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Production, Purification and efficacy determination of Epsilon toxin from *Clostridium perfringens type 'D'* IVRI native culture

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Abstract - Epsilon toxin of C.perfringens type 'D' IVRI native strain was produced by Batch type flask culture and further purified by using APS salt precipitation, Dialysis, DEAE sepharose separation and SDS PAGE analysis. It was also subjected to determine the toxicity by sing mouse toxicity test. Purified toxin showed the potent toxicity even at lowest dilution of 1:10,00,000 (toxin:Phosphate buffered saline).These findings suggested that purification increases the potency of toxin and thus lower the need of dose of vaccine administration which produced by the conversion of this toxin to toxoid. After purification a band of 32 kDA was obtained in SDS acrylamide gel autoradiograph showed the homogeneity with previously obtained results in this area of work. Epsilon toxin can penetrate the blood brain barrier so it could be prove as a potent vehicle for drug delivery system.

Key words – C.perfringens type 'D', Enterotoxaemia, Epsilon toxin, DEAE sepharose purification, Mouse toxicity test

INTRODUCTION

Enterotoxeamia is a bacterial infection caused in Sheep by Bacterium ***Clostridium perfringens type 'D'***, due to the production and accumulation of its exotoxin called Epsilon toxin[6].The toxin was first characterized as extracellular protease of ***C.perfringens type 'D'*** formerly ***C.welchii type 'D'*** [12]. The toxin is the extracellular secretion of bacterium and is secreted as inert prototoxin which is then converted in activated true toxin Epsilon by the action of Intestinal trypsin. The bacterium ***C. perfringens type 'D'*** is a Gram positive, rod shaped, strict anaerobic and high gas producing, soil microorganism[13].Epsilon toxin of ***C. perfringens type 'D'*** can be produced in controlled laboratory conditions using complex nutrient medium providing anaerobiasis.[4][14] Institute of Animal Health and veterinary biologicals produces the toxin for preparation of vaccine against enterotoxeaemia. The method is very conventional which rely only on the production of prototoxin from ***C. perfringens type 'D'*** using typical anaerobic media formulation and the medium thus is used as crud toxin prepared by trypsin treatment.[12] Modern methods of protein purification facilitate the high purity of the Epsilon toxin. Salt precipitation, Column purification and PAGE molecular weight determination was used in this study to increase the efficacy of toxin produced in batch type culture process.

Native strains of ***C. perfringens type 'D'***gives low potential of toxin so that the minimum lethal dose standardization will be high in reference to concentration to be injected and thus the dose of vaccine will be higher due to the low concentration of toxin yield.

MATERIALS AND METHODS

C. welchii type 'D' culture was obtained from IVRI Ijjatnagar, Raibaraily U.P., while Thioglycollate broth, DEAE sepharose, Ammonium per sulfate and SDS PAGE kit was purchased from Himedia labs Mumbai India.
Healthy adult colony bred pure line Male albino mice were obtained from Small animal breeding section of Institute of Animal health and veterinary Biologicals MHOW India.
All the animal testing protocols were followed as per guidelines of CPCSEAE India and according to IVRI Ijjatnagar Raibarely U.P.

Preparation of microorganism

It was done by previously well described method [11].One loop full suspension from ***C. welchii type 'D'*** master seed culture was inoculated in small working seed flask of Thioglycollate broth, Closed tightly and incubated on 37^{o} C for 36 hrs to obtain a gaseous flock forming growth.

Purity test of Organism

Purity protocol for anaerobic organism was followed according to standard protocol of IVRI and a previous method for purity [8]. One loop full suspension from seed flask was transferred to Nutrient Agar plates and incubated at 37° C for 24 hrs and screened for no growth of aerobic organism**.**

Gram Staining

This was done according to protocol described previously [3]and screened for presence of Gram positive organisms.

Production of Epsilon toxin

One loop full suspension from seed flask was inoculated in three production flasks of Thioglycollate Broth and incubated at 37° C for 6 hrs to obtain the maximum growth of microorganism. Prototoxin was secreted at this stage from completely vegetative form of ***C. welchii type 'D'***[3].

Preparation and Purification of Epsilon Toxin

Toxin was separated and prepared from culture supernatant from the method earlier described. Culture supernatant containing secreted toxin was centrifuged at 1000 rpm at Room temperature and supernatant was taken as crude prototoxin[3].

