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Purification and Characterization of Cytotoxin Produced by a Clinical Isolate of *Vibrio cholerae* O54 TV113

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Abstract *Vibrio cholerae* O54 TV113 isolated from a diarrheal patient produces an extracellular cytotoxin that caused alteration in the morphology of Chinese hamster ovary cells manifested as cell shrinkage with intact cell boundaries and finally causing cell death. Syncase medium supplemented with lincomycin (50 μg/ml), pH 7.2, and 18 h incubation with shaking at 37 °C supported optimal cytotoxin production. We isolated and purified this cytotoxin to homogeneity by ultrafiltration, 40–80 % ammonium sulfate precipitation, gradient–anion exchange chromatography, stepwise-anion exchange chromatography, and size exclusion chromatography increasing the specific activity by 866-fold. The cytotoxin is heat-labile, sensitive to protease and papain, and has a molecular weight of 64 kDa determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and enterotoxic activity in rabbit ileal loop assay. Both cytotoxic and enterotoxic activity could be inhibited or neutralized by antiserum raised against purified cytotoxin but not by preimmune serum. Immunodiffusion test between purified cytotoxin and its antiserum gave a single well-defined precipitin band showing reaction of complete identity and a well-defined single band in an immunoblot assay. This study thus indicate that the cytotoxin expressed by strain TV113 has both cytotoxic and enterotoxic activity and appears to contribute in pathogenesis of non-O1, non-O139 strains.

Keywords Purification · Characterization · Cytotoxin · Chromatography · SDS-PAGE · *Vibrio cholerae* · O54 TV113

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Introduction

Vibrio cholerae, an autochthonous inhabitant of brackish water and estuarine systems, is the causative agent of the disease cholera [1]. The symptoms of cholera include profuse watery diarrhea accompanied by abdominal pain, vomiting, fever, hypervoluminous shock, and acidosis. Whereas toxigenic *V. cholerae* O1 and O139 serogroups caused epidemic cholera [2], the non-O1, non-O139 *V. cholerae* were associated with sporadic cases of diarrhea and extra-intestinal infections [3]. Unlike epidemic *V. cholerae* O1 and O139, the non-O1, non-O139 produced a variety of putative virulence factors including choleralike enterotoxin [4], El Tor hemolysin [5–7], thermostable direct hemolysin [8], Shigalike toxin [9], cytolysin [10], heat-stable enterotoxin (NAG-ST) [11], new cholera toxin (CT) [12], a nonmembrane-damaging cytotoxin (NMDCY) [13], hemagglutinin protease [14], and serine protease [15] of which few or all had been reported playing a role in clinical manifestation of non-O1 cholera. Morris [3] demonstrated that, in the presence of adequate colonization factor(s), *V. cholerae* non-O1, which do not produce CT, can cause choleralike illness in humans.

Until recently, it was thought that clinical manifestation of cholera results primarily due to production of CT. However, occurrence of mild to moderate diarrhea in human volunteers fed with genetically engineered mutant *V. cholerae* strains, incapable of producing biologically active CT, prompted investigators to search for additional toxins that can contribute to the pathogenesis of cholera. In this study, we report production of a cytotoxin that evoke cytotoxic effect on Chinese hamster ovary (CHO) cells and caused fluid accumulation and hemorrhage in rabbit ileal loop assay by a clinical isolate of *V. cholerae* non-O1, non-O139 TV113 belonging to serogroup O54. We also present the method of purification of this cytotoxin and characterization of its biological and physicochemical properties.

Materials and Methods

Bacterial Strain

A clinical isolate of *V. cholerae* non-O1, non-O139 TV113 belonging to serogroup O54, isolated from a patient with diarrhea in Trivandrum, India, in 2000 and characterized biochemically and serogrouped at the National Institute of Cholera and Enteric Diseases, Kolkata, India, was used in this study. This strain was negative by PCR for the *ctx* (CT), *zot* (zonula occludens toxin), *ace* (accessory cholera enterotoxin), *tcpA* (toxin coregulated pilus), *ompU* (outer membrane protein), and *st* (heat-stable toxin) genes, but possessed the structural gene for El Tor hemolysin (*hlyA*) and *toxR* [16], and did not produce CT when checked by CT-enzyme linked immunosorbent assay, but produced a cytotoxic effect on CHO cells [17]. *V. cholerae* 569B biotype classical from laboratory stock was used as positive control in rabbit ileal loop assay. These strains were maintained in Tryptic Soy Broth (TSB) supplemented with 20 % glycerol at −80 °C.

Optimization of Growth Conditions for Production of the Cytotoxin

(i) *Medium*—Different media, such as Brain Heart Infusion Broth (BHIB, Difco, USA), TSB (Difco), AKI medium [18], and Syncase medium [19], were used to determine the most suitable medium for scaling up the production of cytotoxin by clinical isolate of *V. cholerae* O54 TV113.

(ii) *Culture conditions*—Three different culture conditions, namely, shaking, static, and both (6 h of static followed by 18 h of shaking incubation) [20], were used to determine the optimal condition for production of cytotoxin. For each culture condition, strain was incubated either at 30 or 37 °C to determine the suitable temperature for cytotoxin production.

