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Characterization of *Clostridium perfringens* isolated from mammals and birds from Guwahati city, India

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Abstract: Of the 102 samples collected from mammals and birds, both domestic and captive wild, 48 were found to be positive for *Clostridium perfringens*. Most of the mammal isolates (84.38%) appeared to have been collected from clinically affected animals, while 33.33% of the bird samples were from clinically affected and 21.43% from apparently healthy birds infected with *C. perfringens*. Isolates revealed high sensitivity to ciprofloxacin, enrofloxacin and norfloxacin. Among the isolated *C. perfringens,* 30 (62.50%) showed DNase production. Hemolytic activity was recorded in 14 (24.16%) of the isolates and 28 (58.33%) showed phospholipase C production. All the phospholipase C positive isolates revealed the presence of *cpa* gene encoding alpha (α) toxin. Of the 102 samples collected from mammals and birds, both domestic and captive wild, 48 were found to be positive for *Clostridium perfringens*. Most of the mammal isolates (84.38%) appeared to have been collected from clinically affected animals, while 33.33% of the bird samples were from clinically affected and 21.43% from apparently healthy birds infected with *C. perfringens*. Isolates revealed high sensitivity to ciprofloxacin, enrofloxacin and norfloxacin. Among the isolated *C. perfringens,* 30 (62.50%) showed DNase production. Hemolytic activity was recorded in 14 (24.16%) of the isolates and 28 (58.33%) showed phospholipase C production. All the phospholipase C positive isolates revealed the presence of *cpa* gene encoding α toxin.

Key words: *Clostridium perfringens,* PCR, α toxin, *cpa* gene, polymerase chain reaction.

INTRODUCTION

Different cases of acute enteritis or fatal enterotoxemia have been reported in several animal species. Sudden death in bovines, ovines and caprines have also been attributed to *Clostridium perfringens* that produce a number of exotoxins and enzymes, which, in turn, have important roles in the production of various disease manifestations like severe gangrene, diarrhea, dysentery, muscle infection and various forms of enteric diseases. The organism is transmitted directly from infected to healthy animals through contaminated soil, animal feed and litter (1).

The toxins produced depend on the *C. perfringens* strain involved and each type induces a specific syndrome. Therefore, a correct identification of pathovars is critical for epidemiological study. As the diseases caused by this organism are soil-borne, effective vaccination rather than management practices can control the disease. Before vaccination, detailed characterization of the causative agent isolated from diverse sources is of paramount importance.

MATERIALS AND METHODS

Fecal materials, affected muscle tissues, intestinal contents and internal organs were collected from

apparently healthy, clinically affected and dead mammals and birds with suspicion of clostridial infection. The study included both domestic and captive wild mammals and birds, in and around Guwahati city in Assam state, India, including the Assam State Zoo.

Isolation and Identification of *C. perfringens*

Clinical materials were inoculated deep into the Robertson's cooked meat broth (RCMB) medium as per the standard method and incubated under anaerobic condition at 37°C for 48 hours (2). Broth cultures exhibiting typical growth characteristics of *C. perfringens, i.e.* production of acid and gas without digestion of meat, sour odor of the culture and reddening of meat were further sub-cultivated on 5% sheep blood agar plates and incubated at 37°C for 24 hours under anaerobic conditions for proliferation. Isolates were at first identified as *C. perfringens* on the basis of cultural, morphological and biochemical characteristics (2).

Susceptibility to Antimicrobial Agents

C. perfringens isolates presenting *in vitro* susceptibility to 12 different antimicrobial agents were tested by a disc diffusion method (3).

Characterization of *C. perfringens* Isolates

All the isolates recovered in the present study were characterized with respect to their DNase, hemolysin and phospholipase production.

Detection of Deoxyribonuclease (DNase) Production by *C. perfringens* Isolates

Isolates were tested for DNase production according to the standard method (4).

Detection of Hemolysin Production by *C. perfringens* Isolates

Hemolysin produced by the *C. perfringens* isolates was detected by inoculating the bacterial colonies on sheep blood agar, as previously described (1). Inoculated plates were incubated at 37°C anaerobically for 24 hours. Presence of hemolytic zones (double or single) around colonies indicated production of hemolysin.

Detection of Phospholipase C (Nagler's Reaction)

The production of phospholipase C by the *C. perfringens* isolates was verified according to the literature (5). Suspected colonies were inoculated into Wills and Hobb's medium without lecithinase-specific antisera, and added to neomycin sulfate at the concentration of 250 μg per mL. Plates were incubated anaerobically at 37°C for 24 hours and then examined for the presence of a pink zone of opacity around the lecithinase-positive and lactose-positive colonies.

