Properties of Crude Campylobacter jejuni Heat-Labile Enterotoxin

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The amount of crude Campylobacter jejuni enterotoxin present in culture products was quantitated by comparing the response of these preparations with that of pure Escherichia coli heat-labile toxin (LT) in the Chinese hamster ovary assay and in enzyme-linked immunosorbent assays that used GM ganglioside or antisera to LT or both. Maximum C. jejuni enterotoxin production was achieved by growth at 42°C for 24 h under agitation in supplemented GC medium. Adding polymyxin separately to either the broth supernatant or the cells enhanced the recovery of toxin; the yield from cell lysates was much lower. The quantity of C. jejuni enterotoxin produced by clinical isolates obtained locally or provided from Mexico varied widely, over a spectrum from none to large amounts; quantitative values for the amount of C. jejuni enterotoxin determined by the Chinese hamster ovary and enzyme-linked immunosorbent assays correlated with the degree of secretory potency of this material in ligated rat ileal loops. The cytotoxic activity of C. jejuni enterotoxin in Chinese hamster ovary cells was abolished by heating at 96°C for 10 min and by preincubation either with GM ganglioside or with LT or cholera toxin antisera. The secretory activity of C. jejuni enterotoxin in ligated rat ileal loops was passively neutralized by antisera to LT, and immunizing rats with either LT or its B subunit significantly (P < 0.001) reduced fluid response to active challenge with C. jejuni enterotoxin in ligated ileal loops. These observations indicate that strains of C. jejuni vary in their capacity to elaborate a heat-labile enterotoxin that has close immunological homology with LT and cholera toxin.

Campylobacter jejuni is now recognized as a major worldwide cause of acute diarrhea among persons living in either temperate or tropical climates (2, 4, 15, 48) as well as among travelers (38, 42, 47). Enteric infection with this organism is particularly prevalent in children (6, 35), among whom it appears to rank as the third most common cause of acute diarrhea, after rotavirus and enterotoxigenic Escherichia coli (1, 43), in developing countries (3, 5, 13, 18). A significant proportion of children living in these areas are also asymptomatic carriers of C. jejuni (3, 5, 18, 39).

Little is known about the pathophysiology of C. jejuni enteric infection. The organism can clearly be invasive, as shown by either overt colitis or the presence of blood and leukocytes in the stools of infected persons (2, 15, 30) and by studies on experimentally contaminated animals (7). However, there is often only a secretory form of diarrhea, particularly in children (2, 18, 35, 42, 48), suggesting that a different mechanism, such as an enterotoxin, may be involved. Enterotoxin production has not been detected by laboratories testing routinely grown C. jejuni cultures for toxin by the Chinese hamster ovary (CHO), Y1 adrenal cell tissue culture, and suckling mouse assays or by distillation into ligated intestinal loops of rabbits, calves, or piglets (21, 33, 49). In contrast, Ruiz-Palacios and his colleagues in Mexico, using special culture conditions, have recently shown that many C. jejuni clinical isolates from children produce a heat-labile enterotoxin that raises intracellular cyclic AMP levels, causes cytotoxic changes in CHO cells, and induces fluid secretion in ligated rat ileal loops; the secretory activity of this toxin is neutralized by antisera to cholera toxin (CT) (41). Culture supernatants of these toxigenic strains have also been shown to cause intraluminal secretion of fluid and electrolytes when perfused in vivo through rat jejunal segments (16).

In the present study, we quantitated the amount of C. jejuni enterotoxin (CJT) present in various growth products by comparing the response of these preparations with that of pure E. coli heat-labile enterotoxin (LT) in enzyme-linked immunosorbent assays (ELISAs) that used antiserum to LT and in the CHO tissue culture assay. This permitted us to identify the culture media, growth conditions, and procedures for processing growth products that gave maximum CJT production and recovery. The amount of CJT produced by strains of C. jejuni isolated from persons with acute diarrhea, both local Rochester, N.Y., residents and Mexicans, was determined quantitatively by the in vitro tests, and these results were compared with those for secretory activity in ligated rat ileal loops. The relationship between crude CJT and pure LT was further evaluated by determining the neutralizing capacity of antisera to LT on the cytotoxic activity of CJT in the CHO assay and on its secretory effect in ligated rat ileal loops and by determining the protective effect of immunizing rats with either LT or its B subunit against active challenge with CJT in ligated ileal loops.

