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THE EFFICACY OF *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN IN RABBITS

S.Y. EL-NAENAEEY* and M. S. ABOU EL-FETOUH**
Departments of Microbiology* and Pathology**.
Faculty of Veterinary Medicine, Zagazig University, Egypt.

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SUMMARY

The enterotoxin in cell free products of various strains of *Cl.perfringens* type A has the ability to produce ileal loop fluid accumulation in rabbits. Rabbits ileal loops challenged with cell extract and culture filtrates of various strains revealed an intestinal response by accumulation of exudate,distension and histopathologycal changes. The enterotoxin was shown to be heat labile and lost its activity at pH values 1 and 12. It was inactivated by pronase but not by amylase, lipase or trypsin enzymes.

INTRODUCTION

Cl. perfringens type A food poisoning is caused by ingestion of food contaminated with large numbers of Cl.perfringens cells., The cells multiply and sporulate in the intestine to produce an enterotoxin. The enterotoxin is released upon cell lysis and causes increased capillary permeability, vasodilatation and excess fluid movement into the intestinal lumen resulting in diarrhoea as reported by Willis (1977) and Popoff and Jouin (1985) . The ligated intestinal loop technique has been used extensively as a convenient model to study the enterotoxin of Vl. perfringens food poisoning (Hauschild, 1971). Few literatures were available concerning the production of enterotoxin from *Cl.perfringens* in rabbits. Therefore, the current study to investigate the production of enterotoxin of *Cl.perfringens* type A and their effect on intestinal ligation of rabbits.

MATERIAL AND METHODS

Strains: A total number of twenty *Cl.perfringens* type A strains from a total of 68 strains previously isolated and typed from different orgens of wild birds (Egypt), were used. Stock cultures were maintained frozen in cooked meat broth media (Difco). Cultures were activated for use by transferring them into fluid thioglycollate medium (OXOID) with subsequent incubation at 37°C for 18 hours under anacrobic atmosphere. Growth of cells and preparation of cell extracts and concentrated culture filtures were done according to Duncan and Strong (1968 and 19969b) and Duncan et al., (1972), smears were stained by Gram's stain and examined for typical morphological and cultural characters of the strains to ensure its purity.

Surgical operation: Twenty New Zealand white rabbits of both sexes whose weights ranged from 1.4 to 2.2 Kgs at the time of testing were used.
The operative technique for the preparation of ligated ileal loops injection was done according to Duncan and Strong (1969a) where the rabbits were anaexthetized by Kitamin Hcl (Park-Davis, U. S. A.) I. M. in a dose of 40 mg. Kg B. wt. Seven segments of about 10 cm length of each were made and these segments were numbered from 1 to 7 towards the direction of the ileum. The tested material (2ml) was injected into the lopps using, one control segment in the beginning, at the end and with the lested material in between every two loops. The injected material used was saline and strain (cell extracts or culture filtrates) alternating. Every prepared strain (either alone or with additional treatment) was injected into a appropriate rabbit. The experimental rabbits were sacrificed after 20 hours post-injection. The loop fluid accumulation and dilatation were recorded macroscopically. The used part of loop either duodenum, jejunum or ileum were opened and examined histopathologically.

Effect of period of heating: The extract or filtrate contained in screwcapped bottles were heated at 55°C in a constant-temperature water bath for various time intervals (5,10,15,20,25 min). After removal from each period of heating the suspensions were cooled in ice water and 2ml of each time preparation was injected per ileal loop and the results were recorded.

Thermostability under different pH values: the solution was initially made to a double strength, and the pH was adjusted by using concentrated Hcl and 4N NaoH. The different pH values of 1,3,5,6,9, 10, 11 and 12 were used.The appropriately adjusted extract or filtrate was then stored at 4°C for 24 hours prior to injection in ileal loops (Duncan and Strong 1969b).

Effect of enzymes: the foolowing enzmes were tested for their effect on the enterotoxin present in both cell extract and culture filtrates: a-amylast (Sigma), lipase (Sigma), trypsin (Fisher Scientific Co.) and pronase (Calbiochem). Pronase was used in a final concentration of 0.05 mg/ml. All other enzymes were used in a final concentration of 2.5 mg/ml. Pronase and trypsin were tested at pH 7.4 with 0.05M trihydroxy methylaminomethane buffer. a-amylase was tested at pH 7.0 with 0.05M phosphate buffer. To test for the effect of the enzymes on the activiy of enterotoxin, three preparations were used for challenge in each rabbit. The test preparation consisted of either the cell extract or filtrate mixed with the specific enzyme and two control preparations consisting of the cell extract or filtrate alone in the respective buffer and the enzyme alone in the buffer, all preparations were incubated for 24 hours at 37°C prior to testing for ileal loop activity (Duncan and Strong 1969b and Hauschild 1971).

