Liquid chromatography-ultraviolet detection and quantification of heat-labile toxin produced by enterotoxigenic *E. coli* cultured under different conditions

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A R T I C L E   I N F O

A B S T R A C T

Enterotoxigenic *Escherichia coli* (ETEC) is the main bacterial cause of dehydrating infant diarrhoea in less-developed countries. Labile toxin (LT) is the major virulent factor of ETEC. Easy diagnostic tests are necessary to reduce the number of cases. Immunological methods have some drawbacks and also have important limitations. For that reason, a Liquid Chromatography coupled to UV detector technique (LC-UV) has been optimize to a rapid identification and quantification of LT from bacteria cultures. It is also important to know optimal conditions for LT and with this purpose several enterotoxigenic *E. coli* strains have been studied to determine the influence of glucose concentration and different culture media on LT production.

LC-UV technique demonstrated to be a good method for LT quantification showing values of 15 ng/mL and 45 ng/mL for limits of detection and quantification respectively. LT quantification revealed that toxin production is directly related to the concentration of glucose added in the broth medium. Tryptic Soy Broth is the most efficient culture medium for *E. coli* growth and enterotoxin production.

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1. Introduction

Enterotoxigenic *E. coli* (ETEC) is one of the most commonly reported diarrheagenic *E. coli* strains (Lamberti et al., 2014; Levine, 1987). ETEC is the main bacterial cause of dehydrating infant diarrhoea in less-developed countries and is also the agent most frequently responsible for travellers' diarrhoea in people from industrialized countries visiting less-developed countries (WHO, 2009; Sack, 1975). Infections are transmitted through the faecal-oral route and bacteria is acquired by ingesting contaminated food or water. Clinical symptoms of ETEC infection are watery diarrhoea, nausea, abdominal cramps, and low-grade fever (Croxen et al., 2013).

ETEC produces two types of enterotoxins that cause diseases in the man and various domestic animals: low weight, heat-stable enterotoxin (ST), and high weight, heat-labile enterotoxin (LT). ST is considered an important cause of diarrhoea in pigs but is rarely associated with humans (Handl and Flock, 1992). However, LT is the major virulent factor of ETEC (Holmgren and Svennerholm, 1992). For an effective reduction of these events, preventive measures and easy diagnostic tests are necessary.

Optimal conditions for ST and LT toxins production have been widely studied (Gonzales et al., 2013; Rocha et al., 2013; Erume et al., 2010). However, not all the LT toxin is secreted into the culture supernatant during growth, part of it remains cell associated. Therefore, specific culture and extraction conditions are needed to release the toxin produced (Lasaro et al., 2006).

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Currently, detection of LT has been carried out by several immunological methods available in various formats including enzyme-linked immunosorbent assays (ELISA), reversed passive latex agglutination (RPLA) and automated systems combining immunomagnetic separation (IMS) and ELISA (GM1-enzyme-linked immunosorbent assay) (Hegde et al., 2009; Lasaro et al., 2006). These LT quantification methods are widely accepted since they are sensitive, specific, fast and simple to perform. However, immunological methods have some drawbacks and also have important limitations, as false positive results, that occur when the immunological material cross-reacts with unrelated antigens (O'sullivan et al., 2007). The main drawback of current methods to detect enterotoxins based on specific polyclonal or monoclonal antibodies remains its high cost.

The aim of this work is to develop a liquid chromatography coupled to an ultraviolet detector system for the simultaneous identification and quantification of LT from ETEC cultures. Moreover, to study how nutrient media influence the production of LT by ETEC.

2. Materials and methods

2.1. Bacterial strains and culture

The enterotoxigenic strains used in this study were *E. coli* DSMZ 10973 and DSMZ 27503. Both of them were purchased as toxigenic strains producers of heat labile enterotoxin (DSMZ). One non-toxigenic *E. coli* strain CECT 405 was used as a negative control for LT production (CECT).

