MOLECULAR CHARACTERIZATION OF *Clostridium perfringens* ISOLATED FROM FOOD AND FOOD PRODUCTS OF ANIMAL ORIGIN AND ITS PUBLIC HEALTH SIGNIFICANCE

ABSTRACT OF THESIS

BY

DR. RITESH KUMAR

(BVC/M/VPH/005/2017-18)

Submitted to

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DEPARTMENT OF VETERINARY PUBLIC HEALTH & EPIDEMIOLOGY
Bihar Veterinary College, Patna-800014.
(Bihar Animal Sciences University Patna, Bihar)

Title of thesis: Molecular characterization of *Clostridium perfringens* isolated from food and food products of animal origin and its public health significance

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C. perfringens are present in diverse environment including gastrointestinal tract microbiota of diseased and non-diseased human as well as animals. It has been continuously related with clinical systemic and enteric diseases, of both humans and animals. In the present study a total of 251food samples of animal origin viz. meat, milk, fish and their products along with human stools were processed for isolation and identification of *Clostridium perfringens* along with toxotyping, antimicrobial susceptibility and phylogenetic study of isolates. The biochemical reaction and PCR study leads to confirmation of *C. perfringens* in 65.07%and 31.87% of samples. By molecular method *C. perfringens* was detected in 51.42%, 26.67%, 48.57%, 60.00%, 33.33%, 4.00% and 26.00% of raw meats, meat products, raw fish, fish products, pasteurized milk, milk products and human diarrhoeic stool samples, respectively. The toxino-typing profile of *C. perfringens* showed that 77.50% of *C. perfringens* were type A, 2.50% (type B) and 16.25% (type C) and 3.75% (type D). Among *C. perfringens* type A, 4.84%, and 29.03% were found to harbour enterotoxin (cpe^{+}) and β2 toxin (cpb2+) gene, respectively while 6.45% isolates harboured both enterotoxin & β2 toxin (cpe^{+}& $cpb2^{+}$) genes. Among *C. perfringens* type B, 50.00% was found to harbour each of enterotoxin and β2 toxin gene separately. Among *C. perfringens* type C, 7.69% and 30.77% isolates were found to harbour enterotoxin and β2 toxin gene. However, 33.33% isolate of *C. perfringens* type D were found to harbour enterotoxin, β2 toxin and both enterotoxin & β2 toxin genes, respectively. The MIC based antibiotic susceptibility profile showed that 50% *C. perfringens* isolates from raw meat samples were resistance to CD followed by 39% to MOX and TI, 11% to TE and 5.6% to AMC and PI. Whereas, 25% isolates from meat products samples were resistance to CD and TE. 47% of isolates from raw fish samples were resistance to MOX followed by 41% to CD, 29% to TE and 5.9% to CTR as well as PIT whereas 11% isolates from fish product samples were found as resistance to AMC, MOX and PIT. The isolates from pasteurized milk samples showed that 41% isolates were resistance to CD followed by 35% to TE, 31% AMP, 24% from C, 18% TI, 12% CTR and 5.9% from AMC, CTX, MOX and PI. Whereas, the isolates from milk product samples did not showed resistance of any of the antibiotics used under study. The antibiotic susceptibility profile *C. perfringens* isolates from human diarrhoeic samples showed that 23% isolates were resistance to TE followed by 7.7% to MOX.

The phylogenetic study of *C. perfringens* type A isolates using ERIC-PCR showed that 85.15% isolates circulating in both meat and meat products as well as human population were of similar genetic makeup. The genetic linkage of *C. perfringens* type A isolates of fish and fish products with human diarrhoeic isolates suggested that three different clones constituted by ~50% and two each of~13.33%of isolates with >95% genetic similarity circulating in both fish and human population while *C. perfringens* type A isolates of raw and pasteurized milk with human

diarrhoeic isolates ~33% of isolates of similar genetic makeup with 95% genetic similarity circulating in both pasteurized milk as well as human population.

From the finding of present study, it may be presumed that meat and meat products, fish and fish products as well as pasteurized milk and milk products in this area harbour the pathogenic *C. perfringens* type A and serve as reservoir/ source food poisoning by entry into food chain.

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MOLECULAR CHARACTERIZATION OF *Clostridium perfringens* ISOLATED FROM FOOD AND FOOD PRODUCTS OF ANIMAL ORIGIN AND ITS PUBLIC HEALTH SIGNIFICANCE

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(Bihar Animal Sciences University Patna, Bihar)

CERTIFICATE- I

This is to certify that the thesis entitled, "**MOLECULAR CHARACTERIZATION OF *Clostridium perfringens* ISOLATED FROM FOOD AND FOOD PRODUCTS OF ANIMAL ORIGIN ANDITS PUBLIC HEALTH SIGNIFICANCE**" submitted in partial fulfillment of the requirements for the award of the degree of Master of Veterinary Science in the discipline of **Veterinary Public Health** of the faculty of Post-Graduate Studies, Bihar Animal Sciences University, Patna, Bihar is the bonafide research work carried out by **Dr. RITESH KUMAR, Registration No- BVC/M/VPH/005/2017-18,**son of Shri.**HARERAM YADAV** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Dr. Anjay
(Major Advisor)

Place: Patna

Date: 12/10/19

DEPARTMENT OF VETERINARY PUBLIC HEALTH & EPIDEMIOLOGY

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(Bihar Animal Sciences University Patna, Bihar)

CERTIFICATE- II

This is to certify that the thesis entitled, "**MOLECULAR CHARACTERIZATION OF *Clostridium perfringens* ISOLATED FROM FOOD AND FOOD PRODUCTS OF ANIMAL ORIGIN AND ITS PUBLIC HEALTH SIGNIFICANCE**"submitted by **Dr. RITESH KUMAR, Registration No- BVC/M/VPH/005/2017-18,** son of Shri **HARERAM YADAV** to the Bihar Animal Sciences University, Patna, Bihar in partial fulfillment of the requirements for the degree of Master of Veterinary Science in the discipline of **Veterinary Public Health** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

12/10/19

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DEDICATED TO....

MY MUMMY

AND

PAPA

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Date: **(Ritesh Kumar)**

Place: Patna

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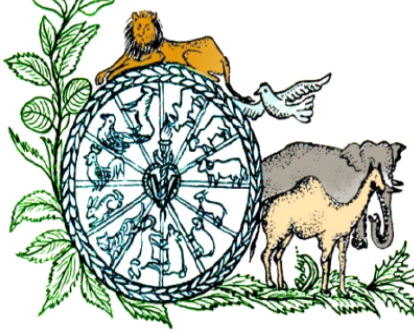
ABBREVIATIONS

%	:	Percentage
µ	:	Micron
µg	:	Micro gr
µl	:	Micro liter
bp	:	Base pair
conc.	:	Concentration
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxy-nucleotide triphosphate
DW	:	Distilled water
et al	:	et alibi
g	:	Gram
h	:	Hour
i.e.	:	That is
M	:	Molar
mA	:	Milli ampere
MCMM	:	Modified cooked meat medium
mg	:	Milligram
ml	:	Milliliter
mM	:	Millimole

Mw	:	Molecular weight
NaCl	:	Sodium chloride
ng	:	Nano gram
NSS	:	Normal Saline Solution
^{o}C	:	Degree centigrade
OD	:	Optical density
p mol	:	Pico mole
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
pH	:	- Log hydrogen ion concentration
rpm	:	Revolutions per minute
SFP	:	Shahidi Fergusan perfrngens agar
TAE	:	Tris Acetate EDTA
Taq	:	*Thermus aquaticus*
Tris	:	Tris-hydroxy methyl aminoethane
UPGMA	:	Unweighted Pair Group Method with Arithmetic mean
UV	:	Ultraviolet
V	:	Volts
Viz.	:	Namely
w/v	:	Weight by volume

Abstract



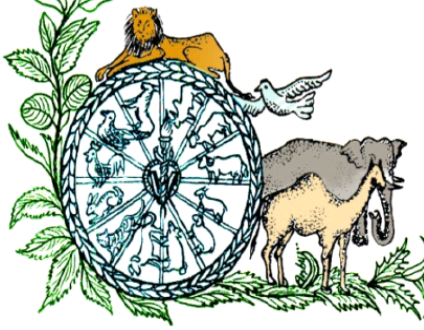
ABSTRACT

C. perfringens are present in diverse environments including gastrointestinal tract microbiota of diseased and non-diseased humans as well as animals. It has been continuously related with clinical systemic and enteric diseases, of both humans and animals. In the present study a total of 251 samples of animal origin foods viz. meat, milk, fish and their products along with human stools were processed for isolation and identification of *Clostridium perfringens* along with toxotyping, antimicrobial susceptibility and phylogenetic study of isolates. The biochemical reaction and PCR study leads to confirmation of *C. perfringens* in 65.07% and 31.87% of samples. By molecular method *C. perfringens* was detected in 51.42%, 26.67%, 48.57%, 60.00%, 33.33%, 4.00% and 26.00% of raw meats, meat products, raw fish, fish products, pasteurized milk, milk products and human diarrhoeic stool samples, respectively. The toxino-typing profile of *C. perfringens* showed that 77.50% of *C. perfringens* were type A, 2.50% (type B) and 16.25% (type C) and 3.75% (type D). Among *C. perfringens* type A, 4.84%, and 29.03% were found to harbour enterotoxin (cpe^{+}) and β2 toxin (cpb2+) gene, respectively while 6.45% isolates harboured both enterotoxin & β2 toxin (cpe^{+} & $cpb2^{+}$) genes. Among *C. perfringens* type B, 50.00% was found to harbour each of enterotoxin and β2 toxin gene separately. Among *C. perfringens* type C, 7.69% and 30.77% isolates were found to harbour enterotoxin and β2 toxin gene. However, 33.33% each isolate of *C. perfringens* type D were found to harbour enterotoxin, β2 toxin and both enterotoxin & β2 toxin genes, respectively. The MIC based generated antibiotic susceptibility profile showed that 50% *C. perfringens* isolates from raw meat samples were resistance to CD followed by 39% to MOX and TI, 11% to TE and 5.6% to AMC and PI. Whereas, 25% isolates from meat products samples were resistance to CD and TE. 47% of isolates from raw fish samples were resistance to MOX followed by 41% to CD, 29% to TE and 5.9% to CTR as well as PIT whereas 11% isolates from fish product samples were found as resistance to AMC, MOX and PIT. The isolates from pasteurized milk samples showed that 41% isolates were resistance to CD followed by 35% to TE, 31% AMP, 24% from C, 18% TI, 12% CTR and 5.9% from AMC, CTX, MOX and PI. Whereas, the isolates from milk product samples did not showed resistance of any of the antibiotics used under study. The antibiotic susceptibility profile *C. perfringens* isolates from human diarrhoeic samples showed that 23% isolates were resistance to TE followed by 7.7% to MOX.

The phylogenetic study of *C. perfringens* type A isolates using ERIC-PCR showed that 85.15% isolates circulating in both meat and meat products as well as human population were of similar genetic makeup. The genetic linkage of *C. perfringens* type A isolates of fish and fish products with human diarrhoeic isolates suggested that three different clones constituted by ~50% and two each of~13.33% of isolates with >95% genetic similarity circulating in both fish and human population while *C. perfringens* type A isolates of raw and pasteurized milk with human diarrhoeic isolates ~33% of isolates of similar genetic makeup with 95% genetic similarity circulating in both pasteurized milk as well as human population.

From the finding of present study, it may be presumed that meat and meat products, fish and fish products as well as pasteurized milk and milk products in this area harbour the pathogenic *C. perfringens* type A and serve as reservoir/ source food poisoning by entry into food chain.

Introduction



1 INTRODUCTION

Clostridium perfringens is a Gram-positive, rod-shaped, anaerobic spore forming ubiquitous bacterium of the genus *Clostridium*, characterized by oval, sub-terminal spores that bulge from the mother cell (Prescott *et al*. 2016). *C. perfringens* is ubiquitous in nature and normally present in decaying vegetation, marine sediments, insects, soil and in the intestinal tract of humans and other vertebrates. (Vinod *et al*., 2014). It able to grows between a temperature range of 15^0C to 50^0C with an optimum of 45^0C. The generation time for most strains is below 20 minutes or sometimes 8 minutes at temperature between 33^0C and 49^0C, (Labbe, 2000).

C. perfringens is considered as an important foodborne zoonotic pathogen and most commonly reported as a contaminant of food. It is considered as the second most common causative agent of food borne diarrhea in United State, after *Salmonella* (Brynestad and Granum, 2002; Scallan *et al*., 2011). Animal and human fecal material are considered the natural source of contamination of food products. However, its transmission to human may occurs via food, water, animal contact, person-to-person and various other way at the point of consumption. Unhygienic handling of food and food products and cross- contamination during and after processing recognized as prime factor for its transmission through foods (Butler *et al.,* 2015). As *C. perfringens* is a spore forming microorganism, hence survive in adverse conditions and may contaminate the various food and food products of animal origin such as milk, meat, fish and their products either before processing with survival during cooking or after processing due to unhygienic handling of processed food (Santos *et al.,* 2002 and Potter, 2001). The consumed *C. perfringens* may produce enterotoxin in the intestine during sporulation of vegetative cells resulting in to watery diarrhea and abdominal pain (McClane, 1996). It may be isolated from animal after slaughtering as post-mortem invasion or externally from contaminated hands, skin of animals, soil, water and processing equipment (Saito, 1990).

On the basis of presence or absence of 04 major toxins *C. perfringens* is classified into 5 types (A, B, C, D, and E). The 4 major toxins produced by it includes alpha (α), beta (β), epsilon (ε), and iota (ι) (Alves *et al*., 2014). Among these toxins α toxin are produced by all *C. perfringens* including type A, type B produces β and ε toxins along with α, type C produces α and β toxins, type D produce α and ε toxins, while type E produce α and ι toxins.

C. perfringens types B to E are recognized as "frank pathogens" for animals and human, while type A strains are recognized as commensal to gastro intestinal tract of animals and human. Certain strains of *C. perfringens* type A produce an exotoxic component known as enterotoxin which recognized as the diarrheagenic toxin responsible for *C. perfringens* food-borne outbreaks (Monma *et al*., 2015). However, strains of *C. perfringens* also reported to produce Beta2-toxin which is a pore-forming cytolytic toxin reported as an accessory toxin in *C. perfringens*-associated non-foodborne diarrhea (Fisher *et al*., 2005). A high number of 10^6–10^8 cells/g of *C. perfringens* type A is required to be ingested for causation of foodborne illness such as food poisoning, sporadic diarrhea, antibiotic-associated diarrhea and nosocomial diarrhea. The food poisoning of *C. perfringens* type A is characterized by the symptoms of diarrhea and abdominal cramps with the rare signs of fever or vomition. The course of disease can be peracute, acute, or chronic with symptom of intense abdominal pain, depression and bloody diarrhea (Shimizu *et al.*, 2002 and Rahimi *et al.*, 2011). Deaths due to *C. perfringens* type A food poisoning are not common but may occur in the elderly and debilitated patients. The food poisoning caused by *C. perfringens* type A reported to kill seven people in the USA and 100 people in the UK (Byrne *et al*., 2008 and Scallan *et al*., 2011).

The toxinotyping of *C. perfringens* can be performed by mouse lethality tests and determining sero-protection with neutralizing antibodies raised against respective toxinotype (Sterne and Batty, 1975). As these methods are time consuming and expensive, in the recent years, various PCR protocols, including multiplex PCR assays, have been established to toxin-typing of *C. perfringens* isolates with respect to the genes *cpa, cpb, etx, iap, cpe* and *cpb2*, encoding the alpha, beta, epsilon, iota, enterotoxin and β_2-toxin, respectively (Al-Khaldi *et al.* 2004; Baums *et al.*, 2004).

The extensive misuse of antibiotics in healthy animals reared for human food has augmented the scope of antimicrobial resistance, globally (FAO, 2016). Antibiotics in animal husbandry practice may be used for metaphylaxis, prophylaxis and

as growth promoters (McEwen and Fedorka-Cray, 2002) which may promote the emergence and spread of antimicrobial resistance in *C. perfringens* (Slavic *et al*., 2011). Resistance of *C. perfringens* isolates to bacitracin, tetracycline, clindamycin, loncomycin, and erythromycin has been reported in many countries (Slavic *et al.,* 2011, Gholamiandehkordi *et al.,* 2009, Chalmers *et al*, 2008, Johansson *et al.,* 2004, Martel *et al.,* 2004).

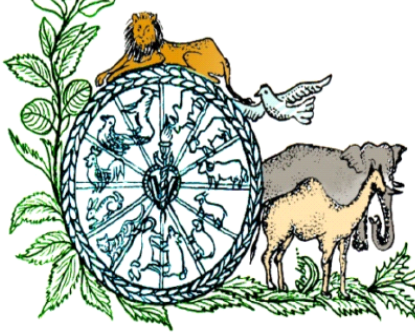
Genotyping has an important role for determination of origin or source of bacterial isolates and also to identify the genetic relatedness between isolates. Recent molecular techniques available for studying genetic diversities of bacterial pathogens may include pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), repetitive extragenic palindromic- PCR (REP-PCR), enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) and multilocus sequence typing (MLST) (Anjay *et al*., 2015). Among various techniques ERIC-PCR has been reported as similar discriminatory power as RAPD-PCR for differentiation of pathogens (Szczuka and Kaznowski, 2004). In addition to this, ERIC-PCR also has an advantage over other genotyping methods, as it requires only a low quantity of DNA for amplification (Thomas *et al*., 2009). Very few epidemiological investigations have been performed to establish a clonal linkage between *C. perfringens* Type A form raw and processed foods of animal origin and human diarrheic stool. With this background information the present research work was performed with the following objectives:

Objectives:

1. To study the occurrence of *C. perfringens* among foods of animal origin and human diarrhea cases.

2. To determine the pathogenic potential of *C. perfringens* isolates and their public health significance.

Review of Literature



2 REVIEW OF LITERATURE

Clostridium perfringens previously recognised as *Bacillus aerogenes capsulatus*, *Bacillus perfringens*, *Bacillus welchii* or *Clostridium welchii* is an anaerobic spore-forming, Gram positive rod-shaped bacterium (Hassan *et al*., 2015). This organism was first isolated and recognized in 1891 by William H. as a novel bacterium.

C. perfringens are present in diverse environments including soils, food, sewage and gastrointestinal tract microbiota of diseased and non-diseased humans as well as animals. *C. perfringens* has been continuously related with various clinical systemic and enteric diseases, of both humans and animals viz., gas gangrene, necrotic enteritis and food poisoning, non-food borne diarrhoea and enterocolitis (Sim *et al*., 2014; Heida *et al*., 2016).