Histopathological : histopathological specimens were taken from the loop and fixed in 10% neutral buffered formalin. Paraffin sections of 5 thickness were prepared and stained with hematoxylin and cosin (H&E) for microscopic examination (Lillie and Fulmen, 1976).

Statisties: statistical analsis was done by "t" test according to Steel and Torrie (1980).

RESULTS

Initial studies made by using Cl. perfringens type Astrains showed that cell extracts and cultrure filtrates prepared from cultures grown in D. S Media contained heat labile enterotoxin that causes distension and fluid accumulation in ileal loops was noticeable. Heating for 10 minutes at 60·C always inactivated the enterotoxin, whereas heating for 5 minutes at 55°C never prevented dilatation and fluid accumulation of ileal loops.

Rabbit ileal loops injected with cell extracts for detection of enterotoxigenicity induced congestion, petechae, enteric hyperaemia, haemorrhagic inflammation and much dilatation of loops due to accumulation of exudate and

Table (1): Ability of Cl. *perfringens* enterotoxin to produce ileal loop fluid accumulation and dilatation in rabbits

The tested material	Strain I[1]			Strain I[2]			Strain I[3]			Strain I[4]			Strain I[5]			Strain I[6]		
	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	L
Cell extract [b]	6	2.7	2.7	4.5	2	2.3	5	2.1	2.4	6	2.3	2.6	4	1.6	2.5	4.5	2.1	
Culture filtrate [c]	1.5	2	0.75	2.9	1.7	1.7	2	1.5	1.3	2	1.9	1.1	2.5	1.8	1.4	3.5	1.8	

Cont. Table (1)

The tested material	Strain I[7]			Strain I[8]			Strain I[9]			Strain I[10]			Strain I[11]			Strain I[12]		
	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	LFV/L ratio	LFV*	L**	
Cell extract [b]	4.5	2.3	2.8	3.9	1.7	2.3	4.2	2	2.1	2.7	1.1	2.5	3	1.1	2.7	4.3	1.9	
Culture filtrate [c]	3.3	2.3	1.4	4.5	1.9	2.4	2	1.5	1.3	3.5	1.6	2.2	2.5	2	1.3	2.5	1.9	

* L.F.V.= Loop fluid volume
** L = Length
a = Number of positive strains (12)
a,b,c = Significant difference ($P<0.05$).

inflammatory response of progressively increasing severity. This amount of loop fluid accumulation and dilatation of intestinal loop were much provoke more than in bacteria free fluids and the ratio ranged form 2.1 to 2.8 (Table 1). The ileal loop fluid volume/length ratios obtained were comparable to those control loops challenged with saline.

In rabbits ileal loops inoculated with culture filtrates gave better results responded by producing progressively fluid increasing accumulation and distention but comparatively limited than that injected with live bacterial

Table (2): The effect of heat on the enterotoxin in both cell extract and culture filtrate of *Cl.perfringens*

Heating time (min) at 55°C	Average loop fluid volume/length ratio*	
	Cell extract	Culture filtrate
5	2.4	1.7
10	2.1	1.6
15	1.4	1.2
20	0.3	0.2
25	0.0	0.0

* Average ratio for the effect of 4 tested enterotoxin strains of Cl.perfringens on ileal loops of rabbits.

extract (Table 1). The ileal loop fluid volume and length ratio ranged from 0.75 to 2.4 . It was noticed that all the injected rabbit ileal loops responded positively to *Cl.perfrigens* enterotoxin while the rest of cell extracts and culture filtrates of 8 strains were non enterotoxigenic and could not able to induce any intestinal response. There were significant differences ($P<0.05$) not only between the strains but also between cell extracts and culture filtrates of the same strains (Table1).

The stability of heat on the activity of enterotoxin in both cell extract and culture filtrate was evident (Table 2). Diminished inactivation of enterotoxin occurred within 5 to 10 minutes of heating, since heating for 15 and 20 minutes resulted in decreasing average loop fluid volume/length ratios. No activity was obtained after heating the preparations for 25 minutes.

The effect of pH on the activity of cell extract and

Table (3): Effect of different pH values on the enterotoxin activity of both cell extract and culture filtrate of Cl.perfringens

Values of pH	Average loop fluid volume/length ratio*	
	Cell extract	Culture filtrate
1.0	0.0	0.0
3.0	0.0	0.0
5.0	1.9	1.1
6.0	2.3	1.8
9.0	2.3	1.7
10.0	1.8	0.5
11.0	0.9	0.7
12.0	0.0	0.0

* Average ratio for the effect of 4 tested enterotoxin strains of Cl.perfringens on ileal loops of rabbits.

culture filtrate was shown in (Table 3). The activity of the enterotoxin was not changed appreciable when cell extract or culture filtrate was adjusted at pH 5,6,9, and 10, Less activity was noticed at an acid pH 3 and at alkaline pH 11, however, compete in activation was apparent at pH 1 and 12 values.