Polymerase Chain Reaction (PCR) – Detection of *cpa* Gene

The *cpa* gene that encodes for α toxin in the *C. perfringens* isolates was detected by PCR (6). Two to five colonies from each isolate grown on blood agar were suspended separately into 100 μL of Milli Q water in a 1.5 mL. The reaction was contained in an Eppendorf tube.

Specific primers (*cpa* IFP 5GCTAATGTTACTGCCGTTGA-3/ and *cpa* IPR 5/CCTCTGATACATCGTGTAAG -3/) as previously described were used for the detection of the *cpa* gene (324 bp) (6). The PCR reaction was performed in a thermocycler (BioRad, USA). Amplified bands were visualized by gel documentation.

RESULTS

On bacteriological examination, 48 (47.06%) of the 102 samples revealed the presence of *C. perfringens*, including 44 (51.76%) from domestic and captive wild mammals, and 4 (23.53%) from birds (Table 1). Frequency of isolation was found to be higher (84.38%) from clinically affected compared to apparently healthy (32.08%) animals. Similarly, 33.33 and 21.43% of the clinically affected and apparently healthy birds, respectively, were found to be positive for *C. perfringens*. *C. perfringens* isolates showed highest sensitivity to ciprofloxacin followed by enrofloxacin and norfloxacin.

Among the *C. perfringens* isolates, 30 (62.50%) were found to be positive for DNase production. Of these, 29 were from mammals and one from a bird. The majority of the isolates positive for DNase production were from clinically affected birds and mammals. Hemolysin production could be detected in 14 (29.16%), including 13 of non-avian origin (Table 1). From these, ten were from clinically affected mammals, as was the bird isolate (Table 1). A total of 28 (58.33%) isolates were positive for phospholipase C production and all

Table 1. Prevalence and characterization of *Clostridium perfringens* of mammal and bird origin in relation to deoxyribonuclease (DNase), hemolysin, phospholipase C production and presence of *cpa* gene in Guwahati city during 2009 and 2010

Sample source	Examined samples	*C. perfringens* presence	Isolates showing production/presence of			
			cpa gene	DNase	Hemolysin	Phospholipase C
A. Mammals (apparently healthy)						
Bovine	10	1	1	0	1	0
Goat	10	2	2	2	2	0
Sheep	2	2	2	0	0	0
Elephant	9	5	5	3	0	0
Tiger	2	1	1	0	0	0
Lion	2	0	0	0	0	0
Pig-tailed monkey	1	0	0	0	0	0
Stump-tailed monkey	5	1	1	0	0	0
Bonnet monkey	1	1	1	0	0	0
Lion-tailed monkey	1	1	1	0	0	0
Golden langur	3	1	1	1	0	0
Sambar	2	1	1	0	0	0
Hog deer	4	0	0	0	0	0
Serow	1	1	1	1	0	0
Total (%)	53	17 (32.08)	17	7	3	0
B. Mammals (clinically affected)						
Cattle	5	4	4	4	0	4
Pigmy hog	2	2	2	2	1	2
Goat	14	12	12	12	5	12
Sheep	3	3	3	0	2	3
Dog	2	2	2	2	0	2
Elephant	6	4	4	2	2	4
Total (%)	32	27 (84.38)	27	22	10	27
C. Birds (apparently healthy)						
Macaw	1	1	1	0	0	0
Cassowary	2	1	1	0	0	0
Peacock	1	1	1	0	0	0
Chicken	10	0	0	0	0	0
Total (%)	14	3 (21.43)	3	0	0	0
C. Birds (clinically affected)						
Pigeon	3	1	1	1	1	1
Total (%)	3	1 (33.33)	1	1	1	1
Grand Total (%)	102	48 (47.06)	48	30 (62.50)	14 (29.16)	28 (58.33)