MATERIALS AND METHODS

Bacterial strains. C. jejuni strains C1 to C6 were clinical isolates obtained consecutively from persons with acute diarrhea in Rochester; strains INN-1-79 and INN-73-83 were cultured from Mexican children with diarrhea and kindly provided by G. M. Ruiz-Palacios, Instituto Nacional de la Nutrición, Tlalpan, Mexico. Some of the enterotoxigenic properties of strain INN-1-79 have been described previously (41). As a routine precaution, multiple copies of each isolate were made and stored at −60°C in brucella broth (Difco Laboratories, Detroit, Mich.) plus 15% glycerol upon receipt so that each isolate underwent no more than two passages before being tested.

Enterotoxin production. C. jejuni isolates were grown for 24 h on GC plates made from GC medium base (Difco) plus 0.1% IsoViteX (BBL Microbiology Systems, Cockeysville, Md.) at 42°C under reduced atmospheric conditions in
a GasPak system (BBL Microbiology Systems) without a catalyst. Two growth media were compared: brucella broth, which was used by Ruiz-Palacios et al. (41), and GC medium, which is a highly enriched medium designed for the growth of fastidious organisms. Growth from each plate was harvested in 5 ml of sterile saline, of which 4 ml/liter of liquid medium was used to inoculate either brucella broth supplemented with 0.25% L-asparagine, L-serine, and L-glutamic acid as described previously (41) or GC medium without the agar base but with 0.1% IsoVitalex. All liquid cultures were grown at 42°C in the presence of 8% CO₂.

All C. jejuni growth fractions were processed with equipment and membranes that had not previously been exposed to either LT or CT. After culture, the cells were centrifuged at 13,000 × g for 10 min at 4°C, and the supernatants were sterilized by passage through a 0.22-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.) and, except where specified, concentrated 10-fold on a YM-10 ultrafiltration membrane (Amicon, Corp., Danvers, Mass.). For polymyxin-treated supernatants, 2 mg of polymyxin B sulfate per ml of medium was added to the whole growth culture, which was then incubated for an additional 10 min at 42°C under agitation; the supernatant was then obtained by centrifugation.

Cells from cultures that had not been exposed to polymyxin were also processed. After separation, the cells were either treated with polymyxin or lysed by sonication. Cells treated with polymyxin were suspended in 50 ml of 0.15 M Tris buffer plus 0.9% NaCl, pH 6.6, containing 2 mg of polymyxin B sulfate per ml and incubated at 42°C under agitation for 10 min; they were then removed by centrifugation, and the supernatant was filtered through a sterile membrane and concentrated to 5 ml on a YM-10 ultrafiltration membrane.

The LT holotoxin was purified in a form by previously described methods (9) from E. coli PDF82, a transformed strain K-12 derivative bearing the LT gene(s) of the Ent plasmid from the human E. coli strain H10407 (11). The LT toxin B subunit was purified by chromatographic techniques (12) from E. coli PDF87, a transformed strain K-12 derivative bearing the B subunit plasmid of the human E. coli strain H10407 (10). The amounts of LT and B subunit were used based on their protein concentration, determined by the method of Lowry et al. (32).

Enterotoxin assays. (i) CHO assay. The CHO assay was performed by a modification (8) of the techniques described by Guerrant et al. (20), except that 96-well microtiter plates (Costar, Inc., Cambridge, Mass.) were used. All samples were tested in duplicate with working volumes of 100 μl per well. Morphological alteration (elongation) of more than 50% of the cells in a well was considered a positive response.

