Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(3): 176-181

Received: 28-03-2018
Accepted: 29-04-2018

Najimaana Wani
Division of Veterinary Public Health and Epidemiology, Faculty of Veterinary Sciences and Animal Husbandry, Shuhama, SKUAST-K, Jammu and Kashmir, India

Shakeel Ahmad Wani
Director Education, SKUAST-K, Jammu and Kashmir, India

ZH Munshi
Division of Veterinary Public Health and Epidemiology, Faculty of Veterinary Sciences and AnimalHusbandry, Shuhama, SKUAST-K, Jammu and Kashmir, India

Showkat Ahmad Shah
Division of Veterinary Pathology, FVSc &AH, Shuhama, SKUAST-K, Jammu and Kashmir, India

Mudasir Rather
Division of Veterinary Public Health and Epidemiology, Faculty of Veterinary Sciences and Animal Husbandry, Shuhama, SKUAST-K, Jammu and Kashmir, India

Akram Hussain
Division of Veterinary Public Health and Epidemiology, Faculty of Veterinary Sciences and AnimalHusbandry, Shuhama, SKUAST-K, Jammu and Kashmir, India

Zahid Kashoo
Division of Veterinary Microbiology and Immunology FVSc &AH, Shuhama, SKUAST-K, Jammu and Kashmir, India

Nusrat Nabi Khan
Division of Animal Genetics and Breeding, FVSc &AH, Shuhama, SKUAST-K, Jammu and Kashmir, India

Correspondence
Najimaana Wani
Division of Veterinary Public Health and Epidemiology, Faculty of Veterinary Sciences and Animal Husbandry, Shuhama, SKUAST-K, Jammu and Kashmir, India

Isolation and virulence gene profiling of *Clostridium perfringens* from freshwater fish

Najimaana Wani, Shakeel Ahmad Wani, ZH Munshi, Showkat Ahmad Shah, Mudasir Rather, Akram Hussain, Zahid Kashoo and Nusrat Nabi Khan

Abstract
The present study was undertaken to isolate and study the virulence gene profile of *C. perfringens* from freshwater fish in Srinagar city (local fish: *Schizothorax spp, Cyprinus carpio* and exotic carp like common carp, silver carp, Rohu, Pangus). Of the total of 110 randomly collected fish samples processed anaerobically for the presence of *C. perfringens*, 13 (11.81%) samples were found positive for the organism. The higher prevalence was observed in locally available fish collected from different markets of the Srinagar city (18.18%) as compared to fish imported from other states of the country (5.45%). The level of contamination among two types of fish samples was statistically significant with higher levels of contamination in locally available fish ($p<0.05$). The virulence gene profiling of the isolates was studied using multiplex-PCR targeting *cpa, cpb, etx, iap, cpb2* and *cpe* genes. The *cpa* gene was detected in all 13 isolates (100%), either alone in 7 isolates (53.84%) or in combination with the *cpb2* gene in 5 isolates (38.46%) and with *cpe* gene in 1 isolate (7.69%). None of the isolates screened carried *cpb, etx* or *iap* gene confirming the occurrence of *C. perfringens* toxinotype A and thereby the absence of toxinotypes B, C, D and E. The study regarding isolation and molecular characterization of *C. perfringens* from fish especially from Srinagar was conducted for the first time 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.

Keywords: *C. perfringens*, Fish, m-PCR, virulence

1. Introduction
The *C. perfringens* is a Gram positive, sporulating anaerobic bacterium belonging to genus *Clostridia* [1]. The organism has large rods (0.6-2.4 x 1.3-9.0 µm), encapsulated and is non-motile. Most *C. perfringens* isolates are catalase negative, oxidase negative and grow optimally around 42-50 °C [1]. It is known as the most rapidly multiplying organism, with some strains possessing less than 10 minute doubling time [2]. A distinguishing characteristic of the *C. perfringens* is their ability to form endospores, typically initiated under conditions of nutrient depletion that helps to preserve genetic content until favourable conditions leading to ubiquitous nature of organism. *C. perfringens* is a prolific toxin producer with a capacity to secrete at least 17 types of toxins [3]. The *C. perfringens* mediated enteric diseases can be attributed to the well characterized, lethal, membrane active toxins which include alpha (α), beta (β), epsilon (ε), iota (ι), enterotoxin and perfringolysin O (θ) [4]. Based on toxin production, *C. perfringens* are classified into five toxinotypes viz; type A (α- toxin), type B (α, β and ε toxin), type C (α and β toxins), type D (α and ε toxin) and type E (α and ι toxins) [5]. *C. perfringens* is the most widespread pathogen responsible for a number of disease syndromes in both humans and animals [6].