(iii) *pH*—Upon selection of the suitable medium and optimal cultural conditions, the pH of the chosen medium was adjusted in the range between 4.5 and 9.5 with 5 N HCl or 5 N NaOH. *V. cholerae* O54 TV113 was examined at 37 °C under shaking condition to establish optimal pH supporting better production of cytotoxin. Uninoculated medium was concurrently added into the cell culture assay as a control to assess whether the pH per se of the medium also affect the cell line.

(iv) *Supplements*—Syncase medium, which was selected as the suitable basal medium for the production of cytotoxin, was supplemented with NaCl (0.5 %–1.0 %), sucrose (0.5 %–1.0 %), and antibiotic lincomycin (20 μg/ml–200 μg/ml) separately and together to check if any of the supplements or its combination enhanced cytotoxin production.

Effect of Cytotoxin on CHO Cells

The cytotoxic activity of the secreted cytotoxin was assayed in 96-well flat-bottomed tissue culture plates (Nunc, USA) with CHO cells (National Center for Cell Science, India) grown in Dulbecco's Modified Eagle Medium (Gibco-BRL, USA), supplemented with 10 % FBS, Penicillin G and streptomycin sulfate (Sigma, USA), and incubated at 37 °C with 5 % CO_2 (Haereus, Germany). CHO cells were grown with Eagle-modified minimum essential medium (Gibco-BRL) containing 10 % FBS. Culture supernatant was sterilized by filtration (0.22 μm pore size diameter, Millipore, USA) and diluted in tissue culture medium containing 2 % FBS. Neat and diluted culture supernatant of *V. cholerae* O54 TV113 was then added to CHO cells, after aspiration of the medium and incubated in a CO_2 incubator. After 24 h, cells were examined for morphological and cytopathic changes with a phase contrast inverted microscope (Axiovert, Carl Zeiss, Germany). The titer of cytotoxic activity in a sample was defined as the reciprocal of the maximum dilution of the sample that showed 100 % cytopathic changes in CHO cells. The specific activity of a sample was defined as the ratio of the reciprocal titer of cytotoxic activity to the protein concentration. The viability of toxin-treated cells was determined by trypan blue dye exclusion technique.

Hemolysin Assay

A hemolysis assay was performed with 1 % rabbit erythrocytes as described by Rowe and Welch [21]. Culture supernatant, crude toxin, partially purified toxin, and purified cytotoxin were serially diluted in phosphate-buffered saline (PBS, pH 7.4) and incubated with equal volume of 1 % rabbit erythrocytes or PBS at room temperature for 1 h and then centrifuged at $800 \times g$ for 10 min to remove unlysed cells. Released hemoglobin was assayed spectrophotometrically at 540 nm. Complete hemolysis was defined as the absorbance of the same number of erythrocytes lysed with detergent.

Purification of Cytotoxin

Entire purification was performed at 4 °C. Fresh modified syncase medium containing lincomycin (50 μg/ml) with seed culture of *V. cholerae* O54 TV113 were incubated at

37 °C for 18 h with shaking at 180 oscillations/min. Culture supernatant was recovered by centrifugation at 10,000 rpm and filtered through 0.22 μm membrane filter (Millipore). To rule out the action of metalloproteases, a cocktail of inhibitors containing 0.5 M EDTA, 0.2 M PMSF, and general protease inhibitor cocktail (Sigma) was added to the culture filtrate. The culture filtrate was concentrated tenfold by ultrafiltration (PM-10 membrane filter, Amicon, USA) followed by 40 % ammonium sulfate saturation, the precipitate discarded. Further ammonium sulfate saturation was done from 40 % to 80 %, kept overnight at 4 °C, and centrifuged at 20,000×*g* for 30 min at 4 °C in a Beckman ultracentrifuge (Model J2-MC, USA). The precipitate was dissolved in 10 ml of 0.02 M sodium PBS (0.02 M PBS, pH 7.2), and dialyzed against 0.04 M sodium phosphate buffer (pH 7.2) at 4 °C until complete removal of ammonium salts. This crude toxin was applied to an anion exchange column (1.6×40 cm column, Bio-Rad, USA) of DE52 resin (Whatman Laboratory, England) previously equilibrated with 10 mM Tris–HCl buffer, pH 7.2, and eluted at 4 °C with same buffer containing 0.0–2.0 M KCl. Fractions exhibiting cytotoxic activity were pooled, dialyzed, and concentrated by ultrafiltration. The concentrated DE-52 fractions were then subjected to stepwise anion exchange DE52 and then eluted with buffer containing 0.4–1.2 M KCl. Dialysed fractions exhibiting cytotoxic activity in cell lines were pooled, concentrated by ultrafiltration and then eluted with Sephadex G-100 column with 10 mM sodium phosphate buffer containing 0.3 M NaCl at a flow rate of 10 ml/h. Fractions constituting the peaks were pooled, dialyzed, concentrated, and then examined for cytotoxic and enterotoxic activity. At all steps of the purification, protein was estimated with Bradford's reagent (Sigma) at 37 °C according to the manufacturer's instruction. Bovine serum albumin was used as control.

