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BIOLOGICAL DETECTION OF CLOSTRIDIUM PERFRINGENS ENTEROTOXIN ORIGINATED FROM CAMEL (*CAMELUS DROMEDARIUS*) ENTEROTOXAEMIA

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SUMMARY

An outbreak of enterotoxaemia in breeding camels in Belbais area was reported. A total of 68 organ samples (liver, kidney, spleen, heart, small intestine, colon, lymph node, abomasum and rumen) collected from 10 dead animals were clinicaly and bateriologicaly examined. Of 40 bacterial isolates, 23 (57.5%) were identified as *Clostridium perfringens* type A, 8 (20%) as Bacillus cereus and 9 (22.5%) as other *Bacillus spp*. The biological detection of *C. perfringens* enterotoxin was done in rabbits ileal loops. Rabbits ileal loops challenged with cell extract or culture filtrates of various strains actively responded by fluid accumulation and dilatation. The enterotoxin was shown to be heat labile and lost its activity at pH 1 and 12. It was inactivated by pronase but not by amylase, lipase or trypsin enzymes.

INTRODUCTION

Outbreaks of enterotoxaemia in both breeding and racing camels in different locations in Dubai was reported by Seifert et al. (1993). In racing camels, intermittent diarrhoea was the predominant symptom which progressed to severe watery diarrhoea during the course of the disease. Four of 10 sick camels died within four days after the onset of symptoms and these with gross pathological lesions were typical for enterotoxaemia in camels. They isolated *C. perfringens* type A from enterotoxaemic camels and prepared bacterin-toxoid (cells and toxoid) as a local specific vaccine for enterotoxaemia in camels. They stated that no subsequent outbreaks of enterotoxaemia have occurred in vaccinated camels. Also Wernery et al. (1993) attributed the haemorrhagic disease (HD) in racing camels in United Arab Emirates to Bacillus cereus as a possible cause of the disease, beside digestive toxaemias especially enterotox-

aemia caused by *C. perfringens* (Jones and Hunt, 1983). El-Naenaeey and Abou El-Fetouh (1993) studied twenty *C. perfringens* type A strains isolated from wild birds to their efficacy of enterotoxin production among rabbits. 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 Jestin (1985). Clostridium perfringens type A enterotoxin induces tissue damage and fluid accumulation in rabbit ileum was reported by Wieckowski et al. (1998), Netherwood et al. (1998) and Siarakas et al. (1997). The ligated intestinal loop technique has been used extensively as a convenient model to study the enterotoxin of *C. perfringens* food poisoning (Sherman et al., 1994). Further studies on Clostridium perfringens enterotoxin (CPE) on permeability alteratioins in plasma membrane were done by McClane (1994, 1996). Therefore, the current study was directed to investigate and detect the production of enterotoxin of *C. perfringens* type A strains isolated from camel enterotoxaemia, and their effect on intestinal ligatin of rabbits.

MATERIAL AND METHODS

An outbreak of enterotoxaemia in breeding camels in Sharkia Governorate (Belbais area) were clinicaly and bacteriologicaly examined.

Isolation:- A total of 68 organ samples (liver, kidney, spleen, heart, small intestine, colon, lymph nodes, abomasum and rumen) as in (Table 1), were collected from 10 dead animals. The samples were collected under complete asepectic condition in sterile plastic bags and transported to laboratory as soon as possible for bacteriological examination. After sterilization, tissue samples from different organs and intestinal mucosal swab were obtained. Each sample was divided into 3 portions. The first portion was inoculated directly onto MacConkey agar, S.S. Agar (Lab. 52 M, Amersham), blood agar and mannitol salt agar plates and incubated aerobically at 37°C for 24-48 hrs. The second portion was inoculated into selenite "F" broth medium and incubated aerobically at 37°C for 12 hrs., followed by subculture onto Hektoen enteric agar medium, MacConkey agar and S.S. agar media. The inoculated plates were incubated aerobically at 37°C for 24-48 hrs. The third portion was inoculated into cooked meat medium (Difco) and incubated anaerobically at 37°C for 24 hrs. It was then subcultured onto neomycin sulphate sheep blood agar (200 ug/ml) as reported by Willis (1977) for purification of the strains. The inoculated plates were incubated anaerobically at 37°C for 48 hrs. The pure growing colonies of the isolates were characterized morphologically and colonial appearance according to Finegold and Martin (1982). The pure colonies were identified biochemically according to Koneman et al. (1983) and Kreig and Holt (1984).

