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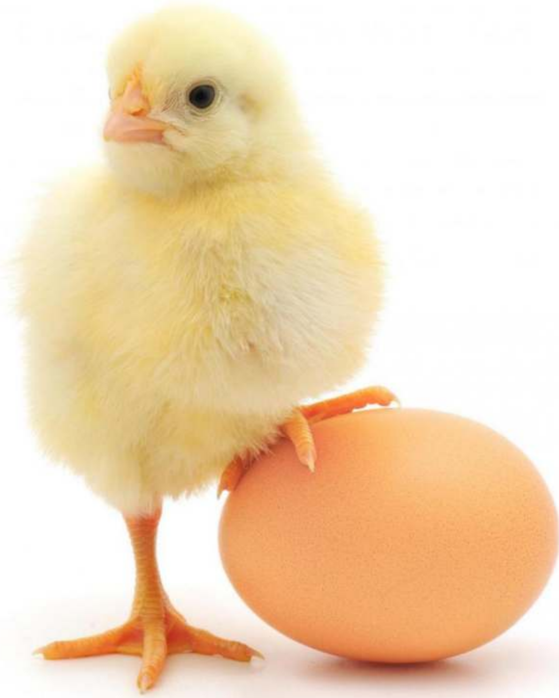

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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Clostridium perfringens Type A from Broiler Chicken with Necrotic Enteritis

Arunava Das[1], Yahya Mazumder[2], Biman Kumar Dutta[3], Bibek Ranjan Shome[4],
Komal Molla Bujarbaruah[5] and Ashok Kumar[6]
[1]Department of Biotechnology, Bannari Amman Institute of Technology,
Sathyamangalam, Tamil Nadu, India
[2]Department of Biotechnology Engineering,
Nagarjuna College of Engineering and Technology, Bangalore, India
[3]Department of Ecology and Environmental Science, Assam University, Silchar, Assam, India
[4]Project Directorate on Animal Disease Monitoring and Surveillance, Hebbal, Bangalore, Karnataka, India
[5]Division of Animal Science, Indian Council of Agricultural Research, Krishi Bhawan, New Delhi, India
[6]Division of Animal Health, Indian Council of Agricultural Research for NEH Region, Umiam, Meghalaya

Abstract: This paper reports the investigation of necrotic enteritis (NE) in six broiler chickens of age two to three weeks old, died in Jowai poultry farm, Meghalaya, India. Initially, scanning electron microscopy (SEM) was performed to observe the morphological changes within the intestine. Intestinal contents and liver samples from dead chicken were investigated for isolation of bacteria and their virulence determinant. The SEM analysis of infected intestine revealed massive necrosis and complete destruction of the intestinal villi within the intestinal mucosa. Bacterial isolation confirmed the causative agent as *C. perfringens* in NE. All the isolates harboured single and double plasmid deoxyribonucleic acid with identical 45.2kb common plasmid. In polymerase chain reaction (PCR) assay all 10 clinical isolates harboured alpha toxin gene (*cpa*) of *C. perfringens*, however, four isolates also carried additional beta2 toxin gene (*cpb*2). None of the isolates were positive for beta, epsilon, iota and enterotoxin genes. PCR analysis revealed that all isolates derived from NE belonged to *C. perfringens* type A. The partial *cpa* gene sequence analysis showed 97.6 to 100% homology among the *C. perfringens* isolates. The study confirmed that *C. perfringens* type A is the most predominant one associated with necrotic enteritis in broiler chickens in Meghalaya, India and the alpha toxin (CPA) might play a significant role in the pathogenesis of the disease in broiler chicken.

Key words: *Clostridium perfringens*, alpha toxin, plasmid, polymerase chain reaction, necrotic enteritis

Introduction

Avian necrotic enteritis (NE), an important sporadic disease was first described by Parish in 1961 (Parish, 1961) and since then it has been reported from most areas of the world (Ficken and Wages, 1997). The estimated cost of NE to the poultry industry globally is nearly $2 billion every year (Anonymous, 2000). The causative agent of NE is *Clostridium perfringens*, an anaerobic, Gram positive and spore forming bacteria that can be readily found in soil, dust, feces, feed, poultry litter and in gastrointestinal tract of healthy birds (Ficken and Wages, 1997).

Mucosal damage inducing factors such as coccidiosis (parasitism), high fiber litters, dietary changes, poor hygienic and housing conditions are considered predisposing factors for NE. The NE is thought to occur when these factors create a favourable environment for *C. perfringens* to multiply rapidly into much higher numbers in the small intestine, leading to the production of potent lethal toxins that damage the intestine (Broussard *et al.,* 1986). Moreover, excessive uses of growth promoting antibiotics enhance *C. perfringens* to induce NE and sub clinical infections, important threats to poultry health (Songer, 1996). *C. perfringens* are toxin typed (A to E) by the presence of four major toxins, alpha, beta, iota and epsilon (Songer and Meer, 1996). Along with four major toxins, enterotoxin and beta2 toxins produced by types of *C. perfringens* are considered as important toxins for enteric diseases (Smedley III *et al.,* 2004). *C. perfringens* strains possessed both high and low molecular weight plasmids (Squires *et al.,* 1984). Eisgruber *et al.* (1996) reported that plasmid profiling could be useful tool for strain differentiation and characterization of *C. perfringens* from different diseased outbreaks. Nowadays, PCR has been used to detect the presence of toxin genes for typing of isolates and to identify the specific strains of *C. perfringens* associated with the particular disease (Songer and Meer, 1996; Gibert *et al.,* 1997). Present study reports the occurrence of fatal necrotic enteritis due to *C. perfringens* in broiler chicken and their virulence determinants.