The pathogenicity of *C. perfringens* may be governed by the toxins and virulent enzymes encoded by virulence genes. The most common toxins type produced by *C. perfringens* includes α-toxin, β-toxin, ε-toxin and ι-toxin, enterotoxin (CPE) and NetB (Petit *et al*., 1999) and recently according to the combination of toxins produced *C. perfringens* are typed into seven toxinotypes: A–G (Kiu and Hall, 2018). The alpha-toxin encoded by cpa gene is produced by all strains of *C. perfringens*, recognised to cause cell necrosis in gas gangrene due to hydrolysis of cell membrane phospholipids. Beta-toxin, encoded by cpb gene, is a plasmid-encoded pore-forming toxin responsible for intestinal necrotic and systemic enterotoxaemia, in humans and neonatal animals (Timoney *et al*., 1988; Uzal *et al*., 2009). *C. perfringens* type C strains that possess the cpb gene were associated with historical Clostridial gut infections such as Darmbrand and Pig bel disease (Murrel, 1983). Beta2-toxin is pore-forming cytolytic toxin encoded on plasmid by cpb2gene recognised as a novel toxin which shares <15% sequence homology with β-toxin (Hunter *et al*., 1993) and responsible for NE in piglets, and enterocolitis in foals (Gibert *et al*., 1997). It is reported as an accessory toxin in C. perfringens-associated non-foodborne diarrhoea (Fisher *et al*., 2005). Epsilon-toxin recognised as a deadly toxin, encoded by plasmid associated etx gene produced by *C. perfringens* type B and D strains responsible for enterotoxaemia in sheep and goat (Popoff, 2011). The iota-toxin is a cytoskeleton-damaging toxin encoded on plasmids by iap and ibp gene and responsible for apoptosis and cell death (Hilger *et al*., 2009). The enterotoxin encoded by *cpe*

gene is recognised as the key toxin to cause food-poisoning and non-foodborne diarrhoea and also responsible for disruption of intercellular claudin tight junctions in gut epithelial cells (Shinoda *et al*., 2016; Eichner *et al*., 2017). The pathogenesis mechanisms in principal food poisoning may be as a result from CPE-induced tight junction rearrangements or pore-formation.

The extensive misuse of antibiotics in healthy animals reared for human food has augmented the scope of antimicrobial resistance, globally (FAO, 2016). Antibiotics in animal husbandry practice may be used for metaphylaxis, prophylaxis and as growth promoters (McEwen and Fedorka-Cray., 2002) cited by the fact of high consumption of antibiotics as growth promoters than therapeutic, globally to meet the growing demand of protein-rich foods of animal origin. The commensal bacteria of livestock may acquire antibiotic resistance genes and accelerate the problem by transmission of resistant microorganisms to humans through livestock products and environment (Marshall and Levy,2011., Allen, 2014). The incidence of antibiotic resistance bacteria in food animals and their food products, such as methicillin-resistant *Staphylococcus aureus* (Price *et al*., 2012), multidrug-resistant *Campylobacter* spp. (Ewnetu and Mihret, 2010), colistin-resistant *E. coli* (Liu *et al*., 2016, Fernandes *et al*., 2016), multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Al Bayssari, 2015), carbapenem-resistant (Fischer *et al*., 2012) and extended-spectrum β-lactamase (ESBL)-producing (Fischer *et al.,* 2012., Boonyasiri *et al.,* 2014) has been reported globally with higher incidence in developing Asian and African countries (Founou *et al*., 2016). The presence of antimicrobial residues in food animal products has been reported from India which indicates the unregulated and extensive use of antibiotics (Ganguly, 2011). Earlier studies reported that most of the *C. perfringens* isolates were sensitive to various antibiotics viz., ciprofloxacin, ofloxacin, enrofloxacin, norfloxacin, tetracycline, nitrofurantoin, amikacin, piperacillin, cefuroxime, ceftazidime, cephoxitin, ceftriaxone, amoxicillin/clavulinic acid chloramphenicol, penicillin G and sulfatriad (Singh *et al*., 2005; Rahman *et al*., 2012; Singh and Bist, 2013; Sarkar *et al*., 2013). It was reported that overuse and misuse of antibiotics in human and veterinary medicine without proper antibiotic susceptibility testing in developing countries, may be a major factor for increased antimicrobial resistance (Wellington *et al*., 2013).

Genotyping has an important role to trace the origin or source of bacterial isolates and also to identify the genetic relatedness between isolates. Recent molecular techniques available for

studying genetic diversities of bacterial pathogens include pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), repetitive extragenic palindromic- PCR (REP-PCR), enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) and multilocus sequence typing (MLST) (Anjay *et al*., 2015).

ERIC-PCR has been reported as similar discriminatory power as RAPD-PCR for differentiation of pathogens (Szczuka and Kaznowski, 2004). In addition to this, ERIC-PCR also showed advantages over other genotyping methods, for instance, it required low quantity of DNA for amplification (Thomas *et al*., 2009).

Distribution of *A. hydrophila* in foods of animal origin and human diarrhoeic samples

C. perfringens is most commonly found in the gastrointestinal tract of both humans and animals and known to cause human and animal diseases along with food poisoning. Food-borne diseases considered as a global health problem and more than 200 different diseases are known to be transmitted via food and food products of animal origin. Among various pathogens *C. perfringens* is responsible for one of the most common types of food poisoning. Foods of animal origin such as milk, poultry meat and fishes are rich in protein and reported with a great importance in the occurrence of food poisoning depending on *C. perfringens*. The study performed by Gurmu *et al*., (2013) to isolate *C. perfringens* from foods of animal origin comprised by chevon, pork, fish, beef and liquid raw milk, curd, pasteurized milk powder, pasteurized liquid milk and ice cream in Guwahati, India along with detection of enterotoxin genes showed a distribution of 8.25% of *C. perfringens* among foods of animal origin with 15.15% isolates harbouring enterotoxin gene (*cpe*) represent its public health importance.

Distribution of *C. perfringens* in chicken meat and meat product samples

Meat is a raw material of high value and the quality of its end-products are highly dependent on the quality of the raw materials. Different important characteristics such as meat composition, sensory quality, bacteriological quality and nutritional value are responsible for meat quality and acceptability by consumers. However, meat quality can be affected by farm rearing of food animals, pre-slaughter handlings of food animals, a series of the postmortem events, as well as by processing and storage conditions. Among various pathogens *C. perfringens* may be a

primary contaminant of chicken meat as it was isolated from 70.4% of chicken meat of slaughterhouses and markets in India (Singh *et al*., 2005)

Stagnitta *et al.* (2002) performed a study to determine the prevalence of enterotoxigenic *C. perfringens* in some non-industrial meat foods in San Luis, Argentina. They analysed 515 samples of meat food (315 fresh sausages, 100 hamburgers and 100 samples of minced meat) and reported an isolation of 24.46% *C. perfringens* with 7.14% as enterotoxigenic all belonged to type A by PCR and RPLA. They also reported that 97.20% of *C. perfringens* isolates belonged to type A, 1.59% to type C and 0.79% to type E.

Nowell *et al*. (2010) performed a study on fresh and frozen chicken thigh or wing samples purchased from retail grocery stores in Ontario and reported a prevalence of 66% and 67% *C. perfringens*, respectively. They also identified netB gene in 21% of isolates along with cpb2 gene in 51% of isolates however, no isolates were reported to harbour cpe gene. Gharaibeh *et al*. (2010) performed a study to isolate *C. perfringens* from Jejunal swabs of clinical cases of commercial broiler flocks, along with typing by multiplex PCR, and to determine the antimicrobial susceptibility by micro-dilution method. They reported that a total of 43.23% samples were positive for *C. perfringens* typed as type A and nonenterotoxin producers. They also reported that *C. perfringens* type A showed sensitivity to penicillins, tetracyclines, quinolones and phenicols as evident by the low MIC_{50}. While, Lincomycin, erythromycins, and tilmicosin showed very high MIC_{50} of ≥256 mg/ml. However, tylosin, amoxicillin, ampicillin, penicillin, florfenicol, danofloxacin, enrofloxacin, chlortetracycline, doxycycline, and oxytetracycline had variable MIC_{50} of 64, 0.5, 1, 1, 8, 4, 8, 4, 8, 0.5 mg/ml, respectively.

Guran and Oksuztepe, (2013) performed a study to investigate the presence of *C. perfringens* in chicken meat parts including breast, wing, drumstick and leg quarter and to detect the *cpa*, *cpb*, *etx*, *iap*, *cpe* and *cpb2* toxin genes and reported that 94% of wing samples 80% of leg quarter, 66% of drumstick samples and 66% of breast samples were contaminated with *Cl. perfringens*. They also reported that 97·6% of *C. perfringens* isolates harboured only *cpa* toxin gene (type A) with 2·1% encoded with both *cpa* and *cpb2* toxin gene (type A-*cpb2*) and 0·1% with both *cpa* and *cpe* toxin genes (type A-*cpe*).

Afshari *et al*. (2015) performed a study to isolate and type *C. perfringens* along with detection of *cpe* gene in *C. perfringens* from broilers' meat from retail meat shops of Mashhad city in Northeastern of Iran. They reported an isolation of 15.50% of *C. perfringens* with 29.03% as

type A and 70.96% as type C. The detection of *cpe*-positive *C. perfringens* was reported in 25.00% of type C isolates. They concluded that *C. perfringens* type C is the most common type in broiler chicken carcasses.

Aras and Hadimli, (2015) isolated 31.7% C. perfringens from meat samples harboured cpa gene with a distribution of 40%, 31% and 24% isolates from beef, chicken meat, and turkey meat samples, respectively. They reported that 88.42 % isolates were type A, with 2.38% carried both cpa and cpe genes (type A-cpe) and 9.52% carried cpa and cpb2 gene (type A-cpb2). While 6, 3, and 2 isolates belonged to beef, chicken and turkey meat samples, were genotyped as type C, type D, and type E, respectively.

Shaltout *et al*. (2017a) analysed 125 random raw and half cooked chicken meat samples collected from various retail stores and supermarkets in Qualyubia governorate for detection of *C. perfringens* and to detect cpa, etx, and cpe toxin genes. They reported that 24% of breast, 32% of raw thigh, 20% of nuggets, 16% of panee, and 16% of frankfurter samples were contaminated with *C. perfringens*. They also reported that 29.6% of C. perfringens isolates carried only alpha toxin gene (type A), and 3.7% of them expressed both alpha and epsilon toxin genes (type D); while cpe gene was not detected in any examined isolate.

Shaltout *et al.* (2017b) collected a total of 100 random samples of raw and cooked beef and chicken from the university student hostel and examined for presence of *C. perfringens* and reported that 15% of samples harboured *C. perfringens* with a distribution of 24% in raw chicken, 12% in cooked chicken, 16% in raw beef and 8% in cooked beef samples. Yadav *et al*. (2017) performed a study to characterize, and determine antibiogram of *C. perfringens* type A isolated from the feces of human and animal diarrhoeal cases, as well as healthy animals, meat of pigs and goats, gills and intestine of fish and samples from fish pond. They reported that toxinotyping of *C. perfringens* type A isolates showed an overall prevalence of 43.2% of *C. perfringens* type A with only cpa+ gene, 45.4% with cpa+ and cpb2+ genes, 4.9% with cpa+ and cpe+ genes, however, 6.6% with cpa+, cpb2+ and cpe+ genes. They also reported that 83.7% of isolates were resistant to three or more antibiotics.

Regan *et al*. (2018) analysed the food samples from New York city for presence of *C. perfringens* along with detection of specific toxin genes and reported that 16% samples were positive for *C. perfringens* constituted by 91.2% as type A or E, 2.9% as type B, and 5.9% as type D.

Zhang *et al*. (2018) performed a study for detection of *C. perfringens* and reported that 23.1% of broiler chickens and 15.1% of retail chicken meat samples in central China harboured *C. perfringens*. They also reported that type A was the preponderant genotype with 3% encoded with enterotoxin (cpe) gene.

Distribution of *C. perfringens* in freshwater fishes

Fish and fish products are recognized as most important dietary nutrients for various health benefits, worldwide. On the other side besides of high nutrition values fishes are highly prone to contamination by large number of water borne pathogens including Aeromonads. They are considered as most important vehicle for transmission of pathogenic bacteria naturally occurring in the aquatic environment may lead to cause human morbidities and mortalities worldwide.

Cai *et al*. (2008) analysed the intestinal content of freshwater fishes caught from water reservoir for the occurrence of *C. perfringens* along with PCR amplification for toxin types, enterotoxin and beta2 toxin. They reported that 17.9% of intestinal contents samples from freshwater fish harboured *C. perfringens*. They also reported that 77.3% of isolates were C. perfringens toxin type C encoded by alpha and beta toxin gene, 17.3% were type A encoded by alpha toxin gene and 5.3% were type B encoded by alpha, beta and epsilon toxin gene. In addition, 62.7% isolates were found to encoded with beta2 toxin gene while none of the isolates were found to encode for enterotoxin gene.

El-Shorbagy *et al*. (2012) carried out a research on processed and unprocessed fish samples collected from different localities in El-Sharkia Governorate, Egypt to isolate and characterize. They reported an incidence of *C. perfringens* in 57.1% of processed fish and 59.6% in unprocessed fish samples. They reported that typing of *C. perfringens* isolates showed that 84.8 isolates were toxigenic with predominance of *C. perfringens* type A.

Das and Jain. (2012a) evaluated the genotypes of *C. perfringens* in fish and fish products from Tamil Nadu and Kerala states of India. They reported that on the basis of biochemical tests and phenotypic determination of virulence factors, 27.24% isolates presumed as *C. perfringens*. Their molecular study showed that 100% isolates harboured *cpa* toxin genes and belonged to C. perfringens type A however, 79.26% isolates also carried additional cpb2 toxin genes while none of the isolates were found to harbour beta, epsilon, iota and enterotoxin genes.

Das and jain. (2012b) evaluated the intestinal contents of fishes for isolation and identification of *C. perfringens* and reported that 18.35% of the samples were positive for *C. perfringens* that harboured cpa gene while 59.18% isoaltes additionally harboured cpb2 gene. However, none of the isolates were found to harbour beta, epsilon, iota and enterotoxin genes.

Nashwa *et al.* (2016) examined seafood samples collected from different supermarkets in El Dakahlya Governorate, Egypt and reported an incidence of 13.3% *C. perfringens*. All isolates were confirmed as *C. perfringens* type "A" (alpha toxin) with distribution of *cpe* genes in 15% and 20% isolates using PCR and real time PCR, respectively.

Sabry *et al*. (2016) performed a study to determine the prevalence of C. perfringens fresh fish, and humans handler and reported that 54.5% fish from aquaculture, 71% from the markets and 63% humans handler were positive for *C. perfringens*. Their genotyping revealed that *C. perfringens* type A with enterotoxin gene negative was the predominant strain among fresh and canned fish, humans and water. They also reported that *C. perfringens* types A with CPE+ were found only in fresh fish while C. perfringens type E strain was also detected in fish, humans, and water.

Yadav *et al*. (2016) studied for isolation of *C. perfringens* from freshwater fish and fish products from Kolkata city of India along with their genotypes and antibiogram profile. They reported a prevalence of 23.52% *C. perfringens* comprising of 24.63% from fresh water fish and 21.21% from fish products by cultural isolation and biochemical characterization. In molecular study they reported that 100% isolates comprising of 70.83% from fresh water fish and 29.17% from fish products harboured 16S rRNA and cpa toxin gene. However, 70.83% of cpa positive isolates comprising of 70.58% from fresh water fish and 71.42% from fish products also harbor additional cpb2 toxin gene, while none of the isolates found to be positive for cpb, εtx, ιap and cpe genes. They reported that 76.47% of the isolates were multidrug resistant and ciprofloxacin as well as amoxicillin/clavulanic acid was the most sensitive drugs.

Distribution of *C. perfringens* in pasteurized milk and milk product samples

Milk is a highly nutritious food but ideal for microbial growth which leads to deterioration of the quality and makes them unsuitable for human consumption (Fao, 2001). The contamination of raw milk may take place by various conditions associated with farm environment like air, milking equipment, rinsing water feed, grass, silage, soil and faeces (Coorevits *et al*., 2008). It is

well known fact that *C. perfringens* is an anaerobic spore former that able to survive the pasteurization process (Garde *et al*., 2011) may leads to various clinical systemic and enteric diseases in humans.

Chaturvedi and Shukla. (2015) performed a study for the presence of *Clostridium* species in raw milk, curd, cheese and paneer samples and reported that 31% samples were positive for Clostridium species of which 35.48% were *C. perfringens.* They also reported the high occurrence of Clostridium perfringens in milk (26%) and milk products.

Osama *et al*. (2015) performed a study for isolation and identification of *C. perfringens* from market milk, damietta cheese, milk powder, ras cheese, kariesh cheese, condensed milk and sterilized milk packs collected from different localities of El-Dakahlia province, Egypt. They reported the prevalence of *C. perfringens* among 20, 60, 20, 60, 36, 0.0 and 0.0% of raw milk, Damietta cheese, milk powder, Ras cheese, Kariesh cheese, sterilized and condensed milk respectively. Their antimicrobial resistance study showed that 100% of *C. perfringens* isolates were resistant to Colistin and Ampicillin followed by 91.8% of resistance to Lincomysin, 75.5% to Erythromycin,73.4% to Ampicillin-Sulbactum, 71.7% to Neomycin, 69.38% to Amoxicillin, 67.34% to Streptomycin, 63.26% to Spiramycin, 53.06% to Clindamycin and Tetracyclin, 42.8% to Cephradine, 40.8% to Pefloxacin, 36.73% to Gentamycin, 30.6% to Norfloxacin and 18.36% to Vancomycin.

Ahsanullah *et al*. (2019) conducted a study to isolate the causal agent of food poisoning in milk samples and dairy products from various zones of Quetta city. They analysed a total 1120 samples and reported that 19.28% were positive for *C. perfringens*. They also reported a distribution of *C. perfringens* as 8.01% in milk, 3.73% in cheese, 2.74% in butter and 4.71% in yogurt. Their antibiotic sensitivity test result showed that isolates were highly sensitivity to penicillin, quinolones, aminoglycosides and chloramphenicol, while resistant to cephalosporin's, lincosamides, macrolides and sulphonamides.