Strains:- The anaerobic isolates were identified biochemically by Nagler reaction and toxin-antitoxin plate test according to Willis and Hobbs (1959). Clostridium perfringens type A isolates were maintained frozen in cooked meat broth medium as stock cultures. Cultures were activated for use by transferring them into fluid thioglycollate medium (oxoid) with subsequent incubation at 37°C for 18 hrs. under anaerobic atmosphere. Growth of cells and preparation of cell extracts and concentrated culture filtrates were done according to Duncan and Strong (1968 and 1969b) 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 three 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) and Sherman et al. (1994) where the rabbits were anesthetized by Kitamin Hcl (Park-Davis, USA) 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 (2 ml) was injected into the loops using, one control segment in the begining, at the end and with the tested 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 an appropriate rabbit. The experimental rabbits were sacrificed after 20 hours post-injection. The loop fluid accumulation and dilatation were recorded macroscopically.

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

The effect 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 following enzymes were tested for their effect on the enterotoxin present in both cell extract and culture filtrates: α-(alpha) amylase (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 0.05 M trihydroxy methyl aminomethane buffer. Alpha (α) amylase was tested at pH 7.0 with 0.05 M phosphate buffer. Lipase was tested at pH 6.0 with 0.05 citrate phosphate bufer. To test for the effect of the enzymes on the activity 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 1969 b and Hauschild, 1970 & 1971).

Statistics: Statistical analysis was done by "t" test according to Steel and Torrie (1980).

RESULTS

The clinical manifestations of affected breeding camels with enterotoxaemia were sweating, shivering, hyperexcitment, ataxia and convulsions. Some animals became aggressive and suddenly died. Untreated animals died within one hour after the onset of symptoms.

Table (1): The bacteriological examinations of organ samples from camels died with enterotoxaemia.

Samples	Number	Clostridium Perfringens type A	Bacillus Cereus	Bacillus Spp.	percentage (%)
Liver	10	5	2	1	80
Kidney	10	-	1	2	30
Spleen	10	4	-	1	50
Heart	5	-	-	-	-
Small intestine	10	6	2	3	110
Large colon	5	2	2	1	100
Lymph nodes*	8	4	-	-	50
Abomasum	5	1	-	-	20
Rumen	5	1	1	1	60
Total	68	23(33.8%)	8(11.76%)	9 (13.2%)	40(58.8%)

* Lymph nodes: Prescapular, jejunal, duodenum, mesentric.

Table 2: Ability of *C. perfringens* enterotoxin to produce ileal loop fluid accumulation and dilatation in rabbits.

The test Materials	Strain 1[a]			Strain 2			Strain 3			Strain 4			Strain 5			Strain 6			Strain 7		
	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration
Cell extract [b]	6	2.7	2.2	4.5	2	2.3	5	2.1	2.4	6	2.3	2.6	4	1.6	2.5	4.5	2.1	2.1	5.5	2.1	2.6
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	1.9	2.1	1.5	1.4

Cont. Table (2)

The test Materials	Strain 8			Strain 9			Strain 10			Strain 11			Strain 12			Strain 13			Strain 14		
	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L ration	LFV*	L**	LFV/L
Cell extract [b]	6.5	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	2.3	4	1.9	2.1
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						

* LFV = Loop fluid volume

** L = Length

a = Number of positive strains (12)

b, c = Significant difference ($P < 0.05$).

The results of bacteriological examinations are summarized in (Table 1). Of 40 (58.8%) bacterial isolates, 23 (57.5%) were identified as *Clostridium perfringents* type A, 8 (20%) as Bacillus cereus and 9 (22.5%) as other Bacillus spp. The initial studies made by using *C. perfringens* type A strains showed that cell extracts and culture 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, where as 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 an inflammatory response of progressively increasing severity. These amount of loop fluid volume and degree of dilatation of intestinal loop were much more distinct than in culture filtrates and the ratio ranged from 2.1 to 2.8 (table 2). 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 extract (Table 2). 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 *C. perfringens* enterotoxin while the rest of cell extracts and culture filtrates of 9 strains (from 23 strains) were non enterotoxigenic and were not be 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 (Table 2).

