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Research Article

ASSOCIATION OF α AND β TOXINS OF *CLOSTRIDIUM PERFRINGENS* WITH ENTERITIS IN BROILER CHICKENS AND *IN VITRO* CYTOPATHIC EFFECT OF CRUDE TOXINS OF CLOSTRIDIUM PERFRINGENS ISOLATES ON VERO CELLS

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ABSTRACT

The present study was done to look for association of α and β toxins of *Clostridium perfringen* using ELISA on intestinal contents of enteritis affected broiler chickens, and also to assess the *in vitro* cytopathic effect of crude toxins of *C. perfringens* on vero cells and quantification of vero cell titre by MTT assay. Out of 70 intestinal fluid samples collected, 62 samples were from enteritis cases and 8 samples were from cases of necrotic enteritis. Out of 70 samples, five samples (7.14%) were positive for alpha toxin alone, two (2.87%) samples were positive for beta toxin alone and three (4.28%) samples were positive for both alpha and beta toxins. Out of 8 necrotic enteritis cases, four (50%) samples were positive for alpha toxin alone and three (37.5%) samples were positive both for alpha and beta toxins and one sample was negative for both of them. Of the 68 samples of intestinal contents from enteritis cases in broiler chicken, 49 intestinal samples (72.05%) were positive for *C. perfringens* by isolation. All the 49 samples gave black wool like colonies on Tryptose Sulphite Cycloserine (TSC) agar enriched with thioglycollate broth. The bacteria isolated were gram positive rod, non-motile with square ends. Crude toxins produced by 28 *C. perfringens* isolates were subjected to Vero cell assay to evaluate the expression of cytopathic effect (CPE). It was observed that 22 isolates (78.5%) expressed CPE in Vero cell culture. Per cent cytotoxicity of toxin on Vero cells varied from 50.2% to 77.3% and per cent dilution of toxin which showed CPE varied from 1:2 to 1:64 dilution.

Key words: *Clostridium perfringens*, Cytopathic effect, MTT Assay, Vero cells

C. perfringens is one of the normal bacterial floras of gastrointestinal tract in both human and animals. It has also been shown to cause number of diseases in humans and animals and reported to be a causal agent of necrotic enteritis, an important avian disease throughout the world. The type of *C. perfringens* in this disease is type A and type C (Parish *et al.,* 1961). *C. perfringens* type A strains produce the chromosomal-encoded alpha toxin, while type C produce alpha toxin together with beta toxin. All types produce α toxin, a phospholipase C that is considered to be a major contributory factor towards development of intestinal mucosal necrosis, the characteristic lesion of necrotic enteritis in poultry (Al-Sheikhly *et al.*, 1976). Alpha toxin hydrolyzes lecithin, a major component of cell membrane and thus destroys red blood cells, platelets and muscles causing myonecrosis. Beta toxin induces hemorrhagic necrosis of the intestinal mucosa. Some *C. perfringens* strains, in addition to alpha toxin, produce beta2 toxin that have been proposed to be important in pathogenesis of intestinal disorders in animals (Thiede *et al*., 2001). Clostridiosis occurs both as an acute clinical disease and subclinical disease. The acute form occurs as necrotic enteritis (NE) in 2-5 week old chicks and sub-clinically causes focal necrosis in intestine and hepatitis with cholangiohepatitis in liver. The present study was done to look for the association of α and β toxins with enteritis in broiler chicken. The study was also done to assess the cytopathic effects of crude toxins produced by *C. perfringens* isolated during the study.

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MATERIALS AND METHODS

Cultures

Reference strains of *Clostridium perfringens* type 'A' and type 'C' were procured from division of biological standardization, Indian Veterinary Research Institute (IVRI), Izzatnagar. The strains were maintained as stock culture in alternative thioglycollate broth and were periodically tested for purity, morphology and biochemical characteristics, after every month. Anaerobic jars were used for anaerobic incubation wherever required. Burning candle was used in anaerobic jar and it was tightly sealed with parafilm to create anaerobic environment. Sixty eight (68) intestinal contents collected from dead broiler birds were processed for *Clostridium perfringens* isolation using one step enrichment and selective plating. Alternative thioglycollate media (Hi media) was used as enrichment medium. 10gm of feacal sample was inoculated in 90ml of enrichment media (1:10) in sterile flask and mixed properly for 2-3 minutes. Homogenized sample was heated at 75°C for 20 minutes, followed by incubation at 37°C for 24 hr for isolation of *Clostridium perfringens*. An agar overlay technique in sterile test tubes was used using TSCagar (contains selective inhibitory agents (sulphite) and cycloserine).