Fish being the major source of dietary protein is often infected with various food borne pathogens particularly bacteria causing health hazards to the consumers. The fish and fish based products get spoiled easily within 40 hrs at <10 °C due to high perishability [7, 8]. The rate of bacterial spoilage of fish is dependent on the initial microbial load, ambient temperature and improper handling; therefore, proper storage is critical in maintaining high standards of safety. The refrigeration temperatures does not prevent spoilage of fish because of autolytic activities and chemical changes occurring in fish after harvest, therefore, creating undesirable changes like off-flavours, texture and appearance [9, 10]. Since the microbiological diversity of the fresh fish muscle depends on the fishing grounds and environmental factors around it, fish

from warm waters mostly harbor mesosphilic Gram negative bacteria while cold waters harbor mostly psychrophilic Gram positive bacteria [11]. Fish acts as a vehicle of transmission to the *C. perfringens* food borne infections. The spores of the organism have been recovered from both raw and cooked fish making it a potential foodborne pathogen [12]. 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, keeping in view the importance of research of *C. perfringens*, the present study was conducted with the following objectives:

- To isolate and determine prevalence of *Clostridium perfringens* from fish
- To determine virulence gene profile of *Clostridium perfringens* isolates.

2. Materials and methods

2.1 Place of work

The study was conducted in the Division of Veterinary Public Health, Faculty of Veterinary Sciences and Animal Husbandry, SKUAST-K, Shuhama, Jammu and Kashmir, India. The period of study was from August, 2015 to January, 2016.

2.2 Sampling

A total of 110 samples of fish were collected from different areas of Srinagar city aseptically in sterile zipper bags that included locally available fish (n=55) (local fish: *Schizothorax spp, Cyprinus carpio)* procured from retail fish markets, Government and private fish farms. Fish imported from outside state (exotic carp like common carp, silver carp, Rohu, Pangus) (n=55) which are mainly brought in the valley from different parts of the country like Punjab, Delhi, Andhra Pradesh and West Bengal via Jammu were also analysed.. The samples were transported in an ice box to the laboratory for microbiological analysis and processed within 2-3 hours for isolation and identification of the *C. perfringens*

2.3 Bacterial strain

The reference strain of *C. perfringens* ATCC-13124 was procured from Hi-media (Mumbai, India) and maintained in the laboratory of Division of Veterinary Public Health. It served as positive control during the study.

2.4 Isolation and Identification of *C. perfringens*

Isolation and identification of *C. perfringens* from fish was carried out as per standard microbiological method [13, 14].

For isolation of *C. perfringens* 25g of the fish meat sample was chopped into very small fine pieces using sterile scissors or scalpel and suspended into 225 ml of sterile 1% peptone water in a 250 ml sterile flask and homogenized for 1-2 min. Fluid thioglycollate medium (FTM) was used for the enrichment of *C. perfringens*. Diluted meat samples (1 ml) were inoculated into each of two tubes containing 10 ml of sterile FTM. To enrich for any *C. perfringens* spores present in the sample, one of these two tubes was heat shocked at 80 °C for 10-15 min. The other tube was not heat shocked to enrich primarily for *C. perfringens* vegetative cells present in the sample. Both were then incubated in 3.5 litre anaerobic jar (Oxoid Limited, Thermo Fisher Scientific Inc., UK) with anaerobic gas-packs at 37 °C for 24 hrs. For selective isolation, sulfite polymyxin sulfadiazine (SPS) agar and tryptose sulfite cycloserine (TSC) agar (Becton, Dickinson and Company, Sparks, MD, USA) were used as the selective isolation medium. A loop full of each FTM and litmus milk-enriched culture medium was streaked on SPS and TSC agar (containing egg yolk). The agar plates were incubated at 37 °C for 18-24 hours anaerobically. The growth of *C. perfringens* was evident by the typical black colored colonies on SPS agar medium and yellowish gray opaque zones caused by lecithinase activity on TSC agar. Such colonies were selected for further purification by sub-culturing onto TSC agar plates. The isolates were stored at -80° C in cooked meat medium containing 40% glycerol until used for PCR.

