

## Isolation, Genotyping and Antibiogram Profile of *Clostridium perfringens* Isolates Recovered from Freshwater Fish and Fish Products from Kolkata Region

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The present study reports the isolation of *Clostridium perfringens* from samples comprising of freshwater fish and fish products from Kolkata city of India, and determining their genotypes and antibiogram profile. A total of 102 samples consisting of intestinal and gill samples from fresh water fish (n=69) and fish products including fish pickles, fish curry and fried fish (n=33) were collected randomly from retail shops and restaurants. On cultural isolation and biochemical characterization, 24 (23.52%) samples [17 (24.63%) from fresh water fish (n=69) and 07 (21.21%) from fish products (n=33)] were presumptively identified as *C. perfringens* positive. The genotyping of the recovered *C. perfringens* isolates was done by amplifying species specific 16S rRNA gene and four major lethal toxin genes viz., alpha toxin gene (*cpa*), beta toxin gene (*cpb*), epsilon toxin gene (*etx*) and iota toxin gene (*itx*). Apart from these, enterotoxin gene (*cpe*) and beta2 toxin gene (*cpb2*) were also targeted. In PCR assays, all the 24 (100%) isolates [17 (70.83%) from fresh water fish and 07 (29.17%) from fish products] were found to harbor species specific 16S rRNA and *cpa* toxin gene, however, 17 (70.83%) *cpa* positive isolates [12 (70.58%) from fresh water fish and 05 (71.42%) from fish products] were also found to harbor additional *cpb2* toxin gene, while none of the isolates were found to be positive for *cpb*, *etx*, *itx* and *cpe* genes. Based on these results, all the isolates were confirmed as *C. perfringens* type A (containing only alpha toxin gene). On antibiotic sensitivity testing, 76.47% of the isolates were found to be multidrug resistant with ciprofloxacin and amoxicillin/clavulanic acid as being the most sensitive drugs. Report regarding isolation and molecular characterization of *C. perfringens* from fish and fish products especially from Kolkata does not exist as well as from other regions of India are very scanty. The present findings suggest that fish may be considered as a potential source of *C. perfringens* type A infection to human populations through food chain and the high antibiotic resistance observed may pose serious public health concerns. Further detailed molecular epidemiological and antibiogram studies are suggested for designing and adapting appropriate prevention and control strategies for countering this important pathogen and its food-borne zoonotic concerns.

**Keywords:** *C. perfringens*, isolation, genotyping, 16S rRNA, lethal toxin genes, PCR, antibiotic sensitivity, fish, fish products

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India is the second largest producer of fresh water fish in the world. Fisheries sector contributed about 0.9% to the National Gross Domestic Product (GDP) and 5.17% to the agricultural GDP (2014-15). West Bengal ranked second in the total production of fish after Andhra Pradesh; it has total fish production as increases from 1,447.26 x 10<sup>3</sup> tonnes (2007-08) to 1678.33 x 10<sup>3</sup> tonnes (2014-15)<sup>1</sup>. This aberrant growth in fish industry require careful monitoring since there is risk of bacterial contamination in fish, especially in the post harvesting storage period, a critical stage prior to human consumption. Whenever bacterially contaminated raw or partially cooked food is consumed by susceptible population, then there is a chance of detrimental impact on public health<sup>2,3</sup>.

*Clostridium perfringens* is one of the most important food-borne pathogen of humans and animals causing both histotoxic diseases and intestinal infections<sup>4-7</sup>. It is Gram-positive, anaerobic, straight rod, spore former, found in the soil, dust, sewage, marine sediments and in the gastro-intestinal tract of humans and animals<sup>8-10</sup>. Based on the production of four major lethal toxins i.e. alpha ( $\alpha$ ) beta ( $\beta$ ), epsilon ( $\epsilon$ ) and iota ( $i$ ), *C. perfringens* is divided into five major toxinotypes/biotypes (A-E)<sup>6,10,11</sup>. Apart from these, it also produces enterotoxin (CPE) and beta 2 toxin (CPB2)<sup>12</sup>. Symptoms of *C. perfringens* type A food poisoning include abdominal cramping, nausea and diarrhea, which usually begin 6–24 h after ingestion of contaminated food and then persist for 12–24 h<sup>7,9</sup>. *C. perfringens* associated symptoms are caused by an enterotoxin which is produced during sporulation of the organism in the small intestine following ingestion of large number of vegetative cells of enterotoxin positive *C. perfringens*<sup>9,13</sup>.

