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Full Length Research Paper

Clostridium perfringens type A beta2 toxin in elephant (*Elephas maximus indicus*) and pygmy hog (*Sus salvanius*) with haemorrhagic enteritis in Assam, India

Arunava Das[1], Yahya Mazumder[2]*, Biman K. Dutta[3], Bibek R. Shome[4], Komal M. Bujarbaruah[5] and Gauri D. Sharma[6]

[1]Department of Biotechnology, Bannari Amman Institute of Technology, Sathyamangalam, Tamil Nadu, India.
[2]Department of Biotechnology, 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]Department of Life Science, Assam University, Silchar, Assam, India.

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This paper reported the investigation of haemorrhagic enteritis in female elephant (*Elephas maximus indicus*) and pygmy hog (*Sus salvanius*) at the Assam State Zoo, Guwahati, Assam, India. An eight year old female elephant and two and half year old female pygmy hog developed haemorrhagic enteritis of unknown cause maintained at the zoo died within four days. Bacteriological investigation revealed that the causative agent *Clostridium perfringens* was associated with the disease. Erythromycin, clindamycin and metronidazole were effective, however, ampicillin or penicillin G was more effective and probably the drug of choice for *C. perfringens* associated haemorrhagic enteritis. Isolates derived from elephant harboured four plasmids (4.1, 14.4, 38.8 and 48.2 kb), while that from pygmy hog carried two plasmids (42.8 and 51.9 kb). PCR analysis of *C. perfringens* isolates revealed presence of alpha toxin gene (*cpa*) and beta2 toxin gene (*cpb*2). None of the isolates were positive for beta, epsilon, iota and enterotoxin genes. The sequence analysis of partial *cpa* gene showed 98.6 to 100% homology among the isolates studied. The study confirmed the involvement of beta2 toxin producing *C. perfringens* type A associated with the haemorrhagic enteritis.

Key words: *Clostridium perfringens*, haemorrhagic enteritis.

INTRODUCTION

The Indian elephant (*Elephas maximus indicus*) and pygmy hog (*Sus salvanius*) are considered among the endangered mammals in the world. The International Union for the Conservation of Nature and Natural resources, Morges, Switzerland has accorded the Indian elephant with criteria: EN A1 cd and pygmy hog with Criteria: A1c, B1+2cd, E as critically endangered. The Clostridial enteritis in captive pygmy hog until now appeared to be rare; however it has been reported in captive African elephants (Bacciarini et al., 2001). *Clostridium perfringens* is ubiquitous, gram positive anaerobe anaerobe commonly associated with the enteric diseases in humans, domestic animals and wildlife (Songer, 1996). Depending on the production of four major toxins (alpha, beta, iota and epsilon), strains of *C. perfringens* can be classified into five genotypes A to E (Songer and Meer, 1996). Along with four major toxins, enterotoxin and beta2 toxin produced by types of *C. perfringens* also considered as important toxins for enteric diseases (Smedley III et al., 2004). There are reports of beta2-toxigenic *C. perfringens* type A associated with enterocolitis in horses (Herholz et al., 1999), ulcerative enteritis in African elephant (Bacciarini et al., 2001) and enterotoxaemia in goat (Dray, 2004). Differentiation between different strains of *C. perfringens* from the clinical cases is not trivial. Therefore, PCR has been used to detect the

*Corresponding author. E-mail: drym@rediffmail.com.

used to detect the presence of toxin genes and to identify the specific strains of *C. perfringens* (Yamagishi et al., 1997). Marks and Kather, (2003) reported that ampicillin, erythromycin, metronidazole and tetracycline are very effective antibiotics for the treatment of *C. perfringens* associated diarrhoea. Eisgruber et al., (1996) reported that strains of *C. perfringens* isolated from different diseased outbreaks possess a high molecular weight identical plasmid which is useful for the strain different-tiation and characterization of *C. perfringens* isolates. The involvement of *C. perfringens* in African elephant causing ulcerative enteritis has been reported earlier but probably rare reports or unnoticed from pygmy hog. This study reports the occurrence of fatal haemorrhagic enteritis due to *C. perfringens* type A in Indian elephant and pygmy hog.