2.5 Morphological and biochemical characterization

Isolates were identified on the basis of their colony characteristics, Gram's staining, morphological features, capsular staining, spore staining and motility testing, catalase test, triple sugar iron test as per the standard procedure. The isolates were further characterized by double haemolysis on blood agar, lecithinase and proteolytic activity as well as gelatin liquefaction, nitrate reduction and lactose fermentation tests [15, 16].

2.6 Molecular characterization

The virulence gene profiling of the isolates was carried out by targeting alpha toxin gene (*cpa*), beta toxin gene (*cpb*), epsilon toxin gene (*etx*), iota toxin gene (*i*A), β2-toxin (*cpb-2*) and enterotoxin (*cpe*) using specific primers by multiplex PCR assay as described by (Songer and Meer [17]; Baums *et al.* [18]; Van Asten *et al.* [19].

2.7 Preparation of DNA template by boiling and snap chilling method [20]

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 3 µl of the supernatant was used as the template DNA in the PCR assays. Purity of the extracted DNA was checked by agarose gel electrophoresis and concentration was checked spectrophotometrically. One microlitre of extracted DNA was diluted in 1 ml of sterile double distilled water and the optical density was measured at 260 nm and 280 nm against sterile double distilled water blank. The purity of the sample was estimated by determining the ratio of absorbance at 260 nm and at 280 nm. For a reasonably pure sample the ratio should be 1.65 to 1.85. The DNA extracted from isolates was stored at -20ºC till further use.

All the *C. perfringens* isolates were screened for six different toxin genes using a multiplex PCR. These six toxin genes include α-toxin (*cpa*), β-toxin (*cpb*), ε-toxin (*etx*), ί-toxin (*cpi*), β2-toxin (*cpb-2*) and enterotoxin (*cpe*). The primers used for the amplification of the genes are listed in Table 1. In a final volume of 25 µl, the reaction mixture consisted of 2.5 µl of 1X PCR buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 200 µM of each dNTPs, 1 U of *Taq* DNA polymerase (MBI-Fermentas), 3.0 µl template DNA.0.2 mM of each primer except beta2 primers. Concentration of each beta2 primer was 0.4 mM. The PCR conditions consisted of initial denaturation at 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 53ºC for 90 sec and extension at 72ºC for 90 sec. This was followed by final extension at 72ºC for 10 min. Sterilized distilled water with no template DNA was used as negative control and

ATCC-13124 strain of *C. perfringens* as standard positive control in every reaction. PCR products were analyzed by gel electrophoresis in 1.5% agarose (SRL) stained with ethidium bromide (5 µg/ml). Gene Ruler 100-bp DNA Ladder (MBI Fermentas) was used as a molecular size standard to estimate the size of amplified products.

