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ALTERNATIVE PROCEDURE FOR THE PURIFICATION OF THE HEAT-STABLE ENTEROTOXIN OF ENTEROTOXIGENIC *ESCHERICHIA COLI* PATHOGENIC FOR CALVES

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SUMMARY

A method for purification of the heat-stable enterotoxin (ST) of enterotoxigenic *Escherichia coli* (ETEC) strains (C1444 and B41) pathogenic for calves and some physiochemical properties of the ST are described. The method involved ultrafiltration on PM-10 and UM-2 Diaflo membranes, acetone fractionation, ion-exchange chromatography on AG 1-X2, chromatofocusing and a combination of hydrophobic interaction chromatogrpahy on octyl-Sepharose CL-4B and gel-permeation on Bio-Gel P-2. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of fluorescamine-labeled purified, reduced and alkylated ST preparations revealed a single band with approximate molecular masses of 2500 and 2200 for the C1444 and B41 STs, respectively. For the C1444 ST, the final purification achieved was approximately 27 000-fold on the basis of absorbance at 280 nm per mouse effective dose. However, it was 2000-fold when calculated on the basis of mg protein per effective dose (5 ng). Amino acid composition of the C1444 ST was found to be different from that of the B41 ST suggesting that the ST produced by bovine isolates may be heterogeneous in their structure.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) play an important role in the etiology of diarrhoea in humans and animals [1–4]. The microorganisms cause diarrhoea by producing either a high-molecular-mass heat-labile enterotoxin (LT) and/or a low-molecular-mass heat-stable enterotoxin (ST) [1–3]. *E. coli* LT is

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immunochemically related to cholera enterotoxin, not only in its quaternary structure but also in its mechanism of action [1, 5], viz. activation of the adenylate cyclase–cyclic adenosine monophosphate system. In contrast, ETEC have been shown to produce one or more ST molecules [6, 7] and very little is known about their mechanism of action, although it has been suggested that intestinal fluid accumulation induced by ST may be mediated through an increase in the cyclic guanosine monophosphate levels [8]. In addition, ST generally has been reported to be non-immunogenic [2, 3, 9]. The prevalence of disease caused by ST-producing ETEC strains, the prevalence of these strains in food [10] and the uncertainty regarding the mechanism of action of ST has until recently been partly due to the lack of availability of a sensitive test for the detection of ST, the development of which depends on the availability of at least a highly-purified, if not homogeneous, preparation of ST. Recently, methods for purification to apparent homogeneity of the ST of ETEC of human and bovine origin have been described [11–13]. Methodology for isolating porcine STa (infant mouse positive toxin) in a highly purified state has also been described [14], although its homogeneity still needs to be demonstrated. In our hands, the procedure described for the purification of human ST [11] was found to yield bovine ST only in an enriched state. The method for preparing bovine ST had the disadvantage of employing relatively expensive high-performance liquid chromatographic (HPLC) equipment [13]. We describe here an alternative procedure for the purification of bovine ST to apparent homogeneity without the use of HPLC and some physico-chemical properties of that ST.

EXPERIMENTAL

Bacteria

Two ETEC strains (C1444 and B41) were used in this investigation. One (C1444) was isolated from an outbreak of calf scours in Australia. Its serotype was 020:KX106:K99$^+$,H$^-$ and it produced both ST and LT. The second strain, B41, was kindly donated by Professor H. William Smith (Houghton Poultry Research Station, Huntingdon, U.K.).

Growth conditions

ETEC strains were grown at 37°C in a batch fermenter (New Brunswick) containing 10 l of defined medium, pH 8.0 [14] for 12 h, since maximum levels of ST activity were detected at that period post-inoculation. The bacterial cultures was aerated at the rate of 3 l/min.

Preparation of crude toxic filtrate

The bacterial cultures were subjected to centrifugation at 12 350 *g* for 40 min in a Sorvall centrifuge (RC-2B) in order to sediment the intact ETEC, which were discarded. The supernatant was subjected to PM-10 and UM-2 ultrafiltration (Amicon, Australia) and the UM-2 ultrafiltration (Amicon, Australia) and the UM-2 retentate further subjected to acetone treatment as described previously [9].

Chromatography

While the ion-exchange chromatography was carried out on AG 1-X2 resin (Bio Rad, Australia) with formic acid as eluent [6], gel permeation chromatography was carried out using Bio-Gel P-2 (55.0×1.0 cm I.D.) equilibrated with distilled water. Removal of formic acid from various fractions was accomplished by neutralization with ammonium hydroxide followed by acetone refractionation and rotary evaporation. Hydrophobic interaction chromatography was carried out on Octyl-Sepharose CL-4B under conditions described in the text.

Chromatofocusing

Chromatofocusing was performed according to the instructions of the manufacturer (Pharmacia, Sydney, Australia) employing Polybuffer exchanger 94 (PBE94) (20.0×1.0 cm). The eluents employed in order were (i) Pharmalyte, pH range 3–10; (ii) Pharmalyte, pH range 2.5–5.0; and (iii) 1 *M* acetic acid. Removal of Pharmalytes from the active fractions was accomplished by a combination of procedures involving acetone fractionation and hydrophobic interaction chromatography, details of which will be reported in a separate communication.

Analytical polyacrylamide gel electrophoresis and molecular mass determination

Polyacrylamide gel electrophoresis in sodium dodecylsulphate (SDS) was done in 15% gels [15] using toxin preparations labelled with fluorescamine [16]. Following electrophoresis, the gels were placed on a UV transilluminator and distances of migration of marker proteins (BDH: M_r range 2512–16949; insulin and insulin B) and the ST measured. Confirmation of the band representing ST was obtained by repeated freezing (−70°C) and thawing (37°C) followed by determination of the ST activity using the infant mouse assay. The unhydrolyzed component of fluorescamine served as a tracking marker. An estimate of the relative mobility was calculated by dividing the distance of migration of the protein band by the distance migrated by the fluorescence tracking marker.

Amino acid analysis

Lyophilized purified C1444 and B41 ST preparations were dissolved in 6 *M* hydrochloric acid and hydrolyzed at 110°C for 24 h under vacuum. The amino acid composition was determined in an automated amino acid analyzer (JLC-6AH). Cysteine was determined, following performic acid oxidation, as cysteic acid.

Biochemical analysis

Protein concentration was determined by the method of Lowry et al. [17]. Methods used for the detection of DNA and RNA were those of Ceriotti [18], and Mukkur and Pyliotis [19], respectively. The lipopolysaccharide content was determined by using 2-keto-3-deoxyoctonate as a marker according to a modified procedure [19] developed from those of Weissbach and Hurwitz [20] and Osborn [21]. Reduction and alkylation were carried out using 2-mercaptoethanol and neutralized iodoacetic acid [22], respectively.

Infant mouse assay

The ST activity was determined using the infant mouse assay [23] which was standardized using ETEC C1444 and B41 strains. It was observed that 2–3 day old infant mice on intragastric inoculation with 0.1 ml of crude ST followed by incubation at ambient temperature (21–23°C) for 3 h yielded the best gut weight-to-residual body weight ratios. A ratio of equal to or greater than 0.090 was considered as positive and constituted one effective dose.

Assay for LT

The LT activity was assayed using Y-1 adrenal tumor cells [24]. The samples to be assayed were serially diluted in Ham's F-10 maintenance medium and 100 μl of each dilution was dispensed into two wells of a tissue culture plate which displayed a confluent monolayer of Y-1 cells. The tissue culture plates were incubated for 18 h in a tissue culture incubator set at 37°C and 5.1% carbon dioxide. The monolayers were examined using an inverted Olympus microscope. Rounding of more than 75% of the Y-1 monolayer was taken as a positive response.

RESULTS

The results described below were first obtained using ETEC C1444 and later extended to ETEC B41. Ultrafiltration on PM-10 membranes of the crude bacterial culture supernatant resulted in the removal of LT from the filtrate indicating that the molecular mass of this enterotoxin was greater than 10 000. Subsequent filtration of the PM-10 untrafiltrate on a UM-2 membrane resulted in a retention of the ST activity (UM-2 retentate). The UM-2 filtrate was devoid of ST activity. Based on absorbance at 280 nm a 1.4-fold purification was achieved (Table I) whereas a 4-fold purification was achieved based on protein concentration/effective dose (Table II). Recovery of activity was 87% (Tables I and II). However, the UM-2 retentate was dark brown in color. Although acetone-treatment of the retentate resulted in a loss of some ST activity, a substantial proportion of the contaminating proteins and macromolecules precipitated and were removed. Further, the color of the acetone-treated UM-2 retentate became orange-yellow instead of being brown. A purification of 65-fold and 44-fold was achieved based on $A_{280\,nm}$ units/effective dose and mg protein/effective dose, respectively (Tables I and II). When the acetone-soluble UM-2 retentate was subjected to chromatography on AG 1-X2, the majority of activity was eluted with 1.0 and 2.0 *M* formic acid, a small proportion being eluted with 3.0–5.0 *M* formic acid (Tables I and II). Therefore, furher data presented in this communication were deduced from experiments carried out with pooled fractions eluted with 1.0 and 2.0 *M* formic acid. The pool was subjected to chromatofocusing (Fig. 1). Whereas five peaks were obtained on elution with Pharmalyte (pH range 3–10), only one asymmetrical peak was eluted with Pharmalyte (pH range 2.5–5.0). There was no ST activity in the material comprising any of the peaks. When the pH of the eluted Pharmalyte reached 4.2, the gel was washed extensively with 0.025 *M* ethanolamine, pH 8.6, and then with 1.0 *M* acetic acid when a single, symmetrical peak possessing ST activity was realized. Residual Pharmalyte was removed using

TABLE I

PURIFICATION SUMMARY FOR HEAT-STABLE ENTEROTOXIN BASED ON ABSORBANCE PER EFFECTIVE DOSE

Preparation	Total absorbance ($A_{280\,nm}$ units)	Total effective dose	Absorbance per effective dose ($A_{280\,nm}$ units)	Purification factor	Recovery (%)
Crude supernatant★	129173	$5 \cdot 10^5$	$2.58 \cdot 10^{-1}$	1.0	100
PM-10 ultrafiltrate	109674	$4.8 \cdot 10^5$	$2.28 \cdot 10^{-1}$	1.1	96
UM-2 retentate	82382	$4.4 \cdot 10^5$	$1.89 \cdot 10^{-1}$	1.4	87
Acetone-treated UM-retentate	1379	$3.5 \cdot 10^5$	$3.94 \cdot 10^{-3}$	65.5	70
AG 1-X2 chromatography with:					
(i) water	285.5	–	–	–	–
(ii) 1.0 *M* formic acid	219.3	$1.28 \cdot 10^5$	$1.72 \cdot 10^{-3}$	150	26
(iii) 2.0 *M* formic acid	117.53	$1.46 \cdot 10^5$	$8.05 \cdot 10^{-4}$	320.5	29
(iv) 3.0 *M* formic acid	76.32	$0.24 \cdot 10^5$	$3.18 \cdot 10^{-3}$	81.1	4.8
(v) 4.0 *M* formic acid	40.23	$0.09 \cdot 10^5$	$4.52 \cdot 10^{-3}$	57.1	1.8
(vi) 5.0 *M* formic acid	52.51	$0.045 \cdot 10^5$	$1.18 \cdot 10^{-2}$	21.9	0.9
Pooled ST-positive fraction obtained by chromatofocusing	30.12	$1.5 \cdot 10^5$	$2.01 \cdot 10^{-4}$	1280	30
Gel-permeation on Bio-Gel P-2, of the ST-positive fraction obtained by chromatofocusing and hydrophobic interaction chromatography	0.35	$0.7 \cdot 10^5$	$9.7 \cdot 10^{-6}$	26598	14

★The crude supernatant used in this experiment was prepared using 50 l of bacterial culture grown in the defined medium.

a procedure which first involved mixing this fraction with ten volumes of cold acetone followed by centrifugation [25]. This resulted in substantial percentage of the contaminating Pharmalyte sedimenting to the bottom of the test tube, while the ST activity remained in the supernatant. The latter was concentrated by rotary evaporation, dissolved in distilled water and subjected to molecular sieve chromatogaphy on Bio-Gel P-2 equilibrated with water. One symmetrical peaks (I), one bimodal peak (III) and one broad peak (II) were realized (Fig. 2). While peaks I and III did not possess any ST activity, peak II did. Peak I was bluish in coloration, peak III yellowish, and the ST-positive peak II was colourless. Peak II was subjected to hydrophobic interaction chromatography on Octyl-Sepharose CL-4B equilibrated with 2 *M* ammonium sulphate. The fraction of Pharmalyte not precipitated by treatment with acetone eluted in the starting ammonium sulphate solution. No ST activity was detectable in this fraction. The solvent was changed to 1% ethylene glycol, and a single peak containing ST activity eluted. Following concentration by rotary evaporation, the ST-active fraction was subjected to gel filtration on Bio-Gel P-2 equilibrated with 1 *M* acetic acid. A single ST-positive peak free of ethylene glycol was obtained. The active mate-

TABLE II

PURIFICATION SUMMARY FOR HEAT-STABLE ENTEROTOXIN BASED ON PROTEIN CONTENT PER EFFECTIVE DOSE

N.D = Not determined.

Preparation	Total protein (mg)	Total effective dose	Protein content per effective dose (mg)	Purification factor	Recovery (%)
Crude supernatant★	5293	$5 \cdot 10^5$	$1.06 \cdot 10^{-2}$	1.0	100
PM-10 ultrafiltrate	3253	$4.8 \cdot 10^5$	$6.78 \cdot 10^{-3}$	1.6	96
UM-2 retentate	1116.3	$4.4 \cdot 10^5$	$2.56 \cdot 10^{-3}$	4.1	88
Acetone-treated UM-retentate	84.76	$3.5 \cdot 10^5$	$2.42 \cdot 10^{-4}$	43.8	70
AG 1-X2 chromatography with					
(i) water	28.75	–	–	–	–
(ii) 1.0 *M* formic acid	23.38	$1.28 \cdot 10^5$	$1.83 \cdot 10^{-4}$	57.7	26
(iii) 2.0 *M* formic acid	6.82	$1.46 \cdot 10^5$	$4.67 \cdot 10^{-5}$	226.6	29
(iv) 3.0 *M* formic acid	4.68	$0.24 \cdot 10^5$	$1.95 \cdot 10^{-4}$	54.3	4.8
(v) 4.0 *M* formic acid	2.79	$0.09 \cdot 10^5$	$3.13 \cdot 10^{-4}$	33.8	1.8
(vi) 5.0 *M* formic acid	2.95	$0.04 \cdot 10^5$	$6.62 \cdot 10^{-4}$	16.0	0.9
Pooled ST-positive fraction obtained by chromatofocusing	N.D.	$1.5 \cdot 10^5$	N.D.	N.D.	30
Gel-Permeation on Bio-Gel P-2, of the ST-positive fraction obtained by chromatofocusing and hydrophobic interaction chromatography	0.35	$0.70 \cdot 10^5$	$5.0 \cdot 10^{-6}$	2120	14

★The crude supernatant used in this experiment was prepared using 50 l of bacterial culture grown in the defined medium.

rial was tagged with fluorescamine and subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulphate. A single band with a molecular mass of 2511 ± 200 was obtained (Fig. 3). The final recovery of ST activity was 14% and the total yield of purified ST was 35 μg/l of culture medium. A purification of 26 598-fold and 2120-fold on the basis of $A_{280\ nm}$ units/effective dose and mg protein/effective dose, respectively, was calculated (Tables I and II). Purified C1444 ST exhibited a typical dose response curve (data not shown) 5 ng comprising one effective dose. The UV absorption profile of purified ST had a maximum at 275 nm (Fig. 4) in contrast to 260 nm for the UM-2 retentate. Amino acid analysis of purified C1444 ST (Table III) revealed the presence of four cysteine residues per mole of ST. Other amino acids present included glycine, aspartic acid, alanine and tyrosine. Amino acid composition of purified B41 ST (prepared by using a method identical to that described in the text for the purification of C1444 ST) was different from that of C1444 ST although it was similar to that reported previously [12]. The UV absorption profile, however, was similar to that obtained

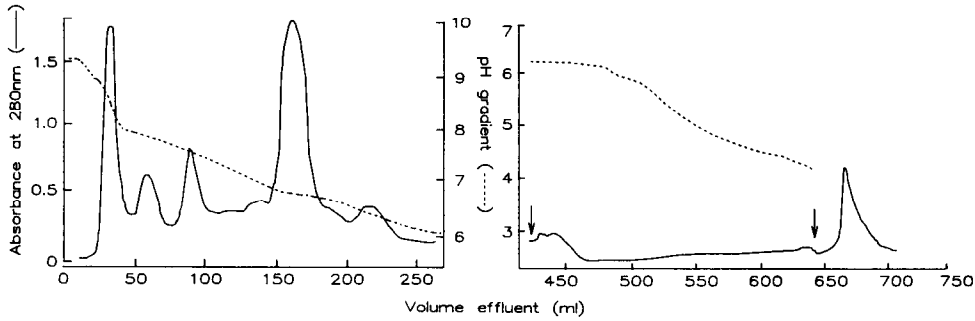

Fig. 1. Chromatofocusing of the ST-enriched fraction obtained by elution of ST activity from AG 1-X2 with 1.0 and 2.0 *M* formic acid. —, $A_{280\,nm}$; - - - -, pH gradient. The first arrow (413 ml) denotes change of eluting Pharmalyte from 3-10 to 2.5-5.0. The second arrow at 643 ml denotes change of eluting solvent to 1.0 *M* acetic acid.

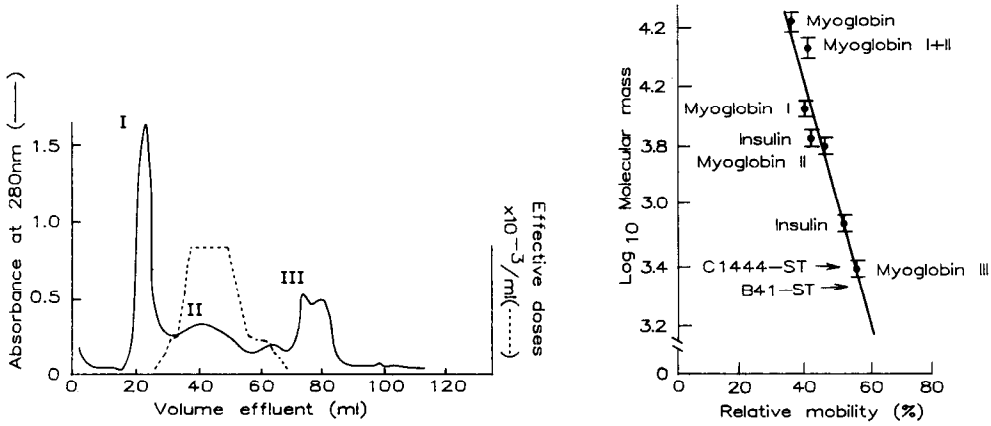

Fig. 2. Gel-permeation of ST-enriched fraction obtained by chromatofocusing, on Bio-Gel P-2 equilibrated with distilled water. —, $A_{280\,nm}$; - - - -, ST activity.

Fig. 3. Molecular mass calibration curve constructed using BDH molecular mass markers, insulin and insulin B.

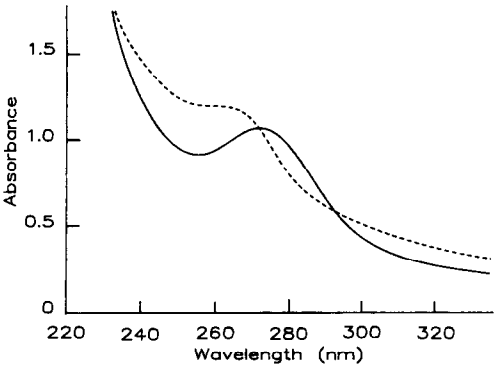

Fig. 4. Ultraviolet absorption spectrum for UM-2 retentate (C1444) (broken line) and purified ST (C1444) (solid line).

TABLE III

AMINO ACID COMPOSITION OF ST PURIFIED FROM BOVINE ETEC STRAINS C1444 AND B41

Amino acid	Moles amino acid residues per mole of ST purified from bovine ETEC	
	C1444	B41
Alanine	2	2
Aspartic acid	3	2
Cysteine	4	6
Glutamic acid	1	1
Glycine	4	1
Leucine	1	1
Phenylalanine	1	1
Proline	1	1
Threonine	1	1
Tyrosine	2	2

for C1444 ST. The molecular mass of purified B41 ST, determined by polyacrylamide gel electrophoresis was 2219 ± 160.

DISCUSSION

Treatment of the darkbrown UM-2 retentate above with acetone proved to be an important step in that a significant amount of colour and substantial contamination with ST-negative proteins and other macromolecules was removed in the insoluble fraction, thus confirming previously reported results [14]. The acetone-soluble fraction of the UM-2 retentate was orange-yellow, and attempts to purify ST free of this colour by a combination of procedures, such as gel permeation chromatography (Sephadex G-25, Bio-Gel P-2, P-4 and P-10), ion-exchange chromatography on DEAE Bio-Gel A, adsorption chromatography on hydroxyapatite, and preparative isoelectric focusing, were unsuccessful (data not shown). Although the use of dye-ligand chromatography yielded some purification of ST activity, particularly using red A and green A (Amicon, Australia), the ST-active preparations were still associated with the original colour (data not shown). Attempts to remove the contaminating colour with cartridges containing supports such as Sep-Pak C_{18}, silica and Fluorosil frequently used in HPLC were ineffective (data not shown). Majority of the ST activity eluted form AG 1-X2 with 1 and 2 *M* formic acid in contrast to a previous report [6] whereby a significant proportion of ST was eluted with 3.7 *M* formic acid. This disparity confirms the heterogeneity in the ST produced by different strains of ETEC. Although our attempts to purify ST by preparative isoelectric focusing according to Madsen and Knoop [26] were unsuccessful, we did determine its isoelectric point (p*I*) to be 3.9 (data not shown). This value was in agreement with that reported for ST of human origin [26, 27]. In contrast no ST activity with a p*I* of 1.5, as reported previously for an ST fraction of human origin [26] was observed. On

chromatofocusing, the ST activity could be eluted with 1 *M* acetic acid after the pH of the Pharmalyte eluted from the PBE94 column reached 4.2. It should be noted that many additional ST-negative contaminants were removed by chromatofocusing. Following removal of Pharmalytes by a acetone-treatment and fractionation on Octyl-Sepharose CL-4B, C1444 and B41 STs were found apparently to be homogeneous as judged by the presence of a single band on polyacrylamide gel electrophoresis in SDS of the fluorescamine-labeled toxins. A major problem was encountered in the photography of fluorescent gels. Purified ST which appeared as a highly fluorescent band in the gel could not be photographed. In contrast the fluorescent tracking marker represented by unhydrolyzed fluorescamine appeared as a faint band in the gel but was photographed as a dark-band (photograph not shown). Several different types of films and filters were employed but without success. This difficulty in photography also was reported by Madsen and Knoop [26], although the application of this technique for the calculation of molecular mass was not attempted. The molecular masses of purified C1444 ST and B41 ST calculated from the amino acid compositions were 2400 and 2284, respectively. These values essentially agree with those reported by Staples et al. [11] for human ST and Saeed et al. [12] for bovine ST, but were lower than the values reported by Takeda et al. [27] for human ST and that reported for a porcine ST by Alderete and Robertson [14]. The total purification achieved was generally within a similar order of magnitude as reported previously [13] although a 13-fold greater purification of ST was calculated on the basis of absorbance at 280 nm per effective dose than mg protein per effective dose. This pointed out the usefulness of techniques such as chromatofocusing and hydrophobic interaction chromatography in the removal of non-protein contaminants. Amino acid composition of the B41 ST was similar to that reported previously [12] whereas that of C1444 ST was different in the number of aspartic acid, cysteine and glycine residues. However, its properties were similar to those described previously [13, 28] in spite of the fewer number of cysteine residues. Although the percentage recovery of ST activity and the final yields were lower than that reported previously [12], our method is a useful alternative for purification of bovine ST. The amino acid composition of B41 ST obtained in this investigation was identical to that reported earlier [13] where the use of HPLC was essential for removal of contaminants including Pharmalytes. The fact that amino acid composition of the C1444 ST was different from that reported for four other bovine ST preparations (including that of B41) clearly suggests that the STs of bovine origin may be structurally heterogeneous which is in contrast to the observations reported previously [13]. As a first priority, therefore, it will be necessary to determine if the epitopes responsible for the toxicity of C1444 ST and B41 ST are partially or completely identical. It would then be appropriate to modify purified ST of bovine origin into an immunogenic form for the development of sensitive tests, similar to those reported for ST of porcine [29] and human origin [30], for its detection in contaminated food and fecal matter of infected animals, and to evaluate the possible contribution of antitoxic immunity in the control of the diarrheal disease syndrome.

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