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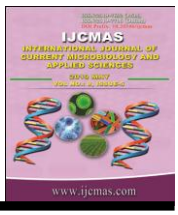

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Characterization of Toxins in the Culture Supernatants of *Clostridium difficile* and their Immunogenic Potential in Mice

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A B S T R A C T

The *Clostridium difficile* toxins, toxin A and Toxin B were characterised in respect to the influence of glucose and incubation period on release of toxins, cytotoxic activities of the toxins in Verocell line and the immunogenic potential of *C. difficile* toxoids. A gradual increase in protein concentrations was observed in the cell free supernatants of toxin A and B positive *C. difficile* isolate with increase in incubation period and found to reach the highest at 48 hr of incubation, *i.e.*, 5.24 µg/µl and 5.06 µg/µl, respectively. Release of *C. difficile* toxins in the culture supernatants was suppressed by glucose supplement in the nutrient media. Both the partially purified toxins were found to be cytotoxic for vero cells at both 1:10 and 1:100 dilutions. Cytotoxic activity of toxin B was more prominent than toxins A. However, similar immune-protective efficacy (100.0%) was exhibited by both the toxoid preparations (Toxoid A and B) in immunized animals against homologous challenge with $6.0x10^8$ CFU, while 75.0 percent protection against $9.0x10^8$ CFU of homologous strains of *C. difficile* was observed in both the groups, immunized separately with toxoid A and B. The affected mice, following challenge with $9.0x10^8$ CFU dose could show clinical symptoms, suggestive of intestinal disorder, without mortality. However, all the mice of control group died on 48 hr of post challenge with both 6.0×10^8 and $9.0x10^8$CFU / dose of homologous strain of *C. difficile*.

Introduction

Clostridium difficile, the anaerobic, spore-forming, Gram-positive bacillus was first identified in 1935 from the healthy newborns (Hall and TO'Toole, 1935). The organism is recognised as the most common nosocomial pathogen responsible for a most important disease condition in human, commonly known as antimicrobial-associated diarrhea. The organism produces several virulence factors including toxin A (*tcd*A), toxin B (*tcd*B), tissue degrading enzymes (proteases, collagenase, hyaluronidase, heparinase chondroitin-4-sulfatase), binary toxins, A (*cdt*A) and B (*cdt*A), endospores, surface

layer proteins and fimbriae (Borriello, 1998; Seddon and Borriello, 1992)**.** The pathogenic mechanism of the *C. difficile* isolates entirely depends upon the optimum *in vivo* release of toxins and their biological activities. The releases of both the toxins are reported to be growth phage dependent (Hundsberger *et al.*, 1997). Various environmental and physio-chemical factors are also reported to have an influence on the optimum release of *C. difficile* toxin in the environment. Production of *C. difficile* toxins was reported to be more during the late logarithm growth phase and the stationary phase (Ketley *et al.,* 1986 and Kamiya *et al.,* 1992). Limiting nutrient levels (e.g. glucose, amino acids, biotin), results in up regulation of toxin expression (Dupuy and Sonenshein, 1998; Haslam *et al.,* 1986; Karlsson *et al.,* 1999, 2000; Yamakawa *et al.,* 1994, 1996). Toxin A of *C.difficile* is primarily an enterotoxin and causes hemorrhage and fluid secretion, while toxin B is lethal and cytotoxic for most cell lines. Considering the probable zoonotics importance of *C. difficile*, there is a growing interest in prevention and in the development of non-antibiotic based approaches to manage *C. difficile* associated disease (Salnikova *et al.,* 2007). Toxoids A and B appears as a promising approach to prevent CDAD and to control recurrences (Giannasca and Warny, 2004; Sougioultzis *et al.,* 2005). Considering the importance of *C. difficile* toxins in disease production and control, the present study was undertaken to determine the influence of growth and nutritional factors on the release of the toxin and to determine cytotoxic activities of *C. difficile* toxins in Vero cells as well as the immune-protective potential of partially purified toxoids in mice.

Materials and Methods

Clostridium difficile strains

Six toxigenic field isolates of *Clostridium difficile*, Pig and Dog origin were randomly selected from the repository of Department of Microbiology, College of veterinary science, Assam Agricultural University, Khanapara, Guwahati -22, Assam. The pig isolates were positive for *tox* A gene, while *tox* B was detected in the dog isolates.