Materials and Methods

History of broiler chickens and sample collection: The outbreak of necrotic enteritis in two to three weeks old broiler chickens reared in the poultry farm of Jowai, Meghalaya, India occurred in the month of April, 2006 were investigated. The atmospheric temperature and humidity was recorded between 25-25^{o}C and 60-70% respectively with heavy rainfall during that period. Out of 268-broiler chicken, 6 (2.2%) were died within two days. In every case, postmortem was performed within one to two hours duration after death. All the internal organs were thoroughly examined and any macroscopic and gross lesions observed were recorded. Intestinal contents and liver samples collected from each died broiler chickens during postmortem examination and from healthy broiler chicken (control) during slaughtering were scientifically processed for microbiological investigation.

Scanning electron microscopy of intestine samples: The intestinal pieces from NE infected chickens and normal intestine collected from healthy chicken (control) were subjected for SEM analysis. The SEM was done commercially from Regional sophisticated instrumentation centre (RSIC) under North Eastern Hill University (NEHU), Shillong, Meghalaya. The specimens were observed, photographed and analysed under the JEOL JSM-6360 (Tokiyo, Japan) scanning electron microscope.

Isolation and identification of *Clostridium perfringens*: All the samples were inoculated in sterile Robertson's cooked meat (RCM) broth medium supplemented with glucose, hemin and vitamin K (Himedia, Mumbai), overlaid with neutral oil and incubated at 37^{o}C for 48hr. The inoculums from each RCM media were seeded onto 10% goat blood agar and incubated anaerobically with an anaerobic gas-pack system (BBL Microbiology Systems Cockeysville, Md.; Div. Becton Dickinson and Co.) for 24hr at 37^{o}C. Bacterial colonies were purified based on the size, shape, color and patterns of haemolysis on blood agar and were subjected to motility test, Gram's and malachite spore staining. The isolates were identified based on the litmus milk test, gelatinase, deoxyribonuclease (DNase), lecithinase and fermentation of glucose and lactose (Holt *et al.,* 1994).

Detection of plasmid: One milliliter of bacterial culture grown overnight anaerobically in brain heart infusion broth at 37^{o}C was used for the extraction of plasmid DNA by alkali lysis method (Birnboim and Doly, 1979). The plasmid DNA was finally dissolved in 35µl of TE-RNase (1mg/ml in 10mM tris-hydrochloric acid and 1mM ethylenediamine tetraacetic acid; pH 8.0) solution, electrophoresed in 0.7% agarose dissolved in 1X TAE (tris-acetate-EDTA; pH 8.0) buffer and stained with 0.4 µg/ml ethidium bromide. The molecular weight of the plasmids was determined by comparing with known DNA ladder (λ DNA / Hind III digest; GENEI, Bangalore). The plasmid DNA bands were visualized and photographed in gel doc system (Image Master® VDS, Pharmacia Biotech, Sweden).

Detection of toxin genes by polymerase chain reaction: To study the virulence of the organism, isolates of *C. perfringens* were tested to detect the alpha toxin gene (*cpa*), beta toxin gene (*cpb*), epsilon toxin gene (*etx*), iota toxin gene (*i*A), enterotoxin gene (*cpe*) and beta2 toxin gene (*cpb*2) by PCR (Songer and Meer, 1996). Freshly grown bacterial colonies from solid media plates were suspended in 200µl of Milli-Q water in a microcentrifuge tube, gently vortexed and boiled for 10 min in a water bath. Supernatant after centrifugation at 10000g for 5 min was used as a template DNA. The amplification was carried out in 25µl reaction volume containing 12.5µl of 2× PCR master mix (Promega, USA) containing 4mM magnesium chloride, 0.4mM of deoxynucleotide triphosphates (dNTPs), 0.5U of *Taq* DNA polymerase, 150mM tris-hydrochlroric acid, pH 8.5 (Promega, USA), 0.5µM primers and 2.5µl of template DNA. The PCR reactions were performed in iCycler (BioRad, USA). After initial denaturation at 94^{o}C for 4 min, the amplification cycle had denaturation, annealing and extension at 94^{o}C, 55^{o}C and 72^{o}C for 1 min each respectively. Final extension was done at 72^{0}C for 10 min. The specific forward and reverse primer pairs for *cpa* gene of 324bp were $5^{/}$-gctaatgttactgccgttga-$3^{/}$ and $5^{/}$-cctctgatacatcgtgtaag-$3^{/}$ (Titbal *et al.,* 1989), *cpb* gene of 180bp were $5^{/}$-gcgaatatgctgaatcatcta-$3^{/}$ and $5^{/}$-gcaggaacattagtatatcttc-$3^{/}$ (Hunter *et al.,* 1993), *etx* gene of 655bp were $5^{/}$-gcggtgatatccatctattc-3$^{/}$ and $5^{/}$-ccacttacttgtcctactaac-$3^{/}$ (Hunter *et al.,* 1992), *i*A gene of 446bp were $5^{/}$-actactctcagacaagacag-$3^{/}$ and $5^{/}$-ctttccttctattactatacg-$3^{/}$ Perelle *et al.,* 1993), *cpb*2 gene of 567bp were $5^{/}$-agattttaaatatgatcctaacc-$3^{/}$ and $5^{/}$-caataccettcaccaaatactc-$3^{/}$ (Gibert *et al.,* 1997) and *cpe* gene of 233bp were $5^{/}$-ggagatggttggatattagg-$3^{/}$ and $5^{/}$-ggaccagcagttgtagata-$3^{/}$ (Czeczulin *et al.,* 1993) were commercially synthesized (GENSET, USA). *C. perfringens* type A positive for *cpa* and *cpb*2 genes isolated from atypical blackleg in cattle was used as positive control (Shome *et al.,* 2006) and *C. septicum* strain negative for *cpa* and *cpb*2 genes was used as negative control. The PCR amplicons (5µl) were electrophoresed in 1.5% agarose gel in TAE buffer, stained with ethidium bromide and observed in gel doc system.

