Production and release of heat-labile toxin by wild-type human-derived enterotoxigenic *Escherichia coli*

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Received 27 April 2006; revised 8 June 2006; accepted 13 June 2006.
First published online 17 August 2006.

DOI:10.1111/j.1574-695X.2006.00134.x

Editor: Patrick Brennan

Keywords
heat-labile toxin; enterotoxigenic *Escherichia coli*; labile toxin production/secretion; diarrheal disease.

Abstract

Production and release of heat-labile toxin (LT) by wild-type enterotoxigenic *Escherichia coli* (ETEC) strains, isolated from diarrheic and asymptomatic Brazilian children, was studied under *in vitro* and *in vivo* conditions. Based on a set of 26 genetically diverse LT^+ enterotoxigenic *E. coli* strains, cell-bound LT concentrations varied from 49.8 to 2415 ng mL^{-1}. The amounts of toxin released in culture supernatants ranged from 0% to 50% of the total synthesized toxin. The amount of LT associated with secreted membrane vesicles represented $< 5\%$ of the total toxin detected in culture supernatants. ETEC strains secreting higher amounts of LT, but not those producing high intracellular levels of cell-bound toxin, elicited enhanced fluid accumulation in tied rabbit ileal loops, suggesting that the strain-specific differences in production and secretion of LT correlates with symptoms induced *in vivo*. However, no clear correlation was established between the ability to produce and secrete LT and the clinical symptoms of the infected individuals. The present results indicate that production and release of LT by wild-type human-derived ETEC strains are heterogeneous traits under both *in vitro* and *in vivo* growth conditions and may impact the clinical outcomes of infected individuals.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains are food-borne pathogens that cause diarrhea among travelers and military personnel visiting or living in endemic regions (WHO, 1998). Global estimations indicate that ETEC strains cause 380 000 deaths annually, primarily among children below 5 years of age, in places where safe food and water sources are in short supply and deficient sanitation conditions prevail (WHO, 1998; Black *et al.*, 2003). To cause disease, ETEC strains must express two sets of virulence-associated factors: colonization factors (CF or CFA), represented by a diverse group of fimbrial or afimbrial adhesins conferring host and tissue specificities, and at least one enterotoxin type, including the heat-stable toxins (ST) and the heat-labile toxins (LT) (Nataro & Kaper, 1998). The type I LT and cholera toxin produced by *Vibrio cholerae* belong to a family of structurally, immunologically and phylogenetically related multimeric AB toxins, which bind to the respective glycosidic receptors via the pentameric B subunits (Merrit *et al.*, 1994; Holmes *et al.*, 1995). These toxins are internalized by epithelial cells and the processed enzymatic active A1 subunit catalyzes the ADP-ribosylation of the adenylate cyclase Gsα subunit, resulting in enhanced cAMP levels and, consequently, altering the ion and water fluxes at the intestinal lumen, ultimately leading to diarrhea (Hirst *et al.*, 1984; Spangler, 1992).

An interesting feature of the ETEC infectious process is the variable symptoms expressed by patients colonized by different strains. In endemic regions, only a fraction of the infected individuals display clinical symptoms, ranging from a mild self-limited diarrhea to a fatal cholera-like syndrome (Finkelstein *et al.*, 1976; Steinsland *et al.*, 2003). Indeed, isolation of ETEC strains from asymptomatic children commonly occurs, particularly for LT^+ ETEC strains (Reis *et al.*, 1982; Viboud *et al.*, 1999; Steinsland *et al.*, 2003). The variable symptoms expressed by ETEC-infected patients might reflect acquisition of protective immunity after repeated exposure to strains displaying

similar or identical virulence-associated factors (Black *et al.*, 1981). Nonetheless, the isolation of ETEC strains from asymptomatic children < 2 years old is intriguing and quantitative differences in the enterotoxicity of wild-type ETEC strains may also affect the clinical symptoms of the infected subjects (Escheverria *et al.*, 1977).