Table 1: Target toxin gene and oligonucleotide primer sequences for multiplex PCR

S. No.	Target gene	Primer Sequence (5′-3′)	Primer conc. (pM)	Product size (bp)
2.	*Cpa*	F-GCTAATGTTACTGCCGTTGA R-CCTCTGATACATCGTGTAAG	10	324
3.	*cpb*	F–GCGAATATGCTGAATCATCA R–GCAGGAACATTAGTATATCTTC	10	195
4.	*etx*	F–TGGGAACTTCGATACAAGCA R–AACTGCACTATAATTTCCTTTTCC	10	376
5.	*iap*	F-AATGGTCCTTTAAATAATCC R-TTAGCAAATGCACTCATATT	10	272
6.	*cpb2*	F–AAATATGATCCTAACCAACAA R-CCAAATACTCTAATCGATGC	10	548
7.	*cpe*	F-TTCAGTTGGATTTACTTCTG R-TGTCCAGTAGCTGTAATTGT	10	485
				486

3. Statistical analysis

The statistical analysis of the data was done by using standard statistical tests *i.e* Chi-Square 'χ^2' test by using standard software package SPSS Version 10 and 16.0 (Statistical Package for Social Sciences) for Windows, Chicago USA for values. The test was referenced for 'P' values for their significance. Any 'p' value less than 0.05 ($p<0.05$) was taken to be significant [21]

4. Results

4.1 Prevalence of *C. perfringens* in Fish

Of the 110 fish samples processed for *C. perfringens,* 13 (11.81%) samples were found positive for the organism. The higher prevalence was observed in locally available fish collected from different markets of the Srinagar city (18.18%) as compared to fish imported from other states of the country (5.45%). The level of contamination among two types of fish samples was statistically significant with higher levels of contamination in locally available fish ($P<0.05$) (Table 2).

Table 2: Prevalence of *C. perfringens* in fish

Nature of sample	Type of sample	No. of samples tested	No. positive (*C. perfringens*)	Per cent positive (*C. perfringens*)	χ^2-value (p-value)
Fish	Locally available *(Schizothorax spp, Cyprinus carpio)*	55	10	18.18	4.414 (0.036)
	Imported from outside state (exotic carp like common carp, silver carp, Rohu, Pangus).	55	3	5.45	
Total		110	13	11.81	

4.2 Isolation and identification of *C. perfringens*

Isolation and identification of *C. perfringens* was done as per the standard procedure [13]. The organism produced typical black colonies on the selective SPS/TSC agar (without egg yolk) due to the reduction of sulfite (Plate 1). On egg yolk agar plates the *C. perfringens* produced opalescence around the colonies indicating the lecithinase activity which is characteristic of the organism. The organism was found non motile in semisolid agar during the motility test which is characteristic of *C. perfringens.* In Gram staining test, large Gram positive, straight parallel rods were observed microscopically suggestive of *C. perfringens* (Plate 2).

4.3 Biochemical characterization

The isolates were further characterized and confirmed biochemically. In Triple Sugar Iron test *C. perfringens* changed the colour of both butt and slant from red to yellow confirming the utilization of all the three sugars. In Litmus milk media stormy fermentation was observed with gas formation in the positive samples (Plate 3). The positive isolates showed nitrate reduction indicated by the red discoloration of the nitrate broth, characteristic of the organism. The cultural, morphological and biochemical characteristics of the isolates were compared with the standard *C. perfringens* ATCC-13124. All the isolates presented similar biochemical characteristics when compared with the standard *C. perfringens* culture. The biochemical characteristics of the isolates and those of the standard strain are presented in Table 3.

Table 3: Biochemical characteristics of *C. perfringens* isolates

S. No.	Name of the test	Characteristics	ATCC-13124	Isolates
1	Growth on SPS Agar	Demonstration of black colonies	+	+
2	Growth on selective TSC Agar (without egg yolk)	Demonstration of black colonies	+	+
3	TSC (with egg yolk)	Lecithino-Vitallin reaction	+	+
3	Gram staining	Gram positive, large rods	+	+
4	Motility test	Non motile	_	_
5	Malachite green staining	Presence of spores	+	+
6	Litmus milk test	Stormy fermentation with gas	+	+
7	Blood agar (5% sheep blood)	β haemolysis, double zone of haemolysis	+	+
8	Nitrate reduction test	Reduction of nitrates to nitrites	+	+
9	Gelatin liquefaction test	After 48 hours	+	+
10	Carbohydrate fermentation	Glucose, lactose, maltose (acid and gas production)	+	+

4.4 Molecular characterization of *C. perfringens* isolates

4.4.1 Genomic DNA

The DNA extracted from the different isolates was found intact and without smearing in gel electrophoresis. The purity of DNA was checked by measuring $OD_{260/280}$ in a spectrophotometer which yielded a value between 1.7- 1.8 confirming it to be free from any contamination of protein or RNA. The concentration of DNA was observed from 1.41 to 4.61µg/µl in different isolates.