Sequencing of PCR amplified product: Six isolates (CP54, CP56, CP59, CP61, CP63 and CP66) derived from intestinal contents of six broiler chicken died in NE were subjected for sequencing. PCR amplified products of partial *cpa* gene of *C. perfringens* were purified using QIA quick® PCR purification kit (QIAGEN, USA) and sequenced in an automated DNA sequencer (Microsynth, Switzerland and Genei, Bangalore, India). Partial sequences obtained were submitted to BLAST analysis (Altschul *et al.,* 1990) to determine the similarities to other sequences available in Gen Bank.

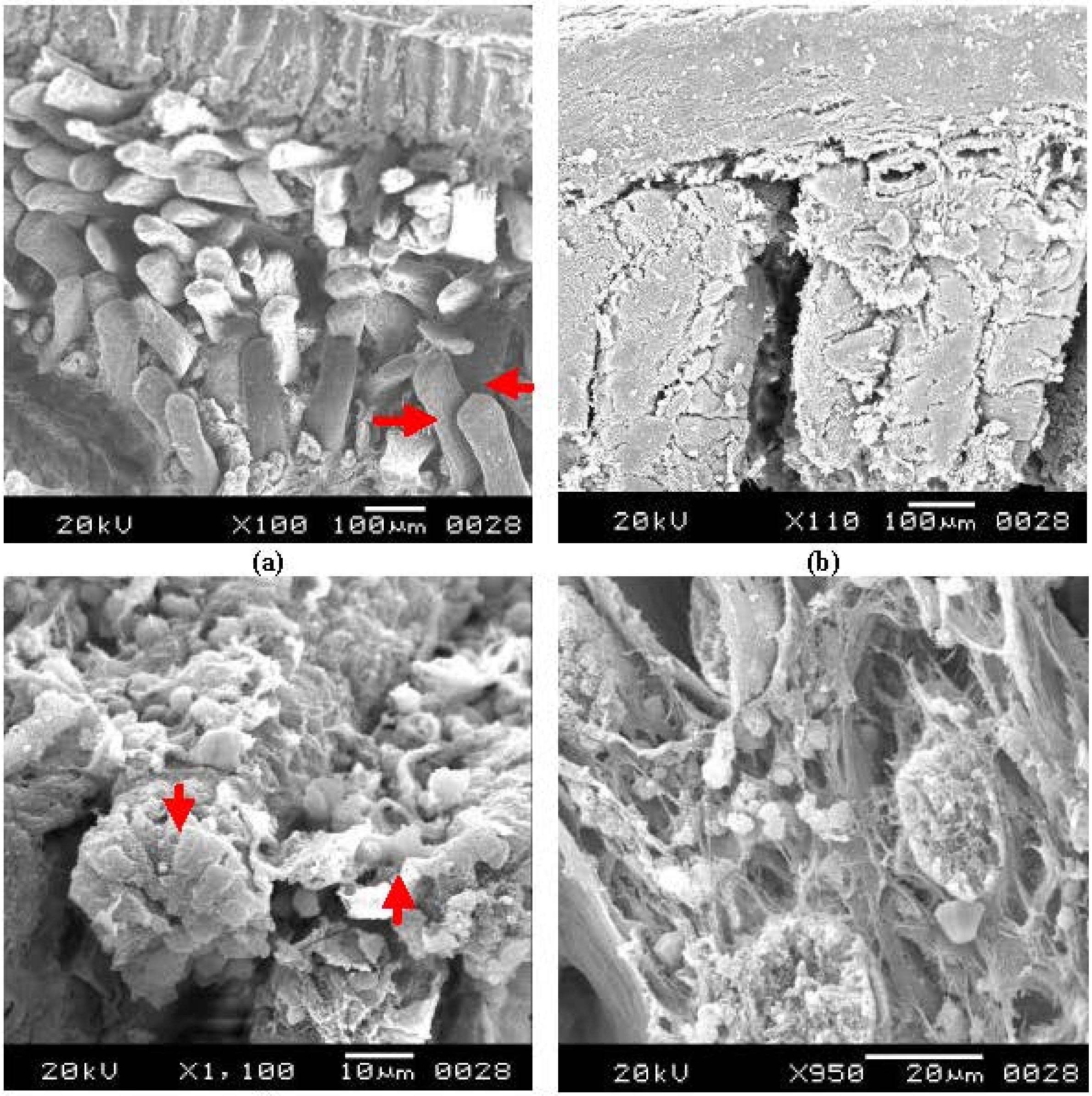

Fig. 1: Scanning electron microscopy of intestines. (a) intestine from control chicken showing normal and healthy architecture with smooth surface of lengthy villi (arrow right) at ×100; (b-d) intestine from necrotic enteritis infected chicken. Arrow left showing shortening of villi with rough surface, fusion of two or three adjacent villi at ×110; arrow down showing intestinal mucosa with stumpy and necrosed villi with blebs on the tip and massive infiltration of inflammatory cells at ×1100; arrow up showing intestinal mucosa with massive necrosis and complete destruction of villi ×950. Bar range varies from 10µm, 20µm and 100µm. Acceleration voltage is 20 KV.

