



## GENOTYPING OF *CLOSTRIDIUM PERFRINGENS* FROM FRESH WATER FISH AND FISH PICKLES

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### ABSTRACT

This study aims to evaluate the genotypes of *Clostridium perfringens* in fish and fish based products from Tamil Nadu and Kerala states of India. A total of 301 samples consisting intestinal contents of freshwater fish (234) from various dams, freshwater lakes, ponds, retail shops and markets and fish pickles (67) obtained from randomly selected retail shops and supermarkets were investigated. Bacterial isolations, identifications and phenotypic characterization of virulence factors were carried out as per standard microbiological procedures. Genotyping of the *C. perfringens* isolates were done by amplifying four major lethal toxin genes namely- alpha toxin gene (*cpa*), beta toxin gene (*cpb*), epsilon toxin gene (*etx*), iota toxin gene (*iA*) in a Thermal Cycler. Isolates were also screened for the presence of enterotoxin gene (*cpe*) and beta2 toxin gene (*cpb2*) by single step PCR. Biochemical tests and phenotypic determination of virulence factors tentatively identified 82 (27.24%) isolates of *C. perfringens*. In PCR assay, all 82 (100%) isolates harbored *cpa* toxin genes of *C. perfringens*, however, 65 (79.26%) isolates also carried additional *cpb2* toxin genes. None of the isolates were found positive for beta, epsilon, iota and enterotoxin genes. Genotyping of the 82 isolates by PCR revealed that all the isolated bacteria were belonged to *C. perfringens* type A and both *cpa* and *cpb2* toxin genes were prevalent among the isolates of *C. perfringens* type A, impending the risk of pathogenicity to human via freshwater fish and fish pickles.

**Keywords:** *Clostridium perfringens*, alpha toxin gene, beta2 toxin gene, fish, fish pickle

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## INTRODUCTION

Fish production in India has increased multi fold since India's independence in 1947. According to the report of UNFAO (2011), fish output in India doubled between 1990 and 2010. As food items, fish and fish based products contribute to country's economy and people's well being by meeting the dietary protein needs of the common man. This is due to the easy availability of fish in the river based states and plains of India. Due to their high perishable characteristic, fish and fish based products easily get spoiled and contaminated by the food borne pathogens particularly bacteria if not properly stored. When such contaminated foods are consumed either raw or inadequately cooked by human population, there is a detrimental impact on public health.

According to the annual report of DAHDF (2009), Kerala and Tamil Nadu states of India are ranked the 4<sup>th</sup> and the 5<sup>th</sup> respectively among the leading fish producing states in India. Presently, there is an increasing demand of locally processed fish and canned fish products like fish pickles in these two states of Southern India. Due to existing poor storage facilities in retail shops or even in local market, the risk of food borne infection is ever present. Among the food borne pathogens that thrives in high protein and canned food items, *Clostridium perfringens* always comes foremost.

*C. perfringens* is ubiquitous, anaerobic, gram positive rod shaped bacteria which causes numerous enteric diseases caused by food infections to human, wildlife and domestic animals (Songer, 1996). *C. perfringens* thrives in high-protein foods of animal origin such as meat and meat products, meat dishes, stews, soups, gravies, and milk. On the basis of production of four major toxins (alpha, beta, iota and epsilon), isolates of *C. perfringens* are classified into five genotypes A to E (Songer and Meer, 1996). Along with four major lethal toxins, enterotoxin and beta2 toxin produced by types of *C. perfringens* also considered as important toxins for enteric diseases in domestic animals (Smedley III et al., 2004; Sting, 2009). However, it is not so easy to differentiate between different strains of *C. perfringens* from clinical cases. Therefore, PCR has been used to detect the presence of toxin genes and to identify the specific strains of *C. perfringens* (Yamagishi et al., 1997).

In last five to six years, the detection of *C. perfringens* type A from black leg in cattle (Shome et al., 2006a), gangrenous dermatitis in broiler birds (Shome et al., 2006b), necrotic

enteritis in broiler chicken (Das et al., 2008a), acute diarrhea in pigs and piglets (Das et al., 2009a), haemorrhagic enteritis in elephant and pygmy hog (Das et al., 2008b; Shome et al., 2010), chronic enteritis in goats (Das et al., 2009b,c), diarrhea in cattle (Das et al., 2012) have been reported from India. But none of the Clostridial cases from India have been reported from fish and fish based products.