MATERIALS AND METHODS

History of animals and sample collection

One female elephant of age eight year and one female pygmy hog of age two and half year at the Assam State Zoo, Guwahati, Assam, India, suddenly developed off feed, weakness, poor growth, anorexia, pyrexia and prolonged episodes of watery followed by bloody diarrhoea. The atmospheric temperature and humidity was recorded between 32 - 39°C and 80 - 90% respectively with heavy rainfall during the month of March. Both the animals were isolated and unresponsive to the treatment with 5 mg tetracycline (Himedia, Mumbai) per kg of body weight parentarily for three days. On fourth day, the animals were died. In both cases, postmortem was performed within two to four hours duration after death. Body surfaces were examined for any injuries or wound. All the internal organs were thoroughly examined and any macroscopic and gross lesions observed were recorded. The small and large intestinal contents were aseptically collected during the postmortem examination for microbiological investigation. Samples from feed and water used for the animals were also collected aseptically for microbiological investigation.

Bacterial isolation and biochemical tests

Both aerobic and anaerobic bacterial isolations were attempted. For aerobic isolation, collected samples were first inoculated in nutrient broth and incubated at 37°C for 4 h. Soon after, inoculum from each tube were transferred to freshly prepared sterile nutrient agar plates and incubated at 37°C for 24 h. Bacterial colonies were purified and identified as per standard bacteriological procedures (Holt et al., 1994). For anaerobic isolation, samples were inoculated in Robertson's cooked meat (RCM) broth medium supplemented with glucose, hemin and vitamin K (Himedia, Mumbai), overlaid with neutral oil and incubated at 37°C for 48 h. The inoculums from the 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 24 h at 37°C. Bacterial colonies were purified based on the size, shape, color and patterns of haemolysis on blood agar and were subjected to 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).

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed by agar dilution method (Sutter et al., 1975) using 10 commercially available antibiotics. The antimicrobial agents used were amoxicillin, ampicillin, clindamycin, doxycycline, erythromycin, metronidazole, penicillin G, cephalexin and tetracycline from Hi media, (Mumbai); and cefazaflur from GSK, (Bangalore). Sets of three laked sheep blood agar plates containing no antibiotic were inoculated before each series of antibiotic-containing plates and incubated anaerobically, 10% CO_2 in air and aerobically to serve as growth and contamination controls. Data were deleted if poor or no growth occurred in anaerobic growth control plate or if any contamination was evident.

Detection of plasmid

One milliliter of bacterial culture grown overnight anaerobically in brain heart infusion broth at 37°C was used for the extraction of plasmid DNA by alkali lysis method (Birnboim and Doly, 1979). The plasmid DNA was finally dissolved in 35 µl of TE-RNase (1 mg/ml in 10 mM tris-hydrochloric acid and 1 mM 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 separated 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 *C. perfringens,* isolates 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 assay (Songer and Meer, 1996). Freshly grown bacterial colonies from solid media plates were suspended in 200 µl of Milli-Q water in a microcentrifuge tube, gently vortexed and boiled for 10 min in a water bath. Supernatant after centrifugation at 10000 g for 5 min was used as a template DNA. The amplification was carried out in 25 µl reaction volume containing 12.5 µl of 2× PCR master mix (Promega, USA) containing 4 mM magnesium chloride, 0.4 mM of deoxynucleotide triphosphates (dNTPs), 0.5U of *Taq* DNA polymerase, 150 mM tris-hydrochlroric acid, pH 8.5 (Promega, USA), 0.5 µM primers and 2.5 µl of template DNA. The PCR reactions were performed in iCycler (BioRad, USA). After initial denaturation at 94°C for 4 min, the amplification cycle had denaturation, annealing and extension at 94°C, 55 and 72°C for 1 min each respectively. Final extension was done at 72°C for 10 min. The specific forward and reverse primer pairs used in this study were commercially synthesized from GENSET (Table 1). *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 (5 µl) were electrophoresed in 1.5% agarose gel in TAE (tris-acetic acid-EDTA, pH 8) buffer, stained with ethidium bromide and observed in gel doc system (Pharmacia Biotech, Sweden).

