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Evaluation of the biological effects of *Clostridium perfringens* type a crude enterotoxin (CPCE) using animal model

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Abstract
The present research was undertaken to study the toxigenic activities of crude enterotoxin of *C. perfringens* type A isolates obtained from raw meat, raw chicken and meat products were studied in blood agar and experimental animal model during 2012-2013. All the 44 (74.58%) *C. perfringens* type A strains produced clear double zone of β-haemolysis on 5% sheep blood agar. In animal model, the field isolates produced maximum vascular permeability reaction within 12 hr of intra-dermal injection of *C. perfringens* crude enterotoxin (CPCE) which subsided completely after 48 hrs. The quantitative response of the ileal loops (fluid volume/loop length ratio) indicated the entero-toxigenic activity of CPCE from field isolates as well as standard ATCC-13124 strain of *C. perfringens* type A. The study confirms the toxigenic activities of *C. perfringens* type A in animal model *in-vivo* and concluded that vascular permeability reaction helps in demonstrating the activities of *C. perfringens* type A enterotoxin over ligated ileal loop techniques without sacrificing the animals.

Keywords: *C. perfringens* type A, entero-toxigenecity, VPR, RLIL

1. Introduction
Clostridium perfringens is ubiquitous in the environment responsible for several important enteric diseases and histotoxic infections in man and animals [1, 2]. Human enteric illness associated with this anaerobic bacterium includes *C. perfringens* type A food poisoning, *C. perfringens* enterotoxin (CPE) associated non-food borne gastro-intestinal (GIT) diseases such as antibiotic associated diarrhea, sporadic non-food borne diarrhoea and enteritis necroticans [3-5]. It is a Gram-positive, anaerobic, sporulating, ubiquitous bacterium categorized into five types A to E according to the production of four major lethal toxins alpha, beta, epsilon and iota toxin [6-8]. Type A produces alpha toxin while type B produces alpha, beta and epsilon toxin; type C produces alpha and beta toxins; type D produces alpha and epsilon; and type E produces alpha and iota toxins [8, 9]. Apart from these, some strains of *C. perfringens* also produce some other toxins like enterotoxin and beta2 toxin causing different syndromes [10, 3]. Type A is responsible for food-poisoning in humans due to the production of enterotoxin during sporulation [11]. Many, but not all, type A strains produced it [12, 13]. The molecular weight of this toxin is about 35–kD containing single polypeptide of 309 amino acids with an isoelectric point of 4.3 [14, 15]. The enterotoxin is heat labile, loses its biological activity when heated at 56°C in 5 minutes and sensitive to extremes of pH which is inactivated at pH between 5 and 12 [2].

In food poisoning outbreaks, the vegetative cells of *C. perfringens* survive stomach passage and the acidic environment may serve to stimulate sporulation of this organism in GIT [16]. The toxin is released into the intestinal lumen when sporulation is complete and the mother cell lysed, releasing a mature spore leading diarrhea [17]. Detection of known or potential toxins produced by *C. perfringens* is crucial for better understanding of *C. perfringens* infections. The enteric pathogenicity of CPE has been evidenced in human volunteer feeding studies, showing that purified toxin causes diarrhea and cramping [18]. Animal model studies to investigate the enterotoxigenicity of *C. perfringens* type A includes ligated loop of the rabbit intestine [19, 20], lamb ligated intestinal loops of lambs, guinea-pig skin test and mouse test etc. [21]. *Clostridium perfringens* is able to produce various toxins and causes different disease conditions. Keeping view of the importance of the activities of toxins the present study was

Conducted with the following objectives:

- To study the haemolytic activities of *Clostridium perfringens* in blood agar.
- To see the toxigenic activities of crude toxins of *Clostridium perfringens* in animal model.

2. Materials and Methods

2.1 Place of work

The study was conducted in the Division of Veterinary Public Health, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-K, Shuhama, Jammu and Kashmir, India. The period of study was from February, 2012 to July, 2012.