To bind to the enterocyte receptors and trigger fluid loss, LT and the closely related cholera toxin must be released from bacteria into the intestinal lumen. Indeed, the more severe symptoms of *V. cholerae*-infected individuals, as compared to those infected with ETEC strains, have been attributed to the ability of *V. cholerae* strains to secrete most of the synthesized toxin, whereas ETEC strains are believed to secrete low amounts of LT (Wensink *et al.*, 1978; Gyles, 1992; Wai *et al.*, 1995; Horstman & Kuehn, 2000). More recent evidence indicated that wild-type ETEC strains can actively secrete LT into culture supernatants but this observation is mainly restricted to the H10407 reference strain and a few other wild-type ETEC strains (Escheverria *et al.*, 1977; Svennerholm & Holmgren, 1978; Kunkel & Robertson, 1979; Fleckenstein *et al.*, 2000; Tauschek *et al.*, 2002). A direct correlation between production and/or release of LT, as determined under *in vitro* conditions, and the symptoms induced by wild-type ETEC strain is still uncertain.

In this study we determined the amounts of LT produced and secreted by a set of different LT-producing ETEC strains isolated from diarrheic and asymptomatic Brazilian children. The results indicated that the production and secretion of LT are independently regulated and represent specific and characteristic traits of wild-type ETEC strains. Additionally, we observed that the capacity of ETEC strains to secrete LT, as determined under *in vitro* conditions, correlates with fluid accumulation in the rabbit ileal loop assay.

Materials and methods

Bacterial strains and growth conditions

The 26 LT^+ ETEC strains included in the present work were derived from case-control studies of acute endemic childhood diarrhea in Brazil from children under the age of 5 (Gomes *et al.*, 1996; Régua-Mangia *et al.*, 2004). Eleven LT^+ ETEC strains were isolated from asymptomatic children, and 15 LT^+ strains were recovered from diarrheic children requiring medical assistance. The reference LT^+/ST^+ ETEC H10407 strain was isolated from a patient with severe cholera-like disease in Bangladesh (Evans *et al.*, 1975). Table 1 lists all tested ETEC strains and respective serotypes, colonization factors, geographic origin, toxigenic profiles, and clinical features. One plasmid-cured nontoxinogenic derivative of the ETEC 4611-4 (O159:H21) strain originally isolated from a diarrheic subject was used as a negative control for LT production. Cultures were routinely prepared in CAYE medium (2% Casamino acids, 0.6% yeast extract, 43 mM NaCl, 38 mM K_2HPO_4, 0.1% trace salt solution consisting of 203 mM $MgSO_4$, 25 mM $MnCl_2$, 18 mM $FeCl_3$) (Mundell *et al.*, 1976) and incubated 18 h at 37 °C with vigorous agitation (200 r.p.m. in a rotary shaker).

LT production and secretion assays

LT production was determined with cultures prepared in CAYE broth after overnight incubation at 37 °C with aeration. After incubation, the same amount of bacterial cells (corresponding to 13–14 $OD_{600\,nm}$) of each sample was centrifuged at 3000 ***g*** for 15 min and the culture supernatants saved for determination of secreted LT. Cell-bound toxin was determined in whole cell extracts prepared after suspension of bacteria in 3 mL of phosphate-buffered saline (PBS) buffer (100 mM phosphate, 150 mM NaCl, pH 7.2) followed by sonic disruption with a Branson sonifier 450D. Cell debris was removed by centrifugation at 5000 ***g*** for 15 min at 4 °C and supernatants, corresponding to cell-associated LT, assayed immediately or stored at -20 °C for up to 7 days. Determination of LT concentrations in culture supernatants and whole cell extracts was based on at least three independent determinations for each tested ETEC strain.

Determination of LT associated with membrane vesicles

The amount of secreted LT associated with membrane vesicle was determined as previously described by Horstman & Kuehn (2000). After cultivation in CAYE broth, bacterial cells were centrifuged (10 000 ***g***, 10 min) and culture supernatants filtered through a disposable 0.22-μm nitrocellulose filter. Half of the filtrate sample was incubated with 100 mM EDTA at 37 °C for 120 min. Both samples were submitted to GM1-ELISA for determination of the total amount of soluble LT. Differences in the amount of LT determined in EDTA-treated and nontreated samples were attributed to the encapsulation of LT into membrane vesicles.