In literature review, it has been noticed that the research on the involvement of *C. perfringens* in freshwater fish is limited (Wen and McClane, 2004; Cai et al., 2008). Equally limited is the research output on the prevalence of Clostridial infection in commercial fish based product particularly fish pickle. In light of the limited research available in this field, it becomes necessary to determine the types of *C. perfringens* prevalent in fish and fish based products sourced from Kerala and Tamil Nadu states of India.

Therefore, the present study is designed to identify the prevalence of *C. perfringens* types in freshwater fishes and fish pickle packed for human consumption.

## **MATERIAL AND METHODS**

### **Sample collection**

A total of 234 fresh water fish consisting live captured fish (167) consisting common carps (55), silver carps (56) and cat fishes (56) and stored fish (67) collected from various dams, freshwater lakes, ponds, retail shops and markets and fish pickles (67) obtained from randomly selected retail shops and supermarkets of Tamil Nadu and Kerala were acquired for the study. All the freshwater fish were collected in a regular consumer packages and immediately transported to the laboratory in an ice chest. Intestinal content samples (234) from the freshwater fish were then removed scientifically and processed for the microbiological analysis. Fish pickle were collected in sterilized sample collecting vials and transported to the laboratory for microbiological analysis.

### **Isolation and biochemical identification of *C. perfringens***

Samples collected from the intestinal contents from freshwater fish and fish pickle samples were inoculated aseptically in Robertson's cooked meat (RCM) medium (HiMedia, Mumbai) with neutral oil overlay and incubated at 37°C for 48 hr. The inoculums from the

RCM media was seeded onto 10% sheep blood agar and incubated anaerobically for 24 hr at 37°C. Bacterial colonies were purified individually based on the size, shape, color, hemolysis pattern and were subjected to Gram's and malachite spore staining followed by array of biochemical tests such as gelatinase, fermentation of glucose, lactose and skim milk and were identified as per **Holt et al. (1994)**.

### **Evaluation of phenotypic virulence factors**

All the isolates of *C. perfringens* were evaluated for the deoxyribonuclease (DNase) enzyme, lecithinase enzyme and hemolysin production as per the protocol described in Bergey's manual of determinative bacteriology (**Holt et al., 1994**) with little modification.

#### **DNase enzyme**

The extracellular DNase enzyme was detected on DNase test agar with toluidine blue medium (HiMedia, Mumbai) under aseptic condition. The cultures were spot inoculated into the media plates and incubated anaerobically at 37°C for 24 hr. The clear white zones surrounding the bacterial colonies on a blue colored DNase test medium after 24 hr of incubation was considered as DNase test positive.

#### **Lecithinase enzyme**

The lecithinase activity of *C. perfringens* was carried out on egg yolk agar base medium (HiMedia, Mumbai) supplemented with 10 % egg yolk emulsion (HiMedia, Mumbai) under aseptic condition. The cultures were spot inoculated into the media plates. The yellowish opalescence (Nagler's reaction) around the bacterial colonies against the colorless background of egg yolk agar after 24 hr of incubation at 37°C in anaerobic condition was considered as lecithinase test positive.

#### **Hemolysin production**

The *In vitro* hemolysin production or hemolytic activity of *C. perfringens* isolates were carried out on solid media plates containing nutrient agar (HiMedia, Mumbai) enriched with 10 % defibrinated blood of sheep. The cultures were streaked into the media plates.

Depending on zone of clearance surrounding the bacterial colonies after 24 hr of incubation at 37°C in anaerobic condition, result was recorded as either  $\alpha$  or  $\beta$  or both the hemolysis.

### **Genotyping by polymerase chain reaction**

The isolates of *C. perfringens* were tested to detect the alpha toxin gene (*cpa*), beta toxin gene (*cpb*), epsilon toxin gene (*etx*), iota toxin gene (*iA*), enterotoxin gene (*cpe*) and beta2 toxin gene (*cpb2*) by PCR assay (Das et al., 2008a).

### **Preparation of template DNA**

A single colony of *C. perfringens* was pilled from blood agar plate taken in 100 $\mu$ l of Milli-Q water, gently vortexed and boiled at 100°C for 10 min in water bath. The cell debris was removed by centrifugation at 10,000 rpm for 5 min at 4°C and the top clear supernatant was used as source of template DNA.