Influence of glucose and incubation hour on release of *C. difficile* and toxins

The influence of glucose in the growth media and influence of incubation period on *in-vitro* release of *C. difficile* toxins were studied as per method of Rolfe and Finegold (1979). A few pure isolated colonies of two randomly selected *tcd*A and *tcd*B gene positive isolates *C. difficile* isolates from Cycloserine-Cefoxitin-Fructose Agar (CCFA) plates were inoculated separately into 30.0 ml Brain Heart Infusion (BHI) broth and incubated anaerobically for overnight at 37^{o}C.The overnight broth cultures were centrifuge at 6000 rpm for 10 minutes. The pellets from the respective tubes were re-suspended separately in 5.0 ml of Phosphate Buffered saline (PBS). A fixed volume (2.0 ml) of each suspension was transferred to a set of fresh tubes, containing 30.0 ml of thioglycolate broth with glucose and without Glucose. Following incubation anaerobically for 48 hr, a 5.0 ml of broth culture from each tube was periodically removed at 8, 12, 24 and 48 hr and the purity of growth were checked. Supernatants were collected from the respective broth cultures by centrifugation at 16,000 rpm for 30 min.

Culture supernatants, extracted at different incubation period and in different nutrient environment were partially purified by ammonium sulphate precipitation technique at 70.0 percent saturation, as described by Mahony *et al.,* (1989). Partially purified culture supernatants were screened for protein concentrations, following the method of Lowry *et al.,* (1951). The partially purified culture supernatants of *C. difficile* were also screened for release of toxins by a

commercial rapid membrane immune assay kit (TechLab, USA).

Cytotoxic activities of the partially purified *C. difficile* toxins

The partially purified toxins (toxin A and Toxin B) of *C. difficile* strains were screened for cytotoxic activities on Vero cells, maintained in the Department of Veterinary Microbiology, College of Veterinary Science, Khanapara, Guwahati, Assam.

Both the partially purified toxins were prepared from the growth of respective isolate in suitable thioglycolate broth and incubation period, determined earlier were filtered through 0.20 µm membrane filter (Millipore, India). Two levels of serial dilution (1:10 and 1:100) were made from each partially purified toxins in PBS and 100 µl of respective diluted toxin was transferred to six wells of 12 well tissue culture plate (Nunc, Denmark) with 24 hr confluent growth of monolayer of Vero cell, while the remaining three wells were kept as negative control with addition of 100 µl of PBS.

Inoculated tissue culture plate was incubated at 37^{o}C in CO_2 incubator with addition of maintenance media (EMEM, Hyclone). All the test wells, including the negative control wells were observed from 18 hr of incubation till 48 hr for cytopathic changes in the cells, *viz.*, rounding of cells, detachment of mono-layers *etc*., if any.

Immuno-protective potential of *C. difficile* toxoids in mice

Immuno protective efficacy of the toxoids, prepared from respective toxins (A and B) was carried out in groups of mice with a prior approval from the Institutional Animal Ethics Committee, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam.

Preparation of toxoid vaccine from detoxified toxins

Both the partially purified toxins of *C. difficile* were detoxified with formaldehyde for production of toxoids, as per the procedure of Kelly *et al.*, (1995). Detoxification was done by addition of 2.8 µl of formaldehyde (37.0%) to each of the 100 µl of partially purified toxins and Incubation at 37^{o}C. After 2 hr of incubation, the excess formaldehyde was removed by dialysis against NSS for 1 hr. The dialyzed detoxified toxins were filter sterilized by passing through 2 µm Millipore filter. Complete detoxifications of the toxins were tested in Vero cell line with two different dilution (1:10 and 1:100), as described above.

Vaccine preparations were made from each of the detoxified toxin of *C. difficile*, by mixing with Freund's (complete and incomplete) adjuvant (Sigma, USA) to give a final protein conc. of 15 µg /ml (Anosova *et al.*, 2013).

Immunization trial

Two groups (A & B) of mice, consisting of six animals of same weight, age and sex, in each group were immunized with 0.2 ml of Freund's complete adjuvanted detoxified toxin A and B (15 µg/ml protein) through intra-muscular (i/m), respectively (Anosova *et al.*, 2013). Another group of six mice (Gr. C) of same weight, age and sex was injected with 0.2 ml of NSS i/mly and kept as control. All the mice, including the control group were bled before immunization (0 day) for pre-immunized serum. Animals of both the vaccinated groups were boosted on 14th day of primary immunization with 0.2 ml of respective toxoid vaccine with Freund's incomplete adjuvant, with same protein concentration and route. Mice of all the three groups were observed till 34th day of primary immunization.

