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Clostridium Perfringens TYPE A FROM FRESH WATER FISHES

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ABSTRACT:

Present research investigation aims to investigate the prevalence of *Clostridium perfringens* type A in fresh water fishes [common carp, cat fish, silver carp] obtained from randomly selected dams, freshwater lakes, ponds, retail shops and local markets of Tamil Nadu, India. Intestinal contents from 267 fishes were processed for the isolation and identification of *C. perfringens*. The ultra structures of the bacterial isolates were carried out in Scanning electron micrograph [SEM] and their virulence determinantion was done by Polymerase chain reaction [PCR]. Bacteriological investigation revealed the presence of *C. perfringens* in 49 [18.35%] samples. The ultrastructure studies of the bacterial isolates in SEM were observed to be in clusters of thick rods with variable in length and mostly occurred in pairs. In PCR, out of four major virulence genes of *C. perfringens*, alpha toxin gene [*cpa*] was harboured by all the isolates. Along with *cpa*, additional beta2 toxin gene [*cpb*2] was also detected in 29 [59.18%] isolates of *C. perfringens*. But none of the isolates were found positive for other major virulence genes [beta, epsilon and iota] and enterotoxin genes. This PCR determination of virulence genes suggested that the isolated *C. perfringens* from fresh water fish samples were belonged to type A.

Keywords: *Clostridium perfringens* type A, alpha toxin gene, beta2 toxin gene, Fish, PCR

[I] INTRODUCTION

Post Indian Independence in 1947, the pisci-culture sector in India has witnessed a phenomenal growth in terms of production. The FAOUN report of 2011 states that there has been a double digit growth in fish production between 1999 and 2010 [1]. Importantly, this vital economic activity is thriving in the river based economy of the state of Tamil Nadu. According to the annual report of DAHDF [2009], Tamil Nadu states is ranked the 5th among the leading fish producing states in India [2]. In the Indian

context, this economic growth is a pointer to the large-scale market demand for fish and fish based products as popular food items. Further, this demand is a logical consequence of the national cognition of the dietary benefits that fish items bring to people's diets, as a commonly available sources of protein.

For scientists and public health authorities in India, this recent unprecedented growth in fish production is a situation requiring careful monitoring as there is an ever present risk of bacterial infection in fish, especially in the post harvesting storage phase, a critical stage prior to human consumption. Often, when bacterially contaminated food in some form or the other is consumed by unsuspecting populace, there is a detrimental impact on public health. More often than not, poor maintenance level of storage facilities, retail shops or local markets is the root cause of endemic bacterial infection in fish. Further, the incidence of bacterial infection also lowers the business credibility of the stakeholders such as pisciculture farmers, entrepreneurs, scold storage houses, packaging houses, besides lowering the credibility of the monitoring government authorities such health inspectors, food certification authorities etc.

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 [3]. *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 [4]. 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 [5, 6]. 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 [7] and to identify the specific strains of *C. perfringens* [8]. From the past six to seven years, identification and molecular characterization of *C. perfringens* isolates from various gastroenteritis cases in animals from India have been reported [9, 10, 11, 12, 13, 14, 15]. But limited research have been carried out in fresh water fishes [16, 17] and therefore, it becomes necessary to determine the types of *C. perfringens* prevalent in fresh water fish species in Tamil Nadu, India useful for human consumption.

[II] MATERIALS AND METHODS

2.1. Collection of fish samples

Two hundred and sixty seven fresh water fishes obtained from common carp [90], cat fish [91] and silver carp [86] were collected from the randomly selected dams, freshwater lakes, ponds, retail shops and local markets across Tamil Nadu, India. All the fresh water fishes were purchased in a regular consumer bags and immediately transferred to the laboratory. The intestine samples from the same number of fishes were collected scientifically processed for the microbial investigation.

2.2. Isolation of *C. perfringens*

Intestinal contents were inoculated aseptically in Robertson's cooked meat medium [RCM] supplemented with glucose, hemin and vitamin K broth [Himedia, Mumbai] with neutral oil overlay and incubated at 37ºC for 24 hr. The inoculum from each RCM media broth were streaked onto nutrient agar with the addition of 10% defibrinated sheep blood and incubated inside the BBL Gas-Jar [Difco, USA] containing CO_2 gas generating kit and the anaerobic indicator [Oxoid, U.K] for ensuring the good anaerobiosis for 24 hr at 37ºC.