PAGE and SDS-PAGE

Native gel electrophoresis was performed by the method of Schagger and von Jagow [22] on 12 % acrylamide gel without sodium dodecyl sulfate (SDS) with a discontinuous buffer system at a constant voltage of 60 V for the stacking gel and 100 V for the resolving gel. Samples were loaded without boiling in loading buffer (10 % glycerol, 0.05 % bromophenol blue, 10 mM Tris–HCl, pH 6.8) without SDS and 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis (PAGE) in 0.1 % SDS was carried by the method of Laemmili [23] on 12 % polyacrylamide gel with a discontinuous buffer system at a constant voltage of 60 V for the stacking gel and 100 V for the resolving gel. Before electrophoresis, samples were boiled for 5 min in the presence of 5 % 2-mercaptoethanol in loading buffer (10 % glycerol, 0.05 % bromophenol blue, 2 % SDS, 10 mM Tris–HCl, pH 6.8). Gel were stained with Coomassie brilliant blue R250 (Sigma) and then destained. All chemicals and molecular weight markers used for this purpose were from GE Healthcare, Sweden.

Polyclonal Antibody Preparation

Antisera to purified cytotoxin was prepared by immunizing New Zealand white rabbits weighing 2.0–2.5 kg by intramuscular and subcutaneous injections with 100 μg of cytotoxin emulsified with an equal volume of Freund's incomplete adjuvant (Gibco-BRL). This was followed by six booster injections of Freund's complete adjuvant at 14-day intervals. Three days after the last injection, the animals were sacrificed and bled. Serum was separated, filtered through 0.22 μm membrane (Millipore), and stored at −20 °C. The reactivity of cytotoxin with serum was examined by Ouchterlony immunodiffusion test [24].

Western Blotting

The proteins separated by SDS-PAGE were transferred to nitrocellulose membrane (0.45 μm Millipore) electrophoretically with a transblot apparatus (Bio-Rad) at 90 V for 1 h at 10 °C in transfer buffer [20 mM Tris, 150 mM glycine, 20 % methanol (v/v), pH 8.3]. Strips were then blocked with 2 % protease-free BSA in Tris-buffered saline (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) at 37 °C for 2 h, washed, and incubated sequentially with polyclonal antibody against cytotoxin [1:100 dilution (v/v)] and goat anti-rabbit immunoglobulin G (Sigma) conjugated to alkaline phosphatase [1:2000 dilution (v/v)]. Color was developed by 5-bromo-4-choloro-3-indolylphosphate and nitroblue tetrazolium solution in dark (BCIP-NBT; Sigma), and reaction was stopped by adding 2 N H_2SO_4.

Characterization of Cytotoxin

The following treatment was performed to further define the cytotoxin. (i) For protease enzyme treatment, 50 μg/ml of the purified cytotoxin was incubated for 1 h with 0.25 mg/ml of trypsin, pronase and papain (Sigma). Soyabean trypsin inhibitor (5 μg/ml, Sigma) was added before the performance of the cell culture assay for cytotoxin. The cytotoxic activity observed was compared with that of control incubated with the same volume of medium or with trypsin that has been previously incubated at 37 °C overnight with trypsin inhibitor. (ii) For checking heat stability, a sample of purified cytotoxic factor (50 μg) was heated at 50, 60, and 100 °C for 10 min. The residual cytotoxic activity was determined and compared with that of the unheated control. (iii) For checking the stability of protein at varying pH, samples of purified cytotoxin were dialyzed against buffers of pH 3.0, 4.0, 5.0, 6.0, 7.4, 8.0, 9.0, and 10.0, and pH was readjusted to 7.2. The residual cytotoxicity was then checked in cell culture as described above and compared with that of the untreated control (iv). Inhibition studies were performed with polyclonal anti-cytotoxin antibody. Serial dilutions of cytotoxin (100 μg) were inoculated in 96-well plates (50 μl/well). Cytotoxic factor was then incubated for 4 h at 37 °C with 50 μl of polyclonal antibody. After the preincubation, the mixture was added to CHO cells and the assay was completed as described above. Inhibition of cytotoxic activity was determined by the ability of the antibody to abolish cytotoxic activity compared to control.

Rabbit Ileal Loop Assay

The rabbit ileal loop assay was performed in New Zealand White rabbits (about 2.0 kg) by the methods of De and Chatterjee [25]. Rabbits were subjected to fasting for 24 h prior to the experiment, providing only water to drink ad libitum. Animals were anesthetized with ketamine (35 mg/kg body weight, Sigma) and xylazine (5 mg/kg body weight, Sigma) intramuscularly. Concentrated culture filtrate (1-ml volume) and partially purified sample obtained at each stage of purification was injected into each 8-cm ileal loop. Cell pellet of overnight culture of *V. cholerae* O54 TV113 in modified syncase medium was washed and resuspended in PBS (pH 7.4), and then 1-ml inoculum was introduced into the ileal loop. Modified syncase medium and PBS were used as negative controls, while purified CT (5 μg) was used as positive control. After 18 h, animals were sacrificed, and the enterotoxicity was expressed as volume-to-length ratio (fluid accumulation ratio) with a ratio greater than 0.4 indicating a positive response. A negative response was defined as loops with no fluid accumulation or a fluid ratio of less than 0.02. Neutralization of biological activity was performed by mixing undiluted anti-cytotoxin serum and a sample of 50 μg/ml of purified

cytotoxin incubated for 60 min at 37 °C, and then the rabbit ileal loop assay was performed as described above.