Capture ELISA (cELISA) and GM1-ELISA

Determination of LT concentrations in culture supernatants or whole cell extracts was carried out with the capture ELISA method (Drevet & Guinet, 1991). Briefly, microtiter plates (Nunc Maxisorp, Nalge Nunc, Roskilde, Denmark) were coated with 100 μL $well^{-1}$ of rabbit anticholera toxin serum (titer of 3.3×10^5) diluted in PBS (1 : 1000) followed by overnight incubation at 4 °C. Plates were washed twice with PBS containing 0.05% Tween-20 (PBS-T) and blocked by

Table 1. Production and release of LT by wild-type ETEC strains isolated from diarrheic and asymptomatic children in Brazil

Strain	Serotype	Other traits	Source	LT (ng mL^{-1})*		Total LT production (ng mL^{-1})†	LT secretion (%)‡
				Cells	Supernatants		
1372-1	O23:H28	CS$^-$	AC	746.7 ± 28.1	3 ± 0.6	749.7	0.4
4092-7	ND:H21	CS$^-$	AC	1206.5 ± 264.1	182.6 ± 42.7	1389.1	13.1
4692-5	ND:H$^-$	CS$^-$	AC	478.2 ± 57.0	55.1 ± 12.4	533.3	10.3
4702-1	O167:H5	CS$^-$	AC	76.0 ± 12.5	71.3 ± 21.9	147.3	48.4
4652-2	O159:H34	CS$^-$	AC	49.8 ± 0.95	< 2	< 51.8	< 0.04
63V	O159:H17	–	AC	696.1 ± 81.1	122.4 ± 45.5	818.5	14.9
121I	O48:H21	–	AC	63.1 ± 11.9	4.6 ± 3.1	67.7	6.8
PE0215	O7:H18	ND	AC	277.1 ± 42.8	52.2 ± 9.1	329.3	15.9
PE0534	O106:H40	ND	AC	182.9 ± 58.7	78.0 ± 6.2	260.9	29.9
PE0690	ONT:H8	ND	AC	258.1 ± 40.1	127.1 ± 14.6	385.2	32.9
PE0415	O152:H2	ND	AC	264.9 ± 26.9	32.8 ± 8.9	297.7	11.0
4811-1	O9:H21	CS$^-$	DC	53.8 ± 6.6	52.7 ± 9.4	106.5	49.5
4101-1	O114:H$^-$	CS$^-$	DC	1463.6 ± 327.3	93.6 ± 13.0	1557.2	6.0
4321-1	ND:H$^-$	CS$^-$	DC	78.7 ± 17.3	7.2 ± 3.2	85.9	8.4
214III	ONT:HNT	–	DC	54.1 ± 12.7	20.8 ± 3.1	74.9	27.8
225IV	O148:H28	CS6	DC	55.4 ± 19.4	13.3 ± 6.4	68.7	19.4
PE0615	ONT:H19	ND	DC	82.5 ± 15.2	49.4 ± 2.3	131.9	37.5
2811-1	O88:H25	CS$^-$	DC	2415.3 ± 706.9	109.8 ± 5.9	2525.1	4.4
136I	O88:H25	–	DC	446.0 ± 22.6	64.7 ± 8.2	510.7	12.7
136II	O88:H25	ND	DC	897.4 ± 74.4	54.4 ± 11.4	951.8	5.7
136III	O88:H25	ND	DC	541.1 ± 36.7	90.3 ± 57.3	631.4	14.3
36III	O159:H21	–	DC	602.4 ± 89.8	166.2 ± 6.6	768.6	21.6
36IV	O159:H21	–	DC	623.4 ± 27.9	161.7 ± 8.1	785.1	20.6
187V	O159:H21	–	DC	337.0 ± 53.7	139.3 ± 18.1	476.3	29.2
PE0260	O8:H9	ND	DC	504.8 ± 54.8	103.0 ± 13.3	607.8	16.9
PE0262	O8:H9	ND	DC	105.9 ± 16.1	102.8 ± 7.1	208.7	49.3
H10407	O78:H11	ST$^+$ CFA/I	DC	526.2 ± 57.0	248.2 ± 65.6	774.4	32.0

*LT values determined with cELISA using whole cell extracts (cells) or culture supernatants of the different ETEC strains cultivated overnight (18 h) in CAYE at 37 °C under aeration. Values expressed by means ± SD of at least three independent experiments.

†Values expressed as means of LT measured in bacterial cells plus LT detected in culture supernatants.

‡LT secretion expressed as the ratio of LT detected in culture supernatant/total LT produced by the strain × 100.