2.3. Biochemical and phenotypical identification of *C. perfringens*

Bacterial colonies from blood agar were subjected to various morphological, cultural and biochemical characters as per the Bergey's manual of determinative bacteriology [18] with little modification. These tests included, motility of the bacteria, Gram's and endospore staining, gelatin liquefaction, litmus milk fermentation, nitrate reduction, urease production, cytochrome oxidase, catalase, indole production, hydrogen sulphide [H_2S] production, Simmon's citrate utilization, triple sugar iron agar for the fermentation of sugar like glucose, lactose and sucrose. All the isolated bacteria were evaluated for the phenotypical tests like deoxyribonuclease [DNase] enzyme, lecithinase enzyme and hemolysin production as per the standered protocol.

2.4. Scanning electron micrograph

The bacterial isolates were grown on sheep blood agar at 37ºC for 24 hr. A single bacterial colony along with agar was cut into small pieces [approx 2-3 mm cubes in size]. The sample specimens were fixed with primary fixative [Karnovsky's fixative, pH 7.2] [Sigma, USA] in an eppendorf tube for a minimum of 4 hr at 4ºC. After discarding the primary fixative, the specimens were washed twice with the washing solution [0.1 M Sodium Cacodylate Buffer, pH 7.4] [Sigma, USA] for 15 min at 4ºC, followed by post fixation with the same for a maximum of 12 hr at 4ºC. The post fixative was discarded carefully from the tube without disturbing the specimens. The specimens were then dehydrated using a series of dehydrating solutions like acetone [gradation of 30%, 50%, 70%, 80%, 90%, 95% and 100%], twice in each case for 15 min at 4ºC. Then the specimens were dried chemically using the drying reagent like Tetra methyl silane [Sigma, USA] for 15 min at 4ºC. Finally the drying reagent was removed [as much as possible] from the tube and the specimens were allowed to air-dry in an air hood for 15 min. After drying, the specimens were mounted on aluminum stubs [10 mm / 30 mm in size] with adhesive tabs and sputter coated with gold for 5 minutes using a polaron [Energy Beam Sciences, Agawam, MA]. The specimens were observed and photographed under the Scanning electron microscope [JEOL JSM-6360, Tokiyo, Japan]. The analysis of the photographs was done with the help of the specific software attached with the SEM.

2.5. Detection of virulence genes by polymerase chain reaction

The isolated bacteria were screened against various virulence gene specific primers by PCR [4]. The PCR was carried out individually for the detection of alpha toxin gene [*cpa*], beta toxin gene [*cpb*], epsilon toxin gene [*etx*], iota toxin gene [*i*A], beta2 toxin gene [*cpb*2] and enterotoxin gene [*cpe*] of *C. perfringens.*

2.5.1. Template DNA preparation

After overnight anaerobic growth on blood agar, a single colony of *C. perfringens* was taken in 100 µl of HPLC grade / MQ water in a 1.5 ml eppendorf tube, gently vortexed and boiled approx 100°C for 10 min in water bath. The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The top clear supernatant was used as a source of template DNA.

2.5.2. Polymerase chain reaction assay

The amplification was carried out in 25µl reaction volume containing 12.5µl of 2× PCR master mix [Promega, USA] containing 4mM magnesium chloride, 0.4mM of deoxynucleotide triphosphates [dNTPs], 0.5U of *Taq* DNA polymerase, 150mM tris-hydrochlroric acid, pH 8.5 [Promega, USA], 0.5µM primers and 2.5µl of template DNA. The PCR reactions were performed in Thermal Cycler [Eppendorf, USA]. After initial denaturation at 94ºC 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. The specific forward and reverse primer pairs for *cpa* gene of 324bp were 5′-gctaatgttactgccgttga-3′ and 5′-cctctgatacatcgtgtaag-3′, *cpb* gene of 180bp were 5′-gcgaatatgctgaatcatcta-3′ and 5′-gcaggaacattagtatatcttc-3′, *etx* gene of 655bp were 5′-gcggtgatatccatctattc-3′ and 5′-ccacttacttgtcctactaac-3′, *i*A gene of 446bp were 5′-actactctcagacaagacag-3′ and 5′-ctttccttctattactatacg-3′, *cpb*2 gene of 567bp were 5′-agattttaaatatgatcctaacc-3′ and 5′-caataccttcaccaaatactc-3′ and *cpe* gene of 233bp were 5′-ggagatggttggatattagg-3′ and 5′-ggaccagcagttgtagata-3′ were commercially synthesized [Eurofins, Bangalore].