Salt precipitation

Crud prototoxin was precipitated at the 60% saturation with ammonium per sulfate and then centrifuged at 35000 rpm. The prototoxin obtained in pellet was taken and dissolved in normal saline phosphate buffer (.89% sodium chloride Phosphate buffer, PH 7.4)[1]

Dialysis

Prototoxin was dialysed against the phosphate buffered saline (PH 7.4) overnight to concentrate the prototoxin.[1]

DEAE sepharose separation

Concentrated prototoxin was applied over DEAE sepharose fast flow column of mm. Elution was carried out with phosphate buffered saline (PH 7.4) [1].

Conversion of Prototoxin to Toxin Epsilon

Prototoxin obtained from DEAE sepharose separation was subjected to .2% trypsin treatment to convert it in to true toxin Epsilon [1].

Mouse Toxicity test/Determination of MLD

Forty two adult healthy male albino mice weighing 25±2 grams were taken for this test and divided in six groups having seven animals in each group. Group one was designated as normal control/ vehicle control injected with phosphate buffered saline and remaining five groups were designated as test groups a, b, c, d and e respectively which were then injected with 1:100, 1:1000, 1:10000,1:100,000 and 1:10,00,000 dilution of toxin Epsilon respectively. Test animals were screened for occurrence of death in relation with time and the concentration of toxin injected [3].

SDS PAGE analysis

This analysis was done for Determination of molecular weight of Epsilon toxin against with standard molecular weight markers available with SDS PAGE kit [8]

RESULTS AND DISCUSSION

In present study, flock and gas forming growth was obtained in seed flask after 36 hrs of incubation at 37° C, which clearly indicates the enough growth of ***C.perfringens type 'D'*** organism. Characteristic gas forming growth of ***C. perfringens type D*** was also obtained previously from lamb dysentery samples from cultivation on Thioglycollate broth.[8]. An evidence of occurrence of ***C.perfringens type 'D'*** in defferent cultivated soils[11]

There was no aerobic organism grown on nutrient agar plates after the incubation of 24 hrs at 37°C indicating the purity of cultured seed organism.

Gram positive Rod shaped organism was seen after performing the gram staining of culture suspension.

Active growth occurred in production flaks after 6hrs incubation seeded with working seed culture having high and rapid gaseouse growth of organism. Previously ***in vitro*** biochemical growth was observed in ***C.perfringens type 'D'*** in anaerobic fermentation [3].

Culture suspension was subjected to prototoxin preparation and purification by Salt precipitation, Dialysis, DEAE sepharose ion exchange.Different types of bacterial proteases were previously reported to be purified by column separation [1].

Gradually increasing toxicity occurred after each steps of purification by attempting mouse toxicity test injecting with concentrated crud toxin.This toxin produced due to conjugative plasmids of ***C.perfringens type 'D'*** which is reported previously [2].

Prototoxin was converted to epsilon toxin by trypsinization and this concentrated toxin was injected directly in lateral tail vein of mouse which showed quick

death of mouse due to toxin accumulation in brain. Previous studies on neurotoxicity was showed that epsilon toxin is a pore forming toxin and it can penetrate the blood brain barrier subsequently causes permanent paralysis and death in experimental rat model in hippocampus via glutamergic system [5].

Mouse toxicity test was performed for each dilution of toxin and for vehicle phosphate buffered saline. Gradual increase in death rate of mice given intravenous toxin with increasing concentration of toxin while no delayed death occurred in group treated with toxin concentration of 1:10,00,000 and no death occurred in vehicle treated group. Quite closer results were shown in previous studies for mouse toxicity analysis as 1:10,000 dilution of toxin was proved lethal in mouse mortality test[3]. The toxin was also proved lethal against cancer cell lines in *in vitro* studies[9].Epsilon toxin was also tested on MDCK cell lines and it was proved a potent pore forming agent to cause lethality on MDCK cells[8].

Purified toxin and crude toxin both were loaded in SDS poly acrylamide gel against molecular weight markers. A band of 32 kDA was appeared for purified toxin sample while a band of 34 kDA was appeared for impure crud toxin. Same result was reported previously for the molecular weight determination of Epsilon toxin [8].