ND, not determined; NT, nontypeable; AC, isolated from an asymptomatic child; DC, isolated from a diarrheic child.

incubation with 5% skim milk in PBS-T for 1 h at 37 °C. After additional washings, 100 μL of crude and serially twofold diluted samples were added to the wells and incubated for 2 h at room temperature. After washings, mouse anti-LT serum (titer of 10^5) diluted in PBS-T (1 : 5000) was added to the wells and incubated for 90 min. Detection of bound antibodies was carried out with 100 μL of PBS-T diluted (1 : 3000) horseradish peroxidase-conjugated antimouse-IgG (Sigma-Aldrich, Poole, UK) incubated for 90 min at room temperature. After a final washing step, color reactions were developed with *o*-phenylenediamine and H_2O_2. After 20 min at room temperature, the reactions were interrupted by the addition of 2 M H_2SO_4 (50 μL well^{-1}) and the $OD_{492\,nm}$ measured in a microplate reader (Multiskan EX, ThermoLabsystems). The final reaction values were obtained after deduction of background absorbance measured in control wells filled with cell extracts of the LT$^-$ derivative of the 4611-4 strain. The GM1-ELISA was carried out as previously described (Ristaino *et al.*, 1983). Briefly, wells of polystyrene 96-well microtiter plate (Nalge Nunc) were coated with PBS-diluted GM1 ganglioside (2 μg mL^{-1}) (Sigma-Aldrich) and incubated overnight at room temperature. In the following day, plates were washed and then blocked with 0.1% bovine serum albumin (BSA) in PBS for 30 min at 37 °C. After washing with PBS-T, undiluted bacterial extracts were dispensed in duplicate wells and then twofold serially diluted. All subsequent steps were identical to cELISA. LT concentrations in tested samples were determined using purified LT isolated from the H10407 strain. Regression analysis ($R^2 > 0.98$) was used to generate a standard curve used for determination of LT concentrations in the tested samples.

Purification of LT

A recombinant *E. coli* DH5α strain harboring the pBSPKS(–) vector (Schweizer, 2001) carrying the complete *elt* operon of the H10407 strain under the control of the

native promoter was used for the expression of LT. The toxin was purified by affinity chromatography on immobilized D-galactose columns (Pierce, Rockford, IL), according to a previously described method (Uesaka *et al.*, 1994). The purified toxin was monitored in SDS-containing 15% polyacrylamide gels (Laemmli, 1970), and the protein concentration determined with the Bradford assay (Bio-Rad Laboratories, Hercules, CA) and BSA (Sigma-Aldrich) as standard.

Production of anticholera toxin and anti-LT antibodies

Cholera toxin-specific serum was obtained after subcutaneous immunization of an adult New Zealand rabbit with four doses of 15 μg of purified cholera toxin (Sigma-Aldrich) in Freund incomplete adjuvant at 2-week intervals. Blood samples were harvested by cardiac puncture of the animal 1 week after the last dose. Sera were separated from cells, pooled and stored at −20 °C. The anti-LT serum was prepared with female C57Bl/6 (6–8-week-old) mice immunized subcutaneously with purified LT (10 μg). The immunization regimen consisted of one dose applied in complete Freund adjuvant followed by two additional doses in incomplete Freund adjuvant given at weekly intervals. One week after the last dose, the blood was harvested, sera collected, pooled, and stored at −20 °C.

Screening for the presence of *gsp* and *leoA* genes

The presence of the *gspD* and *gspK* genes, involved in the type II secretion pathways, in the LT^+ ETEC strains was determined according to the conditions described by Tauschek *et al.* (2002). Genomic DNA of the ETEC strains was prepared by the boiling lysis method and amplification reactions were carried out with primers P78 (5′-TTCGGAAATCGCCCGCGTGC) and P109 (5′-TCCACCTTCGAGACTTCC), for the *gspD* gene, and with primers P5 (5′-GCAGCAGGTGACTAACGGC) and P12 (5′-CAGGGCTTAACCACGGGTC) for the *gspK* gene. The *leoA* gene has been reported to be involved in a specific LT secretion pathway (Fleckenstein *et al.*, 2000). Attempts to detect the *leoA* gene were based on primers 102.wr001 (5′-TCAGCCGCTCATCGTCCATCA) and 102.wr008 (5′-GTAAAACCGCACTGGACT). Amplifications were performed with High Fidelity DNA polymerase (Fermentas, St Leon-Rot, Germany) in a reaction volume of 25 μL in a Mastercycler Gradient thermal cycler (Eppendorf) with an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final extension for 5 min at 72 °C.