Isolates originating from humans with gastrointestinal diseases carry most commonly CPE and sometimes newly discovered CPB2 toxin<sup>12,14</sup>. Enterotoxigenic *C. perfringens* type A is also associated with antibiotic associated diarrhoea (AAD) and sporadic diarrhoea (SD) cases<sup>15,16</sup>. The emerging problem of antimicrobial resistance between pathogenic and commensal bacteria is becoming more intense by intervention of environmental resistance phenomenon of bugs<sup>17,18</sup>. Antibiotics represent one of the most successful forms of therapeutic regimens in medicine. But the

overuse and misuse of antibiotics, mostly in developing countries, lead to growing number of antibiotic-resistant pathogens<sup>19</sup>. Antibiotic resistance, which is associated with increased morbidity and mortality rates as well as increased treatment costs, is considered to be one of the major global public health problems and its magnitude recently prompted a number of national and international bodies to take actions to protect the public health<sup>20</sup>. Problem of antimicrobial resistance in pathogenic and commensal bacteria is increasing by the adoption of mobile genetic elements<sup>21</sup>. Globally, there are reports of earlier studies on epidemiological characterisation of *C. perfringens* from fish samples<sup>7,22-31</sup>, but in Indian scenario there are limited studies carried out earlier on given aspects<sup>2,13,32</sup> and there are no previous reports from the study area, therefore, it is necessary to carry out epidemiological studies in such prone areas to determine the types of *C. perfringens* prevalent in fish and fish products. Considering these facts, the present study was designed to isolate *Clostridium perfringens* from freshwater fish and fish products from Kolkata region of India, characterize these at molecular levels by determining their genotypes and study antibiotic sensitivity patterns.

## MATERIALS AND METHODS

### Collection of samples

One hundred and two samples consisting of intestinal and gill samples from fresh water fish (n=69) and fish products including fish pickles, fish curry and fried fish (n=33) were collected from retail shops and restaurants from Kolkata city of India. All the collected samples were immediately transported to the laboratory in ice pack container and processed within 24 hours for isolation and molecular characterisation of *C. perfringens* using standard bacteriological and molecular procedures. The intestinal and gill samples from the fresh water fish were hygienically collected and processed for the bacteriological analysis. The fish products were aseptically collected in sterile container for the analysis.

### Isolation of *C. perfringens*

All the samples (Intestine, gills and fish products) were inoculated aseptically in Robertson's cooked meat (RCM) medium

supplemented with glucose, hemin and vitamin K broth (HiMedia, Mumbai, India) and heated at 75°C for 20 minute for removal of vegetative cells of other competing microorganisms, followed by incubation at 37°C for 24-48 hours under anaerobic condition in McIntosh anaerobic jar using gas pack (HiMedia, Mumbai, India). Enriched 100 µl inoculums were taken in sterile Petridish, followed by pouring of sterile molten sulfite polymyxin sulfadiazine (SPS) agar over the inoculum with proper mixing by rotating the plate in clockwise and anticlockwise direction. Plates were incubated at 37°C for 24 h under anaerobic condition<sup>33</sup>.

#### **Biochemical and phenotypical analysis of *C. perfringens***

Samples revealing characteristic black coloured colonies on SPS agar plates were subjected to biochemical tests for identification of *C. perfringens*<sup>34</sup>. About 3-5 colonies from the culture plate for each sample were considered for Gram's staining, lactose fermentation, nitrate reduction, gelatin liquefaction, lecithinase and indole tests.

#### **Reference strains**

Reference strains of *C. perfringens* were procured from Biological Standardization Division, ICAR-IVRI, Izatnagar, India.

#### **Molecular characterization / genotyping of *C. perfringens* by PCR**

All the presumptively identified *C. perfringens* isolates recovered from different sources were subjected to polymerase chain reaction (PCR) assays for the detection of species specific 16S rRNA<sup>35</sup> and virulent toxin genes viz., *cpa*, *cpb*, *etx*, *utx*<sup>36</sup>, *cpb2* and *cpe* genes<sup>37</sup>. The oligonucleotides used in present study were synthesized through M/S Xcelris Labs Limited, Ahmadabad, Gujarat (India). The details of primers used in present study are given in Table 1<sup>38-40</sup>.

The snap chill method<sup>41</sup> was used to extract DNA from the isolates. About 200 µl of overnight culture of *C. perfringens* was taken in the microcentrifuge tube and the cell suspension was centrifuged for 10 min at 14,000 × g. The pellet was suspended in 100 µl of nuclease free water (NFW) by vortexing. The microcentrifuge tube was placed in hot water bath for 15 min at 100°C and immediately chilled in ice. An aliquot of 5 µl of the supernatant was used as the template DNA in the PCR assays.