Figures and tables

S.No.	Inoculated flask Number	Observation of Growth
1	Master Seed flask no. 1	High Gas forming growth
2	Master Seed flask no. 2	High Gas forming Growth
3	Master Seed flask no. 3	Moderate gas forming growth

Table1. Comparison of Gas forming growth of ***C. perfringens type 'D'*** (n=3)

S.No.	Inoculated Nutrient agar Plate	Observation for purity
1	Working Seed inoculated plate no.1	No aerobic contaminaton
2	Working Seed inoculated plate no.2	No aerobic contaminaton
3	Working seed inoculated plate no.3	No aerobic contaminaton

Table2. Comparison of pure culture observation for working seed of ***C. perfringens type 'D'*** on Nutrient agar plate for aerobic growth (n=3)

S.No.	Flask for Gram Reaction	Gram reaction (+/-)
1	Working seed flask no.1	+
2	Working seed flask no.2	+
3	Working seed flask no. 3	+

Table3. observation of Gram staining reaction for working seed of ***C. perfringens type 'D'***

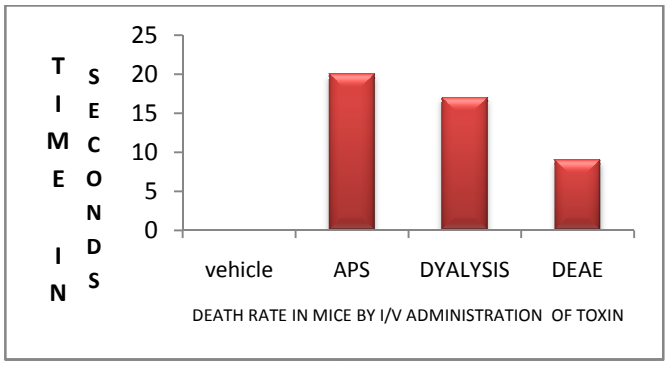

Fig.1 Death rate measurement in relation with time in Sec. by administration of concentrated Epsilon toxin in mouse at each step of Purification

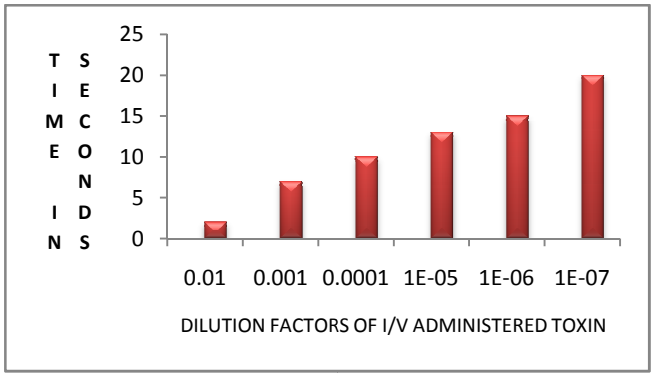

Figure 2. Death rate in mouse by I/V administered dilution series of Toxin Epsilon (.01 to .0000001) in relation with time in Seconds

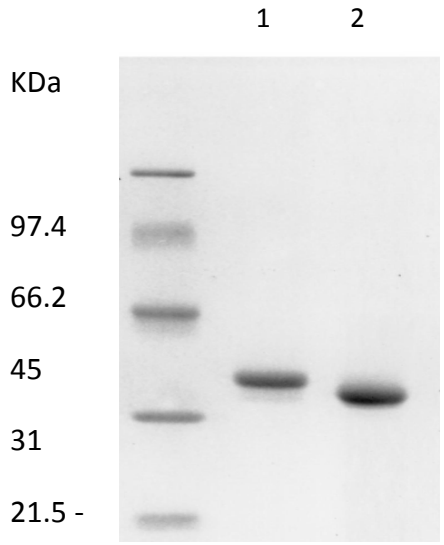

Fig.3 SDS (0.1%)-PAGE (10% polyacrylamide gel; Coomassie blue stained) analysis of purified epsilon-toxin (2.8 mg; lane 1) and Crude epsilon-toxin (2.8 mg; lane 2). The positions of molecular mass standards are shown on the left.

CONCLUSION

Findings in present investigation suggested that large scale purification of epsilon toxin works more effectively on its toxicity as the toxin shows more efficacy and thus lowers the dose of vaccine.

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