Challenged trial

The challenge inoculums were prepared separately from overnight growth of pure colonies of respective toxin gene positive *C. difficile* strains anaerobically in 5 ml BHI broth. Collected pellets after centrifugation were washed three times with sterile NSS and the respective washed bacterial pellets were re-suspended in duplicate tubes with sufficient volume of sterile NSS solution to get final concentration of 6.0×10^8 CFU (MacFarland tube no.1) and 9.0×10^8 CFU per ml (Mac Farland tube no.2).

Animals of all the three groups (A-C) were pretreated with 0.2ml of clindamycin injection with a final concentration of 10 mg/kg through intra-peritoneal (i/p) route on 34^{th} day of primary immunization. Half of the pretreated mice of groups, A1 and B1 were challenged intra-gastrically (i/g) with 0.2 ml of 6.0 x 10^8 CFU of respective homogenous strain of *C. difficile* live culture, while the other half (A2 and B2) were challenged with 9.0 x 10^8 CFU of respective live culture. Equal no. of mice of the control groups (C1 and C2) were also challenged with 9.0 x 10^8 CFU of live strain of toxin A and B positive *C. difficile*. All the challenged mice were observed up to 48 hr post challenge for mortality and gross changes, if any.

The mice died within 48 hr of challenge were examined for any pathological changes in the visceral organ and digestive tract. Re-isolation of inoculated *C. difficile* was attempted from the internal organs of the mice died during challenged trial.

Results and Discussion

Influence of glucose and incubation period on release of *C. difficile* toxins

The release of *C. difficile* (*tox*A and *tox*B) toxins, in terms of protein conc. was found to be increasing in culture supernatants of thioglycolate broth, with or without glucose supplement, and with increase incubation period. Detail results were depicted in Table 1. The culture supernatants of the *tox*A positive *C. difficile,* grown in conventional thioglycolate broth without glucose supplement for initial 8hrs, exhibited protein conc. of 1.43 µg/µl. Almost similar conc. of protein (1.40 µg/µl) could be detected in culture supernatant of the same isolate, while grown in presence of glucose supplement. Increase in incubation period could reveal a gradual increase in the protein conc. in the culture free supernatant of toxin A positive *C. difficile,* grown in presence or absence of glucose. However, the same isolate grown in thioglycolate broth without supplementation of glucose could exhibit a protein conc. (5.24µg/µl) in the culture supernatant at 48hr of incubation. On the other hand, the addition of glucose could show a comparatively low conc. of protein (3.49 µg/µl), released by the same *C. difficile* isolate in the culture supernatant at 48 hr. of incubation. Similar trend was also observed in respect to the protein conc. in the culture supernatant of *tox*B positive *C. difficile* in thioglycolate broth, with or without glucose, and increase in incubation period. The protein concentration in the cell free supernatant of toxin B producing *C. difficile* isolates under the influence of glucose was found to be 1.94 µg/µl at 8 hr of incubation, while absence of glucose in the nutrient medium revealed a protein conc. of 1.28 µg/µl at the same period of incubation. However, 48 hr. of incubation of *tox*B positive *C. difficile* growth in thioglycolate broth without glucose could exhibit protein conc. of 5.06 µg/µl. which was found to be apparently more than that revealed by the growth of the same isolate with glucose supplement (3.99 µg/µl).

Screening of culture supernatants for release of *C. difficile* toxins, A and B by the commercial kit could reveal sharp prominent

bands in respective position for *C. difficile* specific Glutamate Dehydrgenase (GDH) protein and toxins (Fig. 1).

Cytotoxic activities of the partially purified *C.difficile* toxins

The partially purified culture supernatants of *tox*A and *tox*B bearing C. *difficile* strains growth under favourable nutrient environment and incubation period revealed cytotoxic changes in vero cell line. The vero cells treated with the dilutions, 1:10 (4.20mg/ml) and 1:100 (0.42mg/ml) of *tox*A positive isolate could show detachment, rounding, clumping and reduction in cell count. The visible cytopathic effects (CPE) was observed within 18-24 hr and lasted for 48hr of incubation. The partially purified culture supernatant of toxin B positive *C. difficile* also exhibited similar cytotoxic activities in vero cell line at both 1:10 and 1:100 dilutions (Fig. 2). However, the changes in vero cell produced by *tox*B were found to be more distinct at lower protein conc. (2.20mg/ml), while CPE was observed in treated vero cells with 4.20mg/ml protein conc. in *tox*A positive *C. difficile* culture supernatant.

