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Study on *in-vitro* Biochemical Growth Characterization and Assessment of Hemolytic Toxin of *Clostridium perfringens* Type B and D

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Abstract.- The present study was conducted to identify the biochemical characterization, evaluation of growth and hemolytic properties of *Clostridium perfringens* (*C. perfringens*) type B and D in different growth media and physical environment. The reinforced clostridial (RC) and thioglycolate (TG) growth media were observed better for the growth and hemolysin production 12 h post-incubation at 37°C ($P<0.05$). Time, temperature and buffer also illustrated a significant ($P<0.05$) relationship on the hemolytic activity of proto-toxin of *C. perfringens* types B and D. The trypsinization of proto-toxin greatly enhanced hemolytic activity and 1% trypsin in physiological saline at 37°C for one hour showed maximum hemolytic units ($P<0.05$). The reinforced clostridial and thioglycolate growth media were found better for the cultivation of *C. perfringens* and enhanced hemolytic activity with 1% trypsinization. Present results could be helpful in the preparation of an effective bivalent vaccine against enterotoxaemia and lamb dysentery disease of sheep and goat.

Key words: *Clostridium perfringens*, biochemical characterization, growth characteristics, trypsin hemolysin interaction

INTRODUCTION

The *Clostridial* organisms are widely distributed in nature. Their normal habitat includes soil, water and decomposing organic matter, where they occur not only as vegetative cells but also as spore forms and reported as normal flora of human and animal intestinal tract (Phukan *et al*., 1997). They are ingested frequently by the animal along with fodder and some of them are able to adopt temporarily in the intestinal tract. Under favorable conditions, the organisms invade the intestinal mucosa entering the blood stream and produce rapidly killing toxemia and septicemia (Sameera *et al*., 2005). Pathogenic species of *Clostridium* produce disease in man and animal in two ways. First group of clostridial organism produces histotoxic diseases by invading and damaging body tissue and causing diseases such as black quarter (*C. chauvoei*), enterotoxaemia and pulpy kidney (*C. perfringens* type D), lamb dysentery (*C. perfringens* type B), Second group produces toxigenic diseases by secreting harmful substances "TOXINS" in and outside the body and causing diseases *viz.* tetanus (*C. tetani*) and botulism (*C. botulinum*) (Akram *et al*., 2010).

The *C. perfringens* are divided into five types A, B, C, D and E on the basis of four major lethal toxins *i.e.* (alpha, beta, epsilon, and iota) which are produced by these organisms (Harrison *et al*., 2005). Alpha toxin is produced by strains of all types, beta toxin is produced by strain of type B and C, epsilon toxin by strains of type B and D, while iota toxin by strain E of clostridium perfringens (Alex *et al*., 2004).

Enterotoxaemia cum lamb dysentery which is an acute and fatal disease in nature may result in therapeutic failure. The disease can be effectively controlled by ensured satisfactory mass scale vaccination. The vaccine contains the toxoid of the serotypes (B and D) and the toxoid produced by both serotypes differs in the nature (Beta and Epsilon) qualitatively and quantitatively. The bivalent vaccine against enterotoxaemia cum lamb dysentery contains toxoid from both serotypes. To get desired quantity of the toxoid in the vaccine it has to be evaluated for the difference in the toxin production by two different types of *C. perfringens*.

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This difference in the toxin production of the organisms may lower quantity of toxoids in the vaccine and in turn could result incapability of vaccine to effectively produce immunity. The present study is therefore aimed to biochemically characterize, evaluate growth in different media, effect of trypsinization and hemolytic properties of *C. perfring*ens type B and D in different physical environments.

MATERIALS AND METHODS

Seed culture of *C. perfringens* type B and D were provided by Centre for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan.

Culture and biochemical characteristics

The growth characteristics of *C. perfringens* types B and D were recorded on reinforced clostridial (RC), thioglycolate (TG), Robertson's cooked meat (RCM), egg meat (EM), Iron milk (IM) and egg yolk (EY) media respectively. For colony characteristics and haemolytic activity organisms were grown in RC blood agar and RC medium plates incubated under anaerobic conditions for 48 h at 37°C. The bacterial cultures were stained with Gram staining method and biochemically characterized (Cruickshank, 1975; Finegold and Martin, 1982).

Viable counts

Total viable count for the culture of *C. perfringens* types B and D were performed with standard plate count method. The culture was inoculated in triplicate and the bacterial colonies were counted and multiplied with the dilution factor to obtain the bacterial population per ml of the stock culture.