### **PCR assay**

The PCR amplification was carried out in iCycler (BioRad, USA) in 25 $\mu$ l reaction volume 12.5 $\mu$ l of 2 $\times$  PCR master mix [4 mM MgCl<sub>2</sub>; 0.4 mM of each dNTPs (dATP, dCTP, dGTP, dTTP); 0.5 units /  $\mu$ l of *Taq* DNA polymerase; 150 mM Tris-HCl PCR buffer (pH 8.5)]; desired  $\mu$ M of each forward and reverse primers and 2.5 $\mu$ l of template DNA. The details of primer pairs for *cpa*, *cpb*, *etx*, *iA*, *cpe* and *cpb2* genes were commercially synthesized (Eurofins, Bangalore) and were shown in Table 1.

**Table 1** Oligonucleotides and PCR conditions for detection of *cpa*, *cpb*, *etx*, *iA*, *cpe* and *cpb2* genes of *Clostridium perfringens*

Toxin genes	Oligonucleotide sequences 5'→3'	Concentration of Oligonucleotides (μM) each	Amplicon Size (bp)	Reference
<i>cpa</i>	For-gctaagtactgcccgttga Rev-cctctgatacatcgtgtaag	0.50	324	
<i>cpb</i>	For-gcgaatatgctgaatcatcta Rev-gcaggaacattagtatacttc	0.50	180	
<i>etx</i>	For-gcggatgatccatctattc Rev-ccacttactgtcctactaac	0.50	655	<b>Shome et al., (2006a)</b>
<i>iA</i>	For-actactctcagacaagacag Rev-ctttcctctattactatacg	0.34	446	
<i>cpe</i>	For-ggagatgggttgatattagg Rev-ggaccagcagttgtagata	0.36	233	
<i>cpb2</i>	For-agattttaaataatgacctaacc Rev-caatacccttcaccaaatactc	0.36	567	

After initial denaturation at 94°C for 4 min, the amplification cycle had denaturation, annealing and extension at 94°C, 55°C and 72°C for 1 min each respectively. Final extension was done at 72°C for 10 min. *C. perfringens* (MTCC 3296) and *Escherichia coli* (MTCC 723) were used as positive and negative controls respectively.

### Agarose Gel electrophoresis

The PCR amplicons (5μl) were separated by electrophoresis in 1.5% Agarose (Promega, USA) gel with TAE 1X (Tris-Acetate-EDTA; pH 8.0) running buffer at 60 V. The gel was stained with 0.4 μg/ml ethidium bromide (Himedia; Mumbai), visualized and photographed in gel documentation system (Universal Hood, BIORAD, Italy).

## RESULTS AND DISCUSSION

### Isolation and identification of *C. perfringens*

Anaerobic culture process yielded pure colonies of alpha and beta haemolysis on sheep blood agar. The bacterial colonies isolated from the intestinal contents of fresh water fish were observed to be centrally dense and round colonies with rhizoid periphery. However, colonies that originated from pickle were having irregular border with glandular texture. Gram staining showed gram-positive rods, while malachite green staining showed subterminal oval endospores. The isolates produced stormy fermentation, acidity, reduction and coagulation in litmus milk, liquefied gelatin and fermented glucose and lactose (**Das et al., 2008a**). Upon the detailed microbiological investigation, a total of 82 (27.24%) isolates of *Clostridium perfringens* were tentatively identified (Table 2). Out of 234 intestinal contents collected from the same number of fresh water fish, *C. perfringens* were isolated from 43 (18.36%) samples, was almost similar to the previous report from China (**Cai et al., 2008**), but lesser than the report from America (**Wen and McClane, 2004**). The rate of isolation of *C. perfringens* from 67 samples of fish pickles was estimated to be 39 (58.20%), was higher in number than the fresh water fish (Table 2).

**Table 2** Sample details and distribution of virulence genes among the isolates of *Clostridium perfringens* from Tamil Nadu and Kerala

Sample details / No of samples tested	No (%) of <i>C. perfringens</i> isolated from tested samples	No (%) of <i>C. perfringens</i> positive for toxin genes	
		<i>cpa</i>	<i>cpb2</i>
<b>1. Intestinal contents of Fresh water fish (n=234)</b>	43 (18.36)	43 (100)	29 (67.44)
<b>a. Fresh water live fish (n=167)</b>	16 (9.58)	16 (100)	8 (50)
Common carps / 55	3 (5.45)	3	0
Silver carps / 56	5 (8.92)	5	1 (20)
Cat fishes / 56	8 (14.28)	8	7 (87.50)
<b>b. Stored fish (n=67)</b>	27 (40.29)	27 (100)	21 (77.77)
<b>2. Fish pickles / 67</b>	39 (58.20)	39 (100)	36 (92.30)
Total (301)	82 (27.24)	82 (100)	65 (79.26)