Rabbit ileal loop assay

Rabbit ileal loop studies were performed as previously described (De & Chatterje, 1953; Nishibuchi *et al.*, 1992) with 1.5-kg male New Zealand white rabbits. Rabbits were fasted for 48 h prior to surgery, except for ingestion of water with glucose. Laparotomy was carried out aseptically to externalize the intestine of the animals, which were kept under anesthesia by intramuscular administration of telazol (200 μL kg^{-1}) and nilperidol (300 μL kg^{-1}). Isolated 50-cm duodenum loops were spaced by 2-cm interposing loops with ligatures. Strains with different levels of the LT production and secretion (1372-1, 4652-2, 2811-1, 36III), as well as both negative and positive controls (4611-4 and H10407, respectively), were cultivated from single colonies in 30 mL of CAYE broth overnight. Aliquots (500 μL) containing 10^7 CFU mL^{-1} were injected into each loop followed by intestine internalization and incision closure. The volumes of accumulated fluids in each loop were measured *c.* 18 h after the inoculation of the bacterial strains.

Statistical evaluations

Statistical analyses were carried out with the Student's *t*-test and ANOVA for repeated measurements. *P* values < 0.05 were taken as indicative of statistical significance.

Results

Quantification of LT produced and released by wild-type ETEC strains

Twenty-six LT-producing wild-type ETEC strains isolated from asymptomatic (11 strains) or diarrheic children (15 strains) living in Brazil were analyzed for the ability to produce and release LT after growth at 37 °C for 18 h in CAYE medium. Determination of the LT content in whole cell extracts and culture supernatants indicated that these strains were remarkably variable with regard to LT production and secretion (Table 1). Cell-bound LT was produced at concentrations ranging from 50 to 1389 ng mL^{-1} among ETEC strains isolated from asymptomatic patients. The concentration of LT in whole cell extracts of ETEC strains recovered from diarrheic patients varied from 53.8 to 2415 ng mL^{-1}. For comparison, the amount of cell-bound LT produced by the reference H10407 strain cultivated under the same conditions was 526.2 ng mL^{-1}. Eight of 11 (72%) ETEC strains isolated from asymptomatic children and 10 of 15 (71.5%) strains isolated from diarrheic patients produced LT levels above 100 ng mL^{-1} (Table 1). Accordingly, no statistically significant differences ($P = 0.63781$) could be detected after comparing the values of cell-bound LT produced by ETEC strains isolated from symptomatic or asymptomatic subjects (Fig. 1). The same result was

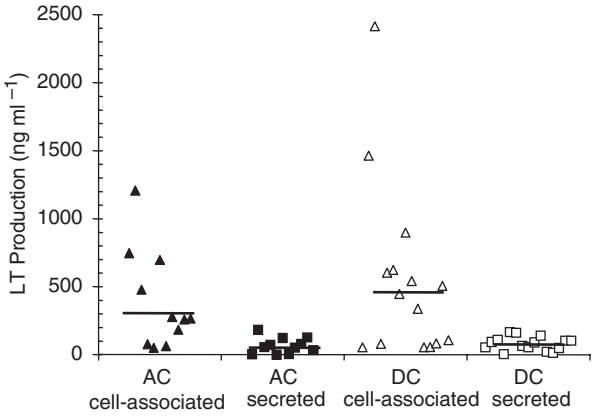

Fig. 1. Production and secretion of LT by wild-type ETEC strains isolated from diarrheic and asymptomatic children. Production of cell-bound (▲, △) and secreted LT (■, □) expressed by each tested ETEC strain recovered from diarrheic (DC) or asymptomatic (AC) children were determined by cELISA and the average values of each strain group represented by bars. The values represent the average of at least three independent experiments.

obtained with LT secreted in culture supernatants. The amounts of LT detected in culture supernatants of ETEC strains isolated from asymptomatic subjects varied from nondetectable levels to 182.6 ng mL^{-1}, and the secreted LT values for strains derived from diarrheic children ranged from 7.2 to 166 ng mL^{-1} (Table 1). In comparison, the amount of LT secreted by the H10407 strain cultivated under the same culture conditions was 248.2 ng mL^{-1}, representing 32% of the total synthesized toxin. Secretion of LT among the tested ETEC strains ranged from 0% to 49% of the produced toxin, indicating that LT secretion is regulated independently of toxin expression. Once again, no statistically significant differences could be demonstrated between the values of secreted LT by wild-type ETEC strains and the clinical symptoms of the infected individuals (Fig. 1). Considering the total amounts of LT (secreted plus cell-bound toxin), wild-type ETEC strains were capable of producing LT at concentrations ranging from *c.* 50 to 2525 ng mL^{-1}. These results clearly demonstrate that both production and secretion of LT by wild-type ETEC strains isolated from human beings are remarkably variable traits, at least as evaluated under laboratory conditions.