(ii) ELISAs. The techniques for raising antiserum to LT in rabbits and goats and for performing double-sandwich ELISAs with these antisera have been described previously (27, 29). All the samples were tested in duplicate with working volumes of 200 μl per well. Because pure GM1 ganglioside was unavailable for these studies, GM ganglioside (type III; Sigma Chemical Co., St. Louis, Mo.) was used in excess. In the GM/LT ELISA 50 μg of GM ganglioside per ml was used for the solid phase, and in the LT/LT ELISA a 1:50 dilution of crude rabbit anti-LT antiserum was used for the solid phase; in both instances, a 1:500 dilution of crude goat anti-LT antiserum was the second antibody, followed by a 1:400 dilution of alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin G antiserum (Miles Laboratories, Inc., Elk hart, Ind.).

(iii) Ligated rat ileal loops. Toxin preparations in 250 μl of buffer were placed for 16 h into single 10-cm-long ligated ileal loops of fasting Sprague-Dawley rats weighing 175 to 200 g as described previously (28). The results reported for each data point are the mean ± the standard error of the mean for the volume/weight ratio (V/W) in four rats.

Immunization procedures. Rats were given primary immunization intraperitoneally with Freund complete adjuvant, followed by two boosters given perorally at 4-day intervals. Peroral immunization was given via intragastric tube 2 h after peroral administration of 50 mg of cimetidine (Tagamet; Smith Kline & French Laboratories, Carolina, P.R.) per kg of body weight to ablate gastric secretion. The dose of LT was 100 μg intraperitoneally for primary immunization and 500 μg for each peroral booster, and that of B subunit was 200 μg intraperitoneally for primary immunization and 1,000 μg for each peroral booster. These immunization dosages were selected because they have previously been shown to provide a significant (P < 0.001) reduction in fluid secretion in immunized rats compared with unimmunized controls challenged by instilling either LT or viable LT-producing E. coli cells into ligated ileal loops (26). Control and immunized rats were challenged simultaneously as described above 4 to 6 days after the final booster immunization by instilling CJT into the ligated ileal loops. The significance of the difference in secretion between the immunized and control groups was determined by Student's t test for two independent means.

RESULTS

Quantitation of CJT. To provide a uniform quantitative value for the amount of CJT produced by individual strains under different culture conditions and obtained by various recovery procedures, this value was expressed as the

![FIG. 1. Response to graded amounts of LT and concentrated broth supernatants of C. jejuni strains C3 and C4 in the GM/LT ELISA. Comparison of the values for LT and the C. jejuni supernatants which gave an optical density of 0.600 at 405 nm gave the amount of CJT.](http://iai.asm.org/Downloaded from http://iai.asm.org)
amount of toxin present per milligram of total protein that was comparable to the activity of LT as determined in the CHO and ELISA assays. The effect of graded concentrations of pure LT (in picograms) and C. jejuni growth products (in micrograms) was determined in each of these assays, and the amount of each material that gave an optical density of 0.600 at 405 nm in the ELISAs (Fig. 1) and in the last positive well in the CHO assay was established. The amount of CJT (i.e., LT activity equivalent) present in the C. jejuni product was calculated by dividing the quantity of LT which gave this result by that of the C. jejuni product which gave the same result in that specific assay; this figure was multiplied by 1,000 to express the amount of CJT in nanograms per milligram of total protein of the culture product.

**Toxin production and recovery.** To determine the optimal growth media and culture conditions, 1-liter batches of C. jejuni INN-73-83 were grown for various periods with or without agitation in supplemented GC medium or in amino acid-supplemented brucella broth. Growth was monitored by measuring the optical density of the whole cultures at 650 nm, and toxin production was measured by both GM/LT ELISA and CHO assays of polymyxin-treated culture supernatants. Bacterial growth was the same under stationary culture conditions, but toxin production was greater after 24 h in the GC medium (Fig. 2). Maximum growth and toxin production occurred after 24 h of growth under agitation in the GC medium.