The effect of different enzymes on the activity of cell extract and culture filtrate of *Cl.perfringens*

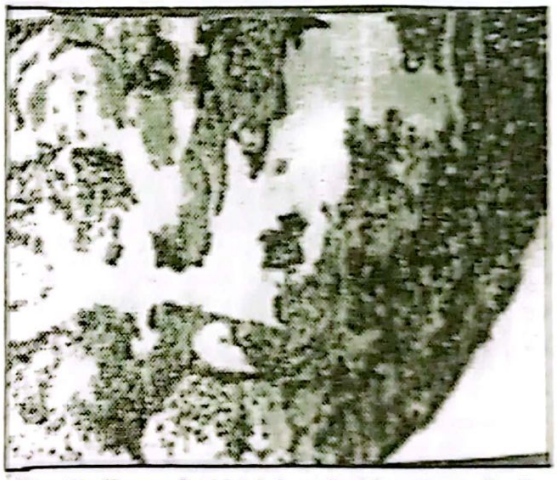

Fig. (1): Ileum of rabbit injected with untreated cell extract, showing haemorrhagic enteritis and ulcer. H & E X 400.

strains was shown in (Table 4). The activity of enterotoxin in both cell extract and culture filtrate was destroyed after treatment with pronase enzyme, however a-amylase, lipase and trypsin did not destroy the activity of enterotoxin.

Table (4): The effect of selected enzymes on the enterotoxin in cell extract and culture filtrate of *Cl perfringens*

Tested material	Average loop fluid volume/length ratio*			
	Amylase	Lipase	Trypsin	Protease
Cell extract with enzyme in physiological saline	2.3	2.6	1.9	0.6
Cell extract in physiological saline	2.1	2.7	2.0	2.5
Enzyme in physiological saline	0.0	0.0	0.0	0.0
Culture filtrate with enzyme in physiological saline	1.7	1.3	1.8	0.0
Culture filtrate in physiological saline	2.1	1.9	1.5	1.7
Enzyme in physiological saline	0.0	0.0	0.0	0.0

* Average ratio for the effect of 4 tested enterotoxin strains of Cl perfringens on ileal loops of rabbits

Histopathological examination showed that the ileum of rabbits injected with cell extracts revealed haemorrhagic exudate in its lumen. This exudate consisted of extravasated erythrocytes, desquamated epithelium and leucocytic infiltration (mostly lymyhocytes). The wall of ileum revealed also congested capillaries as well as lympoid infiltration and ulcer (Fig. 1) Hyperplasia of lymphoid follicles was also noticed. Mared dilatation of the gut, complete shedding of the intestinal villi which were completely effaced and necrosed as well as

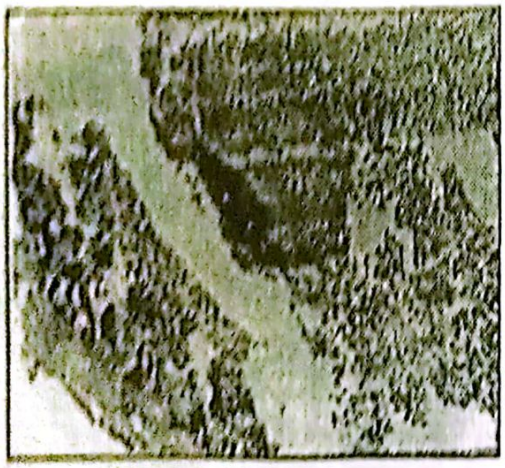

Fig. (2): Ileum of rabbit injected with untreated culture filtrates, showing markedly dilated gut contained exudate and ulcer H & E X 400.

Fig. (3): Ileum of rabbit injected with cell extract trea with trypsin, showing haemorrhage in the gut, shedd and effaced villi H & E X 400.

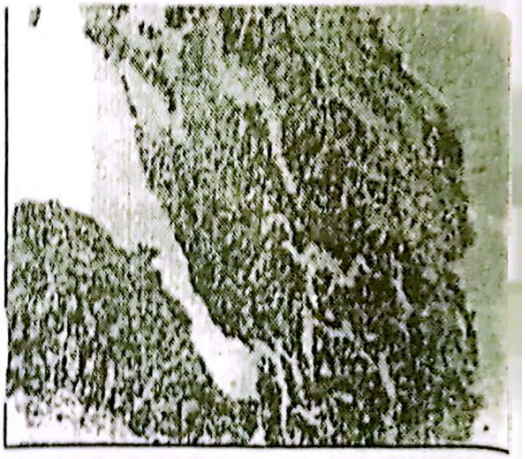

Fig. (4): Ileum of rabbit injected with culture filtrat treated with trypsin, showing dilated gut with desqua mated epithelium and inflammatory cells in the lume and mucosa H & E X 400.