Immuno-protective potential of *C. difficile* toxoid vaccines

The immuno-protective efficacy of the toxoid, prepared from the culture supernatants of both *tox*A and *tox*B positive isolates of *C. difficile* could reveal variable protection level in mice, during 48hr of observation (Table 2).

Both the toxoid preparations could confer 100 percent protection to groups of mice (A1 and B1) against $6.0x10^8$CFU dose of homologous challenge, till 48 hr of observation. On the other hand, 25.0 percent of the immunized mice (A2) with toxoid preparation of toxin A positive isolate showed clinical symptoms of ruffle coat with static movement with loose faeces, within 24 hr of challenge with $9.0x10^8$CFU/ dose of homologous challenge (Fig. 3). The clinical symptoms were found to be subsided on 48 hrs of observation. Fifty percent of mice vaccinated with toxoid, prepared from toxin B positive isolate showed clinical symptoms within 24 hrs of homologous challenge with a dose of $9.0x10^8$CFU. All the clinically affected mice were succumbed to death on 48hr of challenge. The challenge infection with 6.0 $x10^8$ CFU and $9.0x10^8$CFU / dose in the mice of control group (D1 and D2) revealed an initial development of clinical symptoms during 24 hr of observation, suggesting intestinal infection. All the clinically affected mice of control groups were died within 48 hr of observation.

Post mortem of the dead mice from the control group revealed hemorrhage in the intestinal mucosa with gas bubbles and fragile liver (Fig. 3). Detection of *C. difficile*, based on presence of *glu*D gene from the affected liver as well as from the intestinal piece and contents of dead mice confirmed the mortality due to injected *C. difficile* isolates

Influence of glucose and incubation period on release of *C. difficile* toxins

The present study could reveal a negative impact of glucose on protein conc. in the cell free supernatants of *tox*A and *tox*B positive C. *difficile* isolate. Protein conc. in the cell free supernatant of *tox*A bearing *C. difficile* in thioglycolate broth with glucose supplement was found to be 1.40 µg/ml at 8hr of incubation, while 8 hr growth of the same isolate in thioglycolate medium without addition of glucose revealed almost same protein conc. (1.43 µg/ml) in culture supernatant. Although there was an increasing trend in the protein conc. with increase in incubation period, till 48hr of observation, the *tox*A positive isolate in thioglycolate broth

without glucose supplement could show higher protein conc. (5.24 µg/ml) than the thioglycolate broth culture without addition of glucose at 48hr of incubation (3.49 µg/ml). Almost similar trend in protein conc. was revealed by the *tox* B positive isolate, grown in presence glucose with increasing period, *i.e.* 8 hr (1.94 µg/ml) to 48hr (3.99 µg/ml), while growth of the same isolate in thioglycolate broth without glucose supplement showed 1.28 µg/ml and 5.06 µg/ml of protein at 8hr and 28hr of incubation. Based on the present observation, it could reveal that incubation period have a positive impact on release of *C. difficile* toxin in the culture supernatants, while glucose in the nutritional environment was found to inhibitory for release of *C. difficile* toxin in the culture supernatants.

Expression of toxins of *C. difficile* was recorded, mainly during the late exponential growth phase and the stationary phase (Kamiya *et al.*, 1992; Ketley *et al.*, 1986). Release of *C. difficile* toxins was also recorded under limiting nutrient levels, *e.g.* glucose, amino acids, biotin, which lead to upregulation of toxin expression (Dupuy & Sonenshein, 1998). Reports on protein profile of *C. difficile* in cell free extract with different environmental conditions was found to be very scanty in the available literature. Catabolite repression of toxin genes results in reduction in release of *C. difficile* toxins. Dupuy and Sonenshein (1998) revealed catabolite repression of toxin genes of *C difficile* by addition of glucose in the nutrient medium. They could also observe the repression of *tox* mRNA in *C. difficile*, during exponential growth phase and more prominent during in stationary phase with addition of glucose. They also opined during their study that the effect of glucose on release of *C. difficile* toxins might be a general mechanism for many toxigenic isolates.