2.2. Bacterial strain and procedure of study

For this fifty nine (59) *Clostridium perfringens* isolates obtained from different raw meat & meat products maintained in Cooked Meat Medium (CMM, Hi-Media) along with reference *C. perfringens* ATCC-13124 strain procured from Hi-Media were used. Briefly, positive *C. perfringens* type isolates were inoculated in blood agar plates containing 5% sheep blood and incubated at 37°C for 24 hr for demonstration of hemolytic activity of the organisms as per methods of Herbert and Angelotti [22]. In animal model, the toxigenic activities of *C. perfringens* type A enterotoxin was studied by Vascular Permeability Reaction (VPR) in rabbit skin as per method described by Willayat [23] and ligated ileal loop test (*in-vivo*) in rabbit as per methods described by Duncan *et al*. [19] with slight modifications. The Institutional Animal Ethics Committee (F.V. Sc. &A.H., SKUAST-Kashmir) has given kind permission to use the necessary laboratory animals in the study (Appendix 1, copy enclosed).

2.3 Preparation of CPCE

The method of Duncan *et al*. [19] was essentially followed for production of enterotoxin by standard *C. perfringens* type A strain and the field isolates from raw meat and meat products. Cultures maintained in CMM (Hi-media) were sub-cultured in 6 ml of Fluid Thyoglycolate (FTG) medium and incubated overnight at 37°C. The cultures were then heat treated at 70°C for 20 min and transferred to 100 ml of Duncan-Strong Sporulation (DSS) medium. The medium was then incubated anaerobically at 37°C for 24 h for spore formation & production of enterotoxin. Following incubation, the culture was rapidly cooled in an ice bath and subsequently centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and filtered through 0.45 µm membrane filter and then dialysed to its half volume against polyethylene glycol (400) and later collected in a screw capped vials for immediate use or stored at -20^{0}C till further use. This was designated as *C. perfringens* crude enterotoxin (CPCE).

2.4 Vascular permeability reaction in rabbit skin (VPR)

CPCE from representative isolates of raw mutton, raw chicken, mutton kababs and the standard ATCC-12134 strain were used to study the VPR in the rabbit skin by injecting 0.2 ml of the toxin intra-dermally into abdominal areas of two rabbits. For this, the abdominal areas of the rabbits were clean shaved and sterilized by 70% alcohol and subsequently the areas designated for inoculation of the required enterotoxins. Adjacent to the inoculation sites, sterile FTG was also injected as negative control. After two hours of inoculation, 2.5 ml of 0.25% trypan blue dye was injected i/v into the ear vein of each rabbit. An hour after the injection of the dye, VPR was observed as zones of light and dark blue areas surrounding the point of inoculation. Gross observations like intensity of color change and the presence of edema at the site of inoculation were recorded at varying time intervals of 3, 6 and 12 hours post inoculation. The VPR zones were measured by slide caliper and a zone of 8 mm and above were considered as positive.

2.5 Entero-toxigenic activity in rabbit ligated ileal loops (RLIL)

Two male Russian chincilla rabbits weighing 1.5 to 2.0 kg each were fasted for 24 hr with water given freely. The animals were then anesthetized by giving i/v injection of xylazine (5mg /kg.b.wt) and ketamine (25mg/kg, body wt.) A mid-line abdominal incision was made on the shaved abdomen and the ileum was externalized. The ileum was then canulated 90-100 cm proximal to the ileo-caecal junction and washed gently with 300ml of warm sterile Ringer's solution. The ileum was completely cleared of the washing solution by gravity. The washed segment of ileum was then ligated in sections creating 3-5 cm loops (4 loops per rabbit; the first loop was placed 90-100 cm proximal to the ileo-caecal junction at the start of the washed ileal segment), each separated by un-inoculated 5cm inter loops. The ligation was done in such a way to see that the blood supply from the mesenteric vessel to each loop remained intact. An amount of 2 ml each of the CPCE from two isolates and also an equal amount of CPCE from standard ATCC-13124 strain was injected intra-luminally into 1st, 3rd and 4th loops, respectively. Similarly 2 ml of sterile DSS medium was injected into second loop, which served as a negative control. Attempts were made to distribute rapidly the inoculation solution throughout the loop by gently shaking the loops after injection. Saline packs were employed to keep the exposed intestine moist during the surgical procedure. The intestines were placed back into abdominal cavity and the skin was sutured. Following inoculation of CPCE into loops, the animals were sacrificed after 24 hr by i/v injections of thiopentone sodium @50-100mg/kg body weight. The abdomen was opened and the intestines were exposed. The length and fluid volume of the loops were measured to calculate the loop volume /length ratio. A ratio of 0.8 was considered as positive.

