE 4. Duration of the IgG ELISA antitoxin response to LTh in nine persons with diarrhea due to LT *E. coli*^a

Person	Net OD prechallenge	Net OD on month postchallenge:						
		1	6	12	18	24	30	36
1	0.13 ^a	0.31	0.33					
2	0.06	0.51	0.07	0.08	0.04			
3	0.15	0.58		0.33	0.32	0.29	0.34	
4	0.13	0.56	0.24	0.22				
5	0.14	0.58			0.18	0.17		0.13
6	0.01	0.52		0.13	0.09	0.30		
7	0.07	0.68		0.15				
8	0.19	0.38	0.20	0.06	0.16			
9	0.08	0.45		0.12	0.17	0.20		

^a Blank space indicate that no specimen was available.

the presence of a detectable (i.e., significant) level of antitoxin (21). For cholera, a positive/negative (P/N) ratio of 4.0 was used for serodiagnosis; therein, only when the net OD of the test serum exceeded that of the negative control serum by at least four fold was the test serum considered positive (21). For our studies with *E. coli* LTh antitoxin we arbitrarily selected a net OD of ≥ 0.30 as evidence of recent infection with LTh *E. coli*. This number was derived by adding the lowest net OD denoting the presence of antibody (≥ 0.10) to the minimal rise in net OD between two serum samples selected as being significant (0.20).

Sera were available from nine volunteers who were challenged with LTh *E. coli* 6 to 30 months earlier. Analysis of these sera provided insight into the longevity of IgG ELISA LTh antitoxin (Table 4). In prechallenge sera, five of the nine persons had evidence of prior antigenic contact with LTh or related antigens, i.e., five of nine had a net OD of ≥ 0.10 . None had a prechallenge net OD of ≥ 0.30 . One month postchallenge all nine had net OD of > 0.30 (mean, 0.51). Of four persons tested at 6 months postchallenge, only one still had a net OD of ≥ 0.30 . Similarly, by 12 months postchallenge the serum of only one of eight persons tested retained a net OD of ≥ 0.30 in the ELISA. Two persons even had net OD of ≤ 0.10 . These data demonstrate that, in contrast with the elevated levels of antitoxin stimulated by cholera infection, which persist for at least 24 months (21), LTh antitoxin responses in healthy U.S. adults measured by ELISA with purified LTh appear to be more short lived.

Additional data support the usefulness of a net OD of ≥ 0.30 against LTh as evidence for a recent infection with LTh *E. coli*. Only 1 of the 47 healthy U.S. adults who developed diarrhea after challenge with LTh *E. coli* manifested a serum IgG ELISA LTh net OD of ≥ 0.30 in their prechallenge serum, whereas all 36 who exhibited significant rises surpassed this value in their postchallenge specimens.

Similarly, 1 of 14 persons who experienced subclinical infection with LTh *E. coli* had a prechallenge IgG ELISA LTh antitoxin net OD of ≥ 0.30 . Ten of the 11 persons in this group who exhibited significant LTh antitoxin rises had net OD values of ≥ 0.30 in their postchallenge specimens.

Prevalence of antitoxin by age and geographic area. Serum specimens were collected from healthy young children (3 to 5 years of age) and teenagers (15 to 19 years) in Bangladesh (where both cholera and ETEC infections are endemic), Chile (where ETEC, but not cholera infections, are endemic), and Maryland (where neither ETEC nor cholera infections are endemic). These sera were tested against LTh and CT antigens in the IgG ELISA.

In U.S. children 3 to 5 years of age only 7% had IgG ELISA LTh net OD of ≥ 0.10 , and only one (3.3%) had a net OD of ≥ 0.30 (Table 5). By 15 to 19 years of age 33% of the Marylanders had measurable LTh antitoxin levels, but only one (3%) had a net OD of ≥ 0.30 .

In contrast, all of the Chilean 3 to 5 year olds had IgG ELISA LTh net OD of ≥ 0.10 , and 69% had LTh net OD of ≥ 0.30 , implying recent infection. The prevalence of high titers of LTh antitoxin diminished in 15- to 19-year-old Chileans of whom only 31% had LTh net OD of ≥ 0.30 .