Cytotoxic activity of partially purified toxins of *C. difficile* in vero cell line

Both *tox* A and *tox* B bearing *C. difficile* isolates revealed cytotoxic changes, *i.e.*, detachment, rounding, clumping and reduction in cell count in the treated vero cells, within 18-24 hr of incubation. Among the partially purified toxins, more prominent CPA was exhibited by the toxinB positive *C. difficile* isolate with comparatively low protein conc. (2.20mg/ml) than toxin A (4.20mg/ml) at 1:10 dilution. Many previous studies also opined for Vero cell, as a well-established cell line for testing the cytotoxic activities of *C. difficile* (Maniar *et al.*, 1987). However, Torres *et al.*, (1992) could observe variable CPE in different cell lines, exhibited by toxin A and toxin B. More prominent cytotoxic activity could be seen in human colon carcinoma cells (HT-29, epithelial cells) and rhesus monkey kidney cells (MA-104, epithelial cells), exhibited by toxin A of *C. difficile*, while Vero cell line was found to be more suitable for toxin B of *C. difficile*. Variability could be observed among different strains of toxin B positive *C. difficile*, in respect to the type of CPE produced in the Vero cells (Borriello *et al.*, 1992). Comparatively more cytotoxic activity exhibited by toxin B of *C. difficile* in Vero cell line during the present study was in agreement with the previous observation of Lyras *et al.*, (2009). They could provide evidence that the toxin B was more essential than toxin A for virulence. Kuehne *et al.*, (2010) recorded more susceptibility of HT29 (human colon carcinoma) cells towards toxin A, while the toxin B exhibited more prominent cytotoxic activity in Vero cells.

Immuno-protective potential of *c. Difficile* toxoid vaccines

Variable protection level could be conferred in immunized mice by the two toxoids

prepared separately from toxin A and toxin B positive *C. difficile* isolates. Following challenge on 34th day of post immunization with $6x10^8$ cfu of homologous *C. difficile* strain, all the clindamycin pretreated mice were protected. On the other hand, 25.0 percent of mice, immunized with toxoid A could not withstand the challenge with higher conc. ($9x10^8$ cfu) of the same homologous isolate. Similarly, 50.0 percent of vaccinated mice (toxoid B) showed clinical symptoms of intestinal disorder within 24 hr of challenge with a dose of $9x10^8$ cfu of homologous strain, followed by death on 48hr of observation. The challenge infection with 6.0 x 10^8 and $9.0x10^8$ CFU / dose in the mice of control group revealed an initial development of clinical symptoms of intestinal infection during 24 hr of observation and all the clinically affected mice were died within 48 hr of challenge.

Table.1 Protein concentration in the toxins released in nutrient media at different stages of growth

Type of isolates	Protein concentration (µg/µl) in the culture supernatants of							
	Thioglycolate broth with Glucose				Thioglycolate without Glucose			
	Incubation period				Incubation period			
	8 hr	12 hr	24 hr	48 hr	8 hr	12 hr	24 hr	48 hr
ToxA+ve	1.40	2.81	3.12	3.49	1.43	3.65	4.08	5.24
ToxB+ve	1.94	2.67	3.03	3.99	1.28	2.33	3.6l	5.06

Table.2 Protective efficacy of *C. difficile* toxoid in vaccinated mice

Type of toxoid	Gr.	No. of Animal	No. of Vaccinated mice affected following Challenge							
			Clostridium difficile (6 x 10^8 cfu)				*Clostridium difficile* (9 x 10^8 cfu)			
			≥24 hr		48 hr		≥24 hr		48 hr	
			Clinical change	Death	Clinical change	Death	Clinical change	Death	Clinical change	Death
Toxoid A	A1	4	--	--	--	--				
	A2	4					1 (25)	--	--	--
Toxoid B	B1	4	--	--	--	--				
	B2	4					2 (50)	--	--	2(50)
Control	D1	4	4 (100)	--	--	4(100)	4 (100)	--	--	4(100)

Figures in parenthesis indicate percentages

Fig.1 Rapid membrane immune assay (TechLab, USA) for detection of *C. difficile* and toxins