Aliwa and Mulwa, (2019) performed a study to estimate the prevalence of antibiotic resistant *C. perfringens* in camel milk in Isiolo County, Kenya and reported that 48.05% of sample harboured *Clostridium* species with 19.1% as *C. perfringens*. They also reported that 61.02% of isolates were resistant to ampicillin, 47.46% to Sulphamethazole, 45.76% to Cotrimazole, 44.07% to Streptomycin, 42.37% to Chloramphenicol, 40.68% to Kanamycin, 37.29% to Tetracycline and 35.59% to Gentamycin.

Distribution of *C. perfringens* in human diarrhoeic stool samples

Akhi *et al.* (2015) examined stool samples including diarrhea and non-diarrhea and reported an isolation of 61.02% of *C. perfringens* of which 95.18 harboured alpha-toxin (phospholipase C) production gene. They also reported that 9.09% and 4.34% of *C. perfringens* isolates from diarrhoea and non-diarrhoea samples harboured *cpe* genes. In their study they also reported that 43.03% *C. perfringens* isolates showed no resistance to any antibiotic while 22.78% with one and 34.17% showed multiple resistance to imipenem, metronidazole, ceftriaxone, clindamycin, chloramphenicol and penicillin.

Nagpal *et al*. (2015) performed a qPCR assays and detected Alpha-toxigenic *C. perfringens* in 36 % infants and 33 % adults faeces.

Azimirad *et al*. (2019) investigated the prevalence and molecular epidemiology of *C. perfringens* from faecal samples from Iranian diarrheal patients suspected of having antibiotic associated diarrhoea (AAD). They reported a detection of 64.15% *C. perfringens* isolates from AAD and 35.85% from non-AAD, with 84% and 16% of A and F toxinotypes, respectively. They also reported that 41.2% of type F strains were *cpb2*-positive while 13.2% of the isolates from AAD patients were enterotoxigenic *cpe*-positive.

Phylogenetic analysis of *C. perfringens* isolates

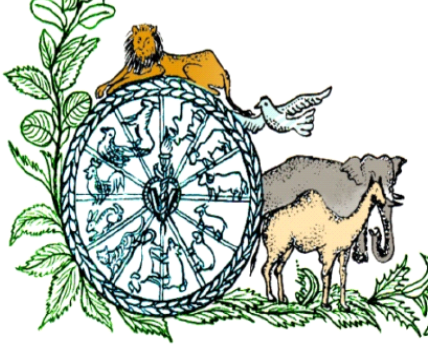
An elementary principle for microbial subtyping is that isolates harboured from an epidemiologically interrelated cluster arise from a common clone which share common characteristics that discriminate them from epidemiologically unrelated isolates categorized as the same species. Since, it is always possible to isolate the similar subtypes of the organisms from different unrelated sources, the epidemiological relationship between subtype can be determined by several typing methods. However, subtyping practices are evaluated on different parameters including typeability, discrimination and utility. The recent techniques using molecular method available for studying genetic relationship of bacterial pathogens included pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), repetitive extragenic palindromic- PCR (REP-PCR), random amplification of polymorphic DNA (RAPD) PCR and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) (Anjay *et al*., 2015). Out of these molecular methods PFGE has been recognised as one of the best typing methods for various microorganisms (Alavandi *et al*. 2001). However, other techniques such as

RAPDs and ERIC-PCR assays for typing of pathogens are easier, inexpensive and faster to accomplish, and are therefore appropriate for epidemiological studies of the pathogenic microorganisms (Versalovic *et al*., 1991; Talon *et al*., 1996).

Xue-qin *et al*. (2009) studied the epidemiology of *C. perfringens* colonizing healthy birds, using isolates from fresh feces and cecal samples of healthy chicken in ten farms of 8 cities in Sichuan province using amplified fragment length polymorphism (AFLP) and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). They reported that 12 AFLP genotypes and 8 ERIC-PCR genotypes were prevalent among 34 C. perfringens type A isolated from 600 bird samples and concluded that *C. perfringens* isolates from healthy chickens in Sichuan province showed a low diversity with close correlation between the regions and epidemiology.

Afshari *et al*. (2016) performed a study to examine the genetic diversity of 49 isolates of *C. perfringens* type A of diverse sources using RAPD-PCR and REP-PCR and reported that RAPD-PCR revealed the most genetic diversity among poultry isolates, while human isolates showed the least genetic diversity while REP-PCR analysis did not show further differentiation between various types of strains.

Materials and Methods



3 MATERIALS AND METHODS

1.1 Design of study

The study was designed for isolation of *Clostridium perfringens* from the samples including foods of animal origin and their products, fish and their products and human stool. Further, the study was also extended for biochemical and molecular confirmation of isolates along with toxin and antimicrobial resistance profiling and their public health significance.

1.2 Sample collection area and experimental samples

A total of two hundred and fifty-one (251) samples related to animal origin foods were collected from different shops located in Patna metropolis area viz., Patliputra, Kankarbagh, Gola road and Maurya lok. Human stool samples were collected from Central Diagnostic Lab., Boring Canal Road, Patna.

1.3 Collection and Transportation of samples

a. Fish & Fish products:

Approximately 100 g of gill and flesh of market fish (35) and cooked fish with gravy (15) sold at Patna Metropolis were collected from different shop in a sterile sample collection bag and kept in transportation box provided with ice pack. The samples were transported to the laboratory of department and processed within an hour of collection.

b. Meat & Meat products:

Approximately 100 g of raw chicken meat (35) and cooked meat with gravy (15) sold at Patna Metropolis were collected from different shops in a sterile sample collection bag and kept in transportation box provided with ice pack. The samples were transported to the laboratory of department and processed within an hour of collection.

c. Pasteurized milk & milk products:

The pasteurized milk (51) packets and milk products (50) including flavored milk, Buttermilk and Lassi of reputed firms were purchased from different milk booth established in Patna Metropolis. All types of milk samples were kept in transportation box provided with ice pack. The samples were transported to the laboratory of department and processed within an hour of collection.

d. Human stool:

Approximately 10-15g of human stool samples (50) were collected from Central Diagnostic Lab., Boring Canal Road, Patna and kept in transportation box provided with ice pack. The samples were transported to the laboratory of department and processed within an hour of collection.

1.4 **Media, buffers and reagents**:

The details of the preparations of media, buffer and other reagents used in the present study have been given in the appendixes. The media used for bacteriological procedures have been presented in appendix 2, 1, reagent for biochemical identification in appendix 2, reagent for genomic DNA isolation in appendix 3 and reagent for Agarose Gel Electrophoresis appendix in appendix 4.

1.5 Chemicals used for molecular studies:

All molecular grade chemicals, kits and bacteriological medias used in this study were procured from Fermentas (USA), Thermo-Scientific (Lithuania), Qiagen (Netherlands), Hi-Media (India) and other reputed firms. The blood and tissue kit and MIC strips were purchased from Qiagen (Netherlands) and Hi-Media (India), respectively.

The chemicals used in the study included *Taq* DNA Polymerase, 10X PCR amplification buffer, magnesium chloride ($MgCl_2$), dNTP mixture (Qiagen and Thermo Scientific), agarose (Hi-media and GenNei India), 6X Gel loading dye (Thermo scientific), master mix (Thermo Scientific), Nuclease free water (Hi-Media, India).

1.6 Other general chemicals–

The other chemicals included under study viz., ethanol, phenol, chloroform, peptone, phenol red indicator, glycerol, gelatin, motility nitrate media, lactose, peptone, beef extract, tris base, EDTA, agarose and sodium chloride were purchased from Hi-media (India).

1.7 Plastic wares and glass wares:

Plastic wares used during this study were obtained from Tarsons (India), Moxcare (India) and Genexy (India). Whereas Glassware's were obtained from Borosil (India) and touff (India). Glass wares were thoroughly washed and sterilized wherever necessary as per the recommended procedures.

1.8 Equipment's –

Some important equipment's used in this study includes PCR machine (sure cycler 8800, Agilent, USA), Electrophoresis unit (Thermo scientific, USA), Spinwin microcentrifuge (Tarsons, India), Vortex shaker (Tarsons, India) Gel-doc XR (Bio-rad, USA), Micropipettes (Thermo scientific, USA), Weighing balance (Precision gold balance, Wencer, India), Vertical laminar flow (Ikon, IK-137, UK), Bio-safety cabinet (MES-139, Matrix eco-solutions, India), Nano-spectrophotometer (Genova nano, Jenway, UK), Incubator (Associated Scientific Technology, India), Refrigerator (LG, South Korea) and -20 Deep fridge (Blue star, India).

1.9 Oligonucleotide Primers:

The Oligonucleotide primers used in this study were custom synthesized from Eurofins Genomics Pvt. Ltd. (India). The details of the primers have been presented in table 1.

2.0 METHODS

2.1 Enrichment:

Approximately 1g of fish and fish products; meat and meat products and human stool and 1 ml of pasteurized milk, and milk product samples were inoculated in test tube containing 10 ml of sterile modified cooked meat medium (MCMM) and incubated for overnight at 37º C under strict anaerobic condition using anaerobic jar with anaero Gas Pack (Hi-Media, India).

2.2 Isolation on selective media:

The tubes showing yellow colored change in MCMM were 10 fold diluted in sterile distilled water (100 µl grown broth and 900µl DW) and 100µl of this diluted broth was plated on the base plate of Shahidi Ferguson Perfringens (SFP) agar overlaid by 5 ml of SFP Agar and incubated at 37º C for 18-24 hrs under strict anaerobic condition using anaerobic jar with anaero Gas Pack (Hi-Media, India).

Table 1: The details of the oligonucleotide primers used under study:

Sr. No.	Primer Sequence	Target Gene	Expected Product Size (bp)	References
1	F: TAACCTGCCTCATAGAGT R: TTTCACATCCCACTTAATC	*16S rRNA*	481	Tonooka *et al*., 2005
2	F: GCTAATGTTACTGCCGTTGACC R: TCTGATACATCGTGTAAG	*cpa*	324	Titball *et al*., 1989
3	F: GCGAATATGCTGAATCATCTA R: GCAGGAACATTAGTATATATCTTC	*cpb*	196	Hunter *et al*., 1993
4	F: GCGGTGATATCCATCTATTC R: CCACTTACTTGTCCTACTAAC	*etx*	655	Hunter *et al*., 1992
5	F: GGAGATGGTTGGATATTAGG R: GGACCAGCAGTTGTAGATA	*cpe*	233	Czeczulin *et al.,* 1993
6	F: AGATTTTAAATATGATCCTAACC R: CAATACCCTTCACCAAATACTC	*cpb2*	567	Garmory *et al*., 2000
7	ERIC1: ATGAAGCTCCTGGGGATTCAC ERIC2: AAGTAAGTGACTGGGGTGAGG	--------	--------	Versalovic *et al*., 1991

F: Forward primer, R: Reverse primer

The characteristic colony of *C. perfringens* from SFP i.e., typical round (2-3mm in diameter) with black colored showing opalescence (Fig. 1) was picked up using straight nichrome wire and inoculated in MCMM and incubated at 37^0C for 18-24 hrs under strict anaerobic condition. All presumptive isolates from MCMM were transferred to 30% glycerol stock for storage at -20^0C.

2.3 Biochemical characterization of *C. perfringens*:

A single characteristic colony of presumptive *C. perfringens* on SFP agar i.e., black coloured (2-3mm in diameter) from each plate were confirmed by Gram staining and biochemical tests viz., motility, nitrate reduction, lactose fermentation, indole and gelatin liquefaction.

Motility nitrate test was performed by inoculation of a loop full of test culture from MCMM in Motility Nitrate medium with incubation at 37º C for 24 hrs under strict anaerobic condition. The isolates of *C. perfringens* showed negative reactions for motility and positive reaction for nitrate reduction (Fig. 2) were further tested for lactose fermentation & gelatin liquefaction.

Lactose fermentation and gelatin liquefaction tests were performed by stab inoculation of test culture from MCMM in Lactose gelatin medium with incubation at 37^0C for 24 hrs followed by incubation at 5^0C for 1 hr. The medium showing gel formation was further incubated for 24 hrs. The isolates of *C. perfringens* showed positive reactions for both lactose fermentation and gelatin liquefaction test (Fig. 2).

Indole test was performed by inoculation of a loop full of test culture from MCMM in peptone broth with incubation at 37^0C for 24 hrs under strict anaerobic condition. The isolates of *C. perfringens* showed negative results for indole test (Fig. 2).

2.4 DNA extraction by snap chill method:

DNA of each individual isolates were isolated by slight modification of modified boiled cell lysis method as described by Chai *et al*., (2007) briefly as follows:

1. About 1.5 ml of overnight grown culture in MCMM broth were taken in 2.0 ml Eppendorf tube and centrifuged at 10,000 rpm for 10 min to pellet the bacterial cell and supernatant was discarded.

2. 1 ml of normal saline solution (NSS) was added to bacterial pellet and vortexed for 15-20 sec. followed by centrifugation at 10,000 rpm for 10 min to get the pellet and supernatant was discarded.

3. Again 1 ml NSS was added and the process was repeated as mentioned in para 2.

4. Then 100 µl of nuclease free water (NFW) was added to the bacterial pellet, vortexed and subjected to boiling for 20 min after proper sealing with paraffin tape.

5. After boiling the tubes were immediately transferred to -20º C for minimum 20 min or till preparation of reaction mixture for PCR assay.

6. Then the tubes were taken out of deep freeze and after thawing centrifuged at 4,000 rpm for 3 min.

7. The supernatants were used as DNA template (bacterial lysate).

2.5 Molecular detection of *C. perfringens* targeting species specific 16S rRNA gene:

A PCR assay was standardized for detection of *C. perfringens* by amplification of 481 bp product size of *C. perfringens* species-specific 16S rRNA gene using primer pair reported by Tonooka *et al.* (2003). To standardized the PCR, DNA templates were prepared by snap chill method (para 2.4) and DNA from approximately 20 presumptive isolates were pooled. The PCR reaction was prepared as 25 µl reaction mixture containing 2.5µl of 10X PCR buffer (500mM KCl, 100mM Tris-HCl, pH 8.3, 15mM MgCl2), 2.0µl of dNTP (2.5mM each), 2.0µl (10 pmol) of 16S rRNA forward and reverse primers, 0.2 µl (1 Unit) *Taq* DNA polymerase, 5 µl of DNA lysate and sterile NFW up to 25µl. The PCR program standardization included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), gradients of annealing ranging from 46ºC to 56ºC for 1 min and extension (72º C for 1 min) with final extension at 72º C for 8 min.

The PCR product amplified at annealing temperature 54.5º C for 1 min abled to produce the specific amplicon size of 481 bp measured with 100 bp DNA molecular weight (Mw) marker (thermo-scientific, USA) in submarine gel electrophoresis of 1.5% agarose gel (Fig. 4A). The

agarose gel was visualized under gel documentation system (Bio-rad, USA) after staining with ethidium bromide (0.5 µg/ml) and images were stored for analysis.

Further all isolates were screened using standardized PCR protocol included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), annealing (54.5º C for 1 min) and extension (72º C for 1 min) with final extension at 72º C for 8 min (Fig. 4B).

2.6 Molecular characterization of Toxin genes

2.6.1 Standardization of PCR targeting alpha toxin (*cpa*) gene:

A PCR assay was standardized for detection of *C. perfringens* alpha toxin (*cpa*) gene by amplification of 324 bp product size using primer pair reported by Titball *et al.* (1989). To standardized the PCR, DNA templates prepared by snap chill method (para 2.4) using 20 16S rRNA confirmed isolates and pooled together. The PCR reaction was prepared as 25 µl reaction mixture containing 2.5µl of 10X PCR buffer (500mM KCl, 100mM Tris-HCl, pH 8.3, 15mM MgCl2), 2µl of dNTP (2.5mM each), 2.0µl (10 pmol) of forward and reverse primers of *cpa* toxin gene, 0.2 µl (1 Unit) *Taq* DNA polymerase, 5 µl of DNA lysate and sterile NFW to make total volume of 25µl. The PCR program standardization included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), gradients of annealing ranging from 50ºC to 60ºC for and extension (72º C for 1 min) with final extension for 8 min at 72º C. The PCR product amplified at annealing temperature 50.2º C for 1 min abled to produce the specific amplicon size of 324 bp measured with 100 bp DNA molecular weight (Mw) marker (thermo-scientific, USA) in submarine gel electrophoresis of 1.5% agarose gel (Fig. 5A). The agarose gel was visualized under gel documentation system (Bio-rad, USA) after staining with ethidium bromide (0.5 µg/ml) and images were stored for analysis.

Further all isolates were amplified using standardized PCR protocol included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), annealing (50.2º C for 1 min) and extension (72º C for 1 min) with final extension at 72º C for 8 min (Fig. 5B).

2.6.2 Standardization of PCR targeting Beta toxin (*cpb*) gene:

A PCR assay was standardized for detection of *C. perfringens* beta toxin (*cpb*) gene by amplification of 196 bp product size using primer pair reported by Hunter *et al.* (1993).

To standardized the PCR, DNA templates prepared by snap chill method (para 2.4) using 20 16S rRNA confirmed isolates and pooled together. The PCR reaction was prepared as 25 µl reaction mixture containing 2.5µl of 10X PCR buffer (500mM KCl, 100mM Tris-HCl, pH 8.3, 15mM MgCl2),2µl of dNTP (2.5mM each), 2.0µl (10 pmol) of forward and reverse primers of *cpb* toxin gene, 0.2 µl (1 Unit) *Taq* DNA polymerase, 5 µl of DNA lysate and sterile NFW to make total volume of 25µl. The PCR program standardization included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), gradients of annealing ranging from 49.3ºC to 59.3ºC for and extension (72º C for 1 min) with final extension for 8 min at 72º C. The PCR product amplified at annealing temperature 55.7º C for 1 min abled to produce the specific amplicon size of 196 bp measured with 100 bp DNA molecular weight (Mw) marker (thermo-scientific, USA) in submarine gel electrophoresis of 2% agarose gel (Fig. 6A). The agarose gel was visualized under gel documentation system (Bio-rad, USA) after staining with ethidium bromide (0.5 µg/ml) and images were stored for analysis.

Further all isolates were amplified using standardized PCR protocol included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), annealing (50.2º C for 1 min) and extension (72º C for 1 min) with final extension at 72º C for 8 min (Fig. 6B).

2.6.3 Standardization of PCR targeting epsilon toxin (*etx*) gene :

A PCR assay was standardized for detection of *C. perfringens* epsilon toxin (*etx*) gene by amplification of 655 bp product size using primer pair reported by Hunter *et al.* (1992).