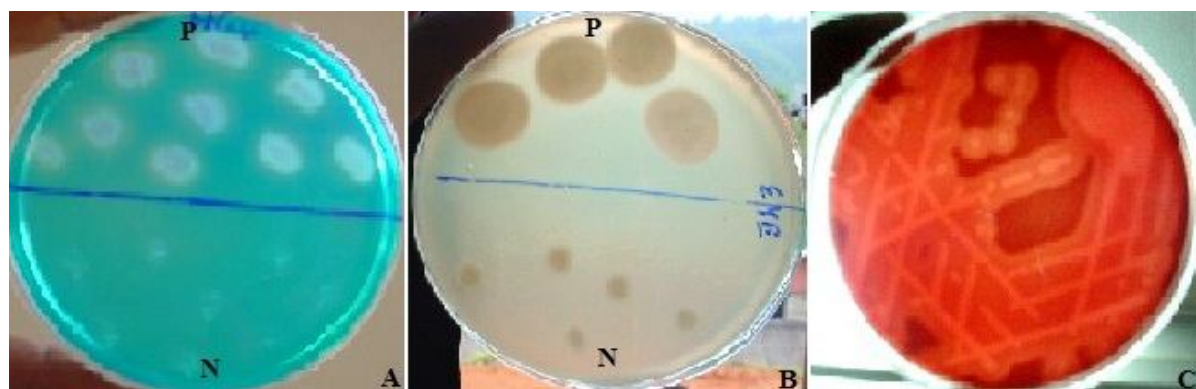
Legend: n = total no of samples tested

### Evaluation of phenotypic virulence factor

The DNase, lecithinase and hemolysis tests are of low cost and could be useful to evaluate quantitatively the phenotypes of *C. perfringens* and for the selection of virulent strains from the samples collected from the fish and fish based products. All the spot inoculated isolates of *C. perfringens* were found to produce DNase and lecithinase activity (Holt et al., 1994; Das et al., 2009a). It has also been observed that the zone of clearance on DNase test agar and zone of yellowish opalescence on egg yolk agar emulsion increased almost double of its size after 48 hr of anaerobic incubation at 37°C (Figure 1). *C. perfringens* (MTCC 3296) and *C. chauvoei* used as positive and negative control respectively.



On 10 % sheep blood agar, majority of *C. perfringens* type A isolates produced double zone of hemolysis (Figure 1), was reported earlier by several researchers (Holt et al., 1994; Shome et al., 2006a). All the colonies were found to be surrounded by a smaller zone of beta hemolysis (complete lysis of blood), which were surrounded by a larger zone of alpha hemolysis (partial lysis of blood). It has also been observed that the hemolysis production become sharpened and prominent after 3-4 days of incubation at 4-8°C.



**Figure 1** Extra-cellular Dnase, Lecithinase and Hemolysin production test of *C. perfringens* type A isolates

A: *C. perfringens* type A isolates positive for Extra-cellular Dnase

B: *C. perfringens* type A isolates positive for Lecithinase

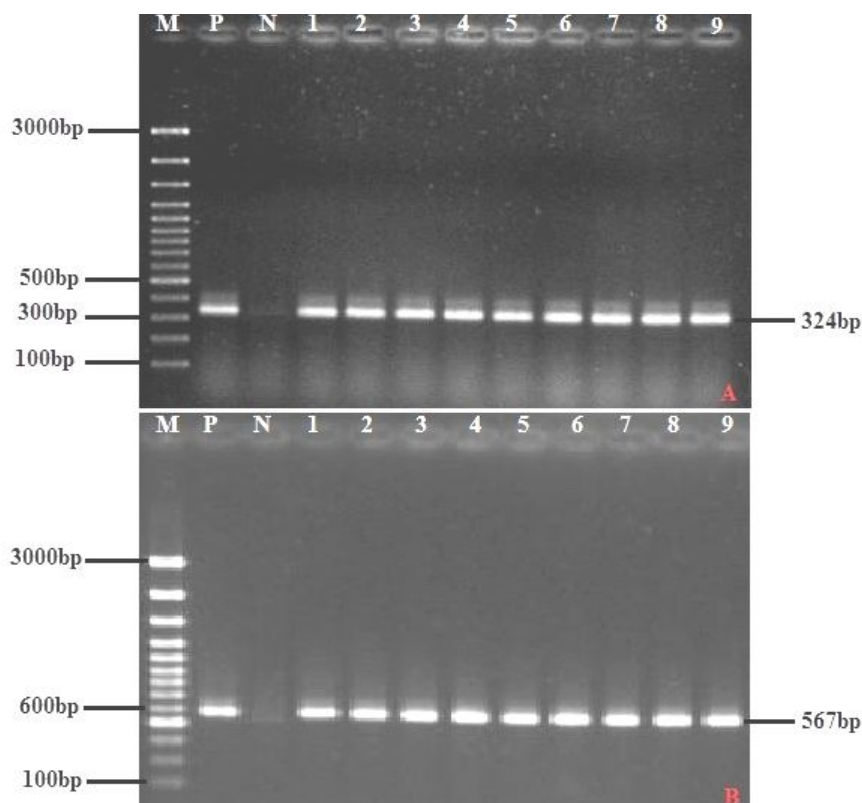
C: *C. perfringens* type A isolates positive for Hemolysin