Lyophilized *E. coli* strains were initially reconstituted in a nutrient broth and incubated at 37 °C with constant shaking. Turbidity was read periodically until arrive at stationary phase and then 1 mL of each strain was plated onto a selective media for *E. coli*, (CHROMagar Microbiology, Paris, France). After 48 h of incubation

at 37 °C, seven colonies from each plate were inoculated into 100 mL of different culture media in 250 mL flanged flasks.

2.2. Bacterial growth

Chopped Meat Broth, Tryptic Soy Broth (TSB), Luria-Bertani Broth and Brain Heart Infusion were purchased from Oxoid (Oxoid, Unipath, Hampshire, United Kingdom) and inoculated with each strain. In selected experiments attempts were made to identify which culture media has chemical factors that favour heat labile enterotoxin production by the *E. coli* strains tested, CECT 405, DSMZ 10973 and DSMZ 27503. In all experiments microorganisms were cultured by triplicate at 37 °C with high aeration by constant shaking at approximately 200 rpm. To test bacterial growth, absorbance of all cultures was measured at different times (0, 4, 8, 24, 32, 48 and 72 h) by optical density at 600 nm.

Since glucose is required for synthesis and release of LT, glucose influence on the production of LT by *E. coli* was tested by inoculation of the strains in TSB spiked with four different concentrations of D-glucose (0.25, 0.5, 0.75 and 1%).

2.3. Toxin production

The VET-RPLA toxin detection kit (Oxoid) is designed for the detection of LT or Cholera Toxin (CT) in culture fluid. The LT has antigenic structures are similar to those found on *Vibrio cholerae* enterotoxin. Antiserum taken from rabbits, immunized with CT, will therefore react with both CT and LT. A positive result given by the test indicates that the bacteria produces the expected enterotoxin.

One ml of each culture was tested for LT production with the VET-RPLA at different times of incubation. Cultures were filtered with 0.22-μm hydrophilic regenerated cellulose filter membranes, and the filtrate was retained for an assay of the toxin content.
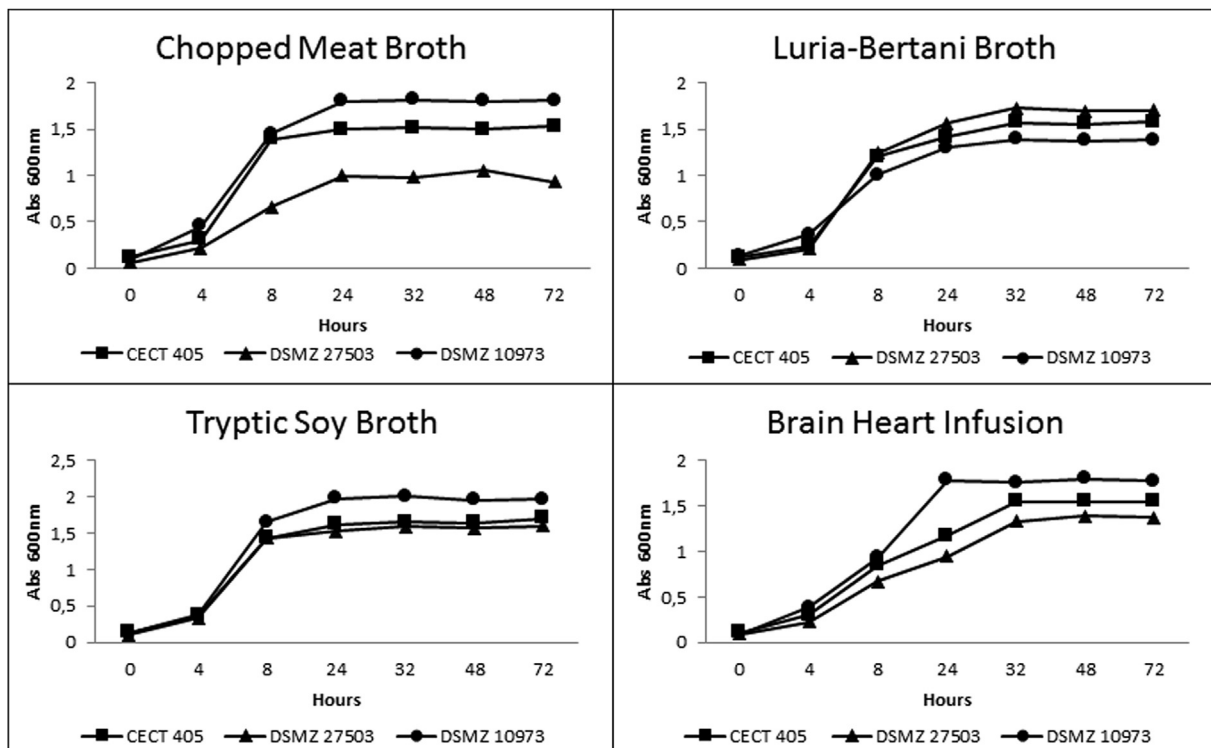