The optimized PCR protocol for all the target genes with 25 µl reaction mixture volume included 1 µl (10 pmol/µl) each of forward and reverse primer sequences, 0.5 µl of 10 mM deoxyribonucleotide triphosphates (dNTPs), 2.5 µl of 10 × PCR buffer [(100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40)], 2.0 µl MgCl<sub>2</sub> (25 mM), 0.20 µl of 5 U/µl of Taq DNA polymerase (Thermo scientific, USA), 5.0 µl DNA template and remaining NFW to make the final volume. The PCR reaction mixture was set for amplification in a thermocycler (Biometra Personal Cycler, Goettingen, Germany) with specific condition for each PCR assay. For 16S rRNA PCR assay, the initial denaturation condition was 94°C for 5 min. then amplified for 35 cycles (1.0 min at 94°C, 1.3 min. at 53°C, 1.3 min. at 72°C for denaturation, annealing and extension phases, respectively), followed by an additional period of extension for 10 min. at 72°C. Regarding multiplex PCR for *cpa*, *cpb*, *etx* and *utx* genes, there was initial denaturation at 95°C for 5 min. then amplified for 30 cycles (1.0 min. at 94°C, 1.0 min. at 55°C, 1.5 min. at 72°C for denaturation, annealing and extension phases, respectively), followed by an additional period of extension for 10 min. at 72°C. Similarly for *cpb2* and *cpe* genes, there was initial denaturation at 94°C for 4 min. then amplified for 35 cycles (1.0 min. at 94°C, 1.2 min. at 55°C, 1.2 min. at 72°C for denaturation, annealing and extension phases, respectively), followed by an additional period of extension for 10 min. at 72°C.

Amplified PCR products (10 µl) were separated by electrophoresis for 45 to 60 min. at 80 V in a 1.2% (w/v) agarose gel with ethidium bromide (0.5 µg/ml) and a 100 bp DNA ladder (Thermo scientific, USA) was included in each agarose run. The resolution of amplified fragments in the gel was visualized by a UV trans-illuminator and digitally recorded by gel documentation system (UVP Gel Sequencing Software). Materials contaminated with ethidium bromide were disposed as per the local guidelines.

#### **Antimicrobial sensitivity test**

Antimicrobial sensitivity test was performed by using disc diffusion method as per the guidelines provided by Clinical Laboratory Standards Institute (CLSI)<sup>42</sup>. The antimicrobials included in the present study were selected based on the information gathered from local

professionals (veterinarians, paediatrician) and available literature. The antimicrobials included were amikacin (AK, 30 µg), co-trimoxazole (COT, 25 µg), ceftazidime (CAZ, 30 µg), norfloxacin (NX, 10 µg), ceftriaxone (CTR, 30 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TE, 30 µg) and amoxicillin/clavulanic acid (AMC, 30µg) (HiMedia Laboratories Ltd, Mumbai, India).

## RESULTS

### Isolation and identification of *C. perfringens* by cultural and biochemical methods

On cultural and biochemical analyses, a total of 24 isolates [17 from fresh water fish (n=69) and 07 from fish products (n=33)] were found to be positive for *C. perfringens*.

### Genotyping by PCR detection of virulence toxin genes

All the biochemically positive isolates were screened by PCR assays for detection of species specific 16S rRNA and virulence toxin genes viz., *cpa*, *cpb2*, *cpe*, *cpb*, *etx* and *tx*. All the

24 (100%) presumptively confirmed isolates of *C. perfringens* [17 (70.83%) from fresh water fish and 07 (29.16%) from fish products] was also shown amplification of species specific 16S rRNA of product size 279 bp (Table 2, Fig 1) and  $\alpha$  toxin gene of product size 402 bp (Table 2, Fig 2). However, 17 (70.83%) *cpa* positive isolates [12 (70.58%) from fresh water fish and 05 (71.42%) from fish products] were also positive for additional beta2 toxin gene (*cpb2*) of 567 bp length (Table 2, Fig 3). The other virulence gene specific primers of *cpb*, *etx*, *tx* and *cpe* genes did not amplify any of the isolates. The virulence gene detection by PCR assay used in this study revealed that all the isolates were belonged to *C. perfringens* type A.