2.5.3. Agarose gel electrophoresis

The PCR amplicons were separated in 1.5 % agarose [Promega, USA] gel. The gel was prepared by dissolving agarose in 1 × tris-acetate-EDTA [TAE] buffer [Genei, Bangalore]. Same buffer was used for the electrophoretic run. A total of 5µl of each amplicons, 1µl of 100bp DNA ladder plus [MBI Fermentas, USA] were mixed with 1 µl of 6 × gel loading dye [Genei, Bangalore] and loaded on the gel. Electrophoresis was carried out at a constant electric current of 60 V until the second dye marker had run 3/4th of the gel. Then the gel was stained with 0.4 µg / ml of ethidium bromide [Genei, Bangalore] for 10 min and was visualized and photographed in the gel documentation system [Universal Hood, BIORAD, Italy].

[III] RESULTS

3.1. Isolation and identification of *C. perfringens*

On 10 % sheep blood agar, after 24 hr of anaerobic incubation at 37^{0}C, the young colonies of *C. perfringens* were observed as hemolytic [both alpha and beta hemolytic], round, 1-3 mm in diameter. The bacterial colonies were observed to be typical umbonate, centrally dense and round with rhizoid periphery and were picked up and purified by repeated streaking and restreaking on fresh blood agar media plates until the pure cultures were obtained. All the isolates of *C. perfringens* were found to be non-motile, gram-positive, thick rods shaped on Gram's staining and sub-terminal oval shaped spores on malachite green spore staining.

All the isolates were found to be liquefied gelatin; stormy fermentation, acidity, reduction and coagulation of skim milk; negative in the production of oxidase, catalase, indole, hydrogen sulphide [H_2S] and Simmon's citrate. However, both the nitrate reduction and urease production were found to be variable in reactions. All the isolates characteristically produced acid [characterized by yellow / yellow color in butt / slant] and gas after fermentation of glucose, lactose and sucrose in triple sugar iron agar slants. After detailed microbiological investigation, 49 [18.35%] isolates were tentatively identified as *C. perfringens*.

3.2. Ultrastucture by SEM

The ultrastructure study of *C. perfringens* in SEM was observed to be in clusters of thick rods [Fig 1]. The rods were observed to be variable in length; sometimes occur either single or in pairs and occasionally in short chains. The total length and breadth of the rods were measured in between 3.8-7.8 µm and 0.6-1.2 µm respectively; total mean length and breadth were measured approx 5.7 µm and 0.9 µm respectively; total area was measured between 9-20.3 sq µm with a mean of approx 15.13 sq µm. The total area of the spores was measured between 2.6-9.4 sq µm with a mean of 4.7 sq µm.

3.3. Detection of virulence genes by PCR

In PCR, all the 49 [100%] suspected isolates of *C. perfringens* irrespective of source and place of isolation amplified the primers specific for alpha toxin gene [*cpa*] of products 324bp [Table 1, Fig 2a]. However, 29 [59.18%] isolates were also

positive for additional beta2 toxin gene [*cpb*2] of 567bp [Table 1, Fig 2b]. The other virulence gene specific primers of *cpb, etx, i*A 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 are belonged to *C. perfringens* type A.