Sequencing of PCR amplified product

Four 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 sequence obtain-

Table 1. Details of primers used in polymerase chain reaction for the detection of toxin genes from *C. perfringens*.

Toxin / toxin genes	Primer sequences	Primer Concentration	Amplicon size (bp)	Reference
Alpha toxin / *cpa*	For 5'-gctaatgttactgccgttga-3' Rev 5'-cctctgatacatcgtgtaag-3'		324	Titball et al., (1989)
Beta toxin / *cpb*	For 5'-gcgaatatgctgaatcatcta-3' Rev 5'-gcaggaacattagtatatcttc-3'	0.5 µM	180	Hunter et al., (1993)
Epsilon toxin / *etx*	For 5'-gcggtgatatccatctattc-3' Rev 5'-ccacttacttgtcctactaac-3'		655	Hunter et al., (1992)
Iota toxin / *iA*	For 5'-actactctcagacaagacag-3' Rev 5'-ctttccttctattactatacg-3'		446	Perelle et al., (1993)
Beta2 toxin / *cpb*2	For 5'-agattttaaatatgatcctaacc-3' Rev 5'-caatacccttcaccaaatactc-3'		567	Gibert et al., (1997)
Enterotoxin / *cpe*	For 5'-ggagatggttggatattagg-3' Rev 5'-ggaccagcagttgtagata-3'		233	Czeczulin et al., (1993)

For: Forward primer; Rev: Reverse primer; bp: Base pair.

obtained were submitted to BLAST analysis (Altschul et al., 1990) to determine the similarities to other sequences available in GenBank. The *cpa* gene sequences derived from the *C. perfringens* isolated from elephant were deposited into the GenBank under accession numbers DQ838706 and DQ838707, while sequences derived from pygmy hog were deposited under accession numbers DQ838708 and DQ838709. The *cpa* sequences up to 292 bases were aligned with the corresponding sequences of four selected *C. perfringens* isolated from bovine enterotoxaemia from USA (DQ184079), healthy chicken from Denmark (AF477009), soil from Japan (NC_003366) and strain C57-1 from China (AY823400) available in the GenBank by using ClustalW algorithm of MegAlign program (DNASTAR, Lasergene, USA). The phylogenetic analysis of the sequences and 97 deduced amino acids residues were also analysed by using same programme.

RESULTS

Postmortem findings

The postmortem examination of elephant and pygmy hog showed diffused haemorrhagic and necrotic enteritis, lesions and edema in both small and large intestines. Discrete areas of necrosis were present on the walls of the caecum and colon. Rest of the organ was found apparently healthy.

Isolation and identification of *C. perfringens*

On aerobic isolation, mixed colonies of both Gram positive and Gram negative bacteria were found, which did not lead to any conclusive evidence. However, on anaerobic culture process, pure colonies showing alpha and beta haemolysis on goat blood agar was clearly observed. The bacterial colonies originated from the intestinal contents of elephant were observed to be flat with irregular thin border. However, colonies that originated from pygmy hog were having radially striated periphery with cremator edges. Gram staining showed gram-positive rods, while malachite green staining showed sub-terminal oval endospores. The isolates produced stormy fermentation, acidity, reduction and coagulation in litmus milk and liquefied gelatin. Isolates also showed both DNase and lecithinase activities and fermented glucose and lactose. Upon the detailed bacteriological investigation, four isolates (two from elephant and two from pygmy hog) were identified as *Clostridium perfringens*. No *C. perfringens* was isolated from the feed or water used for the animals.

Antimicrobial susceptibility and plasmid profiling

The susceptibility test of 10 commercially available antibiotics against the four isolates of *C. perfringens* used in this study is shown in Table 2. All isolates were inhibited by 0.5 µg/ml or less of ampicillin and 0.5 U/ml or less of penicillin G, 1.0 µg/ml or less of erythromycin and 2.0 µg/ml or less of any of the clindamycin and metronidazole tested. Only 77, 42, 43, 52 and 33% isolates were inhibitted by 2.0µg/ml or less of any of the amoxicillin, cefazaflur, cephalexin, doxycycline and tetracycline respecttively tested.