The *cpa* gene sequences derived from the *C. perfringens* isolated from NE infected chickens were deposited into the GenBank under accession numbers-DQ787185, DQ787186, DQ787187, DQ787188, DQ787189 and DQ787190. The partial sequences obtained were in the range of 289 to 301 nucleotide bases. The *cpa* sequences up to 289 bases were aligned with the corresponding sequences of five selected *C. perfringens* isolated from bovine enterotoxaemia from USA (DQ184053), healthy chicken from Denmark (AF477009, AF477010 and AF475144) and soil from Japan (NC_003366) available in the

Table 1: Sample details, plasmid profiles and toxin genes of 10 Clostridium perfringens isolated from necrotic enteritis

Sample number	Sample nature	Isolate number	Source with age (weeks)	Plasmid (kb)	Toxin genes detected by PCR					
					cpa	*cpb*	*etx*	*iA*	*cpb2*	*cpe*
Po-NE1	Intestinal contents	CP54	Broiler chicken (3)	42.8, 45.2	+	-	-	-	+	-
Po-NE2	Liver	CP55		42.8, 45.2	+	-	-	-	-	-
Po-NE3	Intestinal contents	CP56	Broiler chicken (3)	42.8, 45.2	+	-	-	-	-	-
Po-NE5	Liver	CP58	Broiler chicken (2)	42.8, 45.2	+	-	-	-	-	-
Po-NE6	Intestinal contents	CP59		42.8, 45.2	+	-	-	-	+	-
Po-NE7	Liver	CP60	Broiler chicken (2)	42.8, 45.2	+	-	-	-	-	-
Po-NE8	Intestinal contents	CP61		42.8, 45.2	+	-	-	-	+	-
Po-NE9	Liver	CP62	Broiler chicken (2)	42.8, 45.2	+	-	-	-	-	-
Po-NE10	Intestinal contents	CP63		42.8, 45.2	+	-	-	-	+	-
Po-NE11	Intestinal contents	CP66	Broiler chicken (2)	45.2	+	-	-	-	-	-

kb =kilo bases; + = detected; - = not detected

GenBank by using ClustalW algorithm of MegAlign program (DNASTAR, Lasergene, USA). The phylogenetic analysis of the nucleotide sequences and 96 deduced amino acids residues were also analysed by using same programme.

Results

Symptoms and postmortem findings: On the onset of necrotic enteritis, chickens were isolated from the group. All the broiler chickens showed severe depression, decreased appetite, diarrhea, reluctance to move and ruffled feathers prior to death. Post mortem examination showed that birds were dehydrated and had fetid odor with severe lesions and necrosis on the wall of small intestine. In every case, the small intestine was found markedly dilated, with patches of multifocal hemorrhages in the inner wall. Deposition of gas, bile contents in the jejunum and ileum were also noticed. The intestine was friable and easily tore off when handled. Focal necrosis and hemorrhages on the upper surface of liver was also noticed in four chickens. Rest other organs appeared apparently healthy.

Scanning electron microscopy analysis: The SEM analysis of intestinal pieces from control broiler chicken showed normal and healthy architecture with smooth surface of lengthy villi within the intestinal mucosa (Fig. 1a). The intestinal pieces from NE infected chickens under SEM showed shortening of intestinal villi with rough and distorted surface and fusion of two or three adjacent villi. Morphologically, damages were observed highly in intestinal mucosa which showed massive necrosis, porous and collapsed villi tips with several blebs. Intestinal mucosa also showed partial and complete destruction of the microvilli structure (Fig. 1b-d). In some areas, infiltration of deformed and lysed inflammatory cells was also observed within the intestinal mucosa.

Isolation and identification of *Clostridium perfringens*: On goat blood agar, bacterial colonies were found small dew drop like colonies surrounded by an inner zone of complete haemolysis and an outer zone of incomplete haemolysis. Bacteria were observed to be non-motile, Gram-positive, thick rod shaped, forming sub-terminal oval endospores (Fig. 2). Rods were measured approximate length of 0.7-1.9 × 2.9-9.8µm. All the isolates produced stormy fermentation, acidity, reduction and coagulation in litmus milk and liquefied gelatin. Isolates also showed DNase and lecithinase activities and fermented glucose and lactose. Upon detailed bacteriological investigation of six intestine and six liver samples collected from broiler chickens died in NE, 10 (83.3%) *C. perfringens* strains were isolated and identified (Table 1). No *C. perfringens* was isolated from healthy broiler chicken (control).

Plasmid profiling: Out of 10 isolates, 9 (90%) harboured two plasmids of molecular weight approximately 42.8kb and 45.2kb, while one isolate carried single plasmid of 45.2kb (Table 1). The identical 45.2kb plasmid was found common in all *C. perfringens* isolates.

Polymerase chain reaction assay: PCR analysis revealed that out of six virulence genes of *C. perfringens* screened, only alpha toxin gene (*cpa*) of 324bp fragment (Table 1, Fig. 3) was detected from all the clinical isolates. However, the beta2 toxin gene of 567bp fragment was also detected from four clinical isolates originated from the intestinal contents (Table1, Fig. 4). None of the isolates were positive for any of the *cpb*, *etx*, *i*A and *cpe* toxin genes.