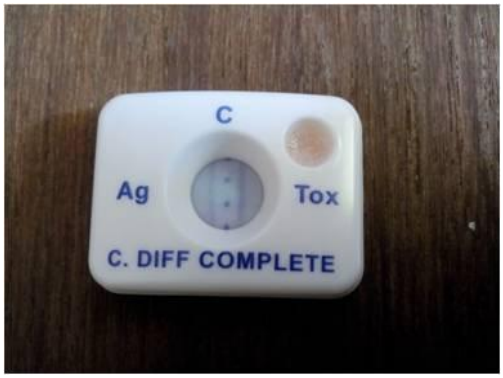
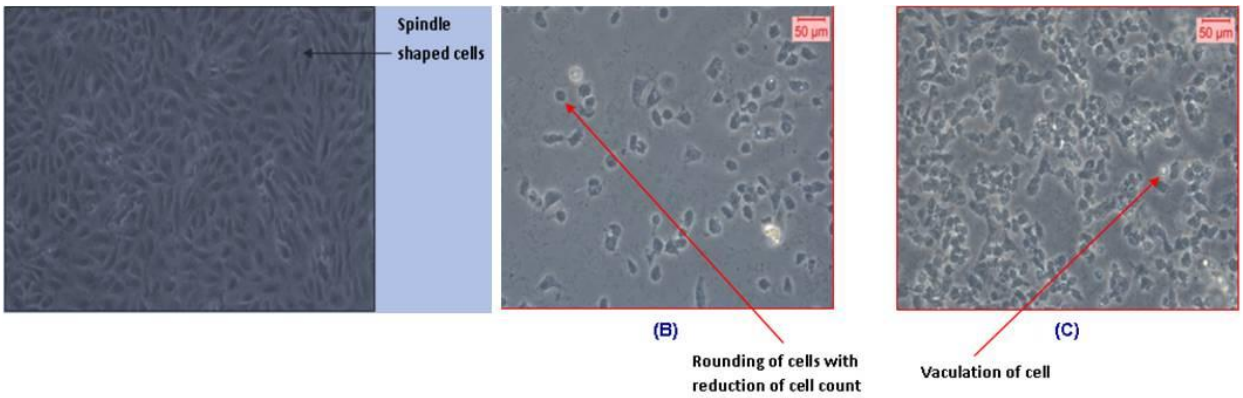
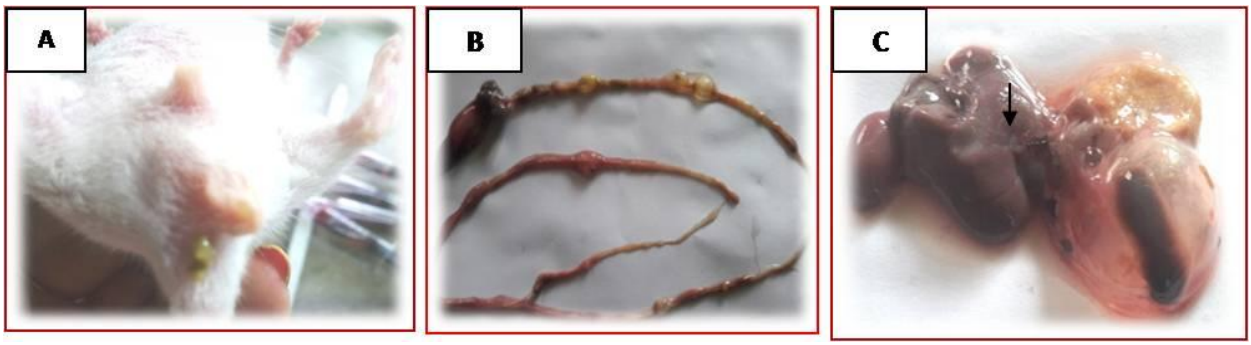


Fig.2 Cytotoxic activity of partially purified toxin in the culture supernatant of *C. difficile* in monolayer of Vero cell



(A) Normal spindle shaped cells in monolayer of Vero cell line.
(B) Rounding and reduction in cell count and in vero cell line treated with partially purified toxin (1:10).
(C) Rounding and reduction in cell count and in vero cell line treated with partially purified toxin (1:100)

Fig.3 Mouse showing clinical symptoms following challenge with *C. difficile*



(A) Clinically affected mouse with loose faeces (arrow head) during 24 hr of challenge
(B) Hemorrhage with gas formation (arrow head) in the affected intestine of dead mouse
(C) Enlarged fragile Liver (arrow headed) of the dead mouse following challenge

Torres *et al.,* (1995), in their study could provide evidence of protection conferred by vaccination with *C. difficile* toxoids (A+B) against challenge with 10^5 cfu of viable *C. difficile*. However, they also opined for an appropriate immunization regimen for an effective immunogenic potential. The route of antigen administration was reported to be an important determinant of mucosal immune effector function. The intragastric and rectal routes of immunization were recorded to be ineffective. They recorded a combination of mucosal and parenteral immunization with *C. difficile* toxoid to be the most effective regimen of immunization for induction of protective immunity against *C. difficile* disease. They also opined for clindamycin treatment before challenge trial, which was found to have an influence on colonization of *C. difficile* strain. In a similar study, Siddiqui *et al.,* (2012) could confirm 100 percent protection in hamsters conferred by the formaldehyde treated *C. difficile* toxin against homologous challenge with 10^6 spores.