4.4.2 Typing of *C. perfringens* by Multiplex PCR

All the 13 isolates obtained during the study carried the *cpa* gene (324bp) while none of the isolates carried *cpb* (195bp), *etx* (376bp) or *iap* (272bp) genes indicating the presence of *C. perfringens* toxinotype A and absence of toxinotypes B, C, D and E (Plate 4; Table 4).

Table 4: Toxin gene pattern of *C. perfringens* type *A* isolates

Type of sample		No of Isolates	Toxin gene pattern		
			***Cpa* (%)**	***cpa /cpb2* (%)**	***cpa/ cpe* (%)**
Fish	Locally available	10	6 (60.00)	3 (30.00)	1 (10.00)
	Imported	3	1 (33.33)	2 (66.66)	0 (00.00)
	Subtotal	13	7 (53.84)	5 (38.46)	1 (7.69)

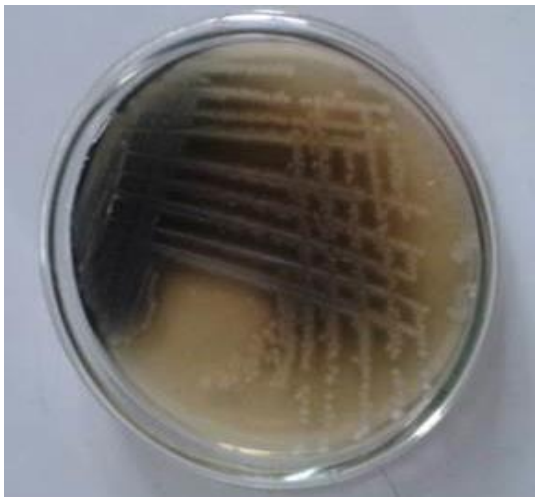

Plate 1: Colony characteristics of *C. perfringens* on Sulphite Polymixin Sulphadiazine media

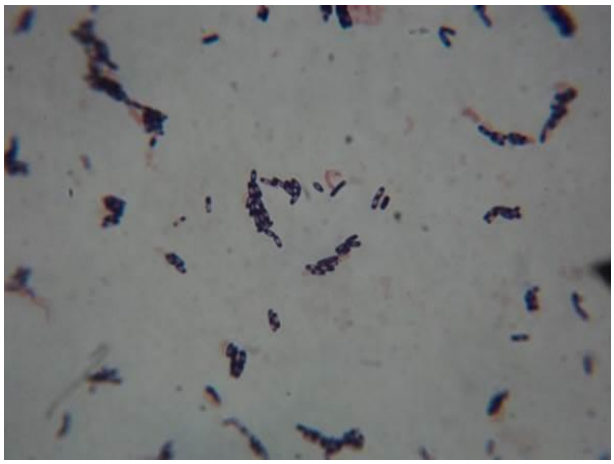

Plate 2: Gram's Staining of *C. perfringens* showing large Gram positive rods under oil immersion (100x)