Sample source [No]	**Sample types**	**No of *C. perfringens* positive [%]**	**No of virulence genes positive by PCR**	
			cpa	***cpb*2**
Common carp [90]	Intestine samples	15 [30.61]	15	10
Cat fish [91]		21 [42.85]	21	12
Silver carp [86]		13 [26.53]	13	7
Total [267]		49 [18.35]	49 [100%]	29 [59.18%]

Table: 1. Sample details and no of *C. perfringens* type A positive for the virulence genes

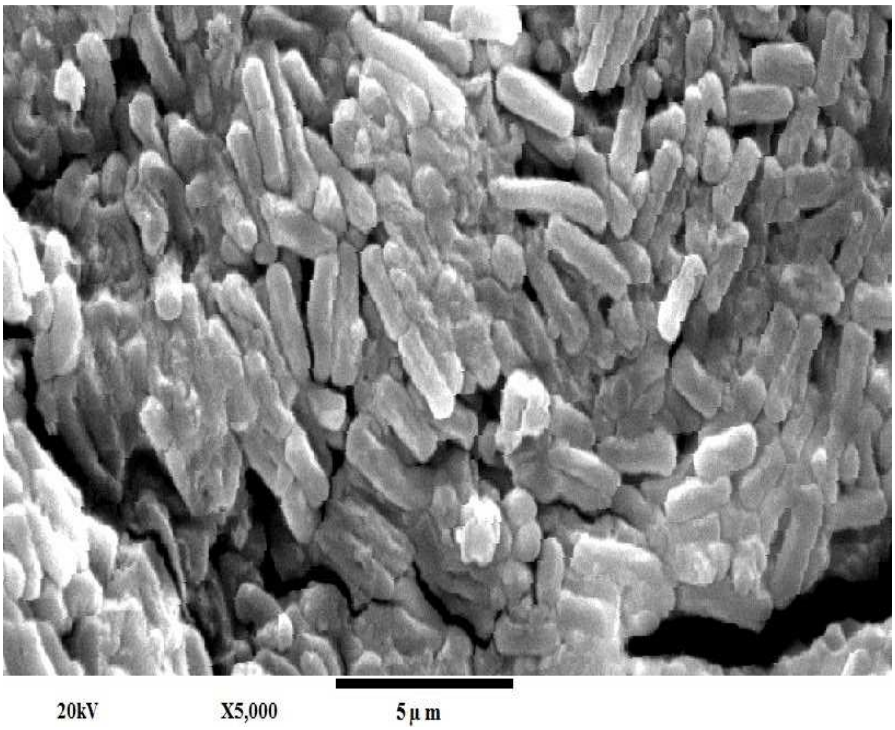

Fig: 1. Ultra structure of *C. perfringens* type A by PCR

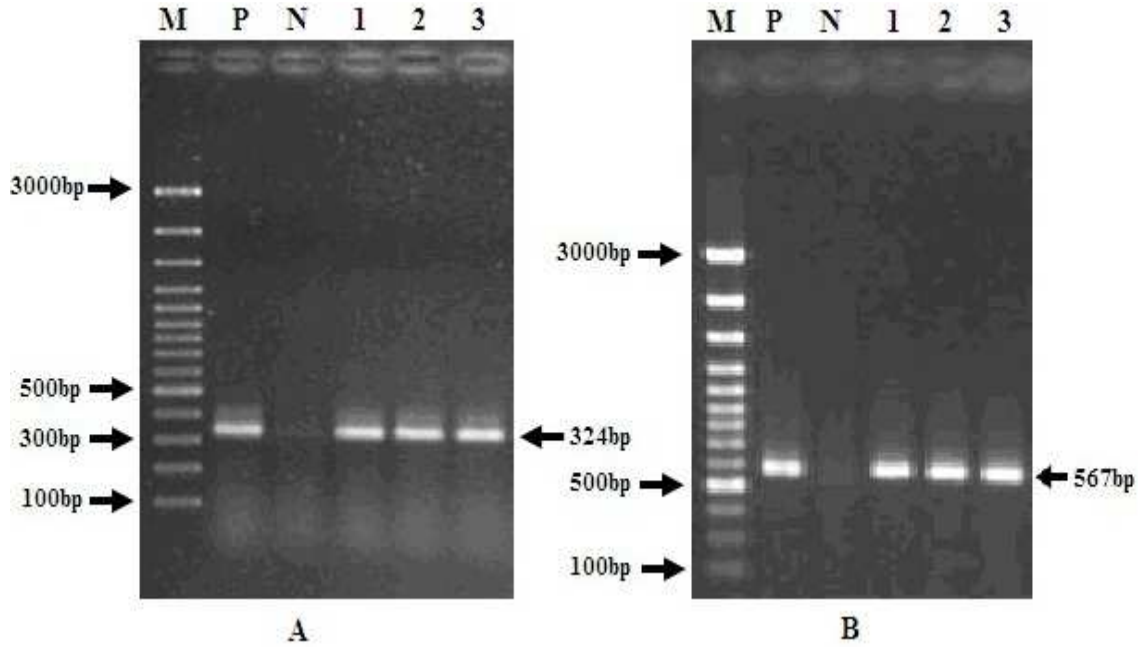

Fig: 2. Detection of virulence genes from *C. perfringens* type A from fish by PCR
A: Detection of *cpa* gene of 324bp fragment, B: Detection of *cpb*2 gene of 567bp fragment
Lane M: High range DNA ruler [Genei, India], Lane P: Positive control, Lane N: Negative control, Lane 1-3: Field isolates positive for virulence genes

[IV] DISCUSSION

C. perfringens is an important food poisoning organism and is ubiquitous in nature, however, there is limited understanding for such obligate and facultative anaerobes in fresh water fishes. In this study, the Robertson's cooked meat media broth supplemented with vitamin-K and haemin and the nutrient agar media enriched with sheep blood under anaerobic atmosphere at 37°C for 24-48 hr were found suitable for the isolation of *C. perfringens* [9, 10, 19]. A total of 49 [18.35%] *C. perfringens* isolates were identified based on the specific cultural, morphological and biochemical characteristics, also reported by other researchers [19]. It has been estimated that 21 [42.85%] isolates of *C. perfringens* were derived from the intestinal samples of cat fish, while 15 [30.61%] and 13 [26.53%] isolates were originated from common carp and silver carp respectively. This suggested that the isolates of *C. perfringens* were most frequent in the intestinal contents of cat fish in compare to common carp and silver carp.