LT secretion pathways in wild-type ETEC strains

In contrast to *V. cholerae*, ETEC strains were initially reported to be defective in LT secretion (Kunkel & Robertson, 1979; Hirst *et al.*, 1984). However, further reports indicated that some ETEC strains are proficient in the secretion of LT by at least three different mechanisms (Fleckenstein *et al.*, 2000; Horstman & Kuehn, 2002; Tauschek *et al.*, 2002). As an initial step toward the analysis of the molecular mechanisms controlling the variable LT secretion expressed by wild-type ETEC strains, we detected the presence of two genes, *gspD* and *gspK*, that played essential roles on the extracellular secretion (EPS) pathway required for LT secretion in the H10407 strain (Tauschek *et al.*, 2002). All 26 LT$^+$ ETEC strains studied, as well as the reference H10407 strain, were positive for the presence of the *gspD* and *gspK* genes, whereas no productive amplification was achieved with *E. coli* K12 strains (data not shown). On the other hand, attempts to amplify the *leo* gene, another gene encoding a putative LT-secretion pathway (Fleckenstein *et al.*, 2000), were unsuccessful in all tested ETEC strains.

In addition to the EPS pathway, ETEC strains have been reported to promote LT secretion by means of membrane vesicles shed during active growth (Horstman & Kuehn, 2000, 2002). One possible explanation for the variable secreted LT levels by wild-type ETEC strains might involve the differential shedding of membrane vesicles. As shown in Fig. 2, the amount LT associated with membrane vesicles represented $< 5\%$ of the total toxin detected in culture supernatants of different ETEC strains. Additionally, no significant differences between the relative amounts of LT associated with membrane vesicles generated by ETEC strains releasing high (4092-7, 36III, and 2811-1 strains) or low LT values (4652-2 and 1372-1 ETEC strains) could be detected. These data suggest that the variable amount of LT secreted cannot be ascribed to the differential shedding of membrane vesicles by wild-type ETEC strains.

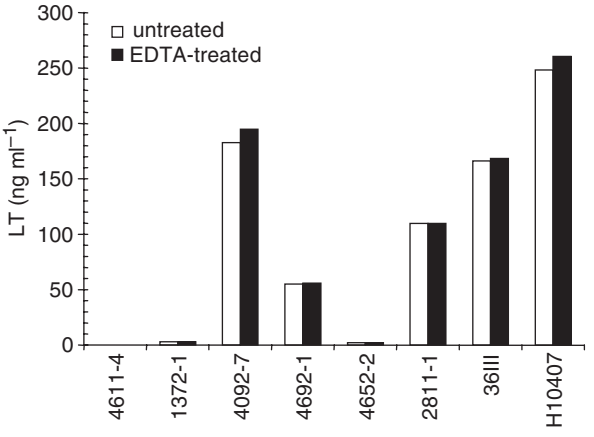

Fig. 2. Association of LT to membrane vesicles secreted by wild-type ETEC strains. Culture supernatants harvested from ETEC cultures kept at 37 °C for 18 h were incubated with 100 mM EDTA for 120 min before measurement of the LT contents by GM1-ELISA. ETEC H10407 and *E. coli* 4611-4 were used as positive and negative controls, respectively, for determination of LT production.

Secretion of LT correlates with fluid accumulation in rabbit ileal loops

Production and release of LT in the intestinal lumen of mammals may differ from values determined with ETEC strains cultivated under ordinary laboratory conditions (Hunt & Hardy, 1991). To determine whether the *in vitro* determined LT values correlate with the *in vivo* behavior of ETEC strains, we tested four wild-type strains (1372-1, 4652-2, 2811-1, 36III) and two reference strains (H10407 and 4611-4) in ligated rabbit ileal loops. Under *in vitro* conditions, 1372-1 and 4652-2 strains secreted low LT levels (3 ng mL^{-1} or non detectable, respectively), and 2811-1, 36III and H10407 strains secreted much higher LT levels (109.8, 166.2 and 248.2 ng mL^{-1}, respectively) (Table 1). On the other hand, the amounts of cell-bound LT produced by 1372-1, 36III and H10407 strains were similar (*c.* 630 ng mL^{-1}), whereas 4652-2 and 2811-1 strains produced the lowest (49.8 ng mL^{-1}) and the highest (2415 ng mL^{-1}) LT values among the tested ETEC strains, respectively. Using the ligated rabbit ileal loop model we observed that ETEC strains secreting low LT levels (1372-1 and 4652-2) caused little or no fluid accumulation even when the amount of cell-bound LT was high, as in the case of strain 1372-1 (Fig. 3). On the other hand, ETEC strains secreting larger amounts of LT, as determined under *in vitro* conditions, caused proportionally more intestinal fluid accumulation, as shown with the 2811-1, 36III and H10407 strains. Interestingly, the 2811-1 strain, although producing approximately three times more toxin than the H10407 strain, induced significantly less fluid accumulation than the H10407 strain, which secretes roughly twice as much LT. These results clearly indicate that the amount of secreted LT is the most relevant determinant of fluid accumulation in the rabbit ileal loop model. Moreover, these results strongly suggest that the values of LT produced and secreted by ETEC strains under *in vitro* conditions correlate with the amounts of toxin produced during *in vivo* growth.