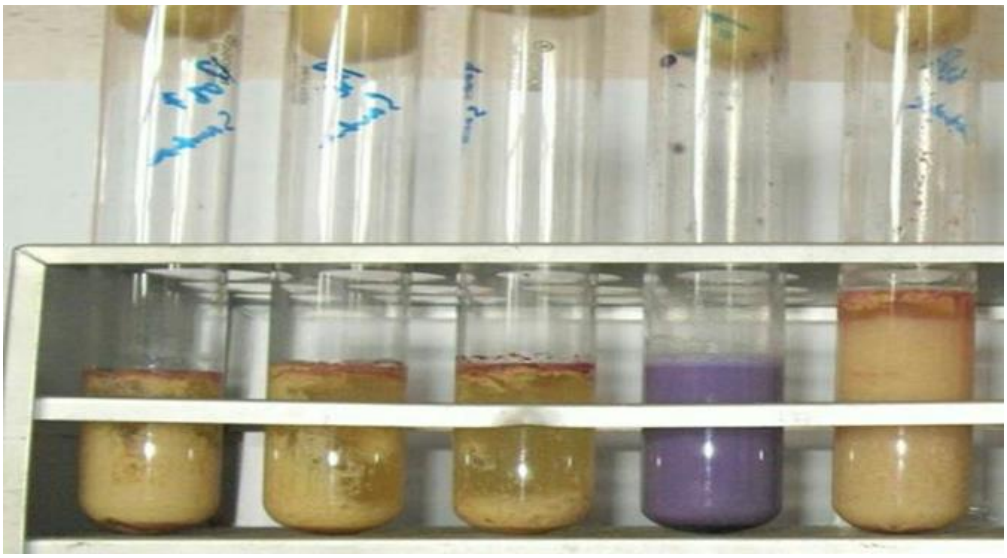

Plate 3: Stormy fermentation produced by *C. perfringens* in Litmus Milk 1, 2, 3, 5 Positive 4 Negative

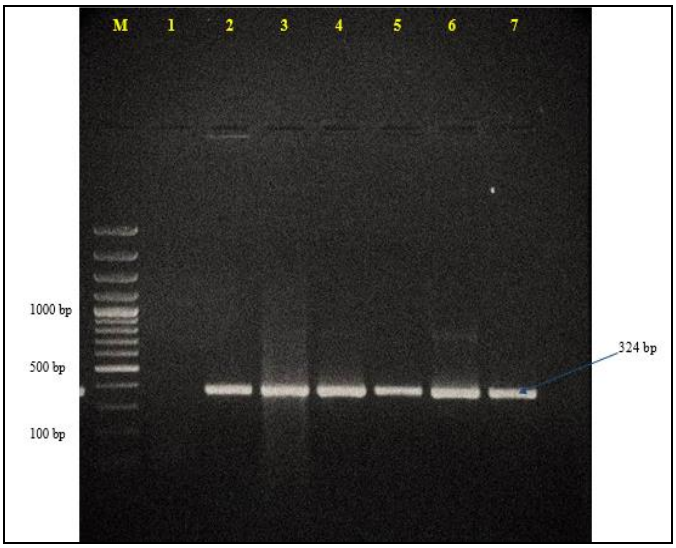

Plate 4: Agarose gel electrophoresis showing amplification of alpha gene of *C. perfringens* type A isolates

Lane M	:	100bp plus DNA ladder
Lane 1	:	Negative control
Lane 2	:	Positive control (ATCC 13124)
Lane 3 -7	:	324 bp product corresponding to alpha gene confirming the presence of *C. perfringens* type A isolates.

5. Discussion

Fish is considered as one of the most popular and nutritionally balanced food. However, the quality of fish and spoilage has always been the concern for human health. *C. perfringens* in raw fish and its products leads to human infections when improperly stored or cooked fish is consumed. In the present study, the *C. perfringens* was detected in 11.81% fish samples. The locally available fish revealed significantly higher level of contamination (18.18%) in comparison to fish imported from other states of the country (5.45%) indicating high amount of organic pollution of human and animal origin in local water bodies of Srinagar city. *C. perfringens* an anaerobe grows best under anaerobic conditions of polluted waters and become pathogenic which is a matter of great public health concern. Presence of toxins in fish imply high risk of occurrence of *C. perfringens* type A disease in human beings and to prevent such diseases the better methods of cooking the fish and their storage should be adapted. In previous studies, Cai *et al.* [22] reported prevalence of the organism in 17.9% freshwater fishes of China. Similar findings related to the prevalence of *C. perfringens* in fish have been reported by Matches *et al.* [23] who found *C. perfringens* in 18% samples from fish gut contents collected in the state of Washington.