Thioglycollate enriched inoculum (0.1ml) was taken in a sterile test tube and 5 ml of sterile TSC agar was added with thorough mixing. It was allowed to solidify for

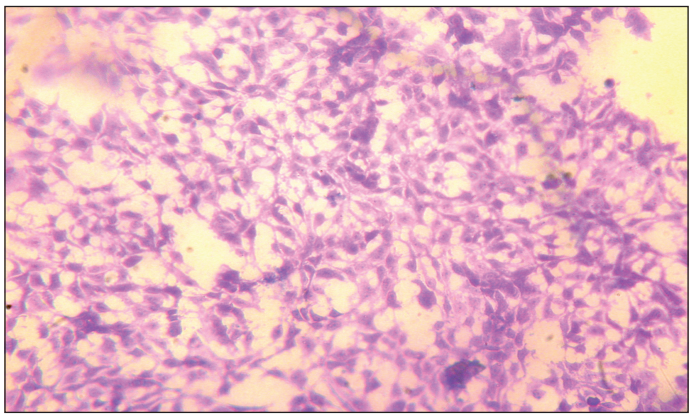

Fig. 1 : Vero cell culture (Control)

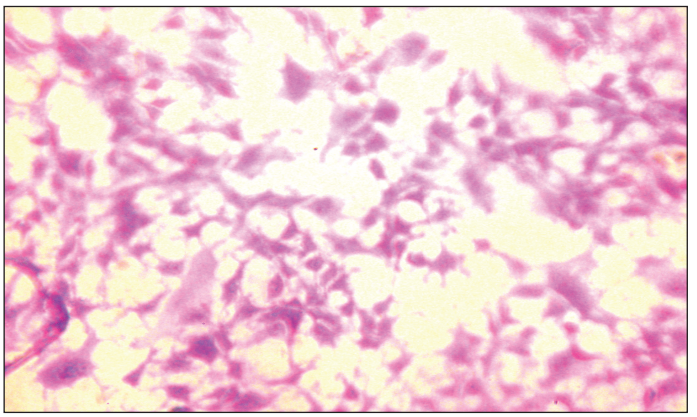

Fig 2 : Toxin exposed Vero cell culture showing CPE (200X)

5-10 min and again 2-3 ml of TSC agar was overlaid. The test tube was then incubated at 44-45° C for 18-24 hr. The black cotton wool like colonies of 2-3 mm in size suspected for *C. perfringens* were taken out with the help of a loop and incubated at 37°C in thioglycollate broth for 24 hr and the suspected colonies were subjected to biochemical tests for confirmation.

Enzyme linked immuno sorbent assay (ELISA)

Monoclonal antibody based Elisa kit (Biox diagnostics) was used for detection of alpha and beta clostridial toxins, as per the protocol of manufacturer. Aliquots (100µl) of the undiluted sample was added to the wells as follows: sample 1 in wells A1 and B1, sample 2 in wells C1 and D1 and so on. The plate was incubated at room temperature for 1 hour. The plate was rinsed with the washing solution three times. Diluted conjugate (100 µl) of the solution was added to each well. Plate was incubated at room temperature for 1 hour. The plate was washed as described above. Then, chromogen + substrate (12 drops of chromogen in 9.5 ml of enzyme substrate for one plate) was added in the amount of 100 µl per microwell immediately. Plate was incubated for 10 minutes at room temperature. 50 µl of stop solution was added per microwell. The optical density of each well was read at 450 nm filter using ELISA plate reader.

Assay for virulence markers

Crude toxin production from 28 *C. perfringens* isolates and its cytotoxic effect on vero cell line was assessed as per method described by Uemura *et al*. (1984) and Mahony *et al.* (1989) with certain modification.