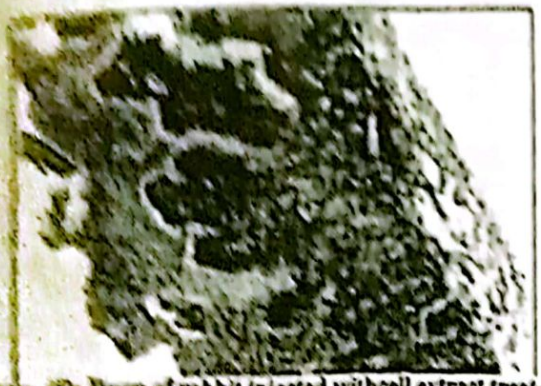

Fig. (5): Ileum of rabbit injected withcell extract treated with lipase, showing shedding of the villi with haemorrhage in the lumen. H & E X 400.

Fig. (6): Ileum of rabbit injected with culture filtrate treated with lipase, showing markedly dilated gut with effaced villi H & E X 400.

haemorrhage in the lumen of the gut had been observed (Figs. 2 and 3).

The ileum of rabbits injected with culture filtrates showed dilated gut, shedding of the lining epithelium, necrosis of the intestinal villi, leucocytic infiltration (mostly neutrophils), lymphocytes and macrophages in the lumen, lamina propria and submucosa (Fig. 4). Haemorrhages in the lumen, shedding of the intestinal villi and congestion were also noticed in these rabbits. These intestinal villi were necrotic and effaced (Figs. 5 and 6).

DISCUSSION

The ligated intestinal loop in rabbits has been used as a model to study *Cl.perfringens* type A enterotoxin as a cause of food poisoning. The suitability of the loop technique for this purpose showed a reasonable results and has been demonstrated in number of publications. Hauschild et al., (1968). Duncana and Strong (1969 a). Haushild (1970) and duncan and Strong (1971).

During this investigation 20 strains from 68 isolates were randomized for studing the production of enterotoxin in ileal loop or rabbits. Table (1) showed the ability of cell extract and culture filtrates of various strains of Cl.perfringens to produce ileal loop fluid acumulation and dilatation. The ability of enterotoxin in cell extract to produce its effect on ileal loop fluid volume/length ratio was much due to large amount of fluid accumulation. It was also noticed that the enterotoxin of the same tested strains (12 Positive strains from 20 by a percentage of 60%) had the ability to produce an active response of ligated intestinal loops. The obtained results (Table 1) showed that a significant differences ($P<0.05$) not only between the strains, but also between the cell free products of the same strains. This results were inagreement with that reported by Duncan et al., (1968). Hauschild et al.,(1970) and Nillo and Dorward (1971), who mentioned that there was a good correlation in the ability of cell extracts and concentrated caulture filtrates of the same strain to produce fluid accumulation and dilatation in the ileal loop. In the mean time Duncan and Strong (1969a) reported a total of 14 of 29 strains isolated from food poisoning outbreaks that produced exudation of fluid and dilatation in the ileal loop when the challenge was made with cell extracts and culture filtrates. The enterotoxin is released upon cell lysis and causes increased capillary permeability. Vasodilatation and excess fluid movement into the intestinal lumen resulting fluid accumulation and dilatation of the intestine, (Hauschild 1971).

The obtained results showed that both preparations (cell extract and culture filtrate) have comparable heat lability of enterotoxin (Table 2). Little inactivation of enterotoxin occurred at 55 C for 5-10 munutes, while no activity wasobtained after heating for 25 minutes. The results in this investigation revealed that complete inactivation occurred at pH ranged from 5,6,9 and 10. There was a complete loss of activity at pH and 12 (Table 3).The enterotoxin in both cell extracts and

culture filtrates were inactivated by pronase enzyme but not affected by amylase, lipase or trypsin (Table 4). These results are inagreement with that reported by Duncan and Strong (1969b) and Hauschild (1971) who concluded that the enterotoxin was shown to be heat labile and was inactivated by pronase but not by steapsin, trypsin, lipase or amylase. They added that loss of activity occurred at pH values 1,3,5, and 12.

The histopathological examiation of the ileum of the rabbits injected with cell extyract and culture filtrate revealed haemorrhagic enteritis, dilated gut and shedding villi which may be necrosed and effaced as well as ulcerated. The forementioned results are inagreement with Duncan and Strong (1968) who stated that similar lesions in addition to oedema had been detected in the ileum of rabbits injected with *Cl.perfringens* strains. On the other hand, Lozano et al., (1970) and Alisdair et al., reported that most of the forementioned lesions were seen in the bovine gastrointestinal tract affected with Cl.perfringens type "A".

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