To standardized the PCR, DNA templates prepared by snap chill method (para 2.4) using 20 16S rRNA confirmed isolates and pooled together. The PCR reaction was prepared as 25 µl reaction mixture containing 2.5µl of 10X PCR buffer (500mM KCl, 100mM Tris-HCl, pH 8.3, 15mM MgCl2),2µl of dNTP (2.5mM each), 2.0µl (10 pmol) of forward and reverse primers of *cpb* toxin gene, 0.2 µl (1 Unit) *Taq* DNA polymerase, 5 µl of DNA lysate and sterile NFW to make total volume of 25µl. The PCR program standardization included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), gradients of annealing ranging from

50.6ºC to 60.6ºC for and extension (72º C for 1 min) with final extension for 8 min at 72º C. The PCR product amplified at annealing temperature 55.1º C for 1 min abled to produce the specific amplicon size of 655 bp measured with 100 bp DNA molecular weight (Mw) marker (thermo-scientific, USA) in submarine gel electrophoresis of 1.5% agarose gel (Fig. 7A). The agarose gel was visualized under gel documentation system (Bio-rad, USA) after staining with ethidium bromide (0.5 µg/ml) and images were stored for analysis.

Further all isolates were amplified using standardized PCR protocol included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), annealing (50.2º C for 1 min) and extension (72º C for 1 min) with final extension at 72º C for 8 min (Fig. 7B)

2.6.4 Standardization of PCR targeting enterotoxin (*cpe*) gene:

A PCR assay was standardized for detection of *C. perfringens* enterotoxin (*cpe*) gene by amplification of 233 bp product size using primer pair reported by Czeczulin *et al.* (1993). To standardized the PCR, DNA templates prepared by snap chill method (para 2.4) using 20 16S rRNA confirmed isolates and pooled together. The PCR reaction was prepared as 25 µl reaction mixture containing 2.5µl of 10X PCR buffer (500mM KCl, 100mM Tris-HCl, pH 8.3, 15mM MgCl2),2µl of dNTP (2.5mM each), 2.0µl (10 pmol) of forward and reverse primers of *cpe* toxin gene, 0.2 µl (1 Unit) *Taq* DNA polymerase, 5 µl of DNA lysate and sterile NFW to make total volume of 25µl. The PCR program standardization included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), gradients of annealing ranging from 43.9ºC to 55.9ºC for and extension (72º C for 1 min) with final extension for 8 min at 72º C. The PCR product amplified at annealing temperature 50.5º C for 1 min abled to produce the specific amplicon size of 233 bp measured with 100 bp DNA molecular weight (Mw) marker (thermo-scientific, USA) in submarine gel electrophoresis of 1.5% agarose gel (Fig. 8A). The agarose gel was visualized under gel documentation system (Bio-rad, USA) after staining with ethidium bromide (0.5 µg/ml) and images were stored for analysis.

Further all isolates were amplified using standardized PCR protocol included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), annealing

(50.2º C for 1 min) and extension (72º C for 1 min) with final extension at 72º C for 8 min (Fig. 8B).

2.6.5 Standardization of PCR targeting beta2 toxin (*cpb2*) gene:

A PCR assay was standardized for detection of *C. perfringens* beta2 toxin (*cpb2*) gene by amplification of 567 bp product size using primer pair reported by Garmory *et al.* (2000). To standardized the PCR, DNA templates prepared by snap chill method (para 2.4) using 20 16S rRNA confirmed isolates and pooled together. The PCR reaction was prepared as 25 µl reaction mixture containing 2.5µl of 10X PCR buffer (500mM KCl, 100mM Tris-HCl, pH 8.3, 15mM MgCl2),2µl of dNTP (2.5mM each), 2.0µl (10 pmol) of forward and reverse primers of *cpb* toxin gene, 0.2 µl (1 Unit) *Taq* DNA polymerase, 5 µl of DNA lysate and sterile NFW to make total volume of 25µl. The PCR program standardization included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), gradients of annealing ranging from 49ºC to 59ºC for and extension (72º C for 1 min) with final extension for 8 min at 72º C. The PCR product amplified at annealing temperature 54.7º C for 1 min abled to produce the specific amplicon size of 196 bp measured with 100 bp DNA molecular weight (Mw) marker (thermo-scientific, USA) in submarine gel electrophoresis of 2% agarose gel (Fig. 9A). The agarose gel was visualized under gel documentation system (Bio-rad, USA) after staining with ethidium bromide (0.5 µg/ml) and images were stored for analysis.

Further all isolates were amplified using standardized PCR protocol included initial denaturation at 95º C for 5 min followed by 30 cycles of denaturation (95º C for 1 min), annealing (50.2º C for 1 min) and extension (72º C for 1 min) with final extension at 72º C for 8 min (Fig. 9B)

2.7. Determination of Minimum Inhibitory Concentration and antibiotic susceptibility profiles of *C. perfringens* isolates

C. perfringens isolates from different samples of fish, meat, milk and human stools obtained from different places of Patna based retail markets and diagnostic laboratories were screened for antibiotic sensitivity by the determination of minimum inhibitory concentration using HiComb^{TM} MIC Test (Hi-Media, India) as recommended by CLSI, 2015 guidelines for anaerobes. The selected groups of antibiotics included penicillins, β-Lactam, cephems, tetracycline,

fluoroquinolones, linosamides and phenicols. The details of the antibiotic concentrations given in **Table 2.** The laboratory procedures adopted for determination of MIC of *C. perfringens* isolates were as follows:

1.Mueller Hinton Agar plates were prepared for *C. perfringens* as per the method recommended by Bauer-Kirby Method. While media preparation care was taken to keep media sterile and the depth of media kept 4mm as recommended by CLSI guidelines.

2. Colonies from stab cultured isolates of different samples were inoculated to MCMM broth and incubated at 37°C for overnight or 24 hrs under strict anaerobic condition.

3.Change in colour of MCMM broth indicated the growth of *C. perfringens* organism.

4. A sterile non-toxic cotton swab was taken dipped it in the standardized inoculum and the soaked swab firmly rotated against the upper inside wall of tube to remove excess soaked fluid. Streaking was performed on entire agar surface of Muller-Hinton plate with inoculum-soaked swab. Then the plate was left aside to dry for 15 minutes with lid in place.

6.The strips of antibiotic both A & B was placed at the center of plate using sterile forceps. Antibiotic strips were placed on plate using aseptic technique.

7. The plates immediately incubated at 37°C for 42-48 hours under strict anaerobic condition.

8. After 48 hours of incubation plates were examined for minimum inhibitory concentration. The MIC of both the strips (A & B) were recorded and interpreted as resistant, intermediate and susceptible

MIC_{50} and MIC_{90} values were calculated using the formula (Schwarz et al, 2010).

2.8 Genetic similarity or diversity *C. perfringens* isolated from divers origin:

Genetic similarity or diversity among *C. perfringens*, isolated from samples were determined by ERIC-PCR using genomic DNA extracted with the help of blood and tissue kit (Qiagen).

2.8.1 Genomic DNA isolation and its purity:

Table 2: Minimum Inhibitory Concentration determination strips of different antibiotics

Sl. No.	Group	Antibiotic	Range(µg)	
			Strip A	**Strip B**
1.	**Penicillins**	Ampicillin (AMP)	240µg-0.01µg	2.048µg-.001µg
		Ticarcillin (TI)	240µg-0.01µg	16µg-.001µg
		Piperacillin (PI)	240µg-0.01µg	5µg-.001µg
2.	**β-Lactam**	Amoxyclav (AMC)	240µg-0.01µg	4µg-.001µg
		Piperacillin - tazobactum (PIT)	240µg-0.01µg	5µg-.001µg
3.	**Cephems**	Ceftriaxone (CTR)	240µg-0.01µg	30µg-.001µg
		Cefotaxime (CTX)	240µg-0.01µg	30µg-.001µg
4.	**Tetracyclines**	Tetracycline (TE)	240µg-0.01µg	5µg-.001µg
5.	**Fluoroquinolones**	Moxifloxacin (MO)	240µg-0.01µg	32µg-.001µg
6.	**Linosamides**	Clindamycin (CD)	240µg-0.01µg	8µg-.001µg
7.	**Phenicols**	Chloramphenicol (C)	240µg-0.01µg	8µg-.001µg

The chromosomal DNA of *C. perfringens* was isolated using Qiagen DNA isolation kit with following steps–

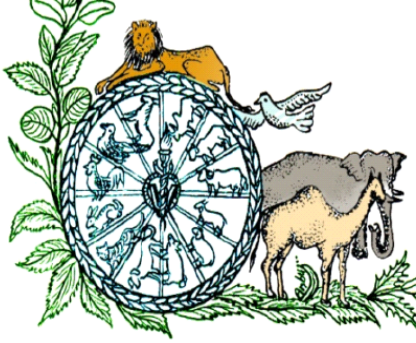
1. A loop full of culture were grown in 2 ml of MCMM broth with overnight incubation at 37º C under anaerobic condition.
2. All grown culture were centrifuge at 10,000 rpm for 10 min to form a pellet and supernatant were discarded.
3. Then the pellet was mixed with 180 µl of ATL buffer and 20 µl of proteinase K and vortexed to mix it properly.
4. The mixture then incubated at 56º C for about half hour in a water bath.
5. Then mixture was vortexed for about 15 sec. and 200 µl of AL buffer was added and vortexed again to mix properly. Then 200 µl of Ethanol was added.
6. The whole mixture was transferred to column tube, provided with Qiagen DNA isolation kit and centrifuged at 8000 rpm for 1 min. The flow through solution was discarded.
7. 500 µl of AW1 buffer was added to the column tube and centrifuged at 8,000 rpm for 1 min and the flow through solution was discarded.
8. Then 500 µl of AW2 buffer was added in the column tube and centrifuged at 14,000 rpm for 3 min and the collection tube with flow through solution was discarded.
9. A new Eppendorf tube was taken and the column was placed on that tube and 30 µl of AE buffer was added.
10. The tube was incubated for 2 min at room temperature and centrifuged at 8000 rpm for 1 min.
11. Again 30 µl of AE buffer was added in the same column tube and incubated for 2 min at room temperature and centrifuged at 8000 rpm for 1 min.
12. The content collected in Eppendorf tube was DNA and stored at -20º C.

Purity and concentration of DNA was checked using Nano-spectrophotometer (Genova Nano, UK).

2.8.2 ERIC-PCR Method

ERIC-PCR was performed using an arbitrary primer ERIC 1 (ATGAAGCTCCTGGGGATTCAC) and ERIC 2 (AAGTAAGTGACTGGGGTGAGG) (Versalovic *et al*., 1991). ERIC-PCR reaction was prepared in PCR tubes containing 25 µl reaction mixture contained 2.5 µl of 10X PCR buffer, 3µl of dNTP mixture(2mMol), 2.5µl of $MgCl_2$, 3µl (30 pmol) of primers each, 0.2 µl (1 Unit) *Taq* DNA polymerase and 3.0 µl of *C. perfringens* genomic DNA (~ 20-40 ng). The PCR cycle conditions included initial denaturation at 94ºC for 5 min, followed by a middle step of 40 cycles at 94ºC for 1 min, 40ºC for 90 sec, 72ºC for 1 min and a final extension of 72ºC for 7 min. The amplified PCR products were electrophoresed in a 1.5 % agarose gel (GenNai, India), at 80 mV for 120 min with 1 kbp DNA Mw marker (MBI, Fermentas) to measure the size of amplicon and ethidium bromide (0.5 µg/ml) to stain the amplified fragments. The stained electrophoresed PCR products were visualized and documented under Gel Documentation system (Bio-rad) (Fig. 16A, 17A, 18A, 19A, 20A, 21A, 22A). The similarity of the banding pattern of different fish, meat, and human isolates were analyzed with PyElph 1.4 software and clustering was analyzed using the unweighted pair group method with arithmetic mean (UPGMA). D value was calculated with help of online available tool of discriminatory power calculator (http://insilico.ehu.es/mini_tools/discriminatory_ power/).

Results & Discussions



4 RESULTS AND DISCUSSION

The finding of study for the isolation of *Clostridium perfringens* by conventional method and identification by biochemical and molecular methods from animal origin food viz. meat, milk and their products along with human stools are presented in the section. The isolates were also subjected for toxotyping for the detection of different types of toxins produced. The isolates were screened for antibiotic susceptibility and phylogenetic analysis and the results as well discussion thereof also presented.

4.1 Cultural isolation of clostridium

For isolation of *C. perfringens* from different food samples, modified cooked meat medium, pH 6.8 (MCMM) has been routinely used as enrichment media and Sahidi fergusan perfringens (SFP) agar was reported as recommended plating media. Accordingly, in this study, a total of 251 foods of animal origin including meat and meat products, milk as well their products, fish and their products and human diarrhoeic stool were allowed for enrichment in MCMM with overnight incubation at 37^0C under strict anaerobic condition followed by streaking on the SFP. By conventional enrichment and plating on SFP, the characteristic colonies of *C. perfringens* i.e., typical round, 2-3 mm in diameter, black colored colonies with opaqueness around the colonies were produced by 146 samples (58.16%) after 24 h incubation at 37^0C under strict anaerobic condition (Table 3, Fig.1). Such typical colony on SFP was yielded in the processed samples as 80% (28/35) of raw meat, 53.33% (8/15) of meat products, 80% (28/35) of raw fish, 66.67% (10/15) of fish product, 82.00 % (41/51) of pasteurized milk, 10% (05/50) of milk product and 52.00% (26/50) of human stools (Table 3).

4.2 Biochemical confirmation of *Clostridium perfringens*

Biochemical reactions have an important role in the biotyping or speciating of bacterial pathogens including *Clostridium*. Some authors have successfully used conventional biochemical tests to distinguished the Genus *Clostridium* up to species level. Based on the previous reports, in this study the biochemical tests recommended by

Rhodehamel and Harmon, (2001) were used for biochemical confirmation of presumptive *C. perfringens* isolate. Accordingly, an individual single characteristic colony from SFP was selected for biochemical characterization using lecithinase test, motility, nitrate reduction test, lactose fermentation and gelatin liquefaction test. The *C. perfringens* isolates showing lecithinase activity indicated by opaqueness and halo around the colonies (Fig. 1) due to the breakdown of lecithin in the egg yolk by lecithinase activity. The nitrate reduction test revealed the presence of *C. perfringens* in samples inoculated into motility nitrate medium shown by its ability to reduce nitrate to nitrite. Further addition of reagent A and B to motility nitrate medium, resulted in the instant development of red colour which showed nitrate reduction. However, in samples unable to show development of red colour, zinc powder was subsequently added which reacts with potassium nitrate to reduce nitrate to free nitrogen and ammonia leads to opaque opalescent colour indicating the presence of *C. perfringens* (Fig. 2A). Motility was observed by growth of the isolates along the side-line of the stab (Fig. 2A). Lactose fermentation test was performed by stab inoculation of broth culture into modified lactose gelatine medium and change in colour from red to yellow indicated lactose fermentation and gelatin liquefaction was observed by gelling of the medium at 5^0C for 1 hr as well as liquefaction of the medium after incubation at 37^0C for 24 hr (Fig. 2B). Indole test was observed by formation of red ring over the rim of Peptone water (Fig. 2C).

In the present study it was found that out of 146 presumptive isolates only 95 (65.07%) were able to produce characteristic biochemical reaction for *C. perfringens* i.e., positive for lecithinase test, nitrate reduction, lactose fermentation and gelatin liquefaction however, negative for motility and indole test (Table. 3 and Fig. 2A, 2B, 2C).

4.3 **Molecular confirmation of *Clostridium perfringens* isolates**

For surveillance, prevention and control of *Clostridium perfringens* infection there is a need of rapid and reliable identification method. The conventional identification methods of *C. perfringens* involved isolation and biochemical characterization that is laborious and time-consuming. Over the last decade, a large number of assays were developed using polymerase chain reaction (PCR) targeted house-keeping genes such as 16S rRNA or 16S rDNA in order to detect the specific sequences of *C. perfringens* DNA from various samples

(Wang *et al*., 1994). Similarly, in this study for identification of *C. perfringens* a PCR assay was standardized using species-specific 16S *rRNA* gene amplification that produced 481bp amplicon size at annealing temperature 54.5º C for 1 min.

Further, all biochemical test confirmed isolates (N=95) were tested for the presence of *C. perfringens* species specific *16SrRNA* gene and 84.21% (80/95) isolates were found positive to give species specific amplicon (481bp) in agarose gel electrophoresis (Fig. 4B). The sample wise distribution of *C. perfringens* showed an occurrence of 31.87% (80/251) of *C. perfringens* among raw and processed meat, fish, pasteurized milk and milk products and human diarrhoeic samples used under study (Table 3).

4.3.1 Distribution of *C. perfringens* in meats and meat products

During the screening of total 50 meats and meat products (meat curry and kabab) *C. perfringens* were confirmed from 51.42% (18/35) and 26.67 % (4/15) of meats and meat products examined under study. The present finding also showed that out of 22 confirmed *C. perfringens* isolates 64.28% (18/28) and 50.00% (4/8) isolates belonged to raw and processed meat samples, respectively (Table 3).

In reference to the finding of present study a higher incidence of C. *perfringens* among 88% of chicken carcasses consumed in Beijing (Guang-Hua and Xiao-Ling, 1994), 70.4% of chicken meat markets in India (Singh *et al*. 2005) and 66% of wing and chicken leg quarter samples in Canada (Nowell *et al*., 2010) were reported. While a lower incidence of 31.7% (Aras and Hadimli, 2015), 24.46% *(Stagnitta et al., 2002),* 18% (Lindblad *et al*., 2006), 16% (Regan *et al*., 2018) and 15.1% (Zhang *et al*., 2018) for *C. perfringens* was also reported. The differences among the various reports from different part of the world may be due to the level of hygienic practices during slaughtering, sampling technique and methodology while a high incidence of C. *perfringens* in meat may be due to the reason that this bacterium are ubiquitous and may present in poultry intestinal flora which lead to cross-contamination during slaughter process, due to lack of sanitation and hygiene conditions (Tekinsen *et al*., 1980; Tschirdewahn *et al*., 1991; Atasever 2001).

The finding of the present study revealed that ~27% of meat products examined harboured *C. perfringens* which was in concordance with the finding of Shaltout *et al*.

(2017a) who reported that raw and half cooked chicken meat samples collected from various retail stores and supermarkets in Qualyubia governorate harboured *C. perfringens* as 24% of breast, 32% of raw thigh, 20% of nuggets, 16% of paneer, and 16% of frankfurter. Similarly, an another study performed by Shaltout *et al.* (2017b) using cooked beef and chicken samples from the university student hostel reported that 12% of cooked chicken and 8% of cooked beef samples harbored *C. perfringens*. The presence of *C. perfringens* in meat products may be attributed to the presence of *Clostridia* in raw meat and the materials such as additives and spices as well as post cooking contamination of cooked meat by cross contamination or from water.

4.3.2 Distribution of *C. perfringens* in fish and fish products

During the screening of total 50 fish and fish products (curry and fried) *C. perfringens* were confirmed from 48.57% (17/35) and 60.00% (9/15) of fish and fish products examined under study. The present finding also showed that out of 38 isolates of cultural isolation 26 confirmed as *C. perfringens* isolates with a distribution of 60.71% (17/28) and 90.00 % (9/10) to fish and fish products, respectively (Table 3).

In concordance with the finding of present study an incidence of 54.5% and 59.6% of *C. perfringens* among fish from aquaculture and unprocessed fish samples respectively was reported by Sabry *et al*. (2016) and El-Shorbagy *et al*., (2012). However, in contrast to the finding of present study a higher incidence of C. *perfringens* among 71% of fresh markets fish 70.83% from fresh water fish respectively were reported by Sabry *et al*. (2016) and Yadav *et al*. (2016). While a lower incidence of 17.9% from intestinal contents freshwater fish (Cai *et al*. 2008), 27.24% from fish and fish products from Tamil Nadu and Kerala, India (Das and Jain, 2012a), 18.35% from intestinal contents of fishes (Das and jain, 2012b) and 13.3% from seafood samples in El Dakahlya Governorate, Egypt (Nashwa *et al*., 2016) were also reported. The finding of the present study revealed that 60% of fish products harboured *C. perfringens* which was in concordance with the finding of El-Shorbagy *et al*. (2012) who reported an incidence of *C. perfringens* in 57.1% of processed fish samples while a lower incidence of 29.17% of *C. perfringens* from fish products of Kolkata city, India was also reported by Yadav *et al*. (2016).

4.3.3 Distribution of *C. perfringens* in pasteurized milk and milk product

In this study, *C. perfringens* were detected in 33.33% (17/51) and 4.00% (02/50) in pasteurized milk and milk products samples, respectively. Further, out of 19 isolates from milk samples 41.46% (17/41) and 40.00% (2/5) isolates of pasteurized milk and milk samples, respectively were identified as *C. perfringens* (Table 3). In concordance with the finding of present study Chaturvedi and Shukla (2015) reported that 35.48% of raw milk, curd, cheese and paneer samples harboured *C. perfringens* with 26.00% in milk. Similarly, Osama *et al*. (2015) reported the prevalence of *C. perfringens* among 20, 60, 20, 60, 36, 0.0 and 0.0% of market milk, Damietta cheese, milk powder, Ras cheese, Kariesh cheese, sterilized and condensed milk respectively collected from different localities of El-Dakahlia province, Egypt. In contrast to the finding of present study Ahsanullah *et al*. (2019) reported an incidence of 19.28% for C. perfringens in milk samples and dairy products from various zones of Quetta city with a distribution of 8.01% in milk, 3.73% in cheese, 2.74% in butter and 4.71% in yogurt.

4.3.4 Distribution of *C. perfringens* in human diarrhoeic stool

The *C. perfringens* showed a distribution of 26.00% (13/50) among human diarrhoeic stool samples analysed under study. Among 26 clostridium isolates from human diarrhoeic 59.00% (13/22) isolates were identified as *C. perfringens* (Table 3). Similar to the finding of present study Nagpal *et al*. (2015) reported an incidence of *C. perfringens* in 36 % infants and 33 % adults faeces. While in contrast to the finding of present study Akhi *et al*. (2015) reported an incidence of 61.02% of *C. perfringens* among diarrhoea and non-diarrhoea stool samples similarly Azimirad *et al*. (2019) reported 64.15% of isolates from AAD and 35.85% from non-AAD faecal samples from Iranian diarrheal patients were *C. perfringens*.

4.4 Distribution of toxin genes and Toxotyping of *C. perfringens* isolates

C. perfringens species is classified into five toxotypes (A - E) on the basis of differential production of the four major toxins α (*cpa*), β (*cpb*), ε (*etx*), and ι (*iap*). All *C. perfringens* type A strains produce α toxin, type B (α, β, and ε toxins), type C (α and β toxins), type D (α and ε toxins), and type E (α and ι toxins). *C. perfringens* type B to E were recognised as frank pathogens for both domestic animals and human, whereas type A strains

are commensals in the intestinal tract of vertebrates, with ability of expression of α toxin which decides their lethal properties (Songer,1996). *C. perfringens* type A strains are implicated in numerous diseases such as necrotic enteritis in broiler chicken, enteritis in piglets, abomasitis and haemorrhagic enteritis in calves, and gas gangrene, food-poisoning, and gastrointestinal illness in humans (Collins *et al*., 1989; Fisher et al., 2005; Hart *et al*., 1983; Long *et al*., 1976; Tominaga *et al*., 1984). *C. perfringens* is capable of producing many additional toxins or enzymes, β2 toxin (*cpb2*) and enterotoxin (*cpe*), whose role in the pathogenesis has been well defined. The toxin genes of *C. perfringens* are located on a chromosome (cpa) and on plasmids (cpb, etx, iap, cpb2) or regarding cpe, they may occur alternatively on both of them. It was proven that acquisition or loss of cpb, cpb2, cpe, etx, and iap genes in the nature is associated with change of ecological niche or host (Borriello *et al*., 1985; Sawiers *et al*., 2006). However, there are affirmative proofs for loss of toxin genes within the same host species. Taking into account epidemiologic evidences it was suggested that there is a possibility of feed-to-animal and animal-to-human route of transmission of *C. perfringens* strains, thus their toxic potential of these anaerobes in particular links of food chain was studied.

4.4.1 Distribution of *cpa* gene in *C. perfringens* isolates from raw and processed food samples of animal origin and human diarrhoeic stool

For identification of *cpa* gene among *C. perfringens* a PCR assay was standardized that produced 324 bp amplicon size at annealing temperature 50.2º C for 1 min. Out of 80 *C. perfringens* isolates tested for the presence of *cpa* gene 100% (80/80) isolates were found positive to give specific amplicon (324bp) in agarose gel electrophoresis (Fig. 5B).

4.4.2 Distribution of *cpb* gene in *C. perfringens* isolates from raw and processed food samples of animal origin and human diarrhoeic stool

For identification of *cpb* gene of *C. perfringens* a PCR assay was standardized that produced 196 bp amplicon size at annealing temperature 55.7º C for 1 min. Further, all 16SrRNA positive *C. perfringens* isolates (N=80) were tested for the presence of *cpb* gene and 18.75% (15/80) isolates were found positive to give specific amplicon (196 bp) in agarose gel electrophoresis (Fig. 6B).

4.4.2.1 Distribution of *cpb* gene in *C. perfringens* isolates from raw and processed meat

The sample wise distribution of *cpb* gene positive *C. perfringens* in raw and processed meat samples, showed that 14.29% (5/35) and 6.67% (1/15) samples, respectively harboured *cpb* gene positive *C. perfringens*. Out of 22 16SrRNA conformed *C. perfringens* isolates 27.28% (5/18) and 25.00% (1/4) isolates belonged to raw and processed meat samples, respectively were identified as *C. perfringens* encoded with *cpb* gene (Fig. 10).

4.4.2.2 Distribution of *cpb* gene in *C. perfringens* isolates from raw and processed fish

The sample wise distribution of *cpb* gene positive *C. perfringens* in raw and processed fish samples, showed that 8.57% (4/35) and 0 (0/15) samples, respectively harboured *cpb* gene positive *C. perfringens*. Out of 26 16SrRNA conformed *C. perfringens* isolates 17.65% (4/17) and 0 (0/9) isolates belonged to raw and processed fish samples, respectively were identified as *C. perfringens* encoded with *cpb* gene (Fig. 10).

4.4.2.3 Distribution of *cpb* gene in *C. perfringens* isolates from pasteurized milk and milk products

The sample wise distribution of *cpb* gene positive *C. perfringens* in pasteurized milk and milk products samples, showed that 1.96% (3/51) and 0 (0/50) samples, respectively harboured *cpb* gen positive *C. perfringens*. Out of 19 16SrRNA conformed *C. perfringens* isolates 5.88% (1/17) and 0.0% isolates belonged to pasteurized milk and milk products, respectively were identified as *C. perfringens* encoded with *cpb* gene (Fig. 10).

4.4.2.4 Distribution of *cpb* gene in *C. perfringens* isolates from human diarrhoeic stool

The sample wise distribution of *cpb* gene positive *C. perfringens* in human diarrhoeic samples, showed that 4.00% (2/50) samples harboured *cpb* gene positive *C. perfringens*. Out 13 16SrRNA conformed *C. perfringens* isolates 15.38% (02/13) isolates were identified as *C. perfringens* encoded with *cpb* gene (Fig. 10).

4.4.3 Distribution of *etx* gene in *C. perfringens* isolates from raw and processed food samples of animal origin and human diarrhoeic stool

For identification of *etx* gene of *C. perfringens* a PCR assay was standardized that produced 655 bp amplicon size at annealing temperature 55.1º C for 1 min. Further, all 16S rRNA positive *C. perfringens* isolates (N=80) were tested for the presence of *etx* gene and 6.25% (5/80) isolates were found positive to give specific amplicon (655 bp) in agarose gel electrophoresis (Fig. 7B).

4.4.3.1 Distribution of *etx* gene in *C. perfringens* isolates from raw and processed meat

The sample wise distribution of *etx* gene positive *C. perfringens* in raw and processed meat samples, showed that 0% (0/35) and 0% (0/15) samples, respectively harboured *etx* gene positive *C. perfringens* (Fig. 10).

4.4.3.2 Distribution of *etx* gene in *C. perfringens* isolates from raw and processed fish

The sample wise distribution of *etx* gene positive *C. perfringens* in raw and processed fish samples, showed that 8.57% (3/35) and 6.67% (1/15) samples, respectively harboured *etx* gene positive *C. perfringens*. Out of 26 16SrRNA conformed *C. perfringens* isolates 17.65% (3/17) and 11.11% (1/9) isolates belonged to raw and processed fish samples, respectively were identified as *C. perfringens* encoded with *etx* gene (Fig. 10).

4.4.3.2 Distribution of *etx* gene in *C. perfringens* isolates from pasteurized milk and milk products

The sample wise distribution of *etx* gene positive *C. perfringens* in pasteurized milk and milk products samples, showed that 0 (0/51) and 0 (0/50) samples, respectively harboured *etx* gene positive *C. perfringens* (Fig. 10).

4.4.3.4 Distribution of *etx* gene in *C. perfringens* isolates from human diarrhoeic stool

The sample wise distribution of *etx* gene positive *C. perfringens* in human diarrhoeic samples, showed that 2.00% (1/50) samples harboured *etx* gene positive *C. perfringens*. Out 13 16SrRNA conformed *C. perfringens* isolates7.69 % (01/13) isolates were identified as *C. perfringens* encoded with *etx* gene (Fig. 10).

4.4.4 Distribution of *cpe* gene in *C. perfringens* isolates from raw and processed food samples of animal origin and human diarrhoeic stool

For identification of *cpe* gene of *C. perfringens* a PCR assay was standardized that produced 233 bp amplicon size at annealing temperature 50.5º C for 1 min. Further, all 16S rRNA positive *C. perfringens* isolates (N=80) were tested for the presence of *cpe* gene and 12.50% (10/80) isolates were found positive to give specific amplicon (655 bp) in agarose gel electrophoresis (Fig. 8B).

4.4.4.1 Distribution of *cpe* gene in *C. perfringens* isolates from raw and processed meat

The sample wise distribution of *cpe* gene positive *C. perfringens* in raw and processed meat samples, showed that 2.86% (1/35) and 6.67% (1/15) samples, respectively harboured *cpe* gene positive *C. perfringens*. Out of 22 16SrRNA conformed *C. perfringens* isolates 5.56% (1/18) and 25.00% (1/4) isolates belonged to raw and processed meat samples, respectively were identified as *C. perfringens* encoded with *cpe* gene (Fig. 10).

4.4.4.2 Distribution of *cpe* gene in *C. perfringens* isolates from raw and processed fish

The sample wise distribution of *cpe* gene positive *C. perfringens* in raw and processed fish samples, showed that 5.71% (2/35) and 0% (0/15) samples, respectively harboured *cpe* gene positive *C. perfringens*. Out of 26 16SrRNA conformed *C. perfringens* isolates 11.76% (2/17) and 0% (0/9) isolates belonged to raw and processed fish samples, respectively were identified as *C. perfringens* encoded with *cpe* gene (Fig. 10).

4.4.4.3 Distribution of *cpe* gene in *C. perfringens* isolates from pasteurized milk and milk products

The sample wise distribution of *cpe* gene positive *C. perfringens* in pasteurized milk and milk products samples, showed that 7.84 (4/51) and 0 (0/50) samples, respectively harboured *cpe* gene positive *C. perfringens*. Out 19 16SrRNA conformed *C. perfringens* isolates 23.50 % (04/17) and 0(0/2) isolates belonged to pasteurized milk and milk products, respectively were identified as *C. perfringens* encoded with *cpe* gene (Fig. 10).

4.4.4.4Distribution of *cpe* gene in *C. perfringens* isolates from human diarrhoeic stool

The sample wise distribution of *cpe* gene positive *C. perfringens* in human diarrhoeic samples, showed that 4.00% (2/50) samples harboured *cpe* gene positive *C.*

perfringens. Out 13 16SrRNA conformed *C. perfringens* isolates 15.38% (02/13) isolates were identified as *C. perfringens* encoded with *cpe* gene (Fig. 10).

4.4.5 Distribution of *cpb2* gene in *C. perfringens* isolates from raw and processed food samples of animal origin and human diarrhoeic stool

For identification of *cpb2* gene of *C. perfringens* a PCR assay was standardized that produced 567 bp amplicon size at annealing temperature 54.5º C for 1 min. Further, all 16S rRNA positive *C. perfringens* isolates (N=80) were tested for the presence of *cpb2* gene and 40.00% (32/80) isolates were found positive to give specific amplicon (567 bp) in agarose gel electrophoresis **(Fig. 9B).**

4.4.5.1 Distribution of *cpb2* gene in *C. perfringens* isolates from raw and processed meat

The sample wise distribution of *cpb2* gene positive *C. perfringens* in raw and processed meat samples, showed that 25.71% (9/35) and 6.67% (1/15) samples, respectively harboured *cpb2* gene positive *C. perfringens*. Out of 22 16SrRNA conformed *C. perfringens* isolates 50.00% (9/18) and 25.00% (1/4) isolates belonged to raw and processed meat samples, respectively were identified as *C. perfringens* encoded with *cpb2* gene (Fig. 10).

4.4.5.2 Distribution of *cpb2* gene in *C. perfringens* isolates from raw and processed fish

The sample wise distribution of *cpb2* gene positive *C. perfringens* in raw and processed fish samples, showed that 17.14% (6/35) and 40.00% (6/15) samples, respectively harboured *cpb2* gene positive *C. perfringens*. Out of 26 16SrRNA conformed *C. perfringens* isolates 35.29% (6/17) and 66.67% (6/9) isolates belonged to raw and processed fish samples, respectively were identified as *C. perfringens* encoded with *cpb2* gene (Fig. 10).

4.4.5.3 Distribution of *cpb2* gene in *C. perfringens* isolates from pasteurized milk and milk products

The sample wise distribution of *cpb2* gene positive *C. perfringens* in pasteurized milk and milk products samples, showed that 11.76% (6/51) and 0 (0/50) samples, respectively harboured *cpb2* gene positive *C. perfringens*. Out 19 16SrRNA conformed *C. perfringens* isolates 35.29 % (06/17) and 0(0/2) isolates belonged to pasteurized milk and milk products, respectively were identified as *C. perfringens* encoded with *cpb2* gene (Fig. 10).

4.4.5.4 Distribution of *cpb2* gene in *C. perfringens* isolates from human diarrhoeic stool

The sample wise distribution of *cpb2* gene positive *C. perfringens* in human diarrhoeic samples, showed that 8.00% (4/50) samples harboured *cpb2* gene positive *C. perfringens*. Out 13 16SrRNA conformed *C. perfringens* isolates 15.38% (04/13) isolates were identified as *C. perfringens* encoded with *cpb2* gene (Fig. 10).

4.6 Occurrence of different toxotypes of *C. perfringens* isolates from raw and processed food samples of animal origin and human diarrhoeic stool

On the basis of production of four major toxins i.e., alpha (α), beta (β), epsilon (ε) and iota 53 (ι), *C. perfringens* isolates were classified divided into five major toxinotypes /biotypes (A-E). Type A and all other *C. perfringens* types produce α toxin encoded by *cpa* gene. Additionally, type B produces β toxin encoded by cpb gene and ε toxins encoded by etx gene, type C produces b toxin encoded by cpb gene, type D produces ε toxin encoded by etx gene and type E produces ı toxin encoded by iap gene (Tutuncu *et al*., 2018). Based on the finding of present study *C. perfringens* isolates of present study showed that 100% (80/80) isolates harboured any one of the toxin gene. Further, toxino-typing profile showed that out of 80 *C. perfringens* isolates 77.50% (62/80) belonged to type A (harbours only *cpa* gene), 2.50% (2/80) to type B (harbours *cpa*, *cpb* and *etx* genes), 16.25% (13/80) to type C (harbours *cpa* and *cpb* gens) and 3.75% (3/80) to type D (harbours *cpa* and *etx* gene) (Fig. 11).

Out of 62 *C. perfringens* type A, 3 (4.84%), 18 (29.03%), and 4 (6.45%) were found to harbour enterotoxin (cpe+), β2 toxin (cpb2+) and both enterotoxin & β2 toxin (cpe+ & cpb2+), respectively (Fig. 12). Among 02 *C. perfringens* type B, 1 (50.00%) was found to harbour enterotoxin (cpe+), while 1 (50.00%) for β2 toxin (cpb2+). Out of 13 *C. perfringens*

type C, 1 (7.69%) was found to harbour enterotoxin (cpe+), while 4 (30.77%) β2 toxin (cpb2+). Among 03 *C. perfringens* type D, 01 (33.33%) each were found to harbour enterotoxin (cpe+), β2 toxin (cpb2+) and both enterotoxin & β2 toxin (cpe+ & cpb2+).

4.6.1 Occurrence of different toxotypes of *C. perfringens* isolates from meat and meat product

The isolates of *C. perfringens* from meat and meat product showed that out of 18 isolates from raw meat samples, 72.22% (13/18) were type A and 27.78% (5/18) type (Table 6). Out of 13 *C. perfringens* type A isolates 38.46% harboured only β2 toxin (cpb2 gene) while 7.69% harboured both enterotoxin (cpe gene) and β2 toxin. However, none of the type A isolates from raw meat samples were found to harbour enterotoxin (cpe gene) alone. Among 05 *C. perfringens* type C isolates 60.00% (03) harboured only β2 toxin (cpb2 gene) while none of the type C isolates were found to harbour enterotoxin (cpe gene). The finding of processed meat samples showed that out of 15 isolates, 75.00% (3/4) were type A and 25.00% (1/4) type C (Table 6). None of the isolates from both meat and meat product samples belonged to type B and D. Out of 03 *C. perfringens* type A isolates 33.33% (1) each were found to harboured enterotoxin (cpe gene) and β2 toxin (cpb2 gene), while 01 isolate was only positive for *cpa* gene (cpa^+, cpe^- & $cpb2^-$). However, none of the type A isolates from raw meat samples were found to harbour enterotoxin (cpe gene) alone. Similar to this study, previously performed studies by other workers also reported *C. perfringens* type A as preponderant genotype (Zhang *et al*. 2018). The study performed by Stagnitta *et al*. (2002) showed that 97.20% of *C. perfringens* isolates from non-industrial meat foods in San Luis, Argentina belonged to type A while 1.59% to type C and 0.79% to type E. Their finding also suggested that 7.14% were enterotoxigenic belonged to type A. Further Guran and Oksuztepe (2013) reported that 97·6% of *C. perfringens* isolates harboured only *cpa* toxin gene (type A) with 2·1% encoded with both *cpa* and *cpb2* toxin gene (type A-*cpb2*) and 0·1% with both *cpa* and *cpe* toxin genes (type A-*cpe*) among *C. perfringens* isolates from chicken meat parts. Afshari *et al*. (2015) found that 29.03% of *C. perfringens* isolates from broilers' meat as type A while 70.96% as type C of which 25.00% were positive for *cpe* gene. Latter on Aras and Hadimli, (2015) reported that 88.42 % of meat isolates were type A, with 2.38% carried both cpa and cpe genes (type A-cpe) and 9.52% carried cpa and cpb2

gene (type A-cpb2). While 6, 3, and 2 isolates belonged to beef, chicken and turkey meat samples, were genotyped as type C, type D, and type E, respectively.

Similarly, other researcher reported that 29.6% of *C. perfringens* isolates from meat samples belonged to type A and 3.7% as type D (Shaltout *et al*., 2017). Further, 43.2% of *C. perfringens* type A with only cpa+ gene, 45.4% with cpa+ and cpb2+ genes, 4.9% with cpa+ and cpe+ genes and 6.6% with cpa+, cpb2+ and cpe+ genes were reported by Yadav *et al*., (2017). While 91.2% isolates were reported as type A or E, 2.9% as type B, and 5.9% as type D by Regan *et al*. (2018).

4.6.2 Occurrence of different toxotypes of *C. perfringens* isolates from fish and fish product

The isolates of *C. perfringens* from fish and fish product showed that out of 17 isolates from raw fish samples, 64.71% (11/17) were type A, 5.88% (1/17) type B, 17.65% (3/17) type C and 11.76% (2/17) type D (Table 6). Out of 11 *C. perfringens* type A isolates 62.50% (5) were found to harboured only β2 toxin (cpb2 gene), while none of the type A isolates were found to harboured enterotoxin (cpe gene). The only one isolate from fish samples that was found as *C. perfringens* type B was also found to harbour β2 toxin (cpb2 gene). Among 02 *C. perfringens* type D isolates 50.00% (01) was found to harboured only enterotoxin (cpe gene) and 50.00% (01) isolate harboured both enterotoxin (cpe gene) β2 toxin (cpb2 gene). The finding of processed fish samples showed that out of 15 isolates, 88.89% (8/9) were type A and 11.11% (1/9) were type D. Out of 08 *C. perfringens* type A isolates 36.36% (04) were found to harboured only β2 toxin (cpb2 gene), while none of the type A isolates were found to harboured enterotoxin (cpe gene). The only one isolate from processed fish samples that was found as *C. perfringens* type D was also found to harbour β2 toxin (cpb2 gene).

Similar to present study Cai *et al*. (2008) analysed the intestinal content of freshwater fishes and reported that 77.3% of isolates belonged to type C encoded by alpha and beta toxin gene, 17.3% to type A encoded by alpha toxin gene and 5.3% to type B encoded by alpha, beta and epsilon toxin gene. In addition, 62.7% isolates were found to encoded with beta2 toxin gene while none of the isolates were found to encode for enterotoxin gene. Similarly, El-Shorbagy *et al*. (2012) reported that *C. perfringens* isolates of processed and

unprocessed fish samples of El-Sharkia Governorate, Egypt showed the presence of 84.8 toxigenic isolates with predominance of *C. perfringens* type A. 100% *C. perfringens* isolates from fish and fish products from Tamil Nadu and Kerala, India were found to harboured *cpa* toxin genes (type A) with 79.26% cpb2 toxin genes while none of the isolates were found to harbour beta, epsilon, iota and enterotoxin genes (Das and Jain, 2012). Further, Sabry *et al*. (2016) reported that *C. perfringens* types A with cpe+ were found only in fresh fish while *C. perfringens* type E strain was detected in fish, humans, and water. In the same year Yadav *et al*. (2016) from Kolkata city, India reported that 100% isolates comprising of 70.83% from fresh water fish and 29.17% from fish products harboured cpa toxin gene with 70.58% isolates from fresh water fish and 71.42% from fish products also harbor additional cpb2 toxin gene, while none of the isolates found to be positive for cpb, εtx, ιap and cpe genes.

4.6.3 Occurrence of different toxotypes of *C. perfringens* isolates from pasteurized milk and milk product

The isolates of *C. perfringens* from pasteurized milk and milk product showed that out of 17 isolates from pasteurized milk samples, 82.35% (14/17) were type A and 17.65% (3/17) type C, while none of the isolates belonged to type B and D (Table 6). Out of 14 *C. perfringens* type A isolates 14.29% (02) each were found to harboured enterotoxin (cpe gene) and β2 toxin (cpb2 gene), while 21.43% (03) isolates were only positive for β2 toxin (cpb2 gene). Among 03 *C. perfringens* type C isolates 33.33% (01) each were found to harboured only enterotoxin (cpe gene) and β2 toxin (cpb2 gene) while none of the isolate harboured both toxin together. The finding of milk product samples showed that all isolates (2/2) were type A and none of the isolates were found to harbour enterotoxin (cpe gene) and β2 toxin (cpb2 gene).

Similar to the finding of present study El Tawab *et al*. (2016) reported that 100% *C. perfringens* isolates from milk and milk products were positive for alpha toxin gene, out of which 28.57% isolates were positive for enterotoxin gene. The presence of toxigenic *C. perfringens* type A harbouring enterotoxin and β2 toxin in pasteurized milk and milk products may contribute to public health hazards to consumers, therefore it is warranted to maintain the strict hygiene during milking, handling and processing to reduce risk of consumers.

4.6.4 Occurrence of different toxotypes of *C. perfringens* isolates from human diarrhoeic stool

The isolates of *C. perfringens* from human diarrhoeic stool samples showed that out of 13 isolates, 84.61% (11/13) were type A, 7.69% (1/13) each type B and type C, while none of the isolates belonged to type D (Table 6). Out of 11 *C. perfringens* type A isolates 18.18% (02) harboured only β2 toxin (cpb2 gene) while 9.09% (01) type A isolates harboured both enterotoxin (cpe gene) and β2 toxin. However, none of the type A isolates from human diarrhoeic stool samples were found to harbour enterotoxin (cpe gene) alone. The only one isolate from human diarrhoeic stool samples that was found as *C. perfringens* type B was also found to harbour enterotoxin (cpe gene). The type C isolate (01) was not found to harbour either enterotoxin (cpe gene) and β2 toxin.

In the present study three toxotypes of *C. perfringens* were recovered from human diarrhoeic stool which is supported by the fact that the healthy humans serve as a rich reservoir for cpe+ C. perfringens type A strains and may play an important role in gastrointestinal diseases caused by this pathogen (Kukier and Kwiatek, 2010). The presence toxic C. perfringens among healthy food handlers was studied by Heikinheimo *et al*. (2006) who reported a prevalence of 18% for enterotoxin gene–carrying (cpe+) *C. perfringens* in the feces. They concluded that humans should be considered as a risk factor for spread of *C. perfringens* type A food poisoning along with possible source of contamination. Further, detection of type B and C in this study also supported by the fact that *C. perfringens* type B – E are recognised as frank pathogens for domestic animals and human (Songer, 1996).

The occurrence of different toxotypes of *C. perfringens* with enterotoxin and β2 toxin among raw and processed foods of animal origin as well as human diarrhoeic cases may pose a public health threats therefore it is warranted to maintain the strict personal hygiene as well as hygienic handling of raw and processed foods of animal origin.

4.7 Determination of Minimum Inhibitory concentration (MIC) and antibiotic sensitivity profile of *C. Perfringens* isolates in different food samples of animal origin, and human diarrhoeic stool

For determination of MIC all 16Sr RNA PCR positive isolates (N=80) harvested from different samples of fish, meat, milk and human diarrhoeic stool were screened using HiComb^{TM} MIC Test (Hi-Media, India) of antibiotics group including penicillins, β-Lactam, cephems, tetracycline, fluoroquinolones, linosamides and phenicols (Table 2). The MIC values of all the isolates (N=80) against above mentioned antibiotic groups were presented in (Table 8). On the basis of finding of MIC Test, the antimicrobial susceptibility pattern of all isolates were represented as resistant, intermediate and susceptible according to interpretative criteria given in CLSI, 2015 guidelines for anaerobes.

The MICs study of ceftriaxone (CTR) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg, at which only 6.25% (05) isolates were inhibited, whereas MIC_{50} and MIC_{90} of CTR for *C. perfringens* isolates were calculated as 0.5µg and 10µg(Table 10), respectively. On the basis of present finding 92.5% (74) isolates were found as sensitive, while 3.75% (3) as intermediate and 3.75% (03) resistant to ceftriaxone (Table 10 , Fig. 15).

The MICs study of amoxyclav (AMC) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg, at which only 6.25% (11) isolates were inhibited, whereas MIC_{50} and MIC_{90} of AMC for *C. perfringens* isolates were calculated as 0.1µg and 0.5µg, respectively. On the basis of present finding 95% (76) isolates were found as sensitive, while 2.5% (02) as intermediate and 2.5% (02) resistant to amoxyclav (Table 10, Fig. 15).

The MICs study of piperracillin/tazobactum (PIT) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg, at which only 5% (04) isolates were inhibited, whereas MIC_{50} and MIC_{90} of PIT for *C. perfringens* isolates were calculated as 0.5µg and 10µg, respectively. On the basis of present finding 96.25% (77) isolates were found as sensitive, while 1.25% (01) as intermediate and 2.5% (02) resistant to piperracillin/tazobactum (Table 10, Fig. 15).

The MICs study of piperacillin (PI) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg at which only 2.5% (02) isolates were inhibited, whereas MIC_{50} and MIC_{90} of PI for *C. perfringens* isolates were calculated as 5µg and 10µg, respectively. On the basis of present finding 97.5% (78) isolates were found as sensitive, while 2.5% (2) resistant to piperacillin (Table 10 , Fig. 15).

The MICs study of moxifloxacin (MO) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg at which only 8.75% (7) isolates were inhibited, whereas MIC_{50} and MIC_{90} of MO for *C. perfringens* isolates were calculated as 4µg and 10µg, respectively. On the basis of present finding 58.75% (47) isolates were found as sensitive, while 8.75% (15) as intermediate and 22.5% (18) resistant to moxifloxacin (Table 10 , Fig. 15).

The MICs study of chloramphenicol (C) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg at which only 8.75% (7) isolates were inhibited, whereas MIC_{50} and MIC_{90} of C for *C. perfringens* isolates were calculated as 0.5µg and 4µg, respectively. On the basis of present finding 95% (76) isolates were found as sensitive, while 5% (04) as resistant to chloramphenicol (Table 10 , Fig. 15).

The MICs study of ticarcillin (TI) for *C. perfringens* isolates (N=80) showed the MIC of 0.1µg at which only 5% (4) isolates were inhibited, whereas MIC_{50} and MIC_{90} of TI for *C. perfringens* isolates were calculated as 8µg and 30µg, respectively. On the basis of present finding 80% (64) isolates were found as sensitive, while 7.5% (06) as intermediate and 12.5% (10) resistant to ticarcillin (Table 10 , Fig. 15).

The MICs study of tetracycline (TE) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg at which only 5% (4) isolates were inhibited, whereas MIC_{50} and MIC_{90} of TE for *C. perfringens* isolates were calculated as 8µg and 30µg, respectively. On the basis of present finding 80% (64) isolates were found as sensitive, while 7.5% (06) as intermediate and 12.5% (10) resistant to tetracycline (Table 10 , Fig. 15).

The MICs study of clindamycin (CD) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg at which only 3.75% (3) isolates were inhibited, whereas MIC_{50} and MIC_{90} of CD for *C. perfringens* isolates were calculated as 2µg and 120µg, respectively. On the basis of present finding 52.5% (42) isolates were found as sensitive, while 16.25% (13) as intermediate and 31.25% (25) resistant to clindamycin (Table 10 , Fig. 15).

The MICs study of ampicillin (AMP) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg at which only 1.25% (01) isolates were inhibited, whereas MIC_{50} and MIC_{90} of AMP for *C. perfringens* isolates were calculated as 0.064µg and 0.256µg, respectively.

On the basis of present finding 95% (76) isolates were found as sensitive, while 5% (04) as resistant to ampicillin (Table 10 , Fig. 15).

The MICs study of cefotaxime (CTX) for *C. perfringens* isolates (N=80) showed the MIC of 0.01µg at which only 7.5% (06) isolates were inhibited, whereas MIC_{50} and MIC_{90} of CTX for *C. perfringens* isolates were calculated as 1µg and 4µg, respectively. On the basis of present finding 97.5% (78) isolates were found as sensitive, while 1.25% (01) each as intermediate and resistant to cefotaxime (Table 10 , Fig. 15)

The detail of the sample wise *C. perfringens* isolates antibiotic susceptibility profile has been presented in fig 14. The present findings highlighted that 50% *C. perfringens* isolates from raw meat samples were resistance to CD followed by 39% to MOX and TI, 11% to TE and 5.6% to AMC and PI. Whereas, 25% isolates from meat products samples were resistance to CD and TE. The antibiotic susceptibility profile also depicted that 47% isolates from raw fish samples were resistance to MOX followed by 41% to CD, 29% to TE and 5.9% to CTR as well as PIT whereas 11% isolates from fish product samples were found as resistance to AMC, MOX and PIT. The antibiotic susceptibility profile of isolates from pasteurized milk samples showed that 41% isolates were resistance to CD followed by 35% to TE, 31% AMP, 24% from C, 18% TI, 12% CTR and 5.9% from AMC, CTX, MOX and PI. Whereas, the isolates from milk product samples did not showed resistance of any of the antibiotics used under study. The antibiotic susceptibility profile *C. perfringens* isolates from human diarrhoeic samples showed that 23% isolates were resistance to TE followed by 7.7% to MOX.

In India, the availability of data regarding antibiotic susceptibility profile of anaerobe specially *C. perfringens* from human and animal origin raw and processed foods as per recommendation of CLSI, 2015 is limited. The present study revealed that none of the antibiotics were sensitive for all *C. perfringens* isolates belonging to type A, type B, type C as well as type D. Similar to the finding of present study Yadav et al. (2017) also reported that irrespective of the source of isolation, majority of the *C. perfringens* type A isolates exhibited resistance to ampicillin, co-trimoxazole, ceftazidime, norfloxacin, ceftriaxone and tetracycline.

On the other hand, other studies reported that that most of the *C. perfringens* isolates were sensitive to ciprofloxacin, ofloxacin, enrofloxacin, norfloxacin, tetracycline, nitrofurantoin, amikacin, piperacillin, cefuroxime, ceftazidime, cephoxitin, ceftriaxone, chloramphenicol, penicillin G and sulfatriad (Singh *et al*., 2005; Rahman *et al*., 2012; Singh and Bist, 2013; Sarkar *et al*., 2013). In developing countries, the available reports support the facts of overuse or misuse of antibiotics in human and veterinary medicine without proper antibiotic susceptibility testing, which could be a major factor for the increased antimicrobial resistance (Wellington *et al*., 2013).

4.8. Phylogenetic analysis of *C. Perfringens* isolates from food samples of animal origin, and human diarrhoeic stool

Genotyping has an important role for determination of origin or source of bacterial isolates and also to identify the genetic relatedness between isolates. Recent molecular techniques available for studying genetic diversities of bacterial pathogens may include pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), repetitive extragenic palindromic- PCR (REP-PCR), enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) and multilocus sequence typing (MLST) (Anjay *et al*., 2015). Among various techniques ERIC-PCR has been reported as similar discriminatory power as RAPD-PCR for differentiation of pathogens. (Szczuka & Kaznowski., 2004). In addition to this, ERIC-PCR also has an advantage over other genotyping methods, as it requires a low quantity of DNA for amplification and also does not require any prior DNA sequence information (Thomas *et al*., 2009). In this segment of study, the results and discussion pertaining to recognize the clonal relatedness along with comparison of genetic similarities of clones among isolates belonged to different fish and fish product, meat and meat product, pasteurized milk and milk products and human diarrhoeal stool specimens are described.

To determine the phylogenetic similarity among different meat, fish, milk and human isolates of *C. Perfringens* ERIC-PCR was performed using an arbitrary primer E1(ATGAAGCTCCTGGGGATTCAC) and E2(AAGTAAGTGACTGGGGTGAGG) (Versalovic *et al*., 1991). In each amplification reaction, the presence (1) or absence (0) of

amplicons generated binary matrix values. The DICE coefficient was used to estimate the genetic similarity of *C. Perfringens* isolates and a dendrogram was obtained by the method of UPGMA (unweighted pair group arithmetic average) with a Bootstrap of 100, using the program PyElph 1.4 (PAVEL & VASILE, 2012). The discrimination power of the assay was determined with on line discrimination power calculator by adding the number of isolates found within a cluster at 95% similarity index.

4.8.1 Phylogenetic analysis of *C. perfringens* isolates from meat and meat product

ERIC PCR amplification of 16 *C. perfringens* type A isolates belonged to meat and meat product samples showed 2 to 7 bands of molecular weight from <250bp to 2000bp (Fig. 16A). The banding pattern observed with UPGMA analysis revealed that all isolates were comes under 3 major clusters naming A to C, illustrating genetic relationships among the isolates. The mostfrequent clusters were A (62.50%), B (25.00%) and C (12.5%) (Figure 1). The discrimination power of the assay was calculated as 0.5667 (Fig. 16B).

4.8.2 Phylogenetic analysis of *C. perfringens* isolates from fish and fish product

ERIC-PCR amplification of 19 *C. perfringens* type A isolates belonged to fish and fish product samples showed 2 to 8 bands of molecular weight from <250bp to 1500bp (Fig. 17A).. The banding pattern observed with UPGMA analysis revealed that all isolates were comes under 5 major clusters naming A to E, illustrating genetic relationships among the isolates. The most frequent clusters were A (57.89%), B (21.05%) and C (10.52%) (6.25%). Cluster D and E were recognized by individual isolate (Figure 1). The discrimination power of the assay was calculated as 0.6374 (Fig. 17B).

4.8.3 Phylogenetic analysis of *C. perfringens* isolates from pasteurized milk and milk products

ERIC-PCR amplification of 16 *C. perfringens* type A isolates belonged to raw and pasteurized milk samples showed 1 to 7 bands of molecular weight from <250bp to 1000bp (Fig. 18A).. The banding pattern oserved with UPGMA analysis revealed that all isolates were comes under 3 major clusters naming A to C, illustrating genetic relationships among the isolates. The most frequent clusters were A (31.25%) and B (62.50%). Cluster Chaving only one isolate. The discrimination power of the assay was calculated as 0.5417 (Fig. 18B).

4.8.4. Phylogenetic analysis of *C. perfringens* isolates from human diarrhoeic stool

ERIC-PCRamplification of 11 *C. perfringens* type A isolates belonged to human diarrhoeic samples showed 3 to 7 bands of molecular weight from <250bp to 750bp (Fig. 19A). The banding pattern observed with UPGMA analysis revealed that all isolates were comes under 5 major clusters naming A to E, illustrating genetic relationships among the isolates. The most frequent clusters were A (45.45%), C and E (18.18%). Cluster D was formed by only one isolate (Figure 1). The discrimination power of the assay was calculated as 0.7818(Fig. 19B).

C. perfringens type A were reported as phenotypically and genetically diverse organism and many conventional methods for identification viz., cultural and biochemical produced contradictory results when compared to the molecular tools. Because of the complexity of methodologies, time taking and difficulty in interpretation, genomic analysis methods have been commonly employed to characterize the microbial pathogens. In the present study all *C. perfringens* type A isolates (N=62) were typable using selected primer with a discrimination power of 0.5417 to 0.7818. ERIC-PCR profile of *C. perfringens* type A isolates generated using ERIC- and ERIC 2 primer pair showed variable polymorphisms and unique DNA bands.

The findings of the present study were in concordance with the finding of Xue-qin *et al*. (2009) who performed a study on the epidemiology of *C. perfringens* type A colonizing healthy birds and reported that all 34 *C. perfringens* type A isolates were typable using enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) with distribution of total 8 ERIC-PCR genotypes. ERIC-PCR fingerprints have been used for typing and diagnosis of bacteria. So, this molecular typing method could be used as a new strategy for epidemiological investigations. This information can be used to improve the quality control and bio-security protocols to check *C. perfringens* type A cases and this concept can be applied to other bacterial pathogens.

4.9.1 Genetic relatedness *C. perfringens type A* isolates of meat and meat products and human diarrhoeic stool

The phylogenetic analysis using the program PyElph 1.4 to estimate the genetic similarity among 16 and 11 of *C. perfringens* type A isolates belonged to meat and meat product samples and human diarrhoeic samples respectively revealed a formation of 5 major

clusters naming A to E. The most frequent clusters were A and B (29.62%) consist of 3 human diarrhoeic and 5 meat and meat product samples. While other Clusters B to E consist of isolates either belonged to meat and meat product and human diarrhoeic or human diarrhoeic. The discrimination power of the assay was calculated as 0.8063 (Fig. 20B).

Above all, the phylogeny of ERIC-PCR assay suggested a high degree (>95%) of lineage between human and meat and meat products isolates of *C. perfringens* type A. The genetic linkage of *C. perfringens* type A isolates of meat and meat products and human diarrhoeic suggested that 4 different clones constituted by ~30%, ~29.62%, ~18.51% and ~7.40% of isolates with >95% genetic similarity circulating in both animal and human population. The above finding also suggested that 85.15% isolates circulating in both meat and meat products as well as human population were of similar genetic makeup.

4.9.2 Genetic relatedness *C. perfringens* type A isolates of fish and fish products and human diarrhoeic stool

The phylogenetic analysis 19 and 11 isolates belonged to fish and fish product samples and human diarrhoeic samples, respectively revealed a formation of 5 major clusters naming A to E. The most frequent clusters were A (50%) consist of 7 and 8 isolates of human diarrhoeic and fish and fish product. While, Clusters C and D (13.33%) consist of 2 each of human diarrhoeic and fish and fish product isolates. However, cluster B and D consist of isolates belonged to fish and fish product. The discrimination power of the assay was calculated as 0.7103 (Fig. 21B).

The genetic linkage of *C. perfringens* type A isolates of fish and fish products with human diarrhoeic isolates suggested that three different clones constituted by ~50% and two each of~13.33% of isolates with >95% genetic similarity circulating in both fish and human population.

4.9.3 Genetic relatedness *C. perfringens* type A isolates of raw and pasteurized milk and human diarrhoeic stool

The phylogenetic analysis 16 and 11 isolates belonged to pasteurized milk and milk products and human diarrhoeic samples, respectively revealed a formation of 6 major clusters naming A to F. The most frequent clusters were A (33.33%) consist of 4 and 5 isolates of human diarrhoeic and pasteurized milk and milk product. While other Clusters

B to F consist of isolates either belonged to pasteurized milk and milk product or human diarrhoeic (Figure1). The discrimination power of the assay was calculated as 0.8063 (Fig. 22B).

The genetic linkage of *C. perfringens* type A isolates of raw and pasteurized milk with human diarrhoeic isolates also suggested that one cluster A ~33% of isolates with 95% genetic similarity circulating in both pasteurized milk as well as human population. The above finding also suggested that ~33% isolates circulating in both pasteurized milk as well as human population were of similar genetic makeup.

From the finding of present study, it may be presumed that meat and meat products, fish and fish products as well as pasteurized milk and milk products in this area harbour the pathogenic *C. perfringens* type A and serve as reservoir/ source of this organism to human beings by entry into food chain. Alternatively, the possibility for transmission of this infection from human to animals may not be ruled out. In such occasion, human carrier may shed the pathogens into the atmosphere and the organism in placing its spore formation get the best opportunity for optimum expression of its genetic characters and they perpetuate therein with longing whenever to get entry in human health through food chain. In these circumstances, our traditional cooking practices not the fast food habit bestowed upon boon to limit probability of occurrence of diarrhoea and gastroenteritis with intake of such suspected foods of animal origin.

Table 3: Cultural isolation, biochemical and molecular confirmation of *C. perfringens* isolates from foods of animal origin and human diarrhoeic samples.

Sl. No.	Samples	Sample type	No. of samples	Cultural isolation of *C. perfringens*	Biochemical confirmation	Species specific 16S rRNA PCR	
						Based on sample	Based on cultural isolation
1.	Meat	Raw	35	80.00% (28)	60.00% (21)	51.43% (18)	64.29% (18)
		Products	15	53.33% (08)	40.00% (06)	26.67% (04)	50.00% (04)
		Total	**50**	**72.00% (36)**	**54.00% (27)**	**44.00% (22)**	**61.11% (22)**
2.	Fish	Raw	35	80.00% (28)	54.28% (19)	48.57% (17)	60.71% (17)
		Products	15	66.67% (10)	66.67% (10)	60.00% (09)	90.00% (9)
		Total	**50**	**76.00 (38)**	**58.00 (29)**	**52.00% (26)**	**68.42% (26)**
3.	Milk	Pasteurized	51	80.39% (41)	39.22% (20)	33.33% (17)	41.46% (17)
		Product	50	10.00% (05)	4.00% (02)	4.00% (02)	40.00% (2)
		Total	**101**	**45.54% (46)**	**21.78% (22)**	**18.81% (19)**	**41.30% (19)**
4.	Human	Diarrhoeic stool	50	52.00% (26)	34.00% (17)	26.00% (13)	50.00% (13)
		Total	**50**	**52.00% (26)**	**34.00% (17)**	**26.00% (13)**	50.00% (13)
		Total	251	**58.17% (146)**	**37.85% (95)**	**31.87% (80)**	**54.79% (80)**

***The percentage value calculated on the basis of total no. of samples in each sample type and cultural isolation.**

Table 4: Distribution of different toxin genes harbouring *C. perfringens* among foods and food product of animal origin and human diarrhoeic samples

Sr. no.	Samples	Types	Total samples	Toxin gene				
				cpa	***cpb***	***etx***	***cpe***	***Cpb2***
1.	Meat	Raw	35	51.43% (18)	14.29% (05)	0% (00)	2.86% (01)	25.71% (09)
		Processed	15	26.67% (04)	6.67% (01)	0% (00)	6.67% (01)	6.67% (01)
		Total	**50**	**44.00% (22)**	**12.00% (06)**	**0% (00)**	**4.00% (02)**	**20.00% (10)**
2.	Fish	Raw	35	48.57% (17)	11.43% (04)	8.57% (03)	5.71% (02)	17.14% (06)
		Processed	15	60.00% (09)	0% (00)	6.67% (01)	0% (00)	40.00% (06)
		Total	**50**	**52.00% (26)**	**8.00% (04)**	**8.00% (04)**	**4.00% (02)**	**24.00% (12)**
3.	Milk	Pasteurized	51	33.33% (17)	5.88% (03)	0% (00)	7.84% (04)	11.76% (06)
		Product	50	4.00% (02)	0% (00)	0% (00)	0% (00)	0% (00)
		Total	**101**	**18.81% (19)**	**2.97% (03)**	**0% (00)**	**3.96% (04)**	**5.94% (06)**
4.	Human	Diarrhoeic	**50**	26.00% (13)	4.00% (02)	2.00% (01)	4.00% (02)	8.00% (04)
		Total	**251**	**31.87% (80)**	**5.98% (15)**	**1.99% (5)**	**3.98% (10)**	**12.75% (32)**

***The percentage value calculated on the basis of total no. of each type samples.**

Table 5: Distribution of different toxin genes among *C. perfringens* isolates from foods and food product of animal origin and human diarrhoeic samples

Sl. no.	Samples	Types	16S rRNA confirmed isolates	Toxin gene				
				cpa	***Cpb***	***Etx***	***Cpe***	***Cpb2***
1.	Meat	Raw	18	100% (18)	27.28% (05)	0.0% (00)	5.56% (01)	50.00% (09)
		Processed	4	100% (04)	25.00% (01)	0.0% (00)	25.00% (01)	25.00% (01)
Total			**22**	**100% (22)**	**27.27% (06)**	**0.0% (00)**	**9.09% (02)**	**45.45% (10)**
2.	Fish	Raw	17	100% (17)	23.52% (04)	17.65% (03)	11.76% (02)	35.29% (06)
		Processed	9	100% (09)	0.0% (00)	11.11% (01)	0.0% (00)	66.67% (06)
Total			**26**	**100% (26)**	**15.38% (04)**	**15.3% (04)**	**7.69% (02)**	**46.15% (12)**
3.	Milk	Pasteurized	17	100% (17)	17.65% (03)	0.0% (00)	23.53% (04)	35.29% (06)
		Product	02	100% (02)	0.0% (00)	0.0% (00)	0.0% (00)	0.0% (00)
Total			**19**	**100% (19)**	**15.79% (03)**	**0.0% (00)**	**21.0% (04)**	**31.58% (06)**
4.	Human	Stools	13	100% (13)	15.38% (02)	7.69% (1)	15.38% (02)	30.77% (04)
Total			**80**	**100% (80)**	**18.75% (15)**	**6.25% (05)**	**12.5% (10)**	**40.00% (32)**

***The percentage value calculated on the basis of no. of confirmed isolates by molecular method.**

Table 7: Distribution of enterotoxin and β_2 toxin among different toxotype of *C. perfringens* isolates

Sl. No.	Toxotypes	Sample	Distribution of toxin genes among *C. perfringens* toxotypes		
			Enterotoxin	β_2 toxin	Enterotoxin+ β_2 toxin
1.	Type A	CM	0	38.46% (5/13)	7.69% (1/13)
		CMP	33.33% (1/3)	33.33% (1/3)	0
		F	0	36.36% (4/11)	0
		FP	0	50% (4/8)	0
		PM	14.29% (2/14)	21.43% (3/14)	14.29% (2/14)
		MP	0	0	0
		HS	0	18.18% (2/11)	9.09% (1/11)
2.	Type B	CM	0	0	0
		CMP	0	0	0
		F	0	100% (1/1)	0
		FP	0	0	0
		PM	0	0	0
		MP	0	0	0
		HS	100% (1/1)	0	0
3.	Type C	CM	0	60.00% (3/5)	0
		CMP	0	0	0
		F	0	0	0
		FP	0	0	0
		PM	33.33% (1/3)	33.33% (1/3)	0
		MP	0	0	0
		HS	0	0	0
4.	Type D	CM	0	0	0
		CMP	0	0	0
		F	50.00% (1/2)	0	50.00% (1/2)
		FP	0	100% (1/1)	0
		PM	0	0	0
		MP	0	0	0
		HS	0	0	0

***The percentage value calculated on the basis of no. of isolates in each toxotype of each sample.**

****CM= Raw meat CMP= meat product F= Raw fish FP= Fish product PM= Pasteurized milk MP= Milk product HS= human stool**

Table 6: Toxotype of *C. perfringens* isolates from foods and food product of animal origin and human diarrhoeic samples

Sl. No.	Samples	Types	16S rRNA confirmed isolates	Toxotypes of C. *perfringens* isolates			
				Type A (*cpa*$^+$)	**Type B (*cpa*$^+$, *cpb*$^+$ & *etx*$^+$)**	**Type C (*cpa*$^+$ & *cpb*$^+$)**	**Type D (*cpa*$^+$ & *etx*$^+$)**
1.	Meat	Raw	18	72.22% (13)	0.0% (00)	27.78% (05)	0.0% (00)
		Processed	4	75.00% (03)	0.0% (00)	25.00% (01)	0.0% (00)
		Total	**22**	**72.73% (16)**	**0.0% (00)**	**27.27% (06)**	**0.0% (00)**
2.	Fish	Raw	17	64.71% (11)	5.88% (01)	17.65% (03)	11.76% (02)
		Processed	9	88.89% (08)	0.0% (00)	0.0% (00)	11.11% (01)
		Total	**26**	**73.08% (19)**	**3.85% (01)**	**11.54% (03)**	**0.0% (00)**
3.	Milk	Pasteurized	17	82.35% (14)	0.0% (00)	17.65% (03)	0.0% (00)
		Product	02	100% (02)	0.0% (00)	0.0% (00)	0.0% (00)
		Total	**19**	**84.21% (16)**	**0.0% (00)**	**15.79% (03)**	**0.0% (00)**
4.	Human	Stools	13	84.61% (11)	7.69% (01)	7.69% (01)	0.0% (00)
		Total	**80**	**77.50% (62)**	**2.50% (02)**	**16.25% (13)**	**3.75% (03)**

*** The percentage value calculated on the basis of no. of confirmed isolates by molecular method.**

Table 8: Minimum Inhibitory Concentration of different isolates of *C. perfringens*																	
		MIC(µg)															
Antibiotic	Sample	0.01	0.05	0.1	0.5	1	2	3	4	5	8*	10	16**	30	60	120	240
AMC	CM	5.56(1)	0(0)	50(9)	38.88(7)	0(0)	0(0)	0(0)	0(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	5.56(1)
	CMP	50(2)	0(0)	50(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	11.76(2)	5.88(1)	41.17(7)	35.29(6)	0(0)	0(0)	0(0)	0(0)	5.88(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	FP	11.11(1)	0(0)	66.67(6)	11.11(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	11.11(1)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	11.76(2)	0(0)	76.47(13)	5.88(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	5.88(1)
	MP	50(1)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	15.38(2)	0(0)	84.6(11)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**11**	**1**	**49**	**15**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**0**	**0**	**2**
TI	CM	0(0)	0(0)	38.85(7)	0(0)	0(0)	11.12(2)	0(0)	11.12(2)	0(0)	11.12(2)	11.12(2)	11.12(2)	0(0)	0(0)	0(0)	5.56(1)
	CMP	0(0)	0(0)	25(1)	0(0)	0(0)	25(1)	0(0)	0(0)	0(0)	25(1)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	0(0)	17.64(3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	41.17(7)	41.17(7)	0(0)	0(0)	0(0)
	FP	0(0)	0(0)	33.33(3)	0(0)	0(0)	11.11(1)	0(0)	22.22(2)	0(0)	22.22(2)	0(0)	11.11(1)	0(0)	0(0)	0(0)	0(0)
	PM	0(0)	0(0)	11.76(2)	0(0)	23.52(4)	5.88(1)	0(0)	35.29(6)	0(0)	5.88(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	MP	0(0)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	0(0)	0(0)	30.76(4)	0(0)	0(0)	0(0)	0(0)	61.52(8)	0(0)	0(0)	0(0)	7.69(1)	0(0)	0(0)	0(0)	0(0)
	Total	**0**	**0**	**21**	**0**	**4**	**5**	**0**	**19**	**0**	**6**	**3**	**11**	**7**	**0**	**0**	**4**
PI	CM	11.12(2)	0(0)	22.24(4)	16.68(3)	16.68(3)	0(0)	0(0)	0(0)	22.24(4)	0(0)	0(0)	0(0)	5.56(1)	11.12(2)	0(0)	5.56(1)
	CMP	0(0)	0(0)	25(1)	0(0)	25(1)	0(0)	0(0)	0(0)	50(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	0(0)	11.76(2)	0(0)	11.76(2)	0(0)	0(0)	0(0)	70.56(12)	0(0)	0(0)	0(0)	0(0)	5.88(1)	0(0)	0(0)
	FP	0(0)	0(0)	33.33(3)	0(0)	0(0)	0(0)	0(0)	0(0)	22.22(2)	0(0)	44.44(4)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	0(0)	0(0)	17.64(3)	5.88(1)	0(0)	0(0)	0(0)	0(0)	23.52(4)	0(0)	41.16(7)	0(0)	5.88(1)	0(0)	0(0)	5.88(1)
	MP	0(0)	0(0)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	50(1)	0(0)	0(0)	0(0)
	HS	0(0)	0(0)	38.45(5)	30.76(4)	0(0)	0(0)	0(0)	0(0)	23.07(3)	7.69 (1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**2**	**0**	**18**	**9**	**6**	**0**	**0**	**0**	**27**	**1**	**11**	**0**	**3**	**3**	**0**	**2**
PIT	CM	11.12(2)	5.56(1)	33.36(6)	5.56(1)	0(0)	0(0)	0(0)	0(0)	38.92(7)	0(0)	0(0)	0(0)	5.56(1)	0(0)	0(0)	0(0)

	CMP	25(1)	0(0)	25(1)	25(1)	0(0)	0(0)	0(0)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	0(0)	29.40(5)	0(0)	0(0)	0(0)	0(0)	0(0)	52.92(9)	0(0)	11.76(2)	0(0)	0(0)	5.88(1)	0(0)	0(0)
	FP	11.11(1)	11.11(1)	11.11(1)	22.22(2)	0(0)	0(0)	0(0)	0(0)	33.33(3)	0(0)	0(0)	0(0)	0(0)	11.11(1)	0(0)	0(0)
	PM	0(0)	0(0)	35.28(6)	23.52(4)	5.88(1)	0(0)	0(0)	0(0)	0(0)	0(0)	23.52(4)	0(0)	11.76(2)	0(0)	0(0)	0(0)
	MP	0(0)	0(0)	0(0)	100(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	0(0)	0(0)	23.07(3)	61.52(8)	0(0)	0(0)	0(0)	0(0)	15.38(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**4**	**2**	**22**	**18**	**1**	**0**	**0**	**0**	**22**	**0**	**6**	**0**	**3**	**2**	**0**	**0**
CTR	CM	5.56(1)	0(0)	44.48(8)	0(0)	33.36(6)	0(0)	5.56(1)	0(0)	5.56(1)	0(0)	5.56(1)	0(0)	0(0)	0(0)	0(0)	0(0)
	CMP	0(0)	0(0)	75(3)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	0(0)	29.4(5)	5.88(1)	5.88(1)	0(0)	17.64(3)	0(0)	5.88(1)	17.64(3)	0(0)	0(0)	5.88(1)	0(0)	0(0)	5.88(1)
	FP	0(0)	0(0)	44.44(4)	0(0)	11.11(1)	0(0)	33.33(3)	0(0)	11.11(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	0(0)	0(0)	52.92(9)	0(0)	11.76(2)	0(0)	5.88(1)	0(0)	0(0)	0(0)	5.88(1)	0(0)	11.76(2)	0(0)	0(0)	11.76(2)
	MP	50(1)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	23.07(3)	0(0)	38.45(5)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	15.38(2)	23.07(3)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**5**	**0**	**34**	**1**	**11**	**0**	**5**	**0**	**2**	**5**	**5**	**0**	**3**	**0**	**0**	**3**
CTX	CM	0(0)	0(0)	33.36(6)	5.56(1)	16.68(3)	0(0)	27.8(5)	0(0)	0(0)	16.68(3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	CMP	0(0)	0(0)	50(2)	0(0)	0(0)	0(0)	25(1)	0(0)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	5.88(1)	0(0)	35.28(6)	0(0)	11.76(2)	0(0)	41.16(7)	0(0)	0(0)	0(0)	0(0)	5.88(1)	0(0)	0(0)	0(0)	0(0)
	FP	33.33(3)	0(0)	44.44(4)	0(0)	22.22(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	5.88(1)	0(0)	35.28(6)	0(0)	23.52(4)	0(0)	16.64(3)	0(0)	0(0)	0(0)	0(0)	5.88(1)	5.88(1)	0(0)	0(0)	5.88(1)
	MP	0(0)	0(0)	0(0)	0(0)	50(1)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	7.69(1)	0(0)	0(0)	0(0)	0(0)	61.52(8)	0(0)	30.76(4)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**6**	**0**	**24**	**1**	**12**	**8**	**17**	**4**	**0**	**4**	**0**	**2**	**1**	**0**	**0**	**1**
TE	CM	0(0)	0(0)	11.12(2)	0(0)	44.48(8)	0(0)	0(0)	5.56(1)	33.36(6)	0(0)	0(0)	0(0)	0(0)	5.56(1)	0(0)	0(0)
	CMP	0(0)	0(0)	0(0)	0(0)	75(3)	0(0)	0(0)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	0(0)	47.04(8)	11.76(2)	0(0)	0(0)	0(0)	0(0)	11.76(2)	0(0)	23.52(4)	0(0)	5.88(1)	0(0)	0(0)	0(0)
	FP	44.44(4)	0(0)	44.44(4)	0(0)	0(0)	0(0)	11.11(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	35.28(6)	0(0)	17.64(3)	0(0)	11.76(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	5.88(1)	0(0)	17.64(3)	11.76(2)

	MP	0(0)	0(0)	100(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	15.38(2)	0(0)	61.52(8)	0(0)	0(0)	0(0)	0(0)	0(0)	38.45(5)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**12**	**0**	**27**	**0**	**13**	**0**	**1**	**1**	**12**	**0**	**4**	**0**	**2**	**1**	**3**	**2**
MO	CM	0(0)	5.56(1)	16.68(3)	5.56(1)	0(0)	0(0)	0(0)	16.68(3)	16.68(3)	38.92(7)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	CMP	0(0)	0(0)	50(2)	25(1)	0(0)	0(0)	0(0)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	5.88(1)	0(0)	23.52(4)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	FP	22.22(2)	22.22(2)	44.44(4)	0(0)	0(0)	0(0)	0(0)	11.11(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	17.64(3)	29.4(5)	41.16(7)	0(0)	0(0)	0(0)	0(0)	5.88 (1/17)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	5.88 (1/17)
	MP	0(0)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	7.69(1)	0(0)	61.52(13)	0(0)	0(0)	0(0)	0(0)	23.07(3)	0(0)	7.69(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**7**	**8**	**21**	**2**	**0**	**0**	**0**	**9**	**12**	**4**	**16**	**0**	**0**	**0**	**0**	**1**
CD	CM	0(0)	0(0)	16.68(3)	0(0)	0(0)	0(0)	0(0)	27.8(5)	0(0)	16.68(3)	0(0)	0(0)	0(0)	11.12(2)	16.68(3)	11.12(2)
	CMP	0(0)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	50(2)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	0(0)	0(0)	0(0)	0(0)	47.04(8)	0(0)	11.76(2)	0(0)	17.64(3)	0(0)	0(0)	17.64(3)	0(0)	5.88(1)	0(0)
	FP	22.22(2)	0(0)	55.56(5)	0(0)	0(0)	11.11(1)	0(0)	11.11(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	5.88(1)	0(0)	0(0)	47.04(8)	0(0)	0(0)	0(0)	5.88(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	5.88(1)	35.28(6)
	MP	0(0)	0(0)	50(1)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	0(0)	0(0)	84.59(11)	15.38(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**3**	**0**	**21**	**11**	**0**	**9**	**0**	**11**	**0**	**7**	**0**	**0**	**3**	**2**	**5**	**8**
C	CM	5.56(1)	0(0)	38.92(7)	27.8(5)	0(0)	11.12(2)	16.68(3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	CMP	0(0)	0(0)	25(1)	0(0)	25(1)	50(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	0(0)	5.88(1)	35.28(6)	0(0)	23.52(4)	0(0)	5.88(1)	5.88(1)	23.52(4)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
		11.11(1)	0(0)	11.11(1)	55.56(5)	11.11(1)	11.11(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	0(0)	5.88(1)	5.88(1)	47.04(8)	11.76(2)	11.76(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	5.88(1)	5.88(1)	5.88(1)	0(0)
	MP	0(0)	0(0)	50(1)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	0(0)	0(0)	23.07(3)	69.21(9)	0(0)	7.69(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**2**	**1**	**15**	**34**	**7**	**12**	**3**	**1**	**1**	**4**	**0**	**0**	**1**	**1**	**1**	**0**

	MIC(µg)	**0.01**	**0.016**	**0.032**	**0.064**	**0.128**	**0.16**	**0.256**	**16**	**32**	**64**	**128**					
AMP	CM	5.56(1)	38.92(7)	0(0)	27.8(5)	16.68(3)	0(0)	11.12(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	CMP	0(0)	25(1)	0(0)	0(0)	50(2)	0(0)	25(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	F	0(0)	41.16(7)	0(0)	23.52(4)	29.4(5)	0(0)	5.88(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	FP	0(0)	77.78(7)	0(0)	0(0)	0(0)	0(0)	22.22(2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	PM	0(0)	29.4(5)	0(0)	5.88(1)	17.64(3)	0(0)	23.52(4)	5.88(1)	5.88(1)	5.88(1)	5.88(1)	0(0)	0(0)	0(0)	0(0)	0(0)
	MP	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	50(1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	HS	0(0)	7.69(1)	15.38(2)	15.38(2)	55.56 (5)	23.07 (3)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Total	**1**	**29**	**2**	**12**	**18**	**3**	**11**	**1**	**1**	**1**	**1**	**0(0)**	**0(0)**	**0(0)**	**0(0)**	**0(0)**

CM=Raw meat ,CMP=processed meat,F=raw fish,FP=processed fish, PM=pasteurized milk, MP= milk product, HS=human stool samples respectively * denotes 7.5µg in case of CTR and CTX ** denotes 15µg in case of CTX

ff **Table 9: Antibiotic susceptibility profile of *C. perfringens* isolates**

Antibiotic	CLSI 2015, MIC Interpretive criteria (µg/ml)			Sample	No. of isolates	Sensitive	Intermediate	Resistance
AMC	S	I	R	CM	18	94.44% (17)	0 (0)	5.56% (1)
	≤4/2	8/4	≥16/8	CMP	4	100% (4)	0 (0)	0 (0)
				F	17	94.12% (16)	5.88% (1)	0 (0)
				FP	9	88.89% (8)	11.11% (1)	0 (0)
				PM	17	94.12% (16)	0 (0)	5.88% (1)
				MP	2	100% (2)	0 (0)	0 (0)
				HS	13	100% (13)	0 (0)	0 (0)
TI	≤32	64	≥128	CM	18	27.78% (5)	33.33% (6)	38.88% (7)
				CMP	4	100% (4)	0 (0)	0 (0)
				F	17	100% (17)	0 (0)	0 (0)
				FP	9	100% (9)	0 (0)	0 (0)
				PM	17	82.35% (14)	0 (0)	17.64% (3)
				MP	2	100% (2)	0(0)	0 (0)
				HS	13	100% (13)	0(0)	0 (0)
PI	≤32/4	64/4	≥128/	CM	18	94.44% (17)	0 (0)	5.56% (1)
				CMP	4	100% (4)	0 (0)	0(0)
				F	17	100% (17)	0 (0)	0 (0)
				FP	9	100% (9)	0 (0)	0 (0)
				PM	17	94.12% (16)	0 (0)	5.88% (1)
				MP	4	100% (2)	0 (0)	0 (0)
				HS	13	100% (13)	0 (0)	0 (0)
PIT	≤32/4	64/4	≥128/4	CM	18	94.44% (17)	0 (0)	5.56% (1)
				CMP	4	100% (4)	0 (0)	0(0)
				F	17	94.12% (16)	0 (0)	5.88% (1)
				FP	9	88.89% (8)	11.11% (1)	0 (0)
				PM	17	100% (17)	0 (0)	0 (0)
				MP	2	100% (2)	0 (0)	0(0)
				HS	13	100% (13)	0 (0)	0(0)

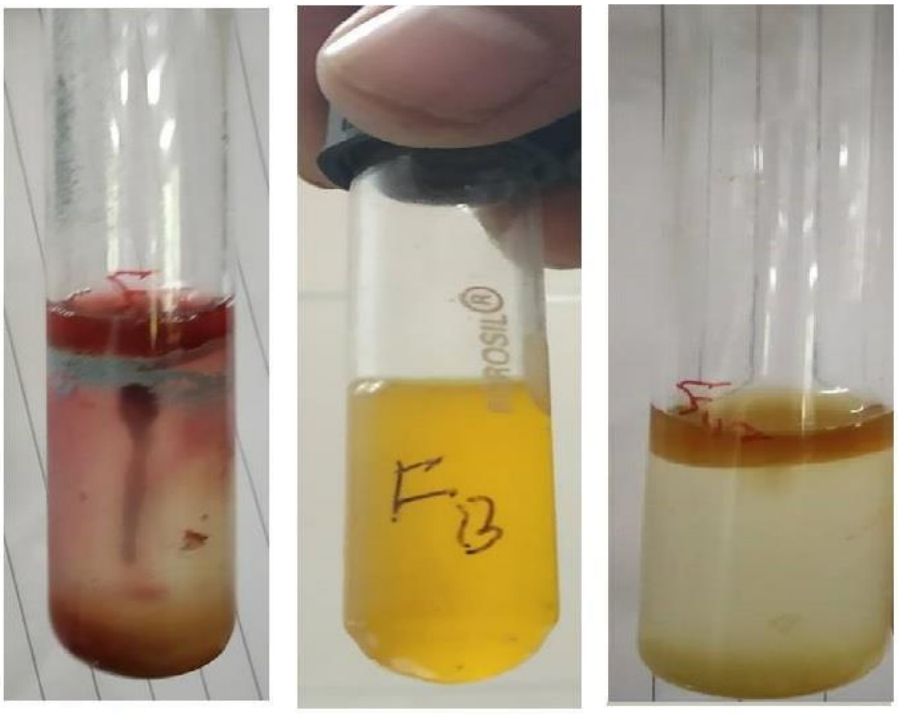
CTR	≤16	32	≥64	CM	18	100% (18)	0 (0)	0 (0)
				CMP	4	100% (4)	0 (0)	0 (0)
				F	17	88.24% (15)	5.88% (1)	5.88% (1)
				FP	9	100% (9)	0 (0)	0 (0)
				PM	17	76.47% (13)	11.76% (2)	11.76% (2)
				MP	2	100% (2)	0 (0)	0 (0)
				HS	13	100% (13)	0 (0)	0 (0)
CTX	≤16	32	≥64	CM	18	100% (18)	0 (0)	0 (0)
				CMP	4	100% (4)	0 (0)	0 (0)
				F	17	100% (17)	0 (0)	0 (0)
				FP	9	100% (9)	0 (0)	0 (0)
				PM	17	88.24%(15)	5.88% (1)	5.88% (1)
				MP	2	100% (2)	0 (0)	0 (0)
				HS	13	100% (13)	0 (0)	0 (0)
TE	≤4	8	≥16	CM	18	55.56% (10)	11.12% (2)	33.33% (6)
				CMP	4	75% (3)	25% (1)	0 (0)
				F	17	58.82% (10)	11.76% (2)	29.41% (5)
				FP	9	88.89% (8)	11.11% (1)	0 (0)
				PM	17	64.70% (11)	0 (0)	35.29% (6)
				MP	2	100% (2)	0 (0)	0 (0)
				HS	13	76.92% (10)	0 (0)	23.07% (3)
MO	≤2	4	≥8	CM	18	33.33% (6)	27.77% (5)	38.88% (7)
				CMP	4	75%(3)	25%(1)	0 (0)
				F	17	29.41% (5)	23.52% (4)	47.04% (8)
				FP	9	88.89% (8)	11.11% (1)	0 (0)
				PM	17	88.24% (15)	5.88% (1)	5.88% (1)
				MP	2	50% (1)	0 (0)	50%(1)
				HS	13	69.23% (9)	23.07% (3)	7.69% (1)

CD	≤4/2	8/4	≥16/8	CM	18	11.12% (2)	38.88% (7)	50% (9)
				CMP	4	25% (1)	50% (2)	25% (1)
				F	17	41.17% (7)	11.76% (2)	41.17% (8)
				FP	9	88.89% (8)	11.11% (1)	0 (0)
				PM	17	52.94% (9)	5.88%(1)	41.17% (7)
				MP	2	100% (2)	0 (0)	0 (0)
C	≤2	4	≥8	HS	13	100% (13)	0 (0)	0 (0)
				CM	18	100% (18)	0 (0)	0 (0)
				CMP	4	100% (4)	0 (0)	0 (0)
				F	17	100% (17)	0 (0)	0 (0)
				FP	9	100% (9)	0 (0)	0 (0)
				PM	17	76.47% (13)	0 (0)	23.52% (4)
				MP	2	100% (2)	0 (0)	0 (0)
				HS	13	100% (13)	0 (0)	0 (0)
AMP	≤0.5	1	≥2	CM	18	100% (18)	0 (0)	0 (0)
				CMP	4	100% (4)	0 (0)	0 (0)
				F	17	100%(17)	0 (0)	0 (0)
				FP	9	100%(9)	0 (0)	0 (0)
				PM	17	76.47% (13)	0 (0)	23.52% (4)
				MP	2	100% (2)	0 (0)	0 (0)
				HS	13	100% (13)	0 (0)	0 (0)

Table 10: MIC_{50} and MIC_{90} values of *C. perfringens* and antibiotic susceptibility profile

Antibiotic	MIC value(µg)	MIC_{50} (µg)	MIC_{90} (µg)	No of isolates		
				Sensitive	Intermediate	Resistance
AMC	0.01	0.1	0.5	76	2	2
TI	0.1	8	30	64	6	10
PI	0.01	5	10	78	0	2
PIT	0.01	0.5	10	77	1	2
CTR	0.01	0.5	10	74	3	3
CTX	0.01	1	4	78	1	1
TE	0.01	1	10	54	6	20
MO	0.01	4	10	47	15	18
CD	0.01	2	120	42	13	25
C	0.01	0.5	4	76	0	4
AMP	0.01	0.064	0.256	76	0	4

Fig 1 : Cultural isolation 2-3mm diameter black colonies with lecithinase activity on Shahidi Ferguson perfringens (SFP) agar with egg yolk emulsion



A **B** **C**

Fig 2 (A): Positive for Nitrate reduction and negative for motility
Fig 2(B): Positive for Lactose fermentation and Gelatin Liquifaction
Fig 2 (C): Negative for Indole