Histopathology

For histopathological studies, intestinal tissue samples were fixed in 10 % formalin for 48 h, dehydrated successively three times in acetone (30 min each), acetone: xylene mixture (1:1 ratio; 20 min), and finally, in xylene (20 min). Tissues were immersed in molten paraffin wax at 65 °C for 1 h and impregnated in fresh paraffin wax overnight and finally embedded in paraffin wax. Sections of tissue were made using a microtome (SIPCON) on to poly L-lysine-coated slides, dewaxed by keeping the slide at 58 °C for 10 min, followed by three changes in xylene for 30 min each. Sections were rehydrated in alcohol in 100 %, 90 %, 80 %, and 75 %, and finally, in distilled water, stained with hematoxylin and washed with running water for 10 min. Slides were dipped in a solution containing 70 % alcohol and 1 % HCl for 3 s and counter-stained with eosin. Slides were mounted in DPX and images were captured using inverted phase contrast microscope (Nikon Eclipse, Japan).

Results

Effect of Cytotoxin on Tissue Culture

Culture supernatant of *V. cholerae* O54 TV113 exhibited alteration in the morphology of CHO cells manifested as cell shrinkage with intact cell boundaries and finally cell death (Fig. 1a, b, c, d). There was complete disruption of the monolayer and dislodgement of the cells from the plate surface and cytotoxicity was accompanied by membrane damage when examined by trypan blue assay. The onset of cytotoxicity was delayed by 4–6 h depending on the concentration of cytotoxin in the supernatant. The cytotoxic activity was not inhibited by antitoxins against CT, El Tor hemolysin and NMDCY, indicating that this cytotoxic factor has no apparent immunological relationships with the abovementioned toxins.

Optimum Condition for Cytotoxin Production

A variety of media and different culture conditions including different incubation temperatures were assessed to determine optimum conditions for production of the cytotoxin. The

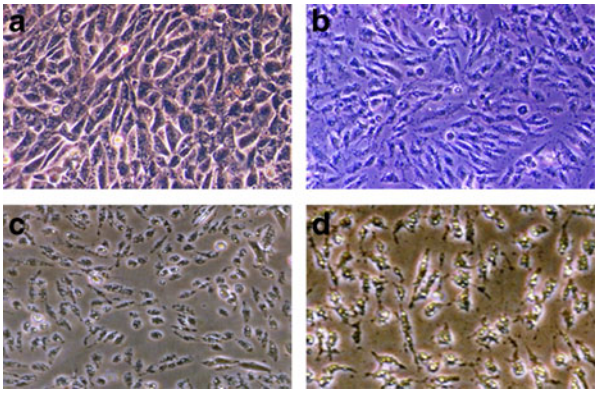

Fig. 1 Normal CHO cell (**a**), and cytotoxic effect which manifest as a kind of shrinkage with cell boundaries intact and cell death evoked by partially purified cytotoxin from *Vibrio cholerae* O54 TV113 after 4 h (**b**), after 8 h (**c**), and after 12 h (**d**), respectively

cytotoxicity of cytotoxin was measured in terms of the highest dilution of cytotoxin showing 100 % cytotoxicity in CHO cells. Syncase medium supplemented with lincomycin (50 μg/ml), pH 7.2, and incubation at 37 °C with shaking for 18 h was optimal conditions for better production of cytotoxin.

Purification of Cytotoxin

The culture supernatant of *V. cholerae* O54 TV113 was concentrated tenfold by ultrafiltration followed by ammonium sulfate precipitation. The dialysed fraction of 40–80 % ammonium sulfate precipitate showing cytotoxic activity in CHO cells were pooled and applied to gradient anion exchange (DE52) column (Fig. 2a). From the elution pattern of gradient anion exchange column (0.0–2.0 M KCl), it was clear that fraction exhibiting cytotoxic activity was eluted in the later fractions with 1.0–1.2 M KCl salt concentration. The cytotoxic active fractions were pooled, concentrated, and subjected to stepwise anion exchange chromatography. Elution curve from this stage of purification shown in Fig. 2b indicates that cytotoxic factor eluted in a single peak with active fractions at around 1.2 M KCl salt concentration. The fractions showing cytotoxic activity were further pooled, concentrated, and subjected to size exclusion (Sephadex G100) column chromatography. The elution pattern (Fig. 2c) indicated that cytotoxin was eluted just after the void volume. At this stage, the specific activity of the cytotoxin was increased about 688-fold and final recovery rate was about 3.1 %. The purification of cytotoxin is summarized in Table 1. The specific activity of the cytotoxin gradually increased with the decline in the amount of protein, suggesting true purification of the cytotoxin. Determination of the homogeneity of purified cytotoxin by native PAGE (Fig. 3a) and structural conformation analysis by SDS-PAGE yielded a band in approximately 64 kDa region (Fig. 3b), indicating that this cytotoxic factor is a single protein.