Toxin production

C. perfringens isolates were inoculated into 10 ml of alternative thioglycolate broth, followed by heat treatment at 75°C for 20 min and incubated overnight at 44-45°C. Five ml of culture was again transferred into newly prepared 10 ml alternative thioglycollate broth and incubated at 44-45°C for 4-5 hr. One ml of this culture was transferred into 15ml of ellner's broth and incubated for 18-24 hr at 37°C followed by centrifugation at 6000 rpm for 10 min. The crude toxin was prepared by filtering the supernatant through Millipore filter (0.22µm) and was stored at -20°C.

Vero cell assay

Vero cell line was procured from Center for Animal Disease Research and Diagnosis (CADRAD), IVRI, Izzatnagar. The cell line was maintained in Glassow minimal essential medium (GMEM) containing 2% fetal calf serum (FCS) and grown in GMEM with 10% FCS. The toxin assay was performed into 96 well microtitre tissue culture plates (Tarsons). The Vero cell culture was grown in tissue culture plate under 5% CO_2 tension for 24 hr at 37° C .The medium was then discarded and cell layer was washed twice with Hank's balanced salt solution (HBSS).

MTT assay for detecting cytotoxicity of Clostridium perfringens toxin

The toxin assay was performed into 96 well microtitre tissue culture plates (Uemura *et al.,* 1984). MEM medium (100 µl) was added in all well. Toxin (100µl) of was added in wells as sample 1 in well A1 and A2, sample 2 in well A3 and A4 and so on up to well A12. Sample (100µl) of was transferred from row A1 to B1 (1:2 dilution), from B1 to C1 (1:4 dilution) and in same way up to G1 (1:128 dilution) except in H1 and H2 which were kept as control. After this, 100µl of Vero cells was added in all the wells including well H1 and H2. Plate was sealed with cellophone tape. Plate was incubated for 2 hr at 37° C at 5% CO_2 tension in incubator at 37° C. Thereafter, 50 µl of MTT dye (3-[4, 5-dimethylthiazoyl-2-y1]-2, 5-diphenyltetrazolium bromide) was added in each well (5µg/ml of PBS). Again it was incubated at 37° C at 5% CO_2 tension in incubator for 3 hr. The contents of the plate were discarded on filter paper thoroughly. Then 100 µl Di methyl sulphoxide (DMSO) was added in all the wells and violet colour appeared. Finally, absorbance was measured by ELISA plate reader at 540 nm wavelength.

Per cent Cytotoxicity was calculated by

{1- (O.D. of cytotoxin treated cells) / (O.D. of untreated cells)} x 100

Table 1
Table showing results of intestinal contents tested for α and β toxin by ELISA

S.No.	Sample No.	α- Toxin alone	β- Toxin alone	α and β Toxin both
-	*C.perfringens* type 'A' (Reference culture)	+ve	-	-
-	*C.perfringens* type 'C' (Reference culture)	+ve	+ve	+ve
1	11693	+ve	-	-
2	11726	+ve	-	-
3	11791	-	-	+ve
4	11831	+ve	-	-
5	11969	+ve	-	-
6	11805	-	-	+ve
7	12008	+ve	-	-
8	12035	-	-	+ve
9	12069	-	+ve	-
10	12080	-	+ve	-
11-70	Other 60 samples	-	-	-
Total tested	No. of samples	70	70	70
Toxin Positive samples		5	2	3
Toxin Negative samples		65	68	67
% positive samples		7.14%	2.87%	4.28%
Necrotic Enteritis samples (Total number of samples-8)		4 (50.00%)	NIL	3 (37.5%)

PCR (Polymerase Chain Reaction) for *cpa* and *cpb2* gene

A total of 28 isolates of *C. perfringens* were tested for the presence of alpha toxin (*cpa* gene) and beta toxin (*cpb2* gene) by polymerase chain reaction (PCR) and has been reproduced here as mentioned by Anshul *et al.,* 2012.