Adding polymyxin to the whole broth culture for the final 10 min of incubation of a 24-h culture in GC medium grown under agitation approximately doubled the concentration of CJT per milligram of protein in the broth supernatant (Table 1). Precipitated cells from broth cultures (which had not been exposed to polymyxin) yielded approximately the same concentration of CJT as the polymyxin-treated broth supernatant did when the cells were resuspended and incubated with polymyxin for 10 min; in contrast, sonicated cells released only a small amount of toxin per milligram of protein. To exclude the possibility that the low concentration of toxin obtained from the sonicated cells was the result of an inadequate cell mass, 10 liters of growth was processed similarly; this did not increase the concentration of toxin. Although the toxin concentration per milligram of protein was approximately the same in both polymyxin-treated broth supernatant and cell preparations, the total yield of toxin, expressed as micrograms of toxin per liter of cells, was considerably greater from the supernatant because of its greater amount of total protein. When measured by CHO assay, 1 liter of cells yielded a total of 81.8 μg of toxin from the polymyxin-treated broth supernatant but only 4.1 μg of toxin from the polymyxin-resuspended cells.

As a result of these tests, the CJT used in subsequent studies was derived from broth supernatants of polymyxin-treated cultures grown for 24 h under agitation in supplemented GC medium.

**Toxin production by clinical isolates.** The amount of CJT produced, as determined by CHO and ELISA assays of 10-fold-concentrated broth supernatants, varied from none to large amounts (Table 2). Preparations shown by these assays to produce moderate or large quantities of toxin also caused fluid secretion in ligated rat ileal loops. In each instance, heating the toxin preparations at 96°C for 10 min abolished their activity in the CHO cell assay and their secretory effect in ligated rat ileal loops. No relationship was apparent between the amount of bacterial growth, as measured by the optical density at 650 nm, and toxin production for five strains, but the growth of one nontoxigenic strain was slight and that of the two strongest toxin producers was the greatest.

**Detection of enterotoxigenic strains of C. jejuni by ELISA.** To determine whether toxigenic strains of C. jejuni can be identified by ELISA of unconcentrated broth supernatants, these fractions of the eight clinical isolates were tested in both the LT/LT and GM/LT ELISAs. We have found in previous studies that unconcentrated broth supernatants of LT-producing enterotoxigenic E. coli strains always give an optical density of >0.200 in both of these ELISAs, whereas those of nontoxigenic strains consistently give values of <0.200 (29). Only the broth supernatants of these three strains

![FIG. 2. Effect of different culture media and growth conditions on growth (A) and toxin production (B) by C. jejuni INN-73-83. BB, Brucella broth. Values for toxin production were determined by the CHO assay.](http://iai.asm.org/)

<table>
<thead>
<tr>
<th>C. jejuni isolate</th>
<th>Bacterial growth (OD&lt;sub&gt;600&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CJT activity (ng/mg of protein) as determined by:</th>
<th>Secretion in ileal loops (mean V/I ± SEM)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>LT/LT</td>
<td>GM/LT</td>
<td>CHO</td>
</tr>
<tr>
<td>C1</td>
<td>0.136</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C6</td>
<td>0.312</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C5</td>
<td>0.326</td>
<td>0.02</td>
<td>0.1</td>
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<tr>
<td>C2</td>
<td>0.238</td>
<td>0.06</td>
<td>1.5</td>
</tr>
<tr>
<td>C3</td>
<td>0.309</td>
<td>0.10</td>
<td>5.1</td>
</tr>
<tr>
<td>C4</td>
<td>0.296</td>
<td>2.06</td>
<td>14.9</td>
</tr>
<tr>
<td>INN-179</td>
<td>0.483</td>
<td>3.05</td>
<td>11.4</td>
</tr>
<tr>
<td>INN-73-83</td>
<td>0.467</td>
<td>3.87</td>
<td>50.6</td>
</tr>
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<sup>a</sup> OD<sub>600</sub>, Optical density of the broth cultures at 650 nm.
<sup>b</sup> V/I, Volume/length ratio of challenged rat ligated ileal loops.