Growth characteristics

The organisms were cultivated in a variety of media such as TG, IM, EM and RC under the optimal conditions. Turbidity, foam formation and hemolysin titer were monitored after 4, 8, 10 and 12 h post-incubation at 37°C. The characteristics for the hemolysin production of the organism were also observed at pH 6.8-7 with 24h of incubation.

Effect of trypsin

The epsilon prototoxin in culture supernatant of *C. perfringens* types Band D was activated with 0, 1, 5 and 10% trypsin solution at the ratio of 9:1 (Lyerly and Wilkins, 1991).

Effect of time, temperature and buffer on prototoxin trypsin interaction

Clostridium perfringens type B and D was cultured in RC medium at pH 6.8-7.0 for 13-14 h. Each of the filtrates was activated with 1% trypsin solution and hemolysin trypsin interaction and resultant hemolytic activity was estimated.

Time

The prototoxin of *C. perfringens* types B and D was activated with 1% trypsin solution for 30, 60, 90, and 120 min at 37°C temperature.

Temperature

The micro-titration plates were incubated at 37°C, 22°C and 4°C temperatures for 60 min with the aim to observe the effect of incubation, temperature and time on the hemolysis.

Buffer

Each of the filtrates was activated with 1% trypsin solution for 60 min and diluted in normal physiological saline (NPS), phosphate buffer solution (PBS) and tris-buffer solution (TBS) @ 0.02M/l, respectively. The sheep RBC (1%) was used and micro-titer plates were then incubated at 37°C for one hour.

Calculation of haemolytic unit (HU)

The hemolytic unit was calculated from the formula HU= A+ (B-A)/2, where B stands for the lowest dilution of the toxin in the well showing the button formation and A stands for the dilution of the toxin in the well showing complete hemolysis. The data was subjected to statistical analysis using Chi square test (cross tab) using SPSS 16 for windows.

RESULTS

Morphology and staining reaction

The microscopic examinations of stained smears reflected Gram positive spore forming bacilli.

Culture characteristics

In RC blood agar the organism growth resulted in the development of circular, low convex, semi translucent, butyrous and easily emulsifiable colonies. The round colonies of both strains were surrounded by a narrow zone of complete haemolysis and a wide zone of partial haemolysis. In cooked meat medium the meat become pink and was not digested. Gelatin was liquefied. In case of filtered EY medium, *C. perfringens* produced a marked opalescence.

Growth characteristics

The results relating the growth of *C. perfringens* types B and D in various growth media (RC, TG, IM, RCM, EY and EM) at different interval of incubation are presented in Table I. The turbidity and foam appeared after eight hours of incubation in different growth media and maximum growth was observed at 12 h; similarly hemolysin production was determined at its maximum 12 h post-incubation for all types of growth media. However, the highest number of HU/ml was observed in RC, which was followed by TG whereas minimum HU/ml was observed in EM ($P<0.05$). In IM growth medium *C. perfringens* type B exhibited better growth than type D. Under constant *pH* conditions, the production of hemolysin (Table II) by both the serotypes were found insignificant ($P>0.05$).

Viable counts

The results of total viable count of the culture of *C. perfringens* type B and D on different growth media are depicted in Table III. The results reflected that both *C. perfringens* type B and D exhibited heterozygous growth in different growth medium. There was a significant difference ($P<0.05$) in the colony count for both types in different growth medium. The highest mean colonies were produced in the RC, which was followed by EM for type B, whereas type D showed the highest bacterial count in RC, followed by IM.

Biochemical characteristics

The results from biochemical tests are presented in Table IV. These organisms fermented glucose, lactose, maltose, sucrose and fructose producing acid and gas. *C. perfringens* type B was unable to ferment mannitol, galactose, dulcitol, while partially fermented salicin. Moreover, all the sugars which were not fermented by type B were fermented by type D of *C. perfringens.*

Table I.- Turbidity, foam and hemolysin production of *C. perfringens* type B and D at different intervals (hours) of incubation in different growth media.