All the isolates harboured multiple plasmids. Two isolate derived from elephant harboured four plasmids (4.1, 14.4, 38.8 and 48.2 kb), while the remaining two isolates from pygmy hog carried two plasmids (42.8b and 51.9 kb). None of the isolates carried identical or common plasmids.

PCR assay and sequence analysis

In PCR analysis, out of six virulence genes of *C. perfringens* screened only alpha toxin gene (*cpa*) of 324 bp fragment and beta2 toxin gene (*cpb*2) of 567bp

Table 2. Activity of ten antibiotics against four isolates of *C. perfringens* derived from haemorrhagic enteritis of elephant and pygmy hog.

Name of antibiotic	Cumulative percentage susceptible to indicated concentration (µg/ml)									
	≤ 0.1	0.5	1.0	2.0	4.0	8.0	16.0	32.0	64.0	≥128.0
Amoxicillin	32	42	65	77	82	87	92	96	100	
Ampicillin	38	100								
Cefazaflur	18	28	38	42	53	64	73	86	94	100
Cephalexin	22	27	33	43	66	77	95	100		
Clindamycin	31	49	79	100						
Doxycycline	14	27	42	52	68	75	81	100		
Erythromycin	32	55	100							
Metronidazole	28	44	84	100						
*Penicillin G	39	100								
Tetracyclin	11	19	26	33	41	47	58	76	89	100

*concentration in U/ml

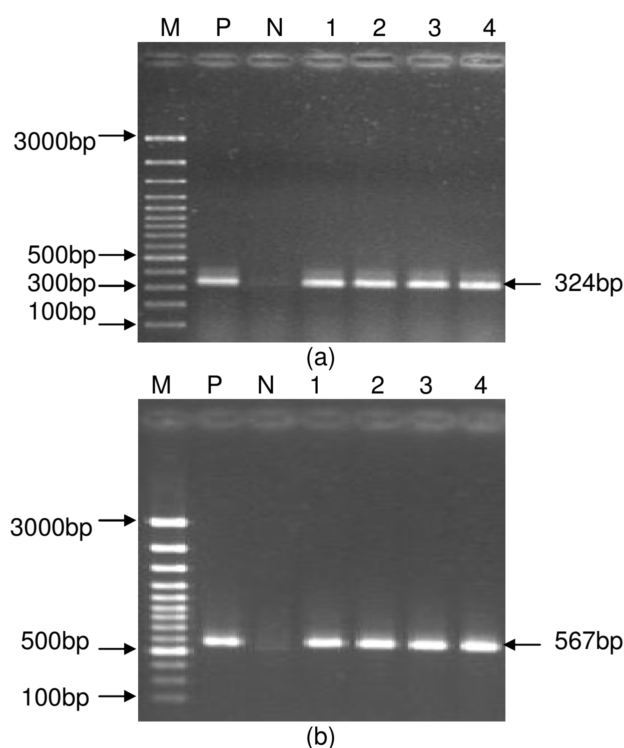

Figure 1. Detection of alpha and beta2 toxin genes of *C. perfringens* by PCR. (a) isolates positive for 324bp fragment of alpha toxin gene; (b) isolates positive for 567bp fragment of beta2 toxin gene. Lane P: positive control; lane N: negative control; lanes 1 to 4: field isolates positive for toxin genes; lane M: 100bp marker DNA.

fragment were detected in all the isolates (Figure 1). None of the isolates were positive for any of the *cpb*, *etx*, *i*A and *cpe* toxin genes. PCR assay thus revealed that the field isolates originated from elephant and pygmy hog were *C. perfringens* type A with an additional *cpb*2 toxin gene.

The partial *cpa* gene sequences from four Indian field isolates in BLAST showed similarity values greater than 99% to the published database sequences of *C. perfringens.* The comparative alignment study of partial *cpa* gene sequences of four Indian field isolates and three reference sequences derived from bovine (USA), chicken (Denmark) and strain C57-1 (China) respectively showed no nucleotide substitution in compared to majority (Figure 2). However, the reference sequence originated from soil (Japan) had four nucleotide substitutions at positions $105_{A\rightarrow G}$, $156_{G\rightarrow A}$, $229_{C\rightarrow T}$ and $255_{G\rightarrow A}$ respectively. The alignment of 97 deduced amino acids revealed no change in proteins. The phylogenetic tree constructed with partial *cpa* gene sequences of *C. perfringens* revealed 98.6 to 100% sequence homology among the isolates irrespective of different source of origin and geographical distribution (Figure 3).