The effect of heat on the activity of enterotoxin in both cell extract and culture filtrate was evident (Table 3). Slight inactivation of enterotoxin occurred within 5 to 10 minutes of heating. However 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 culture filtrate was shown in (Table 4). The activity of the enterotoxin was not changed appreciably when cell extract or culture filtrate was adjusted at pH 5, 6, 9 and 10. Less activity was noticed at an acid pH3 and at alkaline pH11, however, complete inactivation was apparent at pH 1 and 12 values.

The effect of different enzymes on the activity of cell extract and culture filtrate of *C. perfringens* strains was shown in (Table 5). The activity of enterotoxin in both cell extract and culture filtrate

Table (3): The effect of heat on the enterotoxin in both cell extract and culture filtrate of *C. perfringens*.

Heting time (min) at 55°C	Average loop fluid volume/length ration *	
	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 *C. perfringens* on ileal loops of rabbits

Table (4): The effect of pH level on the enterotoxin in both cell extract and culture filtrate of *C. perfringens*.

pH level	Average loop fluid volume/length ration *	
	Cell extract	Culture filtrate
1.0	0.0	0.0
3.0	0.8	0.5
5.0	1.9	1.1
6.0	2.2	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 *C. perfringens* on ileal loops of rabbits

Table (5): The effect of selected enzymes on the enterotoxin in both cell extract and culture filtrate of *C. perfringens*.

Tested material	Average loop fluid volume/length ration *			
	α-amylase	Lipase	Trypsin	Pronase
Cell extract with enzyme in physiological saline	2.3	2.6	1.9	0.0
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 *C. perfringens* on ileal loops of rabbits

was destroyed after treatment with pronase enzyme, however, α-amylase, lipase and trypsin did not destroy the activity of enterotoxin.

DISCUSSION

These data (Table 1) confirm the findings of Seifert et al. (1993) who reported that *C. perfringens* type A is a serious cause of enterotoxaemia in camels and leading to loss in breeding and racing camels in Dubai.

The ligated intestinal loop in rabbits has been used as a model to study *C. perfringens* type A entrotoxin (CPE) as a cause of food poisoning and enterotoxaemia.The suitability of the loop technique for this purpose showed reasonable results and has been demonstrated in number of publications, Duncan and Strong (1971), Sherman et al. (1994), McClane (1994 & 1996), Siarakas et al. (1997) and Wieckowski et al. (1998).

During this investigation 23 strains were used for studying the production of enterotoxin in ileal loop of rabbits. Table (2) showed the ability of cell extract and culture filtrates of various strains of *C. perfringens* to produce ileal loop fluid accumulation 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 (14 positive strains from 23 by a percentage of 60.86%) had the ability to produce an active response of ligated intestinal loops. The obtained results (Table 2) showed that significant differences ($P < 0.05$) were found not only between the strains, but also between the cell free products of the same strains. These results were inagreement with that reported by Duncan et al. (1968), Hauschild et al. (1970), Niilo and Dorward (1971) and El-Naenaeey & Abou El-Fetouh (1993) who mentioned that there was a good correlation in the ability of cell extracts and concentrated culture 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 out of 29 strains isolated from food poisioning outbreaks that produce 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 (Siarakas et al., 1997 and Wieckowski et al., 1998). The obtained results showed that both preparations (cell extract and culture filtrate) have comparable heat lability of enterotoxin (Table 3). Little inactivation of enterotoxin occurred at 55°C for 5-10 minutes, while complete inactivatoin was obtained 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 1 and 12

(Table 4). The enterotoxin in both cell extracts and culture filtrates were inactivated by pronase enzyme but not affected by amylase, lipase or trypsin (Table 5). These results are inagreement with that reported by Wieckowski et al. (1998) who characterized the permeability of membrane associated *C. perfringens* enterotoxin following pronase treatment, and the results which reported by Duncan and Strong (1969b) and Hauschild (1970 & 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.

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