Based on the present observation with support of previous studies, it can be concluded that the formaldehyde treated toxoids of *C.*

difficile can be used as suitable immunogen for control of *C. difficile* infection in individuals.

However, to ascertain its protective efficacy against hetrologous challenge with live bacteria and bacterial toxin, a further study has to be carried out with computation of the dose, protein conc. and selection of a suitable route of immunization.

References

Anosova, N. G., Brown, A. M., Li, L., Liu, N., Cole, L. E., Zhang, J., Mehta, H. and Kleanthous, H. (2013). Systemic antibody responses induced by a two-component *Clostridium difficile* toxoid vaccine protect against *C. difficile* associated disease in hamsters. *J. Med. Microbiol*., 62: 1394-404.

Borriello, S.P. (1998). Pathogenesis of *Clostridium difficile* infection. *J. Antimicrob. Chemother*. 41: 13-19

Borriello, S.P., Wren, B.W., Hyde, S., Seddon, S.V., Sibbons, P., Krishna, M. M., Tabaqchali, S., Manek, S. and Price, A. B. (1992). Molecular, immunological and biological characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile. Infect. Immun*., 60: 4192-4199.

Dupuy, B. and Sonenshein, A. L. (1998). Regulated transcription of *Clostridium difficile* toxin genes. *Mol. Microbiol.*, 27:107-120.

Giannasca, P.J. and Warny, M. (2004). Active and passive immunization against *Clostridium difficile* diarrhea and colitis. *Vaccine*. 22 (7): 848–856.

Hall, I. C. and O'Toole, E. (1935). Intestinal flora in new-born infants with a description of a new Pathogenic anaerobe, *Bacillus difficile. Am. J. Dis. Child.*, 49: 390-402.

Haslam, S. C., Ketley, J. M., Mitchell, T. J., Stephen, J., Burdon, D. W. and Candy, D. C. (1986). Growth of *Clostridium difficile* and production of toxins A and B in complex and defined media. *J. Med. Microbiol.*, 21: 293-297.

Hundsberger, T., Braun, V., Weidmann, M., Leukel, P., Sauerborn, M. and von Eichel Streiber, C. (1997). Transcription analysis of the genes *tcd*A-E of the Pathogenicity locus of *Clostridium diffiile*. *Eur. J. Biochem*., 244: 735-742.

Kamiya, S., Ogura, H., Meng, X.Q. and Nakamura, S. (1992). Correlation between cytotoxin production and sporulation in *Clostridium difficile*. *J. Med. Microbiol.*, 37: 206-210.

Karlsson, S., Burman, L. G. and Akerlund, T. (1999). Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids. *Vet. Microbiol.*, 145: 1683-1693.

Karlsson, S., Lindberg, A., Norin, E., Burman, L. G. and AIkerlund, T. (2000). Toxins, butyric acid and other short chain fatty acids are co-coordinately expressed and down regulated by cysteine in *Clostridium difficile*. *Infect. Immun.*, 68: 5881-5888.

Ketley, J. M., Mitchell, T. J., Haslam, S. C., Stephen, J., Candy, D.C.A. and Burdon, D. W. (1986). Sporogenesis and toxin A production by *Clostridiun difficile*. *J. Med. Microbiol*., 22: 33-38.

Kelly, C. P., Pathoulakis,C., and LaMont, J. T. (1995). *Clostridium difficile* colitis. *N. Engl. J.Med*., 330: 257-262.

Kuehne, S. A., Cartman, S. T., Heap, J. T., Kelly, M. L., Cockayne, A. and Minton, N. P. (2010). The role of toxin A and B in *Clostridium difficile* infection. *Nature*, 467: 711-714.

Libby, J. M., Jortner, B. S. and Wilkins, T.D. (1982). Effects of the two toxins of *Clostridium difficile* in antibiotic-associated cecitis in hamsters. *Infect. Immun*., 36: 822-829.