DISCUSSION

Reports on Clostridial infection of elephant and pygmy hogs are rare due to limited population of these mammals. Of these animals, the pygmy hog is only captive population available in Assam, India. This report now gives a good insight into the susceptibility of these animals to Clostridial disease. The role of *C. perfringens* in present study was made clear by isolation of the organism alone under anaerobic conditions, without any other species of Clostridia. Further, aerobic isolation had not yielded any dominant bacterial type suggestive of enteric infection.

The result of antibiotic susceptibility test clearly revealed that ampicillin or penicillin G appeared to be the drug of choice in *C. perfringens* associated with haemorrhagic enteritis. However, other antibiotics like erythromycin, clindamycin and metronidazole can also be very useful

	A	C	A	A	A	G	A	T	T	T	Majority
										110	
101	.	.	.	.	.	.	.	.	.	.	AF477009
101	.	.	.	.	.	.	.	.	.	.	AY823400
101	.	.	.	.	.	.	.	.	.	.	DQ184079
101	.	.	.	.	.	.	.	.	.	.	DQ838706
101	.	.	.	.	.	.	.	.	.	.	DQ838707
101	.	.	.	.	.	.	.	.	.	.	DQ838708
101	.	.	.	.	.	.	.	.	.	.	DQ838709
101	.	.	.	.	G	.	.	.	.	.	NC_003366

	A	C	A	G	G	G	A	A	A	T	Majority
										160	
151	.	.	.	.	.	.	.	.	.	.	AF477009
151	.	.	.	.	.	.	.	.	.	.	AY823400
151	.	.	.	.	.	.	.	.	.	.	DQ184079
151	.	.	.	.	.	.	.	.	.	.	DQ838706
151	.	.	.	.	.	.	.	.	.	.	DQ838707
151	.	.	.	.	.	.	.	.	.	.	DQ838708
151	.	.	.	.	.	.	.	.	.	.	DQ838709
151	.	.	.	.	.	A	.	.	.	.	NC_003366

	A	G	G	T	A	A	C	T	C	T	Majority
										230	
221	.	.	.	.	.	.	.	.	.	.	AF477009
221	.	.	.	.	.	.	.	.	.	.	AY823400
221	.	.	.	.	.	.	.	.	.	.	DQ184079
221	.	.	.	.	.	.	.	.	.	.	DQ838706
221	.	.	.	.	.	.	.	.	.	.	DQ838707
221	.	.	.	.	.	.	.	.	.	.	DQ838708
221	.	.	.	.	.	.	.	.	.	.	DQ838709
221	.	.	.	.	.	.	.	.	T	.	NC_003366

	C	A	G	C	G	G	G	A	T	A	Majority
										260	
251	.	.	.	.	.	.	.	.	.	.	AF477009
251	.	.	.	.	.	.	.	.	.	.	AY823400
251	.	.	.	.	.	.	.	.	.	.	DQ184079
251	.	.	.	.	.	.	.	.	.	.	DQ838706
251	.	.	.	.	.	.	.	.	.	.	DQ838707
251	.	.	.	.	.	.	.	.	.	.	DQ838708
251	.	.	.	.	.	.	.	.	.	.	DQ838709
251	.	.	.	.	A	.	.	.	.	.	NC_003366

Figure 2. Nucleotide sequence polymorphic sites of 292 bp fragment of *cpa* gene of *C. perfringens*. The 'dots' and 'shade' represent the residues that match the consensus exactly and that differ from the consensus respectively. Accession numbers: AF477009 = chicken, Denmark; AY823400 = strain C57-1, China; DQ184079 = bovine, USA; NC_003366 = soil, Japan; DQ838706 to DQ838707 = elephant, India; DQ838708 to DQ838709 = pygmy hog, India.