3. Results

3.1 Haemolysis on blood agar

In the present study, out of 59 isolates, 44 produced a clear double zone of β-haemolysis and 15 (25.42%) isolates did not cause any type of haemolysis on sheep blood agar plates *per se*. These results indicate the presence of toxigenic factors in 74.58% of the isolates conforming to *C. perfringens* type A (Fig. 1).

3.2 Vascular Permeability Reaction (VPR)

In the present study, the field isolates were compared with *C. perfringens* ATCC-13124 strain, in respect of their enterotoxin production as expressed by VPR in the rabbit skin (Fig. 2). The VPR was mild to moderate in first 3 hours of intra-dermal injection of *C. perfringens* crude enterotoxin (CPCE), but caused marked degree of inflammatory reaction within 6 hours of injection. Thereafter, a declining trend in the inflammatory response was noticed which subsided completely in 48 hr post inoculation. Maximum VPR zone was produced by CPCE of field isolate from raw chicken

(isolate-2), which ranged from 8.7 mm to 12.6 mm with an average of 10.4 mm. However, CPCE from standard ATCC-13124 produced VPR ranging from 7.5 mm to 8.6 mm with an average of 8.06 mm. CPCE from mutton kabab (isolate-3) produced a mild VPR of 3.5 mm to 6.2 mm with an average of 4.83 mm. Both the sites injected with sterile NSS and FTG showed a mild inflammatory reaction initially which subsided within half an hour of inoculation. The results are presented in Table 1.

3.3 Entero-toxigenic activity of CPCE in rabbit ligated ileal loops

The quantitative response of the loops (fluid volume/loop length ratio) indicated the entero-toxigenic activity of CPCE from raw mutton and raw chicken isolates as well as from standard ATCC-13124 strain (Table 2). The CPCE from raw mutton isolate produced a higher fluid volume/loop length ratio (1.12) than that of raw chicken isolates (1.1). The ileal loops injected with CPCE were grossly dark red and flaccid with presence of gas unlike control loops (injected with DSS medium) which failed to show any such positive reaction in either of the experimental animals. Positive loop response was expressed by observing the loops grossly, based on swelling and the accumulation of light brown to bloody fluid (Fig. 3). The pH of the fluids ranged between 6.9 and 7.8.

4. Discussion

The toxigenic activity of *C. perfringens* isolates was observed by inoculating the cultures in blood agar (5% sheep blood). Out of 59 *C. perfringens* isolates, 44 (74.58%) produced a clear double zone of β-haemolysis in blood agar plates which is an inherent characteristic feature of alpha toxin produced by *C. perfringens* type A. Thus, the results indicate the toxigenic activities of the *C. perfringens* type A isolates. Non-hemolytic activity by other 15 (25.42%) isolates of *C. perfringens* may be due to absence of the β-haemolytic toxin. The nature and extent of haemolysis produced by different organisms and different strains may vary according to the species of erythrocytes used [24]. The results are in comparable with report of Herbert and Angelotti[22] where they reported that out of 113 *C. perfringens* isolates recovered from meat and meat products, 91 (80.53%) produced complete or partial haemolysis on horse, ox and sheep blood agar plates.