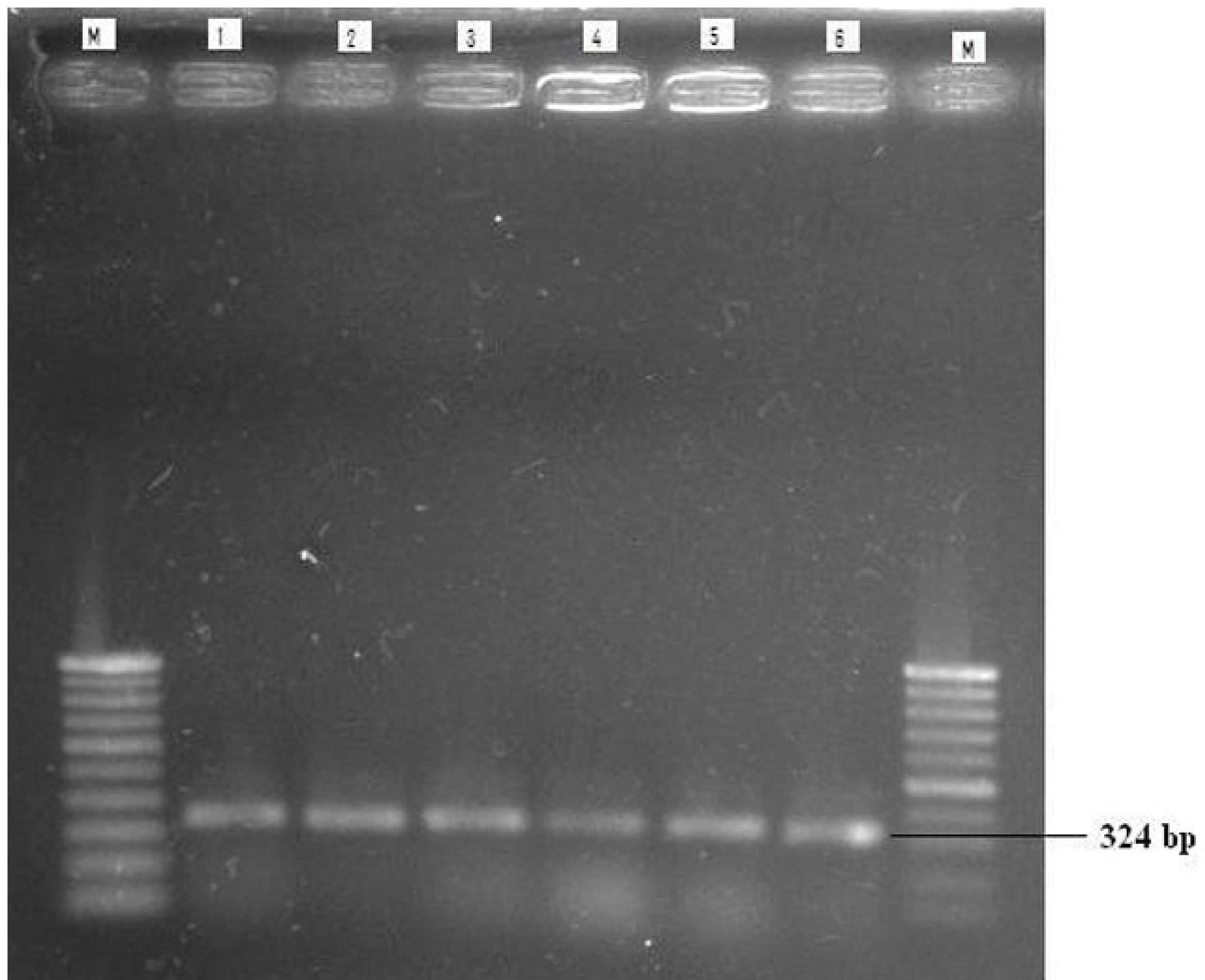

Figure 1. Detection of α toxin (*cpa*) gene in *C. perfringens* by PCR. Lane M: 100 bp molecular marker; lanes 1 to 5: *C. perfringens* field isolates; lane 6: known strain of *C. perfringes* bearing α toxin gene.

were recovered from clinically affected birds and other animals (Table 1). All the 48 *C. perfringens* isolates revealed presence of α toxin gene (Figure 1). All the *C. perfringens* samples presenting the α toxin gene isolated from clinically affected birds and mammals also exhibited phospholipase C activity.

DISCUSSION

Clostridium perfringens is ubiquitous in nature and usually forms a part of the normal intestinal flora of humans and animals. Spores of the organism persist in the soil, sediments and areas subjected to human or animal fecal pollution (7). In its role in the intestinal flora, *C. perfringens* becomes associated with various forms of disease under certain favorable conditions. Therefore, detection of virulence factors including identification of toxin genes in the isolates of *C. perfringens* plays a significant role in the differentiation of pathogenic from non-pathogenic organisms and in establishing its role in the disease condition.