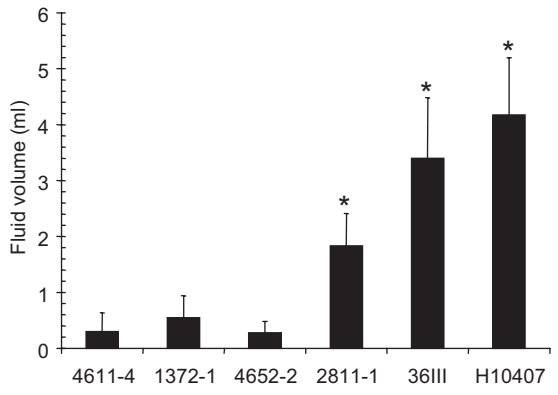

Fig. 3. Fluid accumulation in rabbit ileal loops inoculated with different wild-type ETEC strains. Exponentially growing ETEC cells harvested from cultures prepared in CAYE broth at 37 °C were adjusted to a cell concentration of 10^7 CFU mL^{-1} and 500-μL aliquots inoculated into ileal loops. After 18 h the animals were sacrificed and the volume of liquid in each ileal loop measured. Results were based on experiments carried out with four rabbits and include the H10407 and 4611-4 strains as positive and negative controls, respectively. Error bars indicate standard deviations. Cell-bound and -secreted LT produced by each tested ETEC strain are expressed in ng mL^{-1} and were determined *in vitro* using cELISA. *Statistically different values ($P < 0.01$) as compared to the negative control (4611-4 strain).

Discussion

Since the discovery and characterization of LT, the capacity of production and release of the toxin expressed by ETEC strain has been considered one of the most relevant virulence-associated factors of wild-type strains isolated from human and nonhuman hosts. Moreover, the variable degree of enterotoxigenicity expressed by wild-type ETEC strains has been suggested to contribute to the severity of the disease symptoms of infected patients (Escheverria *et al.*, 1977). In the present report, we carried out a systematic quantitative analysis of LT produced and released by a set of 26 wild LT$^+$ ETEC strains isolated from either diarrheic or asymptomatic children living in Brazil. Our data showed that production and release of LT represent variable but reproducible traits expressed by wild-type ETEC strains cultivated under laboratory conditions. Although no clear correlation could be drawn between the clinical symptoms of infected subjects and the enterotoxigenicity of the tested strains, our evidence indicated that fluid accumulation in rabbit ileal loops is proportional to the amount of secreted LT, thus suggesting that *in vitro* determined LT levels correlate with the *in vivo* effects inflicted by LT$^+$ ETEC strains.

The variable expression of LT by wild-type ETEC strains indicates that regulation of *elt* operon is complex and subject to strain-specific regulation. Regulation of LT production involves the interplay of the histone-like nucleoid structuring (H-NS) repressor protein as the main modulator of gene expression. Available evidence indicates that the plasmidial eltAB operon is silenced by the H-NS protein, which binds to a downstream regulatory element (DRE) blocking the extension of the mRNA for RNA polymerase under lower temperature or osmolarity (Trachman & Maas, 1998; Trachman & Yasmin, 2004). LT expression by wild-type ETEC strains may vary from 50 to values above 2400 ng mL^{-1} under the same culture conditions, which suggests that additional factors, such as promoter strength, plasmid copy number or alternative gene regulation mechanisms, contribute to the activity of the *elt* operon.

Indeed, differences in promoter strength and the presence of trans-acting gene regulation mechanisms have already been demonstrated in *E. coli* K12 strains transformed with LT-encoding plasmids isolated from wild-type ETEC strains (Neill *et al.*, 1983).