Many biological assays have been developed for detecting the *C. perfringens* entero-toxin. Duncan *et al*. [19] suggested ligated loop of the rabbit intestine as a possible experimental model for studying the role of *C. perfringens* in food poisoning. In the present study, the field isolates recovered from meats were compared with *C. perfringens ATCC-13124* strain, for entero-toxigenic activities which were expressed by VPR in rabbit skin as well as ligated ileal loop response in the rabbit ileum. Although VPR was mild to moderate in first 3 hours, marked degree of inflammatory reaction was observed within 6 hours of injection which subsided completely in 48 hr post inoculation of C. perfringens type A crude-enterotoxin (CPCE). Maximum VPR zone average of 10.4 mm was produced by CPCE of field isolate from raw chicken (isolate-2) but CPCE from the isolates of mutton kabab (isolate-3) produced a mild VPR with an average of 4.83 mm. In an experiment Niilo [25] had shown that, when the cell-free extracts of entero-pathogenic strains of C. perfringens type A were administered intravenously to rabbits and guinea pigs, rabbits showed excessive salivation, frequent defecation and dyspnea followed by death. And when the same cell free extract was injected intra-dermally into guinea-pig skin, it caused an immediate increase in the capillary permeability and subsequent erythematous reaction. Reports on the action of bacterial enterotoxins on rabbit skin are well documented [23, 26]. The skin permeability reactions were produced either by purified or partially purified toxins. Earlier in an experiment, Willayat [23] reported moderate VPR as 7.4mm and necrosis in the rabbit skin after 12 hr following inoculation of Bacillus cereus emetic enterotoxin.

Measurable amounts of fluids were accumulated in all three ileal loops, when CPCE from raw mutton isolate, raw chicken isolate and ATCC-13124 were injected. This response of the loops (fluid volume/loop length ratio) indicated the entero-toxigenic activity of CPCE from representative isolates. CPCE from raw mutton isolate produced a higher fluid volume/length ratio (1.12) than that of raw chicken isolates (1.1). Duncan *et al*. [19] reported that 14 out of 29 C. perfringens type A strains isolated from food-poisoning outbreaks consistently produced exudation of fluid and consequent dilatation of the ileal segments (0.8-1.7 loop volume/ length fluid ratio). In another study, where lamb was used as a model animal to investigate the entero-toxigenicity of C. perfringens type A, Hauschild *et al*. [21] reported that the fluid volume of the loops increased up to seven times within seven hours of inoculation of mixture of C. perfringens type A culture and fresh medium. Early histological studies showed shortening of intestinal villi and desquamation of intestinal epithelium causing diarrhea and abdominal cramps [27, 28]. The enteric pathogenicity of C. perfringens enterotoxin has also been evidenced in human volunteer feeding studies showing that purified toxin causes diarrhea and cramps [18].

It was concluded that vascular permeability reaction in rabbits was comparable with the ligated ileal loop techniques in demonstrating the toxigenic activities of C. perfringens type A enterotoxin and can therefore, be used with ease without sacrificing the animals. However, because of the cost, animal welfare considerations and the complexity of toxin detection, use of animals for toxin typing has largely been replaced by genotyping of C. perfringens isolates [29, 30].

Table 1: Vascular Permeability Reaction (VPR) produced by CPCE of field isolates

S. No.	Isolates	VPR zone (mm)			Average (mm)	Result
		After 3 hour	After 6 hour	After 12 hour		
1.	CPCE from Raw mutton (isolate-1)	9.7	10.4	7.3	9.13	Positive
2.	CPCE from Raw chicken (isolate 2)	9.9	12.6	8.7	10.4	Positive
3.	CPCE from Mutton kabab (isolate-3)	4.8	6.2	3.5	4.83	Weakly Positive
4.	CPCE from ATCC-13124 Strain	8.1	8.6	7.5	8.06	Positive
5.	Sterile NSS	0	0	0	0	Negative
6.	Sterile FTG	0	0	0	0	Negative

Table 2: Ligated ileal loop response of rabbits to CPCE of field isolates

Loop No.	Source of CPCE	Dose injected	pH of the fluid (n=2)		Loop fluid volume (ml)/length (cm) ratio (n=2)		Average loop fluid volume (ml)/length (cm) ratio	Result
			Rabbit 1	Rabbit 2	Rabbit 1	Rabbit 2		
Loop-1	Raw mutton (isolate-1)	2 ml	7.5	7.7	0.9	1.34	1.12	Positive
Loop-2	Sterile DSS medium	2 ml	7.1	6.9	0.4	0.6	0.5	Negative
Loop-3	Raw chicken (isolate-2)	2 ml	7.8	7.4	1.0	1.2	1.1	Positive
Loop-4	ATCC-13124 Strain	2 ml	7.7	7.6	1.3	0.8	1.05	Positive