PCR-based technology is considered to be a convenient and highly reliable tool for molecular detection of all the major toxin genes, such as α (*cpa*), β (*cpb1*), ε (*etx*) and ι (*iap*) (8). The α toxin is considered to be one of the major toxins produced by all different types of *C. perfringens*. Given the importance of this organism, it is imperative to characterize the isolates in terms of their virulence factors to establish their pathogenic potential and to detect genes encoding major toxins.

Isolation of *C. perfringens* from both apparently healthy as well as clinically affected birds and mammals confirmed the ubiquitous nature of the organism reported in another work (9). The higher sensitivity of the isolates to ciprofloxacin, enrofloxacin and norfloxacin may be due to infrequent use of these drugs in birds and mammals under study for therapeutic and prophylactic purposes.

Characterization of virulence properties indicated production of DNase by the majority of the isolates, most of which were recovered from clinically affected birds and mammals. This indicates that DNase presence is an important virulence factor in disease production. This observation agrees with previous findings, which reported DNase production in all strains of *C. perfringens* isolated from cases of atypical blackleg in cattle (10).

The high presence of hemolysin in the isolates was also reported in all the strains of *C. perfringens* type C isolated from cases of enterotoxemia in domestic animals (11). All isolates of *C. perfringens* positive for phospholipase C production presented the α toxin gene, which is indicative of *C. perfringens* type A involvement in the disease of clinically affected animals (12).

From the data of the present study, the distribution of *C. perfringens* toxin types could not be ascertained in relation to animals or disease conditions. Additional studies must be carried out with specific primers to detect other major toxins in the isolates to determine the real prevalence of *C. perfringens* types in birds and mammals.

However, the predominance of α toxin in the strains isolated from clinically affected animals suggests the importance of this agent in producing disease in birds and mammals.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS COMMITTEE APPROVAL

The present study was approved by the institutional review board of the Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, India.

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REFERENCES

1. McGinley S. *Clostridium perfringens*: New ways to type strains of a deadly bacteria. Arizona: Arizona Experiment Station Research Report; 1998. Available from: http://ag.arizona.edu/pubs/general/resrpt1998/clostridium.html.
2. Cruickshank R, Duguid JP, Marmion BP, Swain RHA, editors. Medical microbiology. 12th ed. Edinburgh, London: Churchill Livingstone; 1975. 522-35 p. II vol.
3. Wust J. Susceptibility of anaerobic bacteria to metronidazole, ornidazole and tinidazole, and routine susceptibility testing by standardized methods Antimicrob Agents Chemother. 1977;11(4):631-7.
4. Porschen RK, Sonntag S. Extracellular deoxyribonuclease production by anaerobic bacteria. Appl Microbiol. 1974;27(6):1031-3.
5. Baldassi L, Barbosa ML, Bach EE, Iaria ST. Toxigenicity characterization of *Clostridium perfringens* from bovine isolates. J Venom Anim Toxins incl Trop Dis. 2002;8(2):112-26.
6. Titball RW, Hunter SE, Martin KL, Morris BC, Shuttleworth AD, Rubidge T, et al. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect Immun. 1989;57(2):367-76.
7. Taylor AW, Gordon WS. A survey of the types of *C. welchii* present in soil and the intestinal contents of animals and man. J Pathol Bacteriol. 1940;50(1):271-7.
8. Yoo HS, Lee SU, Park KY, Park YH. Molecular typing and epidemiological survey of prevalence of *Clostridium perfringens* types by multiplex PCR. J Clin Microbiol. 1997;35(1):228-32.
9. Harbola PC, Arora BM. Enterotoxaemia due to *Clostridium perfringens* type D in sloth bear. Indian J Comp Microbiol Immunol Infec Dis. 1994;15(1-2): 42.
10. Shome BR, Songer JG, Shakuntala I, Shome R, Kumar A, Chakraborty S, et al. Atypical blackleg caused by *Clostridium perfringens* type A in cattle in Manipur, India. Indian J Anim Sci. 2006;76(5):353-7.
11. Niilo L. Toxigenic characteristics of *Clostridium perfringens* type C in enterotoxemia of domestic animals. Can J Vet Res. 1987;51(1):224-8.
12. Kumar A, Shakuntala L, Devi B. Prevalence of β_2 toxigenic *Clostridium perfringens* in poultry. Indian Vet J, 2011;88(1):52-3.