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In Vitro Production and Detection of Haemolytic Toxin of *Clostridium perfringens* Type-D

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ABSTRACT

Physicochemical factors modulating production of hemolysin in *in vitro* culture of *Clostridium perfringens* (type-D) were evaluated. The hemolysin production was at peak (4184 haemolytic units) on 24 hours post-incubation at 37°C in Reinforced Clostridial Medium-RCM. Anaerobic environment, supply of nitrogen gas and neutral pH during incubation augmented the hemolysin production in the culture. Trypsin in the culture filtrate (0.1%) activated the prototoxin into active toxin which exhibited maximum lytic activity in 60 minutes interaction time. Trypsin solution (1%) alone failed to induce hemolysis while the trypsin containing hemolysin showed maximum haemolytic activity at 37°C. The resultant high titre of hemolysin unveiled the prospects of preparation of combined vaccine against enterotoxaemia for sheep and goats.

Key Words: *In vitro*; Haemolytic; Toxin; *Clostridum perfringens* type-D

INTRODUCTION

Clostridium perfringens (type-D) is normal inhabitant of intestinal tract of healthy animals (Phukan *et al.*, 1997) and is cause of pulpy kidney disease (a form of enterotoxaemia). The disease is common in animals of all ages but young lambs and kids are highly susceptible (Bullen, 1952: Ferrando *et al.*, 1967). The organism produces epsilon toxin which increases permeability of the intestinal mucosae and blood vessels, therefore, is readily absorbed and potentiate absorbance of all the fatal toxins as well (Bullen, 1970). Acute nature of the enterotoxaemia is the main cause of therapeutic failure, therefore the disease is controlled by mass vaccination programs in susceptible animals. The vaccine contains toxoid of *Cl. perfringens* but there are qualitative and quantitative difference in the production of toxins by different type of the *Cl. perfringens* that posses impediment in preparation of an effective vaccine. Mixing of mass culture of these types results in lowering of the toxoid below the level of immunogenicity particularly for preparation of combined vaccine for sheep and goats. Therefore, investigations are required to improve the amount of immunogens in mass culture of each type of *Cl. perfringens*. For this purpose, factors modulating the production and detection of the haemolytic toxin in *in vitro* culture of *Cl. perfringens* (type-D) have been identified.

MATERIALS AND METHODS

Cl. perfringens (type-D) was identified in the Microbiology laboratory, College of Veterinary Sciences, Lahore, on the basis of biochemical tests (Cruickshank, 1975). The organism was grown in Reinforced Clostridial Medium (RCM;Oxoid), thioglycolate (Oxoid) and RCM+K2HPO4 in aerobic and anaerobic environment at 37°C for 24, 48 and 72 hours. The organism was also cultivated in RCM at pH 6.5, 7.0 and 7.5. Each of the culture was centrifuged at 600 g for 10 minutes (Lyerly & Wilkins, 1991) and the supernatant was subjected to haemolytic assay.

Trypsinization of supernatant. The supernatant was activated with trypsin (BDH) solution (Lyerly & Wilkine, 1991). Briefly, trypsin solution (required concentration) was admixed in the culture supernatant at rate of 1:9. The mixture was incubated at 37°C for varying period of time and processed for hemolysin titration (Anonymous, 1983). The haemolytic units were calculated by following formula (Anonymous, 1983).

$$(B-A)/2+ A$$

Where B stands for the lowest dilution of the toxin in the wells showing complete bead formation. A stands for the highest dilution of the toxin in well showing complete hemolysis.