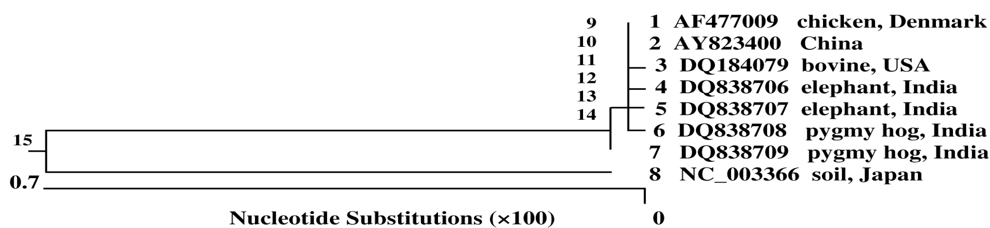

Figure 3. Phylogenetic tree analysis based on the nucleotide sequence of partial alpha toxin gene of *C. perfringens*.

for the treatment of *C. perfringens* associated diarrhoea. This result agrees with the earlier finding reported by Marks and Kather, (2003). But tetracyclines, cefazaflur and even amoxicillin cannot be depended upon and useful in *C. perfringens* infections (Sutter and Finegold, 1976). In plasmid profiling, the isolates showed multiple plasmids of molecular weight ranging from 4.1 to 51.9 kb. None of the isolates in this study shared identical or common plasmid which was previously reported to be common in strains of *C. perfringens* associated diseases (Eisgruber et al., 1996). In PCR, only *cpa* and *cpb*2 genes were detected which revealed that all the isolates used in this study were *C. perfringens* type A. The presence of pathogenic *cpb*2 positive strains of *C. perfringens* type A in occurrence of ulcerative enteritis in typhlocolitis in horses (Herholz et al., 1999) and African elephant (Bacciarini et al., 2001) have been reported earlier. The analysis of *C. perfringens* toxin genes by PCR, replacing elaborate animal experiments is extremely useful for diagnostic purpose (Songer and Meer, 1996; Bacciarini et al., 2001). There was no nucleotide substitution within the 292bp region of *cpa* gene among the Indian field isolates of *C. perfringens* from elephant and pygmy hog and among three reference sequences from bovine, chicken and strain C57-1 from USA, Denmark and China respectively. Hence, all were placed in a single cluster in the phylogram. However, the four nucleotide substitution at position 105 (adenine to guanine), 156 (guanine to adenine), 229 (cytocine to thiamine) and 255 (guanine to adenine) observed only in isolate derived from soil from Japan also resulted in no amino acid residue change. In general, all the nucleotide substitutions occurred in third nucleotide base of codons which is predominantly silent (Rooney et al., 2006). The phylogenetic analysis also revealed that the partial *cpa* gene of *C. perfringens* used in this study is highly conserved among isolates irrespective of different sources of isolation and geographical distribution.

The role of the *cpa* gene in the pathogenesis of haemorhagic enteritis is still not clear because *C. perfringens* type A strains possessing *cpa* gene are also frequently isolated from healthy animals. However, among types A to E of *C. perfringens*, type A isolates carrying *cpb*2 have been implicated to gastrointestinal diseases in various animals (Bueschel et al., 2003). The detection of *cpb*2 gene in *C. perfringens* type A isolated from diarrheic and absence of that in healthy piglet has been reported (Klaasen et al., 1999). Keeping all the above, our findings

clearly revealed that *C. perfringens* type A containing an additional *cpb*2 gene might play a significant role in occurrence of haemorrhagic enteritis in elephant and pygmy hog in Assam. However, the potential synergies of both *cpa* and *cpb*2 gene in association with the disease is required to be understood, therefore further studies have to be conducted to understand the molecular pathogenesis of haemorrhagic enteritis.

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REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Bio. 215(3): 403-410.

Bacciarini LN, Pagan O, Frey J, Grone A (2001). *Clostridium perfringens* beta2 toxin in an African elephant (*Loxodonta africana*) with ulcerative enteritis. Vet. Rec. 149(20): 618-620.

Birnboim HC, Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nuc. Acid. Res. 7(6): 1513-523.