<table>
<thead>
<tr>
<th>TABLE 2. CJT production by clinical isolates</th>
<th>LT/LT</th>
<th>GM/LT</th>
<th>CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0.136</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C6</td>
<td>0.312</td>
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<td>0</td>
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<td>INN-73-83</td>
<td>0.467</td>
<td>3.87</td>
<td>50.6</td>
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</tbody>
</table>

TABLE 1. Recovery of CJT from different growth fractions<sup>a</sup>

<table>
<thead>
<tr>
<th>Assay</th>
<th>Supernatant</th>
<th>Supernatant with FM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sonicated cells</th>
<th>Cells with PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>14.6</td>
<td>29.1</td>
<td>0.9</td>
<td>38.6</td>
</tr>
<tr>
<td>GM/LT ELISA</td>
<td>20.0</td>
<td>50.6</td>
<td>2.5</td>
<td>47.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Obtained from C. jejuni INN-73-83 grown under agitated conditions for 24 h in supplemented GC medium.
<sup>b</sup> PM, Polymyxin.
that produced the most CJT gave a positive response (i.e., an optical density ≥0.200) in the LT/LT ELISA, whereas the GM/LT ELISA distinguished between CJT-producing strains and those which produced either no or a negligible quantity of toxin (Fig. 3).

Properties of CJT. The crude CJT used in these studies was obtained from 10-fold-concentrated polymyxin-treated broth supernatants of strain INN-73-83. The concentration of CJT in the three lots used varied between 29.1 and 44.7 ng of toxin per mg of total protein as determined by the CHO assay; the amount of supernatant used in different studies was expressed as that of the toxin present. All lots were used within 2 weeks of preparation because, as noted by others (41), toxin activity is progressively lost after 2 weeks even in samples to which soybean trypsin inhibitor (Sigma) is added and which are stored at −60°C.

(i) Inhibition by GM ganglioside of CJT activity in CHO cells. From a concentration of 1,000 pg in 25 μl, twofold serial dilutions of GM ganglioside were added to equal volumes containing 2.5 times the minimum amounts of toxins that gave a positive response in the CHO assay (50 pg of pure LT and 82 pg of CJT). The smallest amount of GM ganglioside that gave a negative response in the CHO assay was 125 pg for LT and 250 pg for CJT. This indicated that the inhibitory ratio of GM ganglioside to toxin by weight was 2.5 for LT and 3.1 for CJT.

(ii) Neutralization by antiserum to LT of CJT activity in CHO cells. Twofold serial dilutions of 100 μl of goat hyperimmune antiserum to LT were incubated with 50 pg of LT, 1 ng of CT, or 82 pg of CJT. The antiserum dilution of the last positive well was multiplied by the amount of toxin in the sample; the value obtained was multiplied by 10 to give a per-milliliter value and then divided by 1,000 to obtain the neutralizing capacity of 1 ml of antiserum for nanogram quantities of the toxins. A 1-ml amount of antiserum to LT neutralized 512 ng of LT, 320 ng of CT, and 205 ng of CJT. In another experiment, twofold serial dilutions of purified antiserum to CT (Swiss Serum and Vaccine Institute, Bern, Switzerland) were mixed with the same amounts of the toxins: 1 ml of CT antiserum neutralized 2,560 ng of CT, 256 ng of LT, and 205 ng of CT.

(iii) Neutralization by antiserum to LT of the secretory effect of CJT in ligated rat ileal loops. The results for the secretory response to the instillation of graded amounts of CJT into ligated rat ileal loops was compared with a previously obtained (32) dose-response curve for pure LT (Fig. 4). CJT gave a dose-dependent response, with an ED₅₀ (one-half the dose which yields maximum secretion) of 3.2 ng, versus 0.17 ng for LT. Tenfold serial dilutions of goat anti-LT antiserum in 250 μl were incubated at 37°C for 1 h with 250 μl containing those doses which gave maximum secretion (20 ng of CJT and 1 ng of LT). The secretory effect of CJT was completely neutralized by an antiserum dilution of 1:10 and that of LT was completely neutralized by a dilution of 1:30.

(iv) Protection in rats immunized with LT or B subunit. Four unimmunized control rats and the same number of rats immunized with either LT or its B subunit were challenged simultaneously by instilling 20 ng of CJT into their ligated ileal loops. The volume/length ratio (mean ± standard error of the mean) in the control rats was 304 ± 4; the value decreased by 60%, to 121 ± 2, in rats immunized with LT and by 49%, to 156 ± 4, in those immunized with LT B subunit. Secretion was significantly (P < 0.001) lower in both immunized groups than in the control animals.