All the 13 isolates obtained during the study carried the *cpa* gene (324bp) while none of the isolates carried *cpb* (195bp), *etx* (376bp) or *iap* (272bp) genes indicating the presence of *C. perfringens* toxinotype A and absence of toxinotypes B, C, D and E. Beta2 toxin gene and *cpe* gene were found in 38.46% and 7.69% samples respectively. Similar results were reported by Das and Jain [24] who detected *C. perfringens* in 18.35% of fish samples of Tamil Nadu, India. Out of four major virulence genes, they reported *cpa* gene in 100% and beta2 gene in 59.18% isolates while none of the isolates were found positive for other major virulence genes viz beta, epsilon, iota and enterotoxin genes. Aschfalk and Mulle [25] reported occurrence of *C. perfringens* in 38.9% Atlantic cod samples caught along the northern Norwegian coast. All the isolates were *C. perfringens* toxin type A. The genes encoding for beta, epsilon, iota and enterotoxin genes were not reported whereas in 2.1% samples, the gene encoding for β2 toxin was detected.

Wen and McClane, [26] analysed the non-outbreak American retail foods and reported 34 isolates of *C. perfringens* from fish samples having alpha gene (Type A) while 3 isolates were type A with additional enterotoxin gene (8.82%) but none of the type A isolates were positive for beta2 toxin gene. Similar findings were put forward by Yadav *et al* [27] who reported that all the 24 (100%) isolates from fresh water fish and fish products harbored *cpa* toxin gene confirmed as *C. perfringens* type A during PCR assay however, 17 (70.83%) *cpa* positive isolates were having additional *cpb2* toxin gene, while none of the isolates were found to be positive for *cpb*, *etx*, *iap*, and *cpe* genes.

6. Conclusion

The present study suggested that PCR is a reliable molecular technique and useful tool for the detection of virulence genes and typing of *C. perfringens* isolates recovered from fish. The presence of *cpa* gene in all the isolates of *C. perfringens* suggest that *C. perfringens* type A is the most predominant type in fish in this study area. The *C. perfringens* type A was detected in 11.81% fish samples. The locally available fish revealed significantly higher level of contamination (18.18%) in comparison to fish imported from other states of the country (5.45%). Beta2 toxin gene and *cpe* gene were found in 38.46% and 7.69% samples respectively.

7. Acknowledgement

I owe my deep sense of gratitude to the Division of Vety Microbiology and Immunology, Shuhama SKUAST K for providing the necessary support from NAE project on Anaerobic Bacteriology funded by ICAR.

8. References

1. Adams MR, Moss MO. Food Microbiology, second edition. Cambridge, UK, Royal Society of Chemistry, 2004.
2. Willardsen R, Busta F, Allen C. Growth of *Clostridium perfringens* in three different beef media and uid thioglycollate medium at static and constantly rising temperatures. Journal of Food Protection. 1979; 42:144-148.
3. Bokori-Brown M, Savva CG, Fernandes da Costa SP, Naylor CE, Basak AK, Titball RW. Molecular basis of toxicity of *Clostridium perfringens* epsilon toxin. FEBS Journal. 2011; 278:4599-4601.
4. Uzal F, Freedman J, Shrestha A, Theoret J, Garcia J, Awad M. Towards an understanding of the role of *Clostridium perfringens* toxins in human and animal disease. Future Microbiology. 2014; 9(3):361-77.
5. Songer JG, Meer RR. Genotyping of *Clostridium perfringens* by polymerase chain reaction is a useful adjunct to diagnosis of clostridial enteric disease in animals. Anaerobe. 1996; 2(4):197-203.
6. Canard B, Saintjoanis B, Cole ST. Genomic diversity and organization of virulence genes in the pathogenic anaerobe Clostridium perfringens. Molecular Microbiology. 1992; 6:1421-1429.
7. Musa U, Hati SS, Adamu YI, Mustapha A. Pesticides residues in smoked fish samples from North-Eastern Nigeria. Journal of Applied Science. 2010; 10:975-980.
8. Dewi RS, Nurul Huda G, Ahmad R. Changes in the