Sequence analysis of partial alpha toxin gene: The partial *cpa* gene sequences from six Indian field isolates in BLAST showed similarity values greater than 99% to the published database sequences of *C. perfringens*. The sequencing and phylogenetic relationship of partial *cpa* gene from Indian field isolates and references from Japan, Denmark and USA showed 97.6% to 100% sequence homology among the isolates irrespective of different source of origin and geographical distribution (Fig. 5). A total of nine nucleotide substitutions at positions $9_{(\text{Adenine–Guanine})}$ to $18_{(\text{Adenine–Guanine})}$, to $74_{(\text{Adenine to Cytosine})}$ $82_{(\text{Guanine to Adenine})}$, $102_{(\text{Adenine to Guanine})}$, $153_{(\text{Adenine to Guanine})}$, $207_{(\text{Thymine to Cytosine})}$, $226_{(\text{Thymine to Cytosine})}$ and $252_{(\text{Adenine to Guanine})}$

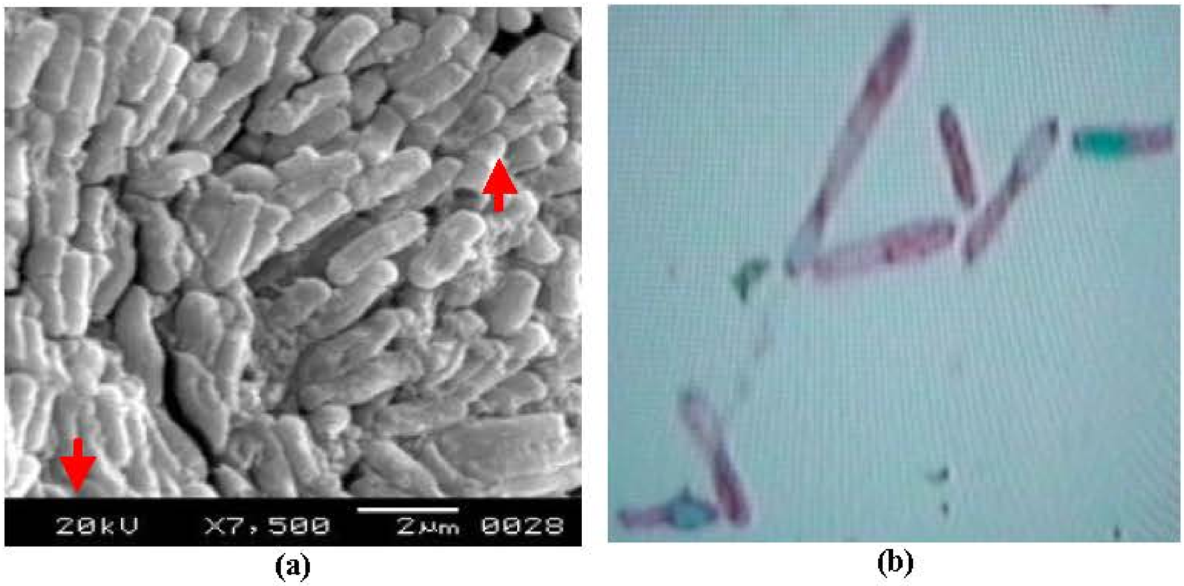

Fig. 2: Ultra structure of *C. perfringens*. (a) scanning electron micrograph showing cluster of rods at ×7500; (b) endo-spore smear showing subterminal oval spores stained with malachite green (arrows) at ×2500 (enlarged) under LEICA compound microscope. Bar range is 2μm. Acceleration voltage is 20 KV.

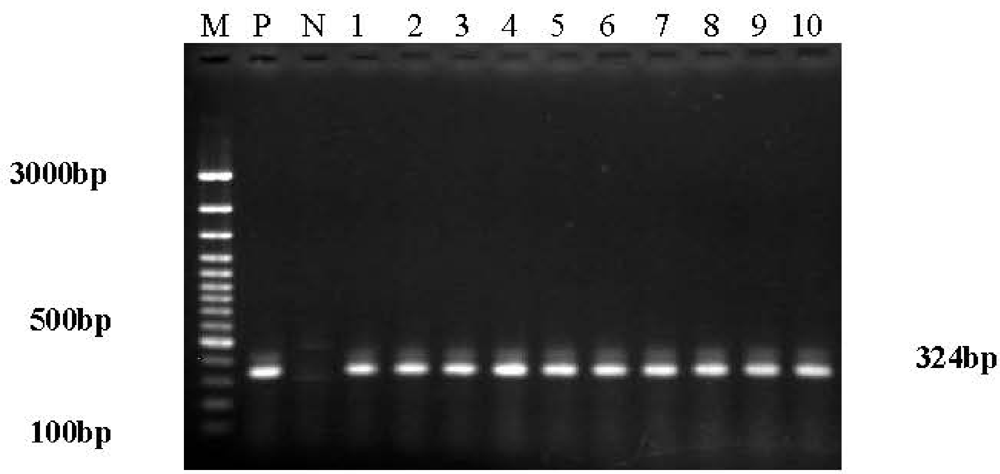

Fig. 3: Detection of 324bp fragment of alpha toxin genes of *C. perfringens* by PCR. Lane P: Positive control; lane N: Negative control; lanes 1 to 10: Field isolates positive for alpha toxin genes; lane M: 100bp marker DNA.

were observed within the 292bp region of *cpa* gene among the isolates. Of these, the nucleotide substitutions $74_{(Adenine\ to\ Cytosine)}$ and $82_{(Guanine\ to\ Adenine)}$ which resulted two corresponding amino acid substitutions at positions $25_{(Aspertic\ acid-Alanine)}$ and $28_{(Alanine-Threonine)}$ in comparison to the consensus were observed in Indian field isolate CP66 (DQ787190) and two references DQ184053 and AF477010 from USA and Denmark respectively (Fig. 6). Remaining nucleotide substitutions have not resulted any corresponding amino acid alternations and were observed only in reference sequences.