Immunological Characterization

Polyclonal antibody raised against purified cytotoxin showed a reciprocal titer of 16 in immunodiffusion test. When the polyclonal antibody was used in immunoblot assay with the cytotoxin antigen, a well-defined single band (Fig. 4a) was observed in the 64 kDa region. Preimmune rabbit serum was used in the immunoblot experiment as the negative control showed no reactivity. Immunodiffusion test carried out between purified cytotoxin as antigen and the polyclonal antibody gave a single well-defined precipitin band against the cytotoxin showing reaction of complete identity (Fig. 4b). This cytotoxin did not give any precipitin band against anti-hemolysin, anti-NMDCY, and anti-CT, indicating that the cytotoxin is antigenically different from these toxins and the El Tor hemolysin (data not shown).

Physiochemical Properties

The cytotoxic activity of the purified cytotoxin was lost on heating at 60 and 100 °C for 10 min, but was stable at −20 °C for 7 days and at 4 °C for 20 days. Treatment of purified cytotoxin with trypsin (1:2 dilution of 0.25 mg/ml) did not show any effect on cytotoxic activity, but treatment with protease and papain (1:64 dilutions of 0.25 mg/ml) caused complete loss of activity. The cytotoxic activity of the purified cytotoxin was markedly reduced after dialysis in buffers of pH 3 and 8 for 18 h but showed maximum biological activity at pH 7.0. Treatment with chemicals like urea and SDS showed complete loss of cytotoxic activity.

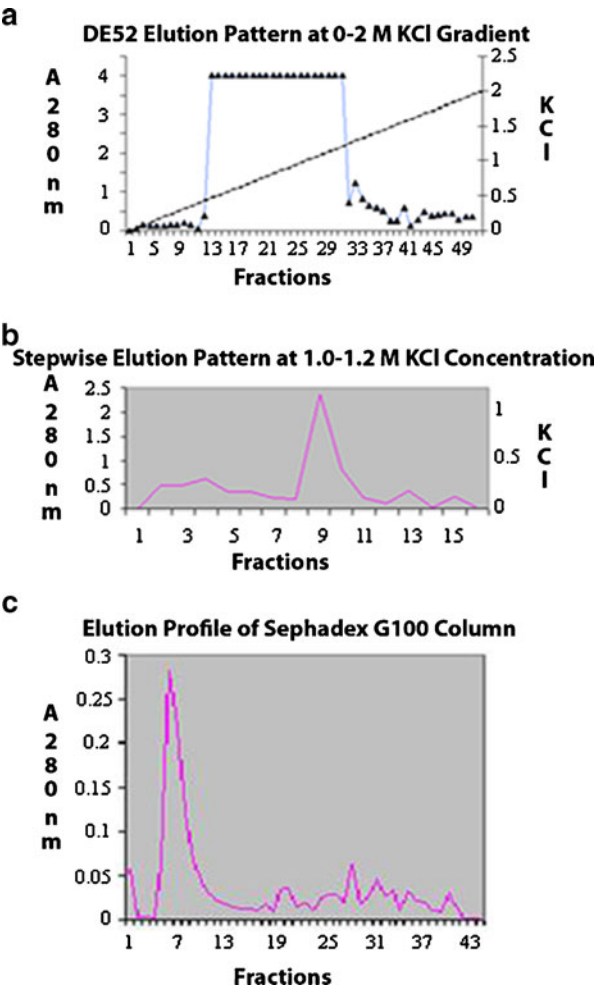

Fig. 2 Chromatography profile of purification of cytotoxin produced by the *Vibrio cholerae* O54 TV113. **a** Elution pattern of tenfold concentrated culture supernatant followed by ammonium sulfate saturation (40–80 %) on a gradient (0.0–2.0 M KCl) anion exchange DE52 column. **b** Stepwise anion exchange DE52 column pooled cytotoxic fraction from gradient DE52. **c** Elution profile of Sephadex G100 column of pooled cytotoxin fraction from stepwise DE52 using 10 mM sodium phosphate buffer, pH 7.2. The active fraction eluted just after the void volume was seen in the graph

Table 1 Summary of purification of cytotoxin produced by *V. cholerae* O54 strain TV113

Stage purification step	Vol. (ml)	Protein		Cytotoxic activity		Specific activity	Recovery (%)	Fold purification
		mg/ml	Total mg	CA/ml	Total CA			
1. Culture supernatant	4000	9.2	36,800.0	1.6×10^{2}	6.40×10^{5}	0.17×10^{2}	100.0	1
2. Ultra-filtration	400	74.2	29,680.0	6.4×10^{2}	2.50×10^{5}	0.08×10^{2}	39.0	0.47
3. Ammonium sulfate precipitation	29	64.4	1288.0	3.2×10^{3}	6.40×10^{4}	0.49×10^{2}	10.0	2.88
4. Anion exchange chromatography with DE52	12 ml	0.54	6.5	1.8×10^{3}	2.16×10^{4}	3.32×10^{3}	3.375	195.29
5. Gel filtration with Sephadex G100	5 ml	0.34	1.7	0.4×10^{4}	2.00×10^{4}	1.17×10^{4}	3.125	688.24

CA Cell shrinking unit defined as the reciprocal of the highest dilution causing 100 % cytotoxic activity