Initial reports have indicated that, in contrast to *V. cholerae*, ETEC strains were not proficient at secreting LT into the extracellular medium (Hirst *et al.*, 1984; Clements *et al.*, 1985). Later, additional evidence demonstrated that ETEC strains are in fact capable of promoting the active secretion of LT by at least three different mechanisms, including the general secretory pathway encoded by *gsp* genes (Tauschek *et al.*, 2002), an ETEC-specific secretion pathway encoded by the *leo* gene (Fleckenstein *et al.*, 2000), and the shedding of LT-containing membrane vesicles (Horstman & Kuehn, 2002). The present data demonstrated that the amount of LT released in the culture supernatants ranged from undetectable levels up to 250 ng mL^{-1}, 0–50% of the toxin produced by the tested strain. All tested ETEC strains harbored the *gsp*D and *gspK* genes involved in the extracellular protein secretion machinery but we failed to demonstrate the presence of the *leo* gene. The amounts of LT associated with membrane vesicles represented only a small fraction of the total released toxin and, thus, could not explain the quantitative differences in the observed secreted LT values. This indicates that the strain-specific differences in LT secretion cannot be explained solely on the basis of the secretion mechanisms so far described and suggests that the additional pathways affecting LT release exist in wild-type ETEC strains. The recent description of a plasmid-encoded two-partner secretion pathway found in the H10407 strains illustrates the complexity and diversity of the protein secretory mechanisms expressed by ETEC strains (Fleckenstein *et al.*, 2006).

The *in vivo* action of LT implies that the toxin is released into the intestinal lumen and binds to receptor molecules on the surface of enterocytes, leading to internalization of the toxin and subsequent activation of adenylate cyclase. The mammalian small intestine environment is clearly distinct from those encountered under ordinary laboratory conditions, differing in pH, oxygen tension, osmolarity and the presence of bile salts (Kunkel & Robertson, 1979; Hunt & Hardy, 1991). In this work we compared the *in vivo* production of LT by wild-type ETEC strains producing different amounts of secreted or cell-bound LT using the tied rabbit ileal loop model. With this *in vivo* model, ETEC strains secreting larger amounts of toxin, but not those producing higher cell-bound toxin, accumulated more liquid in rabbit intestinal loops. These results indicate that amounts of LT produced and secreted *in vitro* by ETEC strains correlate with the *in vivo* expression of the toxin and suggest that host-associated factors, such as the presence of bile salts, did not change the behavior of wild-type strains with regard to LT secretion. The recent finding that LT^+ producing strains more efficiently colonize both gnotobiotic piglets and mice in a newly developed colonization model presents another aspect of the role LT plays in the virulence of ETEC strains (Berberov *et al.*, 2004; Allen *et al.*, 2006). This evidence further supports the notion that the heterogeneous LT production detected among wild-type strains may affect the symptoms of ETEC-infected individuals.

Individuals infected with ETEC strains may develop symptoms ranging from a mild illness to a severe cholera-like diarrhea (Finkelstein *et al.*, 1976). Such differences are not easily accounted for by our present understanding of ETEC pathogenesis. However, the variable enterotoxicity concerning production and release of LT may, at least in part, affect the clinical symptoms of an infected subject, particularly among young children and travellers without previous contact with the pathogen. Nevertheless, no clear correlation could be established between the LT levels produced or secreted by LT^+ ETEC strains and the symptomatic or asymptomatic state of the infected children. As previously shown by other groups, children living in endemic regions are prone to develop LT-specific immunity that confers protection against subsequent infections with LT^+-producing strains (Steinsland *et al.*, 2003). Unfortunately, serum and fecal samples of children infected with the ETEC strains included in the present study were not available and, thus, the presence of LT-specific antibody responses could not be determined. Nonetheless, due to the frequent infections with different ETEC strains, correlation between LT^+ production/secretion and severity of clinical symptoms is unlikely to be demonstrated among children living in endemic regions and would more properly tested in subjects not previously exposed to ETEC.

Acknowledgements

We thankfully acknowledge the contribution of Dr Marcio O. Lasaro (The Wistar Institute) for statistical analyses, critically reading the manuscript and helpful comments. We also thank Carlos A. da Silva and Camila Calderon (University of São Paulo) for the helpful technical assistance. This work was supported by FAPESP and CNPq grants.

Authors contribution

M.A.S.L. and J.F.R. contributed equally to this study.

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