**DISCUSSION**

The results of this study agree with those previously reported by Ruiz-Palacios et al., which indicated that many strains of *C. jejuni* produce a heat-labile enterotoxin (41). Their finding that antiserum to LT neutralizes the secretory activity of crude CJT (41) led us to show that CJT is also immunologically related to *E. coli* LT toxin, which enabled us to quantitate CJT activity by expressing it as a function of the response to pure LT in ELISA and CHO assays. This quantitative approach showed that the amount of CJT produced and recovered depends on the culture conditions and recovery techniques used and that, as with LT production by different enterotoxigenic *E. coli* strains (19, 24), the amount of CJT produced by different *C. jejuni* clinical isolates varies widely. The failure of some laboratories to identify enterotoxin production by strains of *C. jejuni* in the past (21, 33, 49)
was probably due to their using suboptimal culture media and growth conditions, confining their assays to sonicated cells (which we found yielded only a small quantity of CJT), or performing assays that were not sensitive to CJT.

The role of CJT in the pathophysiology of C. jejuni-induced diarrhea has not yet been established, but it is tempting to speculate that the two pathogenic mechanisms now recognized, invasion and enterotoxin production, may account for the variable clinical pattern of diarrheal disease caused by this organism. It remains to be determined whether C. jejuni resembles E. coli in that some strains are nonpathogenic, whereas others have separate plasmid-induced properties of either invasiveness or enterotoxigenicity (22, 40, 45). Strains of C. jejuni that produce either cytotoxic or cytotoxic toxins in various tissue culture assays have been described (23). The relatively simple methods for identifying toxigenic strains of C. jejuni by CHO or GM/LT ELISA assays should facilitate future studies directed at determining whether a correlation exists between enterotoxin production and the presence of a secretory type of diarrhea and whether there is a difference in pathogenicity between strains isolated from sick and from asymptomatic children.

In addition to these and earlier (41) observations, which indicate that CJT is immunologically related to both CT and LT, our findings that CJT is active in a GM/LT ELISA and that its cytotoxic effect in CHO cells is abolished by preincubation with GM ganglioside indicate that CJT shares with CT, LT, and Salmonella enterotoxin the property of attachment to this specific tissue receptor (14, 36); this is known to be a function of the B subunits in LT and CT (34). These observations suggest that, when purified, CJT may turn out to be part of the cholera- E. coli family of immunologically related heat-labile, adenylyl cyclase-stimulating enterotoxins. Recent studies with purified Salmonella typhimurium enterotoxin have established its place in this group (17), and it is perhaps relevant that the initial studies with crude Salmonella enterotoxin which suggested this relationship were similar to ours with crude CJT, in that antisera to CT neutralizes the cytotoxic effect of crude Salmonella enterotoxin in CHO cells (44) and that immunization with procho-leragenoid reduces the secretory effect of viable Salmonella cells in ligated ileal loops (37).

Acute diarrheal disease continues to be a major cause of morbidity and mortality among young children in developing countries (46). Of the three enteric pathogens principally responsible for acute diarrheal disease, vaccines for two, rotavirus and enterotoxigenic E. coli, are currently being developed (25, 31), but no such approach has been considered for C. jejuni. Should toxigenic strains of C. jejuni eventually prove to be a significant cause of diarrhea, then the demonstrated immunological homology between CJT and LT raises the possibility that toxoid vaccines containing LT or its B subunit (27, 28) might also provide some degree of protection against diarrhea caused by toxigenic strains of C. jejuni.

ACKNOWLEDGMENTS

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LITERATURE CITED


ERRATA

Molecular Homogeneity of Heat-Stable Enterotoxins Produced by Bovine Enterotoxigenic Escherichia coli
ABDUL M. K. SAEED, NANCY S. MAGNUSON, N. SRIRANGANATHAN, D. BURGER, AND W. COSAND
Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, and Program in Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-7040

Volume 45, no. 1, p. 246, Fig. 4, row f: Should read “Asn-Ser-Ser-Asn-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Thr-Gly-Cys-Tyr.”

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Volume 45, no. 2: “0.1% IsoVitaleX” should read “1.0% IsoVitaleX” on p. 314, column 2, line 36 and p. 315, column 1, line 10.