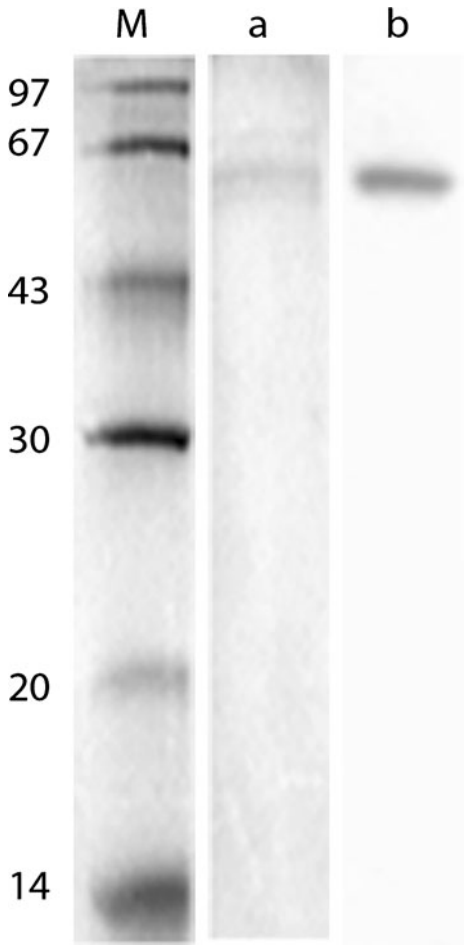

Fig. 3 Migration of purified cytotoxin from *Vibrio cholerae* O54 TV113 obtained after gel filtration chromatography on **a** Native PAGE, **b** SDS-PAGE (12 % acrylamide). *M* Migration of marker protein of known molecular mass (GE Healthcare)

Biological Activity and Histopathology

The biological activity of the purified cytotoxin and the various fractions obtained during purification of cytotoxin is shown in Table 2. Both culture supernatant and purified cytotoxin evoked fluid accumulation in rabbit ileal loop, the nature of fluid was hemorrhagic in comparison to CT. Relatively high dose of cytotoxin was required to evoke a positive fluid accumulation ratio in comparison to CT (Fig. 5). The secretory effect of cytotoxin was completely blocked after incubating the toxin with rabbit anti-cytotoxin at 37 °C for 60 min. The inhibition studies further revealed that the polyclonal antibody could completely inhibit the cytotoxic activity of purified cytotoxin on CHO cells and enterotoxic activity in rabbit ileal loop assay.

Ultrathin sections of the ileum showed damage of the intestinal villi, lacteals, presence of blood cells, and rupture of intestinal capillaries (Fig. 6a–d). Analysis of ion composition of fluid accumulated in rabbit ileum by *V. cholerae* O54 TV113 showed that secretion of Na^+, K^+, and Cl^-, were comparable to that of CT; however, HCO^{3-} ion was more in case of TV113 (Table 3). These observations thus indicate that the mechanism of action of *V. cholerae* O54 TV113 cytotoxin may be different from the O1 strains.

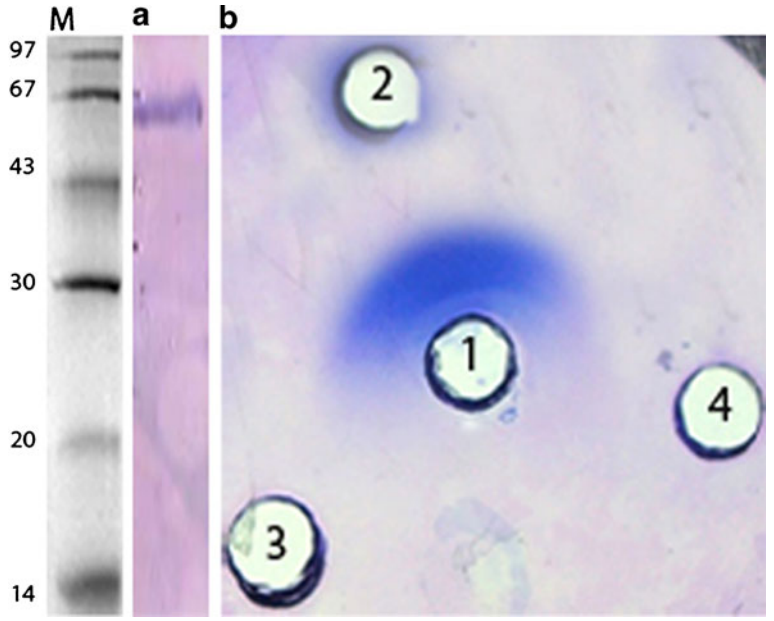

Fig. 4 **a** Immunoblot (Western transfer) of cytotoxin produced by *Vibrio cholerae* O54 TV113. Purified cytototoxic factor after SDS-PAGE was transferred to nitrocellulose membrane electrophoretically with the Bio-Rad transfer cell. The nitrocellulose paper was reacted with rabbit anticytotoxic enterotoxin serum. Preimmune rabbit serum showed no reactivity (not shown). **b** Immunodiffusion test of cytotoxin of *Vibrio cholerae* O54 TV113. *Well 1* (central well) Purified cytotoxin from *V. cholerae* O54 TV113, *well 2* rabbit anti-cytotoxin serum, *well 3* anti-CT, and *well 4* anti-hemolysin. Anti-NMDCY also did not show precipitin band with anti-cytotoxin serum (data not shown). *M* Migration of marker protein of known molecular mass (GE Healthcare)