Effect of trypsin on the integrity of erythrocytes. Effect of trypsin on the integrity of erythrocytes was studied in the immunoplate as described by

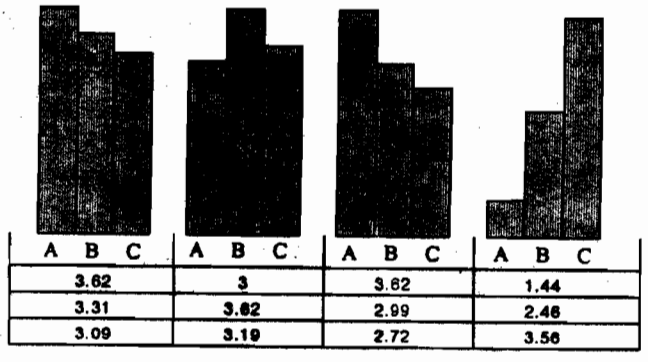
Rinaldini (1959). Moreover, concentration of trypsin, culture trypsin interaction time and source of erythrocytes on the sensitivity of haemolytic assay were evaluated.

RESULTS AND DISCUSSION

Clostridium perfringens (type-D) produced haemolytic toxin in *in vitro* culture. The organism produced higher level of the toxin in RCM than that produced in thioglycolate/RCM with K_2HPO_4 (Fig. 1).

Fig. 1. Effect of medium, pH, incubation time and temperature on activity of hemolysin

Medium	pH	Incubation time	Temperature
A=RCM	6.5	24 hours	4°C
B=Thioglycolate	7.0	48 hours	23°C
C=RCM+Thio.	7.5	72 hours	37°C



A	B	C	A	B	C	A	B	C	A	B	C
3.62			3			3.62			1.44		
3.31			3.62			2.99			2.46		
3.09			3.19			2.72			3.56		

Toxin production seems to be influenced by the ingredients of the medium. Inclusion of barley or wheat grains (10%) in the culture medium potentiates the toxin production (Bohnel *et al.,* 1989). This condition is related with field outbreaks of the disease. The toxin production in the RCM at pH 7.0 was significantly higher than that of at 6.5 and 7.5 (Fig. 1). Acidic or basic pH of the medium mitigated the toxin production. It might be due to accumulation of toxic metabolites. Stirring of the culture with nitrogen supply in the fermenter during incubation increased the level of toxin production that might be due to continuous elimination of such metabolites. The maximum level of the toxin was achieved on 24 hours incubation of the culture while its titre was reduced with further incubation of the culture (Fig. 1). Presumably, in sustained cultures, some proteolytic enzymes secreted by the organism break down molecules of the hemolysin.

Trypsin is an enzyme that alone did not induce hemolysis of sheep erythrocytes. Presumably substrate (receptors) for the said enzyme may not be present on the erythrocytes. Therefore, free trypsin in the culture filtrate had not contributed to haemolytic activity of the toxin. The culture filtrate had 832 haemolytic unit titre which was increased upto 1472 by trypsinization. This could be due to presence of prototoxin in the culture that got activated by the trypsin (Katic *et al.,* 1977). Heyningen (1970) also recorded that the organism secreted epsilon exotoxin in the culture medium as inactive toxin or prototoxin. The proteolytic enzyme convert the prototoxin into active toxin by splitting off small fragment from the precursor molecule. The activated hemolysin might have additive effect on the haemolytic activity of the culture filtrate thus enhanced the haemolytic titre. It was further observed that 60 minutes culture filtrate-trypsin interaction time induced maximum activation of the prototoxin (Fig. 1). The hemolysin is presumably an enzyme that showed maximum hemolysis at 37°C for one hour. The haemolytic activity of the toxin at 4°C was poor which did not improve even on increasing the incubation time. These observations indicate that activity of the hemolysin is temperature dependent. However, there is linear relationship between temperature of incubation and rate of hemolysis of the toxin produced by *Clostridium chauveoi* (Ramachandran, 1969).

CONCLUSIONS

It is concluded from the results that toxin production can be enhanced by constituents of the culture medium, continuous stirring and provision of nitrogen gas to the culture. The resultant high titer of hemolysin unveiled the prospects of preparation of combined vaccine of enterotoxaemia for sheep and goats.

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