physicochemical properties, microstructure and sensory characteristics of shark dendeng using different drying methods. American Journal of Food Technology. 2011; 6:149-157.
9. Ames GR. The kinds and levels of post-harvest losses in Africa in land fisheries. Food agriculture organization of the United Nations. Rome Italy, CIFA Technical, 1992, 19.
10. Jhonston WA, Nicholson FJ, Roger A, Stroud GD. Freezing and refrigerated storage in fisheries. FAO Fisheries Technical Paper. No. 340. Rome, FAO, 1994, 143.
11. Abbas MS. Isolation of bacteria from fish. International Journal of Advanced Research. 2014; 2(3):274-279.
12. Grass JE, Gould LH, Mahon BE. Epidemiology of Foodborne Disease Outbreaks Caused by Clostridium perfringens, United States, 1998-2010. Foodborne Pathogens and Disease. 2013; 10(2):131-135.
13. FDA. (Food and Drug Administration). Food Code - Table of Contents. http://www.fda.gov/Food/ Food Safety/ Retail Food Protection/ Food Code/Food- Code 2009/default.htm
14. Holt JG, Krige NR, Sneeth PHA, Staley JT, Williams ST. Bergey's Manual of Determinative Bacteriology. 9th edn. Williams and Wilkins, Baltimore, USA, 1994, 749-755.
15. ISO (International Organization for Standardization). Microbiology of food and animal feeding stuffs: horizontal method for the enumeration of Clostridium perfringens; colony count technique. International Organization for Standardization, Geneva, Switzerland, 2004.
16. Rhodehamel E, Harmon S. BAM: Clostridium perfringens. US FDA, Rockville, MD.2001 http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/Bacteriological Analytical Manual BAM/ ucm 070878.
17. Songer JG, Meer RR. Multiplex polymerase chain reaction assay for genotyping Clostridium perfringens. American Journal of Veterinary Research. 1997; 58:702-705.
18. Baums CG, Schotte U, Amtsberg G, Goethe R. Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. Veterinary Microbiology. 2004; 100:11-16.
19. Van Asten AJ, AM, Allaart JG, Meeles AD, Gloudemans PWJM, Houwers DJ et al. A new PCR followed by MboI digestion for the detection of all variants of the *Clostridium perfringens* cpb2 gene. Veterinary Microbiology. 2008; 127:412-416.
20. Croci L, Delibato E, Volpe G, Medici DD, Palleschi G. Comparison of PCR, electrochemical enzyme-linked immunosorbent assays, and the standard culture method for detecting *Salmonella* in meat products. Applied Environment Microbiology. 2004; 70:1393-1396.
21. Snedecor GW, Cochran WG. Statistical Methods, 8th edition, State University Press, Iowa, USA, 1982.
22. Cai Y, Gao J, Wang X. *Clostridium perfringens* toxin types from freshwater fishes in one water reservoir of Shandong province of China, determined by PCR. Deutsche Tierarztliche Wochenschrift. 2008; 115:292-294.
23. Matches JR, Liston J, Curran D. *Clostridium perfringens* in the Environment. Applied Microbiology. 1974; 28(4):655-660.
24. Das A, Jain A. Genotyping of *Clostridium perfringens* from fresh water fish and fish pickles. Journal of Microbiology, Biotechnology and Food Sciences. 2012b; 2(1):162-174.
25. Aschfalk A, Muller W. *Clostridium perfringens* toxin types from wild-caught Atlantic cod (Gadus morhua) determined by PCR and ELISA. Canadian Journal of Microbiology. 2002; 48:365-368
26. Wen QY, McClane BA. Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. Applied Environmental Microbiology. 2004; 70:2685-2691.
27. Yadav JP, Das SC, Dhaka P, Deepthi Vijay Kumar M, Pranav C, Singh R *et al*. Isolation, Genotyping and Antibiogram Profile of *Clostridium perfringens* isolates recovered from Freshwater Fish and Fish Products from Kolkata Region. 2017 Journal of Pure and Applied Microbiology. 2016; 10(4):2807-2814.