Leuzzi, R., Spencer, J., Buckley, A., Brettoni, C., Martinelli, M., Tulli, L., Marchi, S., Luzzi, E., Irvine, J. and Candlish, D. (2014). Protective efficacy induced by recombinant *Clostridium difficile* toxin fragments. *Infect. Immun*., 81: 2851-60.

Lyras, D., Connor O', J. R., Howarth, P. M., Sambol, S. P., Carter, G. P., Phumoonna, T., Poon, V. R., Adams, G. V., Johnson, S.,

Gerding, D. N. and Rood, J. I. (2009). Toxin B is essential for virulence of *Clostridium difficile*. *Nature*, 458: 1176-1179.

Mahony, D. E., Gilliatt, E., Dawson, S., Stockdale, E. and Lee, S. H. S. (1989). Vero cell assay for rapid detection of *C. perfringens* enterotoxins. *Appl. Environ. Microbiol*., 55(9): 2141-2143.

Maniar, A. C., Williums, T. W. and Hammond, G. W. (1987). Detection of *Clostridium difficile* toxin in various tissue culture monolayers. *J. Clin. Microbiol*., 25: 1999-2000.

Rolfe, R. D. and Finegold, S. M. (1979).Purification and Characterization of *Clostridium difficile* Toxin. *Infect. Immune*., 25: 191-201.

Salnikova, M. S., Joshi, S. B., Rytting, J. H., Warny, M. and Middaugh, C. R. (2007). Physical Characterization of *Clostridium difficile* Toxins and Toxoids: Effect of the formaldehyde Crosslinking on Thermal Stability. *J. Pharmaceutical. Sci.*, 97(9): 3735-3752.

Seddon, S. V. and Borriello, S. P. (1992). Proteolytic activity of *Clostridium difficile*. *J. Med. Microbiol*., 36(5): 307-311.

Siddiqui, F., O'Connor, J. P., Nagaro, K., C, Adam., Sambol, S. P., Vedantam, G., Gerding, D.N. and Johnson, S. (2012). Vaccination with parenteral toxoid B protects hamsters against lethal challenge with toxin A-negative, toxin B-positive *Clostridium difficile* but does not prevent colonization. *J. Infect. Dis*., 205: 128-133.

Sougioultzis, S., Kyne, L., Drudy, D., Keates, S., Maroo, S., Pothoulakis, C., Giannasca, P.J., Lee, C.K., Warny, M., Monath, T.P. and Kelly, C.P. (2005). *Clostridium difficile* toxoid vaccine in recurrent *C. difficile*-associated diarrhea. *Gastroenterology*, 128: 764-770.

Toma, C., Nakamura, S., Kamiya, S., Nakasone, N. and lwanaga, M. (1999). Detection of *Clostridium difficile* Toxin A by Reversed Passive Latex Agglutination. *Microbiol. Immunol*., 43(8): 737-742.

Torres, J. F., Lyerly, D. M., Hill, J. E., Monath, T. P. (1995). Evaluation of formalin-inactivated *Clostridium difficile* vaccines administered by parenteral and mucosal routes of immunization in hamsters. *Infect. Immun*., 63: 4619-4627.

Torres, J., Camorlinga, P. M. and Munoz, O. (1992). Sensitivity in culture of epithelial cells from rhesus monkey kidney and human colon carcinoma to toxins A and B from *Clostridium difficile*. *Toxicon*, 30: (4) 419-426.

Wang, B., Wang, S., Rustaind, R. R., Wang, F., Mensch, C. D., Hong, L., Kristopeit,A., Secore, S., Dornadula, G., Kanavage, A., Heinrichs, J. H., Mach, H., Blue, J. T. and Thiriot, D. S. (2015). Detecting and preventing reversion to toxicity for a formaldehyde- treated *C. difficile* toxin B mutant. *Vaccine*, 33: 252-259.

Yamakawa, K., Kamiya, S., Meng, X. Q., Karasawa, T. and Nakamura, S. (1994). Toxin production by *Clostridium difficile* in a defined medium with limited amino acids. *J. Med. Microbiol.*, 41: 319-323.

Yamakawa, K., Karasawa, T., Ikoma, S. and Nakamura, S. (1996). Enhancement of *Clostridium difficile* toxin production in biotin limited conditions. *J. Med. Microbiol*., 44: 111-114.

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