### Antibiotic sensitivity testing

A total of 17 *C. perfringens* isolates (both *cpa* and *cpb2* positive) were tested for antimicrobial sensitivity assays. Out of 17 isolates, 13 (76.47%) were found to be resistant to  $\geq$  03 tested groups of antibiotics, indicating multidrug resistance phenomenon. Isolates showed highest resistance against co-trimoxazole (88%), followed by

**Table 1.** Details of the primers for testing of samples for *Clostridium perfringens* by PCR

Target genes	Primer sequence 5' - 3'	Product size (bp)	References
16SrRNA	F-AAAGATGGCATCATCATTCAAC R-TACCGTCATTATCTCCCAAAA	279	Yoo <i>et al.</i> <sup>38</sup>
<i>cpa</i>	F-GTTGATAGCGCAGGACATGTTAAG R-CATGTAGTCATCTGTTCCAGCATC	402	Yoo <i>et al.</i> <sup>38</sup>
<i>cpb</i>	F-ACTATACAGACAGATCATTCAACC R-TTAGGAGCAGTTAGAACTACAGAC	236	Yoo <i>et al.</i> <sup>38</sup>
<i>etx</i>	F-ACTGCAACTACTACTCATACTGTG R-CTGGTGCCTTAATAGAAAGACTCC	541	Yoo <i>et al.</i> <sup>38</sup>
<i>tx</i>	F-GCGATGAAAAGCCTACACCACTAC R-GGTATATCCTCCACGCATATAGTC	317	Yoo <i>et al.</i> <sup>38</sup>
<i>cpb2</i>	F-AGATTTTAAATATGATCCTAACC R-CAATACCCTTCACCAAATACTC	567	Gibert <i>et al.</i> <sup>39</sup>
<i>cpe</i>	F-GGAGATGGTTGGATATTAGG R-GGACCAGCAGTTGTAGATA	233	Czeczulin <i>et al.</i> <sup>40</sup>

**Table 2.** Samples found positive for *C. perfringens* and their virulence genes by PCR

Sample source	Sample types	No. of <i>C. perfringens</i> positive samples (%)	No. of sample positive for virulence gene by PCR	
			<i>cpa</i>	<i>cpb2</i>
Fresh water fish (n=69)	Intestinal and gills	17 (24.63)	17	12
Fish products (n=33)	Whole part	07 (21.21)	07	05
			24 (100%)	17 (70.83%)

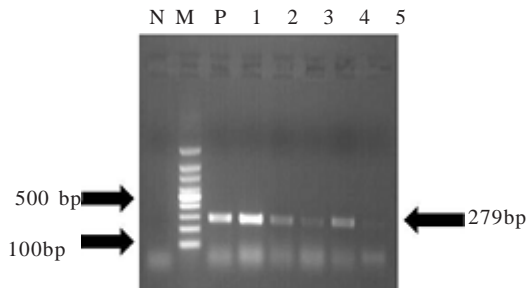
ceftriaxone (87%), cefazidime (53%), tetracycline (44%), norfloxacin (21%) and amikacin (11%), while

all the isolates were found to be sensitive to ciprofloxacin and amoxicillin/clavulanic acid.

## DISCUSSION

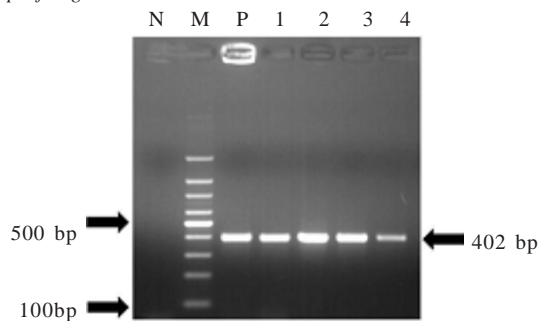
*C. perfringens* is considered as one of the most important food-borne pathogen and is ubiquitous in nature<sup>6-9</sup>, however, in Indian scenario there are limited studies occurred earlier on epidemiological and molecular aspects of *C. perfringens* in fresh water fish and fish products. In the present study, a total of 24 samples (23.52%) [17 (24.63%) from fresh water fish (n=69) and 07 (21.21%) from fish products (n=33)], were found to be positive for *C. perfringens*. It has been estimated that 17/24 isolates of *C. perfringens* (70.83%) were derived from the intestinal and gill samples of fresh water fish and 07/24 (29.17%) from fish products.