Media	Time* (hour)	Turbidity*		Foam formation*		Hemolysin activity* (HU/ml)	
		B[1]	D[1]	B[1]	D[1]	B[1]	D[1]
TG	4	0	0	0	0	32	64
	8	1	1	1	1	144	160
	10	2	2	1	1	544	273
	12	3	3	2	3	1040	1056
IM	4	0	0	0	0	136	132
	8	0	1	0	1	1000	132
	10	0	1	0	1	1088	264
	12	1	2	1	2	2112	528
EM	4	0	0	0	0	32	32
	8	0	0	0	0	160	144
	10	0	1	0	0	264	160
	12	0	0	0	0	580	528
RC	4	0	2	0	1	66	66
	8	1	2	1	1	518	132
	10	1	3	1	2	1056	640
	12	2	3	2	3	2000	4224

TG, thioglycolate; IM, iron milk; EM, egg meat; RC, reinforced clostridium; 0,-; 1, +; 2, ++; 3, +++.
*Significant association (*P<0.05*) between time and turbidity, foam formation and hemolysin formation for *C. perfringens* Type B & D.
B[1] & D[1], *C. perfringens* type B & *C. perfringens* type D

Table II.- Titer of the hemolytic toxin of *C. perfringens* type B and D 24 hours post-incubation at constant *pH* in different growth medium (HU/ml)

Growth medium	*C. perfringens* Type B	D
Thioglycollate	144	144
Iron milk	280	288
Egg meat	264	288
Reinforced clostridium	264	280

*Non-significant association ($P>0.05$) in the HU/ml between *C. perfringens* type B and D at constant *pH* 7.0

Clostridium perfringens types B and D reduced nitrate, produced H_2S, fermented litmus milk and led to Neglar reaction (produced

phospholipase). *Clostridium perfringens* type B also hydrolyzed urea and produced ammonia. Both of the serotypes did not produce indole, acid metabolites from glucose and found negative for catalase.

Table III.- Viable count of *Clostridium perfringens* type B and D in different media.

Growth medium	Total bacterial count	
	C. perfringens B	*C. perfringens* D
Thioglycolate medium (TG)	$5.2x10^{8b}$	$10x10^{8b}$
Iron milk medium (IM)	$3.6x10^{8c}$	$12x10^{8b}$
Egg meat medium (EM)	$7.0x10^{8b}$	$7.4x10^{8c}$
Reinforced clostridium medium (RC)	$31x10^{8a}$	$15x10^{8a}$

[abc]Different superscript in the same column are significantly different (*P<0.05*)

Table IV.- Biochemical characteristics of *Clostridium perfringens* type B and D.

Biochemical Tests	*Clostridium perfringens* serotype	
	B	D
Glucose fermentation	+A	+A
Maltose fermentation	+A	+A
Lactose fermentation	+A	+A
Sucrose fermentation	+A	+A
Fructose fermentation	+A	+A
Galactose fermentation	–	+
Dulcitol fermentation	–	+
Indole production	–	–
Methyl red	–	–
Nitrate reduction	+	+
H_2S production	+	+
Voges Proskauer	–	–
Litmus milk	+	+
Urease	+	–
Catalase	–	–
Phospho lipase reaction	+	+
Salisin fermentation	±	–

A, Acid production; +, Positive; -, Negative; ±, Partial reaction.

Effect of trypsin concentration

The *C. perfringens* types B and D were incubated for different growth media at pH 6.5-7.0 after 13-14 h. The filtrate containing toxins was treated with trypsin (1, 5, and 10%) for the activation of prototoxin and hemolytic titre is quantified (Table V). The clostridial hemolytic property was increased as a result of interaction with 1, 5 and 10% trypsin solution in physiological saline in the ratio of 1:9. *Clostridium perfringens* type D produced maximum HU in RC, which was followed by TG and IM growth media (P<0.05).

Effect of time, temperature and buffers on prototoxin trypsin interaction

The results of the effect of incubation time, temperature and buffers on prototoxin interaction are illustrated in Table VI. The time and temperature were significantly (P<0.05) associated with the hemolytic activity. Maximum HU/ml was observed when interaction time was 60 minutes at 37°C. The incubation temperature of 37°C resulted in maximum production of HU, while minimum HU was recorded at 4°C (P<0.05). Similarly normal physiological saline was found as better buffer resulted in more hemolytic activity of *C.perfringens* types B and D.

DISCUSSION

The present study was designed to biochemically characterize the *Clostridium perfringens* serotypes B and D and to determine the effect of different physical parameters on the toxin production. The culture of *Clostridium perfringens* types B and D was found pure in terms of morphology, cultural characteristics and biochemical tests. All of the present findings were in accordance with the results described by Carter and John (1990).