Discussion

In the present study necrotic enteritis caused 2.2% mortality within two days in the broiler chickens of age between two to three weeks old. Broussard *et al.* (1986) reported that clinical NE cause higher mortality in 2 to 4 weeks old chicken which sometimes exceeds 1% daily. Necrotic enteritis were identified by the symptoms of severe depression, decreased appetite, diarrhea, reluctance to move and ruffled feathers also reported earlier (Songer, 1996; Das *et al.*, 1997). In post mortem examination, chickens were found dehydrated and produced fetid odor and had severe lesions and

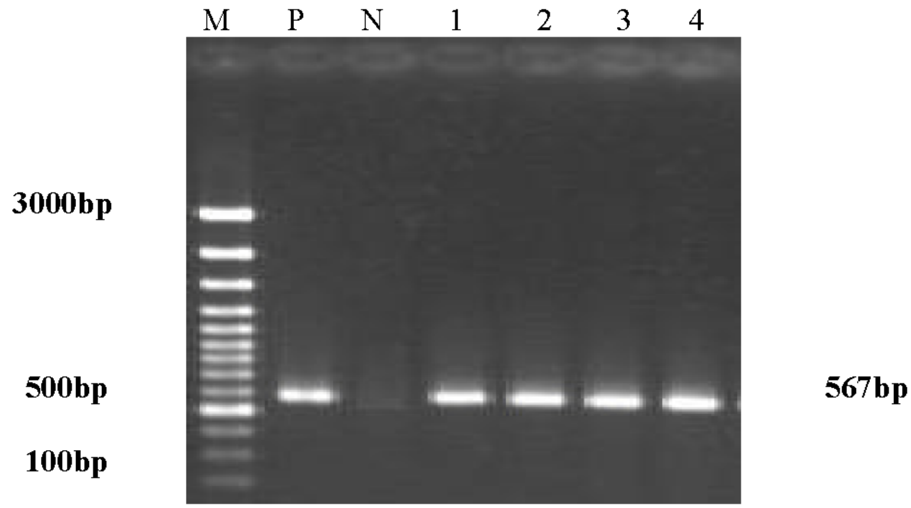

Fig. 4: Detection of 567bp fragment of beta2 toxin genes of *C. perfringens* by PCR. Lane P: Positive control; lane N: Negative control; lanes 1 to 4: field isolates positive for beta2 toxin genes; lane M: 100bp marker DNA.

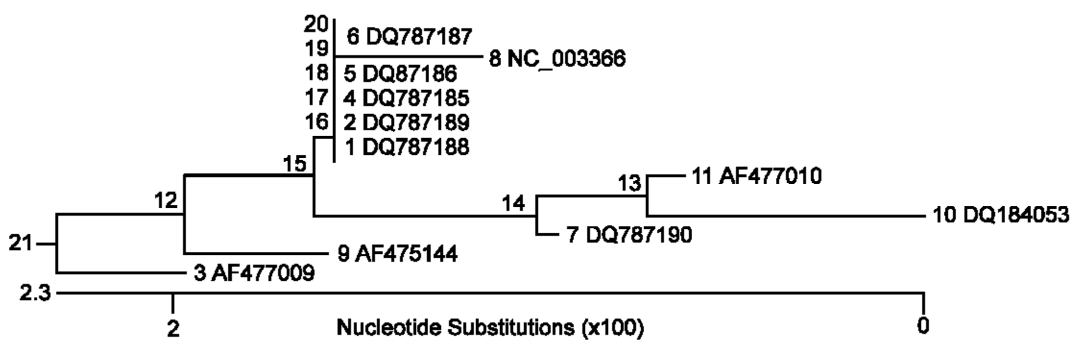

Fig. 5: Phylogenetic tree analysis based on the nucleotide sequence of partial alpha toxin gene of *C. perfringens*.

necrosis on the wall of the small intestine. In some cases focal necrosis and hemorrhages on the upper surface of liver was also observed. Scanning electron microscopy analysis also revealed the occurrence of massive necrosis, porous and collapsed villi tips and complete destruction of microvilli structure within the intestinal mucosa. In the acute form of NE the birds often become dehydrated and emit foul smell. The gross pathological changes were characterized by severe lesions and diffused mucosal necrosis in the small intestine and even in caecum, liver and kidney (Long, 1974; Broussard *et al*., 1986).

The bacteriological investigation revealed the etiological agent *C. perfringens* in association with NE in broiler chickens (Long, 1974). Isolates of *C. perfringens* harboured high molecular weight single plasmid (45.2kb) and double plasmids (42.8kb and 45.2kb) with 45.2kb identical plasmid common in all isolates. The 42.8kb plasmid from VPI 11268 strain of *C. perfringens* was detected earlier (Squires *et al*., 1984) and identical plasmid pattern was reported to be very common in clinical isolates (Eisgruber *et al.,* 1996). Plasmid profiling differentiated the *C. perfringens* isolates into two groups and could be a useful tool for strain differentiation of *C. perfringens*.