In this study, the use of PCR assay for the detection of virulence genes and genotyping of the *C. perfringens* isolates from fresh water fishes revealed that all the isolates [49] were type A, positive for alpha toxin gene [*cpa*] in 49 [100 %] isolates and beta2 toxin gene [*cpb2*] in 29 [59.18%] isolates. In a similar study, 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] [17]. However, none of the isolates of *C. perfringens* in this study were found positive for *cpe* gene or any other major virulence genes, since, the presence of *cpe* gene in *C. perfringens* is very uncommon, and <5% of global *C. perfringens* type A isolates are *cpe* positive [Czeczulin *et al.*, 1993]. In congruence to present result, the *cpe*- negative *C. perfringens* type A from the clinical cases of gastroenteritis was reported previously [12, 19]. In another

study, out of 34 isolates of *C. perfringens* from fish samples, 31 were found 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 *cpb*2 gene. In contradiction, present study reported the occurences of *cpb*2 positive isolates in 59.18% *C. perfringens* type A also suggested the frequent distribution of this gene along with the predominantly distributed *cpa* gene in fresh water fishes in Tamil Nadu India [16].

[V] CONCLUSION

The present study suggested that PCR is a reliable DNA based technique and useful tool for the detection of virulence genes typing of *C. perfringens* isolates from the fresh water fish samples. The present findings also suggested that *C. perfringens* type A is the most predominant type in fresh water fishes used in this study. The presence of alpha toxin gene in all the isolates of *C. perfringens* type A irrespective of source of isolation and geographical location also suggested that the primer specific to alpha toxin gene in PCR assay could be useful for the rapid identification of *C. perfringens*. The PCR assay described in this study might be provided enough clues to the importance and improvement of the present method for the surveillance of *C. perfringens* type A in fresh water fishes. The bacterium is spore forming and both alpha and beta2 toxin can cause these diseases in animal and human by entering in to the food chain. Thus, the consumption of uncooked fish for human has a crucial impact on public health.

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