Among Bangladeshi children, 76% had LTh net OD of ≥ 0.10 , and 57% of 3 to 5 year olds and 38% of 15 to 19 year olds had values of ≥ 0.30 . None of the U.S. or Chilean children with LTh net OD of ≥ 0.30 had CT/LTh ratios of ≥ 0.70 ; in contrast, many of the Bengali children did (Table 5), providing clear-cut seroepidemiological evidence of cholera infection in these children from a cholera-endemic area.

GM₁ ELISA with crude LTh, purified LTh, and CT. The finding that cholera patients often had IgG ELISA net OD values as high or slightly higher against purified LTh as against CT was in disagreement with the observations of Svennerholm et al. (29), who used crude LTh in a GM₁ ELISA. We therefore carried out a GM₁ ELISA of sera from 10 cholera patients with crude LTh and purified LTh and CT as the antigens and using the method of Svennerholm et al. (Table 6). In this assay, cholera patients did show higher net OD against CT than against crude LTh, corroborating the observation of Svennerholm et al. (29). The net OD values of the patients' sera were much lower in the GM₁ ELISA with crude LTh, achieving a mean net OD only one-half as high as when purified LTh was utilized as the antigen. However, the net OD of these sera tested in GM₁ plates sensitized with purified LTh (at the same concentration as CT) showed slightly higher values against LTh than against CT; the CT/LTh ratio always exceeded 0.70. Thus, when purified reagents are used, the results are the same whether the antigens are coated directly onto the solid phase or onto the GM₁ receptor.

TABLE 5. Prevalence and magnitude of IgG ELISA antitoxin to LTh and CT antigen in young children and teenagers in the U.S., Chile, and Bangladesh

Geographic area	Age group (yrs)	n	Mean net OD $\pm$ SEM		% with LTh net OD of:		% with CT/LTh ratio of $> 0.70^a$
			LTh	CT	≥ 0.10	≥ 0.30	
Maryland, U.S.	3 to 5	30	0.04 $\pm$ 0.04	0.01 $\pm$ 0.01	7	3.3	0
Maryland, U.S.	15 to 19	30	0.11 $\pm$ 0.02	0.04 $\pm$ 0.01	33	1	0
Chile	3 to 5	32	0.47 $\pm$ 0.04	0.14 $\pm$ 0.02	100	69	0
Chile	15 to 19	29	0.26 $\pm$ 0.03	0.09 $\pm$ 0.01	97	31	0
Bangladesh	3 to 5	21	0.44 $\pm$ 0.07	0.09 $\pm$ 0.06	76	57	45
Bangladesh	15 to 19	21	0.30 to 0.06	0.22 $\pm$ 0.06	76	38	19

^a Refers only to persons with IgG ELISA net OD to LTh of ≥ 0.30 .

TABLE 6. Serum antitoxin responses against purified LTh and CHT and against crude LTh in pre- and postchallenge sera from nine volunteers with diarrhea due to *V. cholerae* measured by the GM₁ ELISA method of Svennerholm et al. (29)

Volunteer	Net OD of purified LTh (0.1 µg/ml) on day:			Net OD of purified CT (0.1 µg/ml) on day:			Net OD of crude LTh (0.1 mg/ml) on day:		
	0	21	28	0	21	28	0	21	28
5011-1	0.11	0.72	0.72	0.02	0.56	0.55	0.05	0.23	0.24
5011-2	0.12	0.69	0.67	0.03	0.62	0.61	0.09	0.22	0.17
5011-10	0.07	0.67	0.68	0.03	0.65	0.68	0.01	0.19	0.20
5011-22	0.06	0.52	0.45	0.01	0.43	0.39	0.00	0.03	0.04
6001-2	0.06	0.54	0.50	0.05	0.65	0.66	0.03	0.22	0.04
6001-7	0.07	0.50	0.46	0.05	0.45	0.40	0.04	0.10	0.13
6001-11	0.07	0.59	0.54	0.02	0.41	0.38	0.02	0.20	0.16
6001-13	0.02	0.50	0.46	0.01	0.48	0.41	0.04	0.05	0.05
6001-14	0.06	0.47	0.37	0.02	0.39	0.40	0.04	0.16	0.18

DISCUSSION

ETEC are a major cause of diarrhea in infants in the developing world as well as the most important agent of diarrhea in persons from industrialized countries who visit less-developed areas; approximately one-half the isolated strains possess LTh (2, 23). We sought to develop an ELISA to measure anti-LTh as an improved means of studying the immune response in LTh infections, for serodiagnosis of recent infections, and for seroepidemiological studies. Since LTs from porcine ETEC and CT are immunologically related to LTh (3, 4, 8, 9, 11, 13–15, 22, 30), and LTh infections are highly prevalent almost everywhere that cholera is endemic, it was imperative to determine whether the specific antitoxin responses could be differentiated. The preliminary work of Svennerholm et al. (29) with purified CT and crude LTh in a GM₁ ELISA suggested that such differentiation was indeed possible. The observations in this report, utilizing purified LTh, LTp, and CT, show a differential response to the various antigens and provide criteria for the use of the antitoxin assays in serodiagnosis and seroepidemiology.

