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

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http://dx.doi.org/10.22207/JPAM.10.4.40

(Received: 23 August 2016; accepted: 02 October 2016)

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 (*ctx*) 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 (71.43%) from fish products] were also found to harbor additional *cpb2* toxin gene, while none of the isolates were found to be positive for *cpb*, *ctx*, *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 10³ tonnes (2007-08) to 1678.33 x 10³ tonnes (2014-15). 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. Clostridium perfringens is one of the most important food-borne pathogen of humans and animals causing both histotoxic diseases and intestinal infections. 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. Based on the production of four major lethal toxins i.e. alpha (α) beta (β), epsilon (ε) and iota (i), C. perfringens is divided into five major toxinotypes/biotypes (A-E). Apart from these, it also produces enterotoxin (CPE) and beta 2 toxin (CPB2). 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. 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.

Isolates originating from humans with gastrointestinal diseases carry most commonly CPE and sometimes newly discovered CPB2 toxin. Enteroxigenic C. perfringens type A is also associated with antibiotic associated diarrhoea (AAD) and sporadic diarrhoea (SD) cases. The emerging problem of antimicrobial resistance between pathogenic and commensal bacteria is becoming more intense by intervention of environmental resistance phenomenon of bugs. 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. 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. Problem of antimicrobial resistance in pathogenic and commensal bacteria is increasing by the adoption of mobile genetic elements. Globally, there are reports of earlier studies on epidemiological characterisation of C. perfringens from fish samples, but in Indian scenario there are limited studies carried out earlier on given aspects 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 condition33.

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. perfringens34. 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
pecific 16S rRNA35 and virulent toxin genes viz.,
cpa, cph, atx, itx36, cpb2 and cpe genes37. 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 138-40.

The snap chill method41 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 x 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 MgCl₂ (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, cph, atx and itx 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-illator 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
performe by using disc diffusion method as per
the guidelines provided by Clinical Laboratory
Standards Institute (CLSI)42. 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, 30 µg) (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, εtx and ιtx. 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 α 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, εtx, ιtx 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 ≥ 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

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequence 5' - 3'</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16SrRNA</td>
<td>F-AAAGATGGCATCATATTCTACATCAAC</td>
<td>279</td>
<td>Yoo et al.38</td>
</tr>
<tr>
<td></td>
<td>R-TACCCTTGTATTCTTTCCTTTTTAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpa</td>
<td>F-GTTGATAGCGCAAGACATGTTAAG</td>
<td>402</td>
<td>Yoo et al.38</td>
</tr>
<tr>
<td></td>
<td>R-CATGGAATCTCATCTGTCAACATCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpb</td>
<td>F-ACTATACAGACAGATCATCATTACC</td>
<td>236</td>
<td>Yoo et al.38</td>
</tr>
<tr>
<td></td>
<td>R-TTAGGACAGTTAGAATACACAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>εtx</td>
<td>F-CTGCAACTACACTCATACTGTTG</td>
<td>541</td>
<td>Yoo et al.38</td>
</tr>
<tr>
<td></td>
<td>R-CGGTGTCCCTTAAAGAAGACTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ιtx</td>
<td>F-AGATTGAAAGCCTACACAGACTAC</td>
<td>317</td>
<td>Yoo et al.38</td>
</tr>
<tr>
<td></td>
<td>R-GGTATATCCTACGCAATATACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpb2</td>
<td>F-AGATTGAAAGCCTACACACACTAC</td>
<td>567</td>
<td>Gibert et al.39</td>
</tr>
<tr>
<td></td>
<td>R-GGTATATCCTACGCAATATACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cpe</td>
<td>F-GAGATTGTTCCATTGGATTTAGG</td>
<td>233</td>
<td>Czeczulin et al.40</td>
</tr>
<tr>
<td></td>
<td>R-GGCCAGACAGCTTGTGACA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Sample types</th>
<th>No. of C. perfringens positive samples (%)</th>
<th>No. of sample positive for virulence gene by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>cpa cpe</td>
</tr>
<tr>
<td>Fresh water fish</td>
<td>Intestinal and gills</td>
<td>17 (24.63)</td>
<td>17 12</td>
</tr>
<tr>
<td>Fish products</td>
<td>Whole part</td>
<td>07 (21.21)</td>
<td>07 05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17 (70.83%)</td>
</tr>
</tbody>
</table>
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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 cph2 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

C. perfringens is considered as one of the most important food-borne pathogen and is ubiquitous in nature, 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 α 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 (cph2). 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. Cai et al. reported that out of 75 isolates of C. perfringens from fresh water fish, 13 (17.3%) isolates were found to be positive for only α toxin gene, 58 (77.3%) isolates for α & β toxin genes positive (C. perfringens type C) and 04 (5.3%) isolates for α, β and ε 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. In concurrence to present results, the cpe- negative C. perfringens type A from the fish sample and clinical cases of gastroenteritis was reported previously. 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.
positive, but none of the *C. perfringens* type A isolates were found to be positive for *cpb2* gene. 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.

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. 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 α 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.

**ACKNOWLEDGEMENT**

The authors thank The Director, Indian Veterinary Research Institute, Izatnagar for providing necessary facilities for undertaking the research work.

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J PURE APPL MICROBIO, 10(4), DECEMBER 2016.