The organism was grown in different media such as TG, RC, IM and EM growth media, overall better growth was observed in RC and TG medium. This finding was in close agreement with the finding reported by Jain *et al.* (1990), who prepared antigens of *C. perfringens, C. tetani and C. chauvoei* in thioglycollate medium studied the biochemical activity and found high yield of hemolysin. The present study also disclosed that RCM is better growth medium as compared to TG, EM and IM in case of *C. perfringens* type D. Similar results are also described by Dar *et al.* (1992), who used the RC for the growth and toxin production of *C. chauvoei and C. perfringens* and reported higher titer of haemolysin in culture

Table V.- Effect of trypsin concentration on the activation of C. *perfringens* type B and D produced prototoxin into toxin in different growth media (HU/ml).

Growth medium	Trypsin concentration							
	0%		1%		5%		10%	
	B	D	B	D	B	D	B	D
TG	1280	2304	2056	4096	2000	2560	1304	2560
IM	2560	2112	4096	4096	3136	2560	3650	2560
EM	1280	256	2650	1024	1304	264	1376	544
RC	2112	8448	3136	16384	2560	15217	2304	12576

TG, thioglycolate; IM, iron milk; EM, egg meat; RC, reinforced clostridium medium.
*Significant association ($P<0.05$) between trypsin concentration and growth medium for the hemolytic activity of *C. perfringens*

Table VI.- Effect of time, temperature and buffers on prototoxin trypsin (1%) interaction.

Parameter		Hemolytic activity of *C. perfringens* (HU/ml)	
		B	D
Time* (Minutes)	30	320	528
	60	556	2056
	90	518	1040
	120	544	720
Temperature* (°C)	37	2112	8448
	22	640	1040
	4	132	160
Buffers*	NPS	2112	8448
	PBS	1600	4352
	TBS	1080	3644

*Significant association ($P<0.05$) in the hemolysin activity between *C. perfringens* type B and D for time, temperature and buffers

supernatant. It was also noticed that haemolysin titer of *C. perfringens* type B in IM and TG medium was higher than the EM.

The present study indicated high level of hemolysin produced after 12 h of incubation by the organism and was found deteriorated on further incubation. These observations were in line with Farag *et al.* (1982) and Dar *et al.* (1992).

During the propagation of clostridial organisms the metabolic activities resulted in the production of acetic acid, propionic acid and butyric acid resulting into acidic pH of the culture medium. The optimum pH for survival and growth of the organism ranges (5-9) as suggested by Farag *et al.* (1982) and Fuentes *et al.* (1983). Present study indicated that the hemolysin production was higher at pH 4.2 to 5.8 pH after 12 h of incubation. The hemolysin activity was excelled with the acidic environment of the medium. Conversely at pH 6.8 - 7.0, the hemolytic activity was purely hemolysin dependent as reported previously by Fuentes *et al.* (1983) and Dar *et al.* (1992) who reported that *pH* of the medium ranged between 6.8-7.0 during incubation of the *C. septicum* for its toxin production.

The prototoxin from *C. perfringens* types B and D was treated with 1, 5 and 10% trypsin solution in physiological normal saline (ratio of 1:9), all three fractions of trypsin showed an effect on the concentration of hemolytic toxin; however, the result of 1% trypsin treated prototoxin was in agreement with previous result of the study carried out by of Lyerly and Wilkins (1991). They further stated that epsilon prototoxin and epsilon toxoid had the same combining power but varied in antigenecity. In this way, the antigenic efficiency of the epsilon toxin and toxoid could be greatly improved by trypsinization.

In the present study, it was also observed that 60 minutes interaction time was the ideal time for the activation of prototoxin to hemolytic toxin. Present result confirmed the finding of Dar *et al.* (1992) who treated the culture filtrate with trypsin at 37°C for one hour in order to observe the hemolytic activity.

Of the different temperatures used for the growth of the organisms, it was observed that the organisms elaborated more toxins at 37°C. This observation was in harmony with the results of Fuentes *et al.* (1983), who observed the highest titer

of hemolysins at 37°C in case of *C. perfringens, C. haemolyticum and C. chauvoei*.

Moreover, in the present study different diluents such as PNS, PBS and TBS were used. Comparatively marked difference in hemolytic activity of toxin in these diluents was observed. This result was in agreement with the observation of Ramachandan (1969), who reported that haemolysin of *C. chauovei* diluted in physiological saline exhibited highest titer in comparison with other buffers used.

CONCLUSIONS

The present study highlighted that the *C. perfringens* may be cultivated in different growth media but maximum growth is possible with reinforced clostridial medium and thioglycolate medium incubated at 37°C for 12 h. Similarly prototoxin activation into toxin with 1% trypsin yielded more hemolysin units in normal physiological saline incubated at 37°C for one hour. This property of the toxin may be utilized in the vaccine production against *C. perfringens.*

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