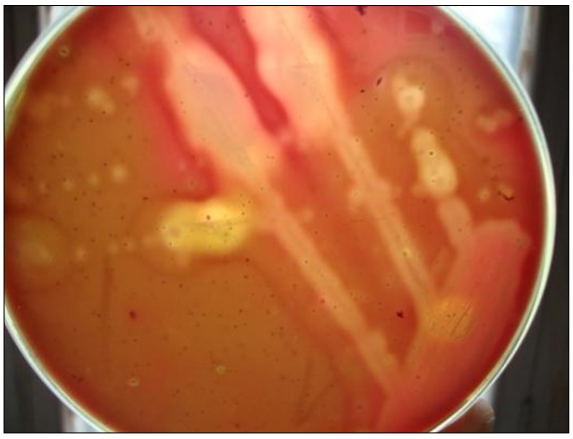

Fig 1: β haemolysis produced by *C. perfringens* type A in 5% sheep blood agar.

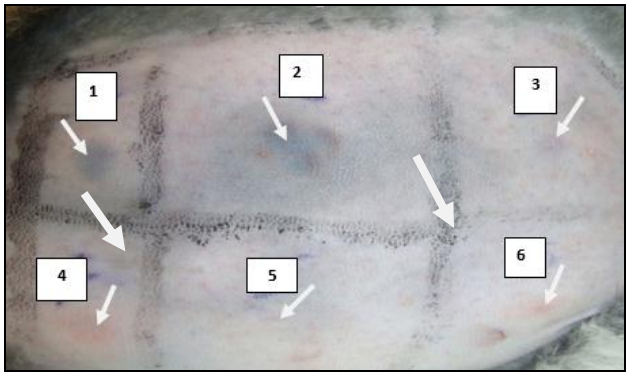

Fig 2: Vascular permeability reaction in rabbit skin 8 hr post inoculation showing bluish inflammatory reaction

Site 1: CPCE from raw mutton (isolate-1) Site 2: CPCE from raw chicken (isolate-2)
Site 3: CPCE from mutton kabab (isolate-3) Site 4: CPCE from ATCC-13124 strain
Site 5: Sterile NSS Site 6: Sterile FTG

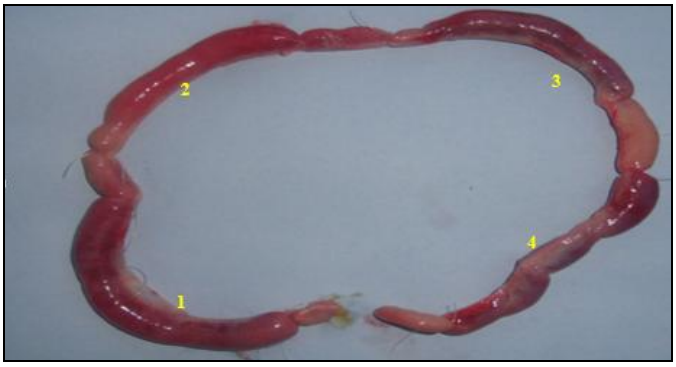

Fig 3: Rabbit ligated ileal loop responses injected with *C. perfringens* crude enterotoxin (CPCE)

Loop-1: CPCE Raw mutton (isolate-1) Loop-2: CPCE Sterile DSS medium
Loop-3: CPCE Raw chicken (isolate-2) Loop-4: CPCE ATCC-13124 Strain

4. Conclusion

The present study concluded that all the field isolates produced maximum vascular permeability reaction in 12hr following intra-dermal injection of *C. perfringens* crude entero-toxin (CPCE), which subsided completely in 48 hr post inoculation. The quantitative response of the ileal loops (fluid volume/loop length ratio) indicated the entero toxigenic activity of CPCE from field isolates as well as the standard ATCC-13124 strain. Vascular permeability reaction in rabbits was comparable with the ligated ileal loop technique and can, therefore, be used with ease without sacrificing the animals.

5. Acknowledgements

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