Bueschel DM, Jost BH, Billington SJ, Trinh HT, Songer JG (2003). Prevalence of *cpb*2, encoding beta2 toxin, in *C. perfringens* field isolates: correlation of genotype with phenotype. Vet. Microbiol. 94(2): 121-129.

Czeczulin JR, Collie RE, Mcclane BA (1996). Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative Type A, B and C isolates of *C. perfringens*. Infect. Immun. 64(8): 3301-3309.

Dray T (2004). *Clostridium perfringens* type A β2 toxin associated with enterotoxemia in a 5-week-old goat. Can. Vet. J. 45: 251-253.

Eisgruber H, Wiedmann M, Stolle A (1996). Plasmid profiling for strain differentiation and characterization of *Clostridium perfringens* isolates. Zentralbl Veterinarmed B. 43(3): 137-146.

Gibert M, Jolivet RC, Popoff MR (1997). Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene. 203(1): 65-73.

Herholz C, Miserez R, Nicolet J, Frey J, Popoff M, Gibert M, Gerber H, Straub R (1999). Prevalence of beta2 toxigenic *C. perfringens* in horses with intestinal disorder. J. Clin. Microbiol. 37(2): 358-361.

Holt JG, Krige NR, Sneeth PHA, Staley JT, Williams ST (1994). Bergey's Manual of Determinative Bacteriology. 9th ed, Baltimore, USA, Williams and Wilkins, pp. 749-755.

Hunter SEC, Brown JE, Oyston PC, Sakurai J, Titball RW (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(9): 3958-3965.

Hunter SEC, Clarke IN, Kelly DC, Titball RW (1992). Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. Infect. Immun. 60(1): 102-110.

Klaasen HLBM, Molkenboer MJCH, Bakker J, Miserez R, Hani H, Frey J, Popoff MR, Van Den Bosch JF (1999). Detection of the beta2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in the Netherlands and Switzerland. Immun. Med. Microbiol. 24(3): 325-332.

Marks SL, Kather EJ (2003). Antimicrobial susceptibilities of canine *Clostridium difficile* and *Clostridium perfringens* isolates to commonly utilized antimicrobial drugs. Vet. Microbiol. 94(1): 39-45.

Perelle S, Gibert M, Boquet P, Popoff MR (1993). Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun. 61(12): 5147-5156.

Rooney AP, Swezey JL, Friedman R, Hecht DW, Maddox CW (2006). A analysis of core housekeeping and virulence genes reveals cryptic lineages of *C. perfringens* that are associated with distinct disease presentations. Genetics. 172(4): 2081-2092.

Shome BR, Songer JG, Shakuntala I, Shome R, Kumar A, Chakraborty S, Mazumder Y, Rahman MM, Das A, Murugkar HV, Rahman H, Bujarbaruah KM (2006). Atypical blackleg caused by *Clostridium perfringens* Type A in cattle in Manipur, India. Indian J. An. Sci. 76(5): 353-357.

Smedley III JG, Fisher DJ, Sayeed S, Chakrabarti G, McClane BA (2004). The enteric toxins of *Clostridium perfringens*. Rev. Physiol. Biochem. Pharmacol. 152: 183-204.

Songer JG (1996). Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. 9: 216-234.

Songer JG Meer RR (1996). Genotyping of *Clostridium perfringens* by polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. Anaerobe. 2(4): 197-203.

Sutter VL, Finegold SM (1976). Susceptibility of anaerobic bacteria to 23 antimicrobial agents. Antimicrob. Agents. Chemother. 10(4): 736-752.

Sutter VL, Vargo VL, Finegold SM (1975). Wadsworth anaerobic bacteriology manual. 2nd Ed., University of California, Los Angeles Extension Division, Los Angeles.

Titball RW, Hunter SEC, Martin KL, Morris AD, Shuttleworth AD, Rubdige T, Anderson DW, Kelly DC (1989). Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect. Immun. 57(2), 367-376.

Yamagishi T, Sugitani K, Tanishima K, Nakamura S (1997). Polymerase chain reaction test for differentiation of five toxin types of *Clostridium perfringens*. Microbiol. Immunol. 41(4): 295-299.