In PCR, *cpa* gene was detected in all the 10 clinical isolates, however four isolates derived from intestinal contents were also found positive for *cpb*2 gene. None of the isolates were positive for any of the *cpb*, *etx*, *i*A and *cpe* toxin genes. The PCR analysis thus revealed that the isolates associated with NE were *C. perfringens* type A. The detection of *cpa* gene (Songer and Meer, 1996) and *cpb*2 gene (Sheedy *et al*., 2004) of *C. perfringens* type A from chicken with NE was earlier reported. Engstrom *et al*. (2003) analysed 53 isolates of *C. perfringens* from NE cases in poultry and healthy poultry by PCR and found that all isolates belonged to type A with the gene coding for alpha toxin production. He also detected beta2 toxin gene in two isolates but none were positive for beta, epsilon, iota and enterotoxin. In the present study, *cpa* gene was detected

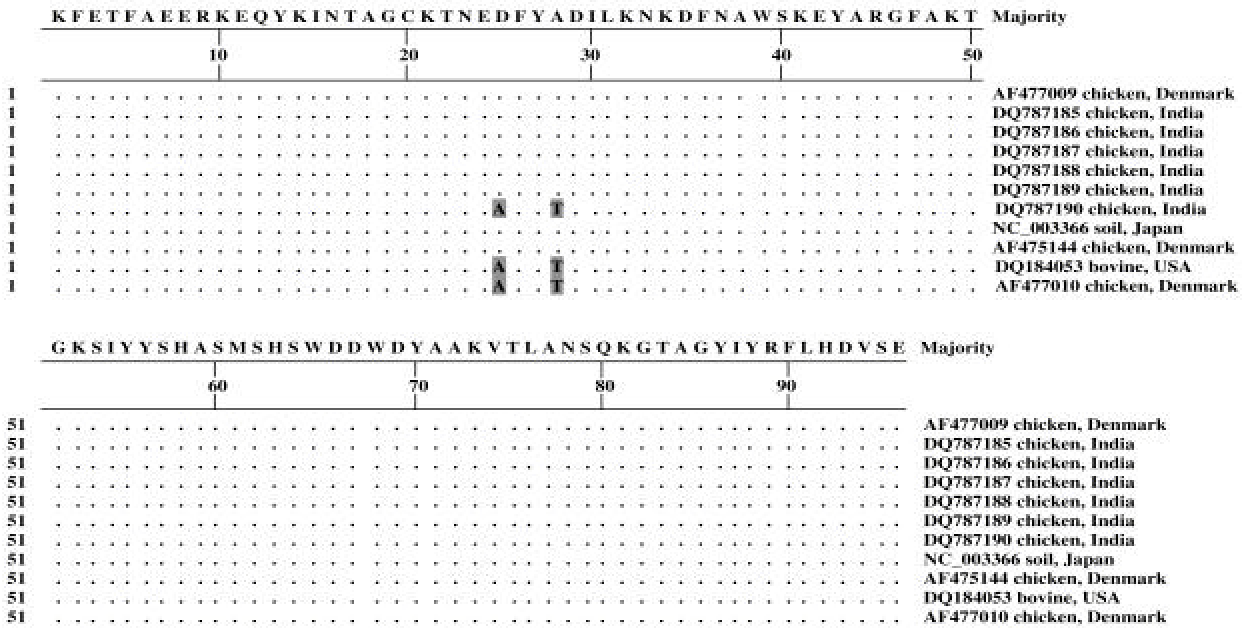

Fig. 6: Deduced amino acid sequences of partial alpha toxin gene of *C. perfringens*. The 'dots' and 'shade' represent the residues that match the consensus exactly and that differ from the consensus respectively.

from all the six intestinal contents and four liver samples. This suggested that alpha toxin of *C. perfringens* type A is the predominant virulence factor in NE in chicken. Necrotic enteritis in poultry is caused predominantly by *C. perfringens* types A and among all other toxins that might be involved in causing NE, alpha-toxin of type A is the most important (Songer, 1996; Das *et al.,* 1997; Sheedy *et al.*, 2004). In this study, beta2 toxin gene was detected only from four intestinal contents and therefore, the occurrence of CPB2 within the intestine did not correlate with the production of NE in chickens. Crespo *et al.* (2007) reported that a large number of healthy birds (90%) carried CPB2-producing isolates and over half of the *cpb2*-positive isolates from diseased birds failed to produce CPB2, did not suggest a causal relationship between beta2 toxin and necrotic enteritis in birds. As classical identification methods are expensive, time consuming and also gives low sensitivity results, PCR can be very useful tool to determine the presence of toxin genes and for typing of *C. perfringens* from the clinical samples.

There was nine nucleotide substitution observed within the 289bp region of *cpa* gene among the isolates studied and were placed in separate clusters in the phylogram. The two nucleotide substitutions which resulted the corresponding two amino acid differences (aspertic acid to alanine and alanine to threonine) were detected in Indian field isolate CP66 (DQ787190) from chicken with NE and two references DQ184053 from bovine enterotoxaemia, USA and AF477010 from healthy chicken, Denmark. All these substitutions were the result of single base substitution and did not significantly alter the physical properties of the encoded proteins. In one nucleotide substitution at position 226 where TTA changed to CTA where both the codons were found coded for the same amino acid leucine. Remaining nucleotide substitutions were occurred at third nucleotide base of codons and any change in third codon is predominantly silent (Rooney *et al.,* 2006). The phylogeny of partial alpha toxin sequences from chicken (India) showed greater than 97.6% to 100% sequence identity with chicken (Denmark), bovine (USA) and soil (Japan). The encoded proteins were also found to be highly conserved in all, irrespective of different source of isolation, health status and geographical distribution. Similarly, Sheedy *et al.* (2004) reported that alpha toxin sequence from chicken isolates closely resembled that of the toxin from human isolates and soil isolate with greater than 98% identity. Ginter *et al.* (1996) found the highly conserved *cpa* sequences and a close homology between the *cpa* sequences of *C. perfringens,* isolated from human and animals.