Discussion

V. cholerae O1 and O139 are often associated with epidemics of cholera, whereas the non-O1, non-O139 strains are associated with localized outbreaks of diarrhea or diarrhealike disease [2]. *V. cholerae* non-O1, non-O139 isolates produce a variety of extracellular

Table 2 Biological activity of the various fractions obtained during purification of cytotoxin

Fraction sample		Amount of protein/no. of cells	F/A ratio (mean)
1	0.04 M Sodium phosphate-buffered saline	–	–
2	Cholera toxin	5 μg/ml	1.34
3	*Vibrio cholerae* O54 TV113	106 cells/ml	0.92
4	Concentrated culture supernatant of *V. cholerae* O54 TV113	9.2 mg/ml	1.00
5	Cytotoxin after anion exchange chromatography DE52	61.1 μg/ml	0.70
6	Purified cytotoxin after gel filtration Sephadex G100	50.0 μg/ml	0.87

F/A Fluid accumulation

Fig. 5 Enterotoxic activity of cytotoxin produced by *Vibrio cholerae* O54 TV113 shown in term of fluid accumulation in rabbit ileal loop assay. *Loop 1* Cholera toxin, *loop 2* purified cytotoxin fraction from Sephadex G100 column of *V. cholerae* O54 TV113, *loop 3* concentrated culture filtrate of *V. cholerae* O54 TV113, *loop 4*, 0.04 M sodium PBS, *loop 5* syncase medium, *loop 6* mixture of cytotoxin and anti-cytotoxin serum, and *loop 7* 0.04 M sodium PBS

products, of which some of them have been reported playing role in the pathogenesis of the disease. Considering the multiplicity of extracellular products produced by non-O1, non-O139 *V. cholerae*, it was important to determine whether cytotoxin examined in this study was distinct from those already reported. It was noted that the morphological changes affected by the cytotoxin under investigation on CHO cells was distinct from the cytotoxic activity exhibited by hemolysin and other toxins and the cytolethal distending effect exhibited by a variety of enteropathogens. The morphological changes on CHO cells shown by the *V. cholerae* O54 TV113 cytotoxin closely resembled the cytopathic effect exhibited

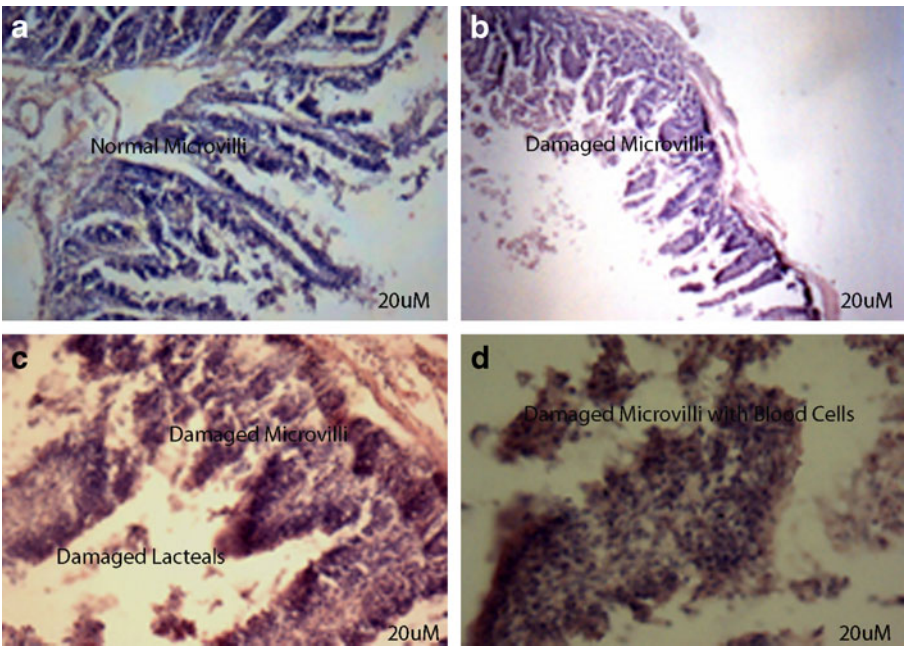

Fig. 6 Effect of cytotoxin of *V. cholerae* O54 TV113 on histopathology of rabbit ileum injected with purified cytotoxin. **a** Normal rabbit ileum showing intact villi and lacteals in 4× magnification. **b** Damaged villi in 20× magnification. **c** Damaged villi and lacteals in 20× magnification. **d** Damaged villi in 40× magnification with blood cells and ruptured blood vessels

Table 3 Electrolyte content of the fluid accumulated in rabbit ileal loop

Sample	F/A ratio	Analysis of ions secreted into the ileal loop (mEq/l)			
		Na^+	K^+	Cl^-	HCO_3^-
Cholera toxin	1.34	369	20.25	18	9760
Vibrio cholerae O54 TV113	0.92	180	10.00	12	1275
Concentrated culture supernatant of *V. cholerae* O54 TV113	1.00	220	14.00	9.2	12200
Purified cytotoxin after gel filtration Sephadex G100	0.87	108	6.00	10.6	5490

by *Clostridium difficile* toxin which involved cell damage and rounding of CHO cell. The possibility of the cytotoxin being a hemolysin can be ruled out, because syncase medium, which does not support the production of hemolysin, was used for its production. Purified El Tor hemolysin has been reported to evoke fluid accumulation in rabbit ileal loop and suckling mouse models, suggesting that it is an enterotoxic factor [26]. However, research at the Center for Vaccine Development, USA reported that recombinant strains carrying the gene for El Tor hemolysin (JBK 70 and CVD101) or *hly* gene knockout vaccine strains (CVD 104 and CVD 105) did not affect efficacy of the vaccine strains, indicating that the El Tor hemolysin does not play a role in the causation of diarrhea [27, 28].