In persons with LTh *E. coli* infections there was a gradient of response depending on the antigen. The rate and magnitude of antitoxin seroconversion was greatest against LTh, intermediate against LTp, and least against CT (Tables 1 and 2, Fig. 1). In fact, only 15 of 61 persons (25%) with LTh *E. coli* infections had significant rises when tested against CT versus 47 of 61 (77%) when tested against LTh ($P < 0.002$). These observations would be expected since LTh has unique antigens as well as common epitopes that are shared with LTp and CT.

Persons with cholera manifested a strikingly different serological pattern, showing potent rises against LTh and LTp as well as CT. Indeed, the magnitude of the mean net OD recorded against LTh was slightly higher than against CT (Table 3). This may imply that *Vibrio cholerae* infection is a more potent stimulus than LT *E. coli* of antibody to the common antigenic determinants shared by LTh, LTp, and CT (22). It is of interest that Svennerholm et al. (29), using crude LTh and purified CT in a GM₁ ELISA, found that OD readings were higher in sera of cholera patients when tested against CT rather than crude LTh. When we ran our sera in the GM₁ ELISA with crude LTh and purified CT, we corroborated the observations of Svennerholm et al. The explanation for this difference between the two techniques is apparently due to less LTh antigen in the crude LTh, since the levels of antitoxin recorded with crude LTh in the GM₁ assay were one-half those obtained with purified LTh. Sera from cholera patients usually gave slightly higher net OD values against purified LTh than against CT whether the antigens were bound directly to the polystyrene or to GM₁.

Although many persons with cholera had slightly higher net OD values against LTh than CT, the net OD values were always quite close. As a consequence, this provided a source of differentiation from LTh *E. coli* infection where the anti-CT response was very meager in comparison with the anti-LTh response. Thus the ratio of the CT/LTh net OD in all 11 persons with cholera was always 0.70 or greater, whereas in only 1 of 47 persons (2%) with LTh *E. coli* infection did the ratio exceed that value (it was 0.71 in that one person) ($P < 0.000000001$).

The rapid fall of the LTh antitoxin levels after 3 months in persons with LTh *E. coli* infection contrasts to the persistence of high-titer anti-CT in North Americans convalescing from cholera (21). Nevertheless, it was possible to select a level of LTh antitoxin (net OD of ≥ 0.30) that was diagnostic of recent (i.e., within the previous 3 months) LTh *E. coli* infection.

Perhaps the most exciting findings followed the application of this IgG ELISA to sera of children and teenagers from Maryland, Chile, and Bangladesh. Only 3% of the 60 U.S. children or teenagers had evidence of recent LTh *E. coli* infection. In contrast, the majority of 3 to 5 year olds in Chile and Bangladesh had serological evidence of recent LTh *E. coli* infection. These data fit well with results of the prospective studies of Black et al. (2) of infants and young children in Bangladesh. These workers showed that in the first few years of life young children in Bangladesh suffer on the average two to three episodes of ETEC diarrhea per child per year; approximately one-half of the *E. coli* strains produce LT. The seroepidemiological studies also showed that the rate of recent infections was one-half or one-third as high in the 15 to 19 year olds as in the 3 to 5 year olds. This agrees with serosurveys of cholera antitoxin in Bangladesh that show a lower prevalence and mean level of antitoxin in older children and adults compared with young children (5). Finally, only in sera from Bangladesh, a cholera-endemic area, did we encounter CT/LTh ratios above 0.70, indicative of cholera infections. Previous investigators have vividly described the limitations of attempting serodiagnosis and seroepidemiological studies with neutralization assays to measure LT and have expressed the need for improved serological assays that use purified reagents (32). The IgG ELISA for LTh and CT that we have developed is a notable improvement in the measurement of LTh for purposes of serodiagnosis, seroepidemiology, and immune response.

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