On PCR detection of species specific 16S rRNA and virulence toxin genes of the *C. perfringens* isolates it was found that all the isolates (24) were shown species specific amplicon of 16S rRNA and positive for  $\alpha$  toxin gene (*cpa*). Apart from these, 17 *cpa* positive isolate (70.83%) [12 (70.58%) from fresh water fish and 05 (71.42%) from fish products], were also positive for beta2 toxin gene (*cpb2*). Previous studies have reported occurrence of *C. perfringens* from fish and sea food samples with a varying isolation rates of 1% to 84% from different countries<sup>2,7,13,22-32</sup>. Cai *et al.*<sup>27</sup> reported that out of 75 isolates of *C. perfringens* from fresh water fish, 13 (17.3%) isolates were found to be positive for only  $\alpha$  toxin gene, 58 (77.3%) isolates for  $\alpha$  &  $\beta$  toxin genes positive (*C. perfringens* type C) and 04 (5.3%) isolates for  $\alpha$ ,  $\beta$  and  $\epsilon$  toxin genes positive (*C. perfringens* type B), while 47 (62.70%) isolates have additional beta 2 toxin gene, however, none of the isolates were found to be positive for *cpe* and other virulence toxin genes, since, the presence of *cpe* gene in *C. perfringens* is very uncommon, and only <5% of global *C. perfringens* type A isolates were found to be *cpe* gene positive<sup>7,9</sup>. In concurrence to present results, the *cpe*- negative *C. perfringens* type A from the fish sample and clinical cases of gastroenteritis was reported previously<sup>2,13,31,32,43</sup>. In another study, out of 34 isolates of *C. perfringens* from fish samples, 31 were found to be positive for *C. perfringens* type A (only *cpa* gene positive) and 3 isolates were *cpa* as well as *cpe* genes



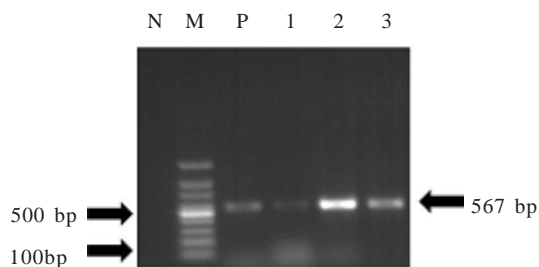
**Fig. 1.** PCR assays for detection of 16S rRNA of *C. perfringens*

Lane N: Negative control; Lane M: 100 bp DNA ladder; Lane P: Reference strain of *C. perfringens*; Lane 1-5: *C. perfringens* isolates



**Fig. 2.** PCR assays for detection of *cpa* gene of *C. perfringens*

Lane N: Negative control; Lane M: 100 bp DNA Ladder; Lane P: Reference strain of *C. perfringens*; Lane 1-4: *C. perfringens* isolates



**Fig. 3.** PCR assays for detection of *cpb2* gene of *C. perfringens*

Lane N: Negative control; Lane M: 100 bp DNA Ladder; Lane P: Reference strain of *C. perfringens*; Lane 1-3: *C. perfringens* isolates

positive, but none of the *C. perfringens* type A isolates were found to be positive for *cpb2* gene<sup>2</sup>. In contradiction, present study reported the occurrence of *cpb2* positive isolates in 70.83% of *C. perfringens* type A isolates. These findings highlights the high prevalence of *cpb2* associated *C. perfringens* type A which is mainly associated with antibiotic associated diarrhoea (AAD) and sporadic diarrhoea (SD) cases<sup>12</sup>.

The antibiogram studies performed against isolates recovered from freshwater fish and fish products unveils an alarming public health concern due to higher resistance in recovered isolates. Highest resistance was observed against co-trimoxazole (88%), followed by ceftriaxone (87%), ceftazidime (53%), tetracycline (44%), norfloxacin (21%) and amikacin (11%), while all the isolates were found to be sensitive to ciprofloxacin and amoxicillin/clavulanic acid. These finding are found to be concurrent with earlier studies reporting a high emerging resistance toward various tested antibiotics<sup>44-52</sup>. These observations are major point of concern from public health perspectives, since most of the antibiotics drugs used in the present study are employed as a first line of treatment in diarrhoeal cases.

### CONCLUSION

The present study suggested that PCR is a reliable molecular technique and useful tool for the detection of virulence genes typing of *C. perfringens* isolates recovered from fresh water fish and fish products. The presence of species specific 16S rRNA and *cpa* gene in all the isolates of *C. perfringens* suggest that *C. perfringens* type A (only have  $\alpha$  toxin gene) is the most predominant type in fresh water fish and fish products in this study area. Primer specific to species specific 16S rRNA and *cpa* gene in PCR assays, used in present study, could be useful for the rapid identification of *C. perfringens* and provided a clues to the importance and improvement of the present method for the surveillance of *C. perfringens* from fresh water fish and fish products. On antibiogram profile, except for ciprofloxacin and amoxicillin/clavulanic acid, a high frequency of multiple drug resistance pattern was observed among the *C. perfringens* type A isolates. These emerging resistance trends

are major point of concern from public health perspectives.

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