P: *C. perfringens* (MTCC 3296) as Positive control

N: *C. chauvoei* as Negative control

### Genotyping by PCR

In this study, PCR was employed for genotyping the 82 *C. perfringens* isolates obtained from fresh water fishes and fish pickles. In PCR, out of six virulence genes screened, alpha toxin gene (*cpa*) of 324bp fragment were detected in all the 82 (100%) isolates. However, additional beta2 toxin genes (*cpb2*) of 567bp fragment were detected in 65 (79.26%) isolates originated from fish pickles (36), stored fishes (21), cat fishes (7) and silver carps (1) (Table 2; Figure 2). None of the *C. perfringens* isolates were positive for any of the *cpb*, *etx*, *iA* and *cpe* toxin genes. PCR assay thus revealed that all the field isolates originated from freshwater fish and fish pickles were *C. perfringens* type A.



**Figure 2** Detection of alpha (*cpa*) and beta2 toxin (*cpb2*) gene of *C. perfringens* by PCR

A: *C. perfringens* type A isolates positive for 324bp fragment of *cpa* gene

B: *C. perfringens* type A isolates positive for 567bp fragment of *cpb2* gene

P: *C. perfringens* (MTCC 3296) as Positive control

N: *E. coli* (MTCC 723) as Negative control

Lanes 1-9: Representatives of *C. perfringens* type A field isolates

M: 100bp DNA ladder plus (MBI Fermentas, USA)

In a similar study, **Wen and Mcclane, (2004)** reported that out of 34 isolates of *C. perfringens* from fish samples 31 were positive for type A (only alpha toxin producer) and 3 isolates were type A with additional enterotoxin gene, but none of the type A isolates were positive for beta 2 toxin gene. However, in another study conducted by **Cai et al. (2008)**, out of 75 isolates of *C. perfringens* from fresh water fish, 13 (17.3%) isolates were found positive for toxin type A, 58 (77.3%) isolates for type C and 4 (5.3%) isolates were type B, and 47 (62.7%) isolates with additional beta 2 toxin gene (seven from type A, two from type B, and 38 from type C). But none of the isolates were found positive for enterotoxin gene. In congruence to present result, the *cpe*- negative *C. perfringens* type A from the clinical cases

of gastroenteritis was reported previously (Das et al., 2009b; Das et al., 2012). In this study, it has also been noted that alpha toxin is the most prevalent toxin among the *C. perfringens* type A isolates (Songer and Meer, 1996), irrespective of sources of isolation and geographical location. From the results and discussions, it could be suggested that the PCR assay is a suitable diagnostics for the detection of toxin genes and genotyping of *C. perfringens* from the fish and fish products. Both alpha toxin and beta 2 toxins of *C. perfringens* type A are lethal and thus the detection of these two toxins in fresh water fish and fish pickles imply high risk of occurrences of clostridial diseases in human.

## CONCLUSION

The Robertson's cooked meat media broth and the nutrient agar media enriched with 10% sheep blood are suitable for the isolation of *C. perfringens*. The evaluation of biochemical tests and phenotypic virulence factors are useful for tentative identification of *C. perfringens*. The genotyping by PCR suggested that unlike alpha toxin, the beta2 toxin is also linked to *C. perfringens* type A in freshwater fish and fish pickles. Since, *C. perfringens* is spore forming and both alpha and beta2 toxins can cause diseases in animal and human by entering via food chain, therefore, consumption of raw fish pickles and uncooked fishes for human might cause serious clostridial infection.

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