Fig. 1. Efficiency of different culture media broth on the growth of *E. coli* strains.

Table 1
VET-RPLA results of different *E. coli* strains cultured in several broth media.

Culture Media	Strains tested	VET-RPLA RESULTS							Pellet
		Hours							
		0	4	8	24	32	48	72	
Luria Bertani Broth	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 27503	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 10973	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chopped Meat Broth	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 27503	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 10973	n.d.	n.d.	n.d.	n.d.	+	+	+	n.d.
Tryptic Soy Broth	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 27503	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 10973	n.d.	n.d.	n.d.	+	+	+	+	n.d.
Brain Heart Infusion	CECT 405	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 27503	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DSMZ 10973	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected.

Cell-bound toxin was also determined in whole cell extracts. Bacterial cultures were centrifuged and the pellet was separated from the supernatant. Bacteria cells were suspended in 3 mL of phosphate-buffer saline (PBS) buffer (100 mM phosphate, 150 mM NaCl, pH 7.2) followed by sonic disruption with a Branson sonifier 450D according to Lasaro et al. (2007). As treatment with chloroform allows a simple, rapid, and quantitative release of periplasmic proteins, this procedure was also tested. Therefore, 10 μl of chloroform for each ml of culture tested were added to the bacterial pellet following the method described by Ames et al. (1984). Cell debris of both methods were removed by centrifugation at 4000 rpm for 10 min at 4ʲ C and supernatants assayed by VET-RPLA kit to test toxin content.

The microtiter plates of the VET-RPLA kit were shaken to mix the contents of the wells and incubated at room temperature on a vibration-free surface. The agglutination reactions were read after 20–24 h by holding the plates against a dark background with indirect lighting, according to the manufacturer's instructions.

2.4. LC-UV analysis

Positive VET-RPLA bacterial cultures were also tested with LC-UV to confirm and quantify the toxin produced. One ml of the culture filtrates was centrifuged at 13,000 rpm for 15 min. Supernatants were lyophilized and denatured at room temperature in a guanidine buffer (6 M guanidine-HCl in 0.5 M Tris/Tris-HCl with EDTA 1 mM) at pH 8. Disulfide bridges were reduced by the addition of DTT during 120 min at 56 °C. Free cysteine residues were then carboxymethylated with an excess of iodoacetamide in the same guanidine buffer, and maintained for 30 min in the dark. The reactant solution was transferred to Centriprep® cartridges having a 3-KDa cut-off membrane a MWCO 3000 filter, centrifuged at 14,000 g for 25 min and washed twice with 400 μl NH_4HCO_3 buffer.