RESULTS AND DISCUSSION

Isolation of C. perfringens

A total of 68 intestinal samples from the broiler birds brought to disease investigation laboratory, Hisar were screened for isolation of C. *perfringens*. Black wool like colonies of 2-3 mm in size was obtained in 49 (72.05%) samples in thioglycollate broth. On TSC agar, colonies were round yellowish-gray, semi-translucent and smooth with entire edge. The organisms in these colonies were confirmed as Gram positive, non-motile, rod shaped bacteria with square end. The organisms were also subjected to biochemical tests for confirmation. In present investigation, enrichment with heat treatment at 75° C for 20 minute followed by inoculation onto TSC agar medium was carried out for isolation of *C. perfringens.* The heat treatment used in this protocol, killed most of the heat sensitive pathogenic and non pathogenic organisms thereby allowing the growth of *C. perfringens*. The use of enrichment before selective plating has been reported to show better recovery percentage of *C. perfringens* (Fujisawa *et al*., 2001).

Elisa for alpha and beta toxins

ELISA results for alpha and beta toxins of intestinal contents are given in Table 1. A total of seventy (70) intestinal fluid samples were collected from dead birds. Out of these 70 samples, 62 samples were from enteritis cases and 8 samples were from cases of necrotic enteritis. These samples were tested for presence of alpha and beta toxins by kits (Bio-X Diagnostic). Toxin produced from reference culture of *C. perfringens* type 'A' and type 'C' was also tested by ELISA kit. Results revealed that toxin produced by type 'A' culture was positive for alpha toxin alone while toxin produced by type 'C' culture was positive for both alpha and beta toxins. The results of intestinal contents revealed that five samples (7.14%) were positive for alpha toxin alone, two (2.87%) samples were positive for beta toxin alone and three (4.28%) samples were positive for both alpha and beta toxins. Songer (1996) and Engstrom *et al*. (2003) reported that necrotic enteritis in poultry is caused by *C. perfringens* type 'A' and to a lesser extent by type 'C'. Petit *et al*. (1999) reported that type 'A' produces alpha toxin and type 'C' produces alpha toxin together with beta toxin. In our results numbers of cases for alpha and beta toxins were less probably due to the fact that most of the cases were of enteritis (62 cases) and not of necrotic enteritis (8 cases). Results might also be less due to low level of toxins production in these samples. Hale and stiles (1999) reported sensitivity of 19 ng/ml with purified toxin in monoclonal antibody (4F2) based alpha toxin sandwich ELISA.

Out of these 70 samples, eight samples were of necrotic enteritis. Out of these eight samples, four (50%) samples were positive for alpha toxin, three (37.5%) samples were positive for both alpha and beta toxins and one sample was negative for both of them (Table 1). The results depicted that in necrotic enteritis cases alpha and beta toxin are involved. As one sample of necrotic enteritis was negative for alpha and beta toxin both by ELISA; It could probably be explained by the findings of Keyburn *et al*. (2008), who reported the presence of NetB, a new toxin that has been found to be associated with avian necrotic enteritis caused by *C. perfringens*. Two other cases of simple enteritis were also positive for alpha toxin by ELISA. These two cases might be positive due to the presence of subclinical form of necrotic enteritis even when coccidiostats and antibiotics are used in most of the feeds in our area, which tends to inhibit the growth of *C. perfringens*. Kaldushdal and Hofshagen (1992); Lovland and Kaldhusdal (2001) reported that a mild subclinical

Table 2
Table showing comparison of results of Vero cell assay and PCR (Anshul *et al.*, 2012) of C. perfringens isolates

					Anshul *et al.*, 2012		
S. No. of the sample	Sample No.	District	Vero Cell Titre	% Cytotoxicity	PCR for α- Toxin (cpa gene) alone	PCR for β-2 Toxin (cpb 2 gene) alone	PCR for α and β Toxin both
1	11325	Hisar	NO CPE	NIL	-	-	-
2	11333	Hisar	1:64	56.4	-	-	+ve
3	11347	Hisar	1:2	55.5	-	-	-
4	11381	Fatehabad	1:32	67.5	-	-	+ve
5	11572	Hisar	NO CPE	NIL	-	-	-
6	11629	Hisar	NO CPE	NIL	-	-	-
7	11646	Sirsa	1:16	50.2	+ve	-	-
8	11660	Bhiwani	NO CPE	NIL	-	-	-
9	11666	Bhiwani	NO CPE	NIL	-	-	-
10	11693	Hisar	1:2	56.2	-	-	+ve
11	11754	Fatehabad	1:7	57.7	+ve	-	-
12	11805	Bhiwani	1:16	55.1	-	-	+ve
13	11819	Fatehabad	1:16	55.1	-	-	+ve
14	11826	Hisar	NO CPE	NIL	-	-	-
15	11831	Hisar	1:8	54.1	+ve	-	-
16	11832	Bhiwani	1:8	50.4	-	-	-
17	11833	Hisar	1:2	52.3	+ve	-	-
18	11850	Hisar	1:2	54.9	+ve	-	-
19	11859	Jind	1:8	63.4	+ve	-	-
20	11962	Hisar	1:16	58.2	+ve	-	-
21	11977	Hisar	1:4	52.5	+ve	-	-
22	11981	Hisar	1:8	56.7	+ve	-	-
23	11998	Hisar	1:2	77.3	-	-	+ve
24	12008	Hisar	1:4	60.6	-	-	+ve
25	12035	Fatehabad	1:16	52.1	-	-	+ve
26	12069	Bhiwani	1:16	52.5	-	-	+ve
27	12054	Hisar	1:8	51.8	+ve	-	-
28	12107	Hisar	1:2	73.1	+ve	-	-
No. positive (n=28)			22		11	Nil	9
% positive			78.5		39.30	Nil	32.10

Where, '-' stands for negative

form of necrotic enteritis is also present in field, leading to decline in performance.

Vero Cell Assay

Vero cell control and Vero cell cytopathic effect are shown in Fig. 1 and Fig. 2, respectively. Vero cell assay was conducted for the toxin produced from 28 isolates of *C. perfringens*. The results (Table 2 and Table 3) revealed that 22 isolates (78.5%) expressed cytotoxin producing cytopathic effect (morphological changes, inhibition of plating efficiency and rounding of cells) in vero cell culture. Percentage cytotoxicity of toxins on Vero cells varied from 50.2% to 77.3% and per cent dilution of toxin varied from 1:2 to 1:64 dilution. Six isolates did not reveal any cytopathic effect on vero cell. These six samples were also negative for presence of *cpa* and *cpb2* genes of alpha and beta2 toxin, respectively. This indicates that these 6 isolates (21.4%) might be having some other toxin producing genes other than genes *cpa* and *cpb2* for alpha

Table 3
Overall comparison of results of Vero cell assay and PCR (Anshul *et al.*, 2012) of C. perfringens isolates

Cytotoxicity on Vero cell line	Total number of isolates	PCR Results (Anshul *et al.,* 2012)			
		Number of isolates positive for alpha toxin gene alone	Number of isolates positive for beta-2 toxin gene alone	Number of isolates positive for both alpha and beta-2 toxin gene	Number of isolates negative for both alpha and beta-2 toxin gene
Number of isolates positive for CPE*	22 (78.5%)	11	Nil	09	02
Number of isolates negative for CPE	06 (21.4%)	Nil	Nil	Nil	06
Total	28	11(39.30 %)	Nil	09 (32.10 %)	08 (28.60 %)

CPE - Cytopathic effects

and beta toxins, respectively. Recently Keyburn *et al*. (2008) reported Net B toxin to be responsible for avian necrotic enteritis. This Net B toxin does not produce any CPE on vero cell line. Two samples (sample no.11347 and 11832; Table 2) showed CPE in dilution 1:2 and 1:8; however they were found to be negative for *cpa* and *cpb*2 genes of *clostridium* by PCR in the present study. McDonel *et al*. (1980) reported that *C. perfringens* enterotoxin also shows inhibition of plating efficiency on vero cell culture. Presence of enterotoxin has also been reported in poultry (Miwa *et al*., 1996). So, these two samples might contain enterotoxin as they were positive for CPE but negative by PCR for *cpa* and *cpb*2 gene of *clostridium*. It can be concluded from the study that crude toxin produced by isolates of *C. perfringens* isolated from broiler farms of some parts of Haryana produced cytopathic effects on vero cell line. Further studies are required to determine the presence of other toxins (Net B and enterotoxin) in these isolates and their correlation with cytopathic effects on Vero cell line.

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