The *cpa* gene was amplified by PCR and was used for the correct identification of the *C. perfringens*. The present findings suggested that *C. perfringens* type A is the most predominant type associated with necrotic enteritis in broiler chickens in Meghalaya, India and the alpha toxin (CPA) might play a significant role in the pathogenesis of the disease in broiler chicken. Since *C. perfringens* is spore forming and alpha toxin can cause diseases in animal and human by entering into the food chain, therefore consumption of broiler chicken meat for human has a crucial impact on public health. As the disease is having public health importance, good management practices, awareness regarding the disease is in first priority. Further, molecular analysis of the pathogen and the role of *cpa* gene in association with the disease is required to be understood for undertaking the development of control measures, especially for the formulation of cost effecting vaccine.

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References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Bio., 215: 403-410.

Anonymous, 2000. World growth continues. Poult. Int., 39: 8-14.

Birnboim, H.C. and J. Doly, 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res., 7: 1513-1523.

Broussard, C.T., C.L. Hofacre, R.K. Page and O.J. Fletcher, 1986. Necrotic enteritis in cage-reared commercial layer pullets. Avian Dis., 30: 617-619.

Crespo, R., D.J. Fisher, H.L. Shivaprasad, M.E. Fernandez - Miyakawa and F.A. Uzal, 2007. Toxinotypes of *Clostridium perfringens* isolated from sick and healthy avian species. J. Vet. Diagn. Invest., 19: 329-333.

Czeczulin, J.R., P.C. Hanna and B.A. Mcclane, 1993. Cloning, nucleotide sequencing and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. Infect. Immun., 61: 3429-3439.

Das, B.C., G.N. Dutta, L.A. Devriese and A. Phykan, 1997. Necrotic entritis in chickens due to field isolates of *Clostridium perfringens* type A. Indian J. Vet. Pathol., 21: 27-29.

Eisgruber, H., M. Wiedmann and A. Stolle, 1996. Plasmid profiling for strain differentiation and characterization of *Clostridium perfringens* isolates. Zentralbl Veterinarmed B., 43: 137-46.

Engstrom, B.E., C. Fermer, A. Lindberg, E. Saarinen, V. Baverud and A. Gunnarsson, 2003. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. Vet. Microbiol., 9: 225-235.

Ficken, M.D. and D.P. Wages, 1997. Diseases of Poultry. Mosby-Wolfe, Ames, Iowa, USA, pp: 261-264.

Gibert, M., R.C. Jolivet and M.R. Popoff, 1997. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene, 203: 65-73.

Ginter, A., E.D. Williamson, F. Dessy, P. Coppe, H. Bullifent, A. Howells and R.W. Titball, 1996. Molecular variation between the alpha-toxins from the type strain (NCTC 8237) and clinical isolates of *Clostridium perfringens* associated with disease in man and animals. Microbiol., 142: 191-198.

Holt, J.G., N.R. Krige, P.H.A. Sneeth, J.T. Staley and S.T. Williams (Eds.), 1994. Bergey's Manual of Determinative Bacteriology. 9th (Edn.), Williams and Wilkins, Baltimore, USA, pp: 749-755.

Hunter, S.E.C., I.N. Clarke, D.C. Kelly and R.W. Titball, 1992. Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. Infect. Immun., 60: 102-110.

Hunter, S.E.C., J.E. Brown, P.C. Oyston, J. Sakurai and R.W. Titball, 1993. Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with alpha-toxin, Gamma-toxin and leukocidin of *Staphylococcus aureus*. Infect. Immun., 61: 3958-3965.

Long, J.R., 1974. Studies on necrotic enteritis in broiler chickens with emphasis on the role of *Clostridium perfringens*. Disser. Abs. Int., 35: 2503.

Parish, W.E., 1961. Necrotic enteritis in the fowl (*Gallus gallus domesticus*). I. Histopathology of the disease and isolation of a strain of *Clostridium welchii*. J. Comp. Pathol., 71: 377-393.

Perelle, S., M. Gibert, P. Boquet and M.R. Popoff, 1993. Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun., 61: 5147-5156.

Rooney, A.P., J.L. Swezey, R. Friedman, D.W. Hecht and C.W. Maddox, 2006. A analysis of core housekeeping and virulence genes reveals cryptic lineages of *C. perfringens* that are associated with distinct disease presentations. Genetics., 172: 2081-2092.

Sheedy, S.A., A.B. Ingham, J.I. Rood and R.J. Moore, 2004. Highly conserved alpha-toxin sequences of avian isolates of *Clostridium perfringens*. J. Clin. Microbiol., 42: 1345-1347.

Shome, B.R., J.G. Songer, I. Shakuntala, R. Shome, A. Kumar, S. Chakraborty, Y. Mazumder, M.M. Rahman, A. Das, H.V. Murugkar, H. Rahman and K.M. Bujarbaruah, 2006. Atypical blackleg caused by *Clostridium perfringens* type A in cattle in Manipur, India. Ind. J. Anim. Sc., 76: 353-357.

Smedley, III, J.G., D.J. Fisher, S. Sayeed, G. Chakrabarti and McClane, 2004. The enteric toxins of *Clostridium perfringens*. Rev. Physiol. Biochem. Pharmacol., 152: 183-204.

Songer, J.G., 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev., 9: 216-234.

Songer, J.G. and R.R. Meer, 1996. Genotyping of *Clostridium perfringens* by polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. Anaerobe., 2: 197-203.

Squires, C.H., D.L. Heefner, R.J. Evans, B.J. Kopp and M.J. Yarus, 1984. Shuttle plasmids for *Escherichia coli* and *Clostridium perfringens*. J. Bacteriol., 159: 465-471.

Titball, R.W., S.E.C. Hunter, K.L. Martin, A.D. Morris, A.D. Shuttleworth, T. Rubdige, D.W. Anderson and D.C. Kelly, 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect. Immun., 57: 367-376.