LC-UV analysis was performed using a HP 1100 modular HPLC apparatus (Agilent, Palo Alto CA, USA) with a Jupiter C18 reversed-phase analytical column (150 × 2 mm, 3 μm, Phenomenex). Solvent A consisted of 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. The conditions for the mobile phase were set up at a constant flow of 0.2 mL/min with the following gradient: 0 min 20% B, at 10 min 40% B and 20 min 60% B and in the next 10 min arrived at the initial conditions. The volume injected of standards and sample solutions was 20 μl. LT was detected at 205 nm and enterotoxin identification was performed by comparing retention time and UV spectra of purified extracted samples to pure standards purchased from Sigma-Aldrich (St. Luis, USA).

3. Results

3.1. Bacterial growth and toxin production

E. coli strains show to be capable of grow in the four media cultures where were inoculated. Nevertheless, bacteria do not achieve the same growth levels in all media tested. In Chopped Meat Broth strains CECT 405 and DSMZ 10973 reach stationary phase with high absorbance values, indicating optimal growth. However, strain DSMZ 27503 does not reach the same density. In the other three culture media, all the strains achieve similar densities but only in TSB cultures reach stationary phase after 24 h (Fig. 1).

The results obtained with the VET-RPLA test indicate that only two culture media induce toxin production under the conditions measured and only in one of the toxigenic strains tested. TSB shows positive results for the DSMZ 10973 strain; the Chopped Meat Broth culture inoculated with the same strain is also positive for LT. Toxin production is only detected in cultures once stationary phase had been achieved (after 24 or 32 h). Although DSMZ 27503 has been described as a toxigenic strain, none of the cultures of this bacteria are positive for TL production. For the control strain (non-toxigenic CECT 405), the results obtained for all culture media tested are always negative (Table 1). Similarly, the analysis of the bacterial pellets from all the media tested have yielded negative results for both extraction methods tested.

TSB has been selected as culture medium for the following tests because of its ability to favour bacteria growth and toxin production.

The study of glucose influence on the production of LT by enterotoxigenic *E. coli* strains shows that the quantity of LT produced by *E. coli* DSM 10973 is directly related to the concentration added in the broth medium (Table 2). Maximum LT production is seen when the strains are grown in TSB medium containing 1% glucose. The LT production influenced by glucose concentration has been detected by VET-RPLA test and confirmed by LC-UV technique. Results are presented as mean concentration ± standard deviation (SD). Toxin quantification has been carried out by comparing peak

Table 2
Glucose influence on the production of labile toxin by *E. coli* DSMZ 10973.

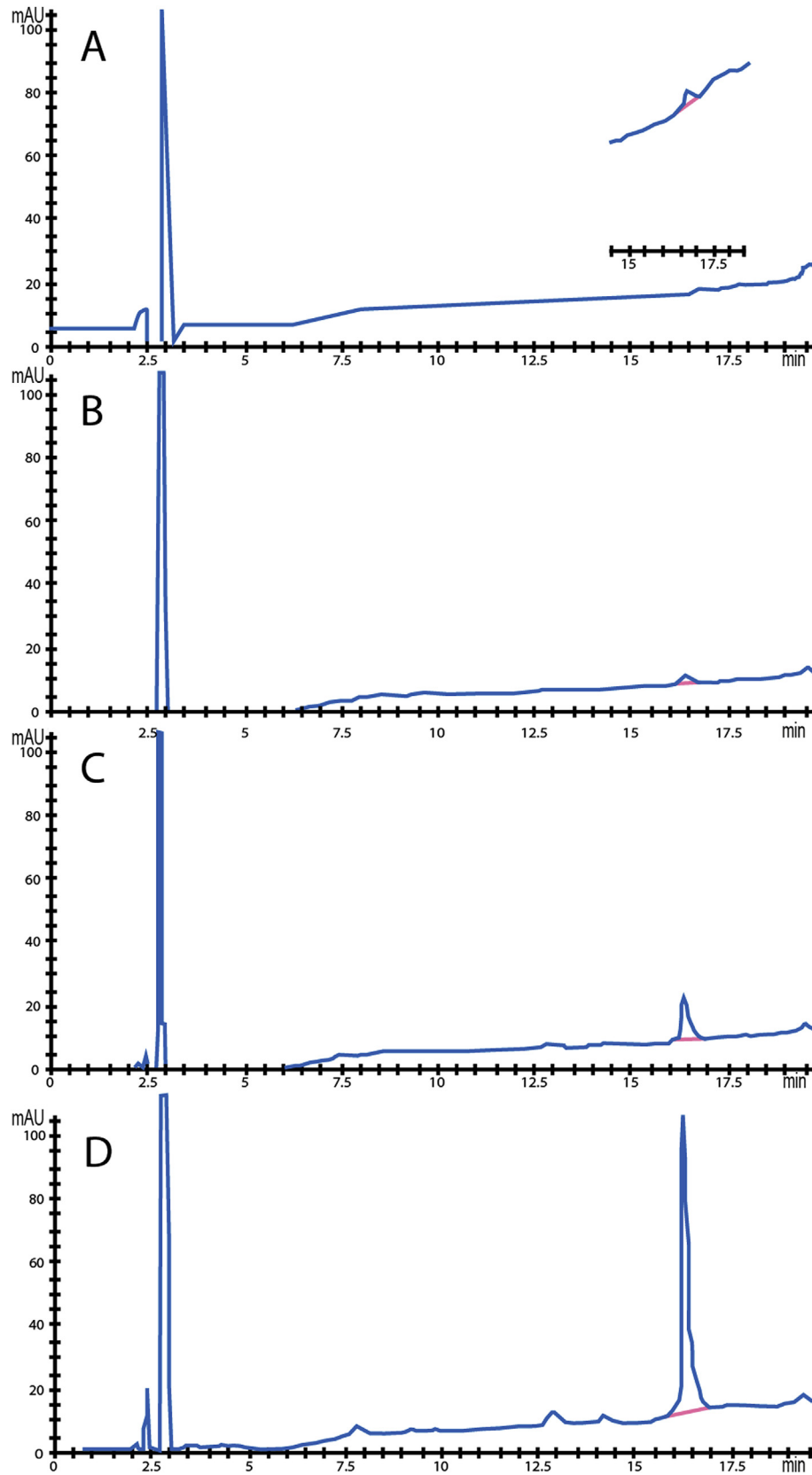| Toxin	% Glucose			
	0.25	0.5	0.75	1
LT (ng/mL)	49 ± 0.7	130 ± 2.1	522 ± 1.9	810 ± 6.1

Fig. 2. LC-UV chromatograms showing *E. coli* labile toxin production after the inoculation of 1 mL of the strain DSM 10973 in TSB media enriched with 0.25% glucose (A), 0.5% glucose (B), 0.75% glucose (C), and 1% glucose (D).

areas of analysed samples with the calibration curve of pure standards.

3.2. LC-UV analysis

Results obtained by VET-RPLA test have been also confirmed by LC-UV technique. Under the chromatographic conditions used in this study, the calibration curve shows good linear regression ($r^2 = 0.998$) and the limits of detection (LOD) and quantification (LOQ) are 15 ± 0.3 ng/mL and 45 ± 0.4 ng/mL, respectively. Both limits have been calculated according to s/n = 3 and s/n = 10, respectively. Retention time for LT is 16.48 min (Fig. 2). The recovery assays of the B chain have been carried out by adding the toxin standard to the bacteria culture inoculated with the non-toxigenic strain tested (CECT 405) and analysed in triplicate. Recovery for toxin assayed was 83.9% with a standard deviation of 1.3%.

4. Discussion

All culture media tested shows to be suitable for *E. coli* growth. Nevertheless, TSB has shown to be the most efficient culture medium for *E. coli* enterotoxin production. Although all our enterotoxigenic *E. coli* strains have proved capable of grow in different culture media according to previous studies (Gonzales et al., 2013; Rocha et al., 2013), not all culture broths tested are suitable to stimulate the production of the toxin.

In our study LT production by *E. coli* vary among strains and depending on the culture conditions; these results agree with the study of Rocha et al. (2013), who also found that results from different *E. coli* strains differs under the same conditions.

According to the works of Kunkel and Robertson (1979) and Hegde et al. (2009) the maximum LT production has been found when the strains are grown in TSB medium containing the maximum concentration of glucose.

Unlike us, Erume et al. (2010) found brain heart infusion broth as the best culture medium for supporting the production of ST. In our study TSB is the best medium to this purpose. It can be explained because although ST and LT toxins are synthetized by the same bacteria, the synthesis and the release of both of them are carried out by different mechanisms, so production conditions are different (Alderete and Robertson, 1977).

TSB contains glucose as a carbon source for bacteria growth, even so, several concentrations of glucose has been added to the broth to check if glucose addition also improves LT production. High levels of LT has been found in media culture with high glucose content. This is an expected outcome according to the work of Rosano and Ceccarelli (2014), which explained that glucose addition to the culture media can help to increase the protein expression in *E. coli*.

Labile toxin has been identified from our culture supernatants, as well as some researchers have done previously (Clements and Finkelstein, 1979; Dorner et al., 1976; Lasaro et al., 2006; Rocha et al., 2013). However, although those authors found LT in supernatant extracts, some of them also observed LT release from cell pellets (Lasaro et al., 2007). The analysis of the bacterial pellets from all the media tested in the present study have given negative results. We expected also to find positive results on pellet samples since literature shows that all LT toxin in general is not secreted into the culture supernatant during growth but remains cell associated being localized to either the periplasm or the cytoplasm. Even though that fact, our finding is supported by Rocha et al. (2013) who did not found significant differences when compared LT release from culture medium without treatment with LT release from culture medium after pellet treatment with three different

procedures. In our study, pellet samples were analysed once bacterial growth was in the stationary phase. Tauschek et al. (2002) also found that when stationary phase is reached, most of the LT can be found in the supernatant but not in the periplasm, demonstrating that some ETEC strains can secrete the toxin and LT does not remains cell-associated. Our results can also confirm the conclusions from previous studies, where is suspected that production and release of LT can vary among different ETEC strains (O'sullivan et al., 2007).

Levels of enterotoxin produced by ETEC strains tested are in the range of levels found by other authors with different growing conditions, and wild and reference strains. Having in account the range of LT concentration found in this and in previous studies (Hegde et al., 2009; Lasaro et al., 2006), LC-UV technique optimized in the present work can be a useful instrument for a rapid and sensitive detection of LT.

According to our results, the production of LT among different LT producing ETEC strains can vary over a wide range, Lasaro et al. (2006) found levels from a minimum of 49.8 ng/mL to more than 2400 ng/mL.

Determination and identification of enterotoxins using traditional biochemical tests are time consuming and one of the problems with antigen assays is that a false positive may set off an inappropriate and potentially expensive investigation (Grys et al., 2009). Although immunoassays are simple, quicker and present high sensitivity, immunological methods have some drawbacks and also have important limitations (Sospedra et al., 2013). However, one of the most advantages of the LC-UV detection is the specificity of the analytical response, that does not depend on the antibody-binding affinity and it is not biased by possible non-specific recognition.

In conclusion, our study demonstrates that the LC-UV technique proposed is simple to perform and avoid the possibility of false positive results because enables elimination of false diagnoses due to interferences or lack of specificity. LC-UV method allows a rapid identification and quantification of LT that could be also adapted to food matrices.

TSB spiked with 1% glucose is the most efficient culture medium for *E. coli* growth and enterotoxin production, even so LT production by *E. coli* can vary among ETEC strains.

Transparency document

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