Because cytotoxin produced by *V. cholerae* O54 TV113 isolated from a patient with diarrhealike illness and it seems distinct from reported *V. cholerae* toxins and hemolysin, we decided to purify and characterize the cytotoxin. We isolated and purified a 64 kDa molecular weight cytotoxin to homogeneity from culture supernatant of *V. cholerae* O54 TV113 grown in syncase medium supplemented with lincomycin by three step purification procedure. The ultrafiltration and the stepwise ammonium sulfate precipitation of cytotoxin indicated that the cytotoxic activity is associated with 40–80 % fraction. Here, we would like to mention that CT is recovered in a 98 % ammonium sulfate fraction from the culture supernatant of *V. cholerae* 569B and El Tor hemolysin in a 60 % fraction from a culture in syncase medium supplemented with 3 % glycerol at 37 °C under 48 h static condition. The observation that cytotoxin was heat-labile, unstable on storage at 4 °C, and sensitive to protease and papain but not trypsin indicate that the active compound is protein in nature and is different from CT, El Tor hemolysin, and NMDCY which were inactivated by trypsin. The cytotoxin had no subunits as shown by SDS-PAGE. Progressive purification of the cytotoxin and its ability to cause fluid accumulation in rabbit gut revealed that it is an enterotoxic factor as well. From this study, it was clear that the enterotoxic activity of cytotoxin was at least tenfold less than that of CT. Inhibition and neutralization studies of the cytotoxin using immune rabbit serum showed that this cytotoxin had both cytotoxic and enterotoxic activities.

Histopathology of the rabbit ileum inoculated with CT showed no damage to the intestinal villi and associated blood vessels. However, concentrated culture filtrate or purified cytotoxin from *V. cholerae* O54 TV113 showed extensive damage to the intestinal villi and the lacteals of the villi with rupture of blood vessels, similar to that caused by the *C. difficile* toxin. *V. cholerae* O1 strains have been reported not to invade the intestinal mucosa and infection is limited to the intestinal tracts, whereas the non-O1strains have been reported to cause extraintestinal infections like cellulites [29–31], wound infection [32, 33], and septicemia [34]. This study thus indicate that, like CT, *V. cholerae* O54 TV113 secreted Na^+, K^+, Cl^-, and HCO^{3-} ions like CT, but the concentration of bicarbonate ions was more

pronounced (Table 2) as compared to that of CT suggesting a different mode of action of the *V. cholerae* O54 TV113 cytotoxin.

It is known that enteric pathogens produce both cytotonic and cytotoxic enterotoxin. CT is prototype of cytotonic enterotoxin, while cytotoxins, which elicit enterotoxic activity, include Vero-toxin [35] and the cytotoxic enterotoxin of *Aeromonas* spp. [36, 37], *Salmonella* spp. [38], *C. difficile* [39], *Bacteroides fragilis* [40]. The *C. difficile* and *B. fragilis* toxins possess both cytotoxic and enterotoxic activity [40–42]. It is interesting to note that, although *V. cholerae* O54 TV113 cytotoxin showed similar effect compared with cytotoxin produced by *C. difficile* and *B. fragilis* in terms of morphological changes elicited on cell culture, fluid accumulation, destruction of intestinal villi, together with rupture of blood vessels in rabbit gut in histopathological studies, this toxin differs genetically from them as we could not amplify clostridium toxin genes from *V. cholerae* O54 TV113. However, multifactorial cytotoxin exhibiting hemolytic and enterotoxic activity in addition to cytotoxicity have been purified from clinical *Aeromonas* spp. [38, 43]. Our data lead us to conclude that the cytotoxin produced by *V. cholerae* O54 TV113, because of enterotoxic potential, has a role in the pathogenesis of non-O1 infections. At this point, however, we are unable to correlate the relevance of the cytotoxic activity with fluid accumulation in rabbit ileal loop. It is possible that the cytotoxic factor of *V. cholerae* O54 TV113 like cytotoxic enterotoxin of *Aeromonas hydrophila* [37] and *C. difficile* toxin possesses both cytotoxic and enterotoxic activity. These observations lead us to conclude that the cytotoxin produced by *V. cholerae* O54 TV113 had both cytotoxic and enterotoxic activity and had distinct effect in vivo and in vitro. Further genetic characterization and exploration of possible mechanism of the cytotoxic and enterotoxic effects of the cytotoxin of *V. cholerae* O54 TV113 is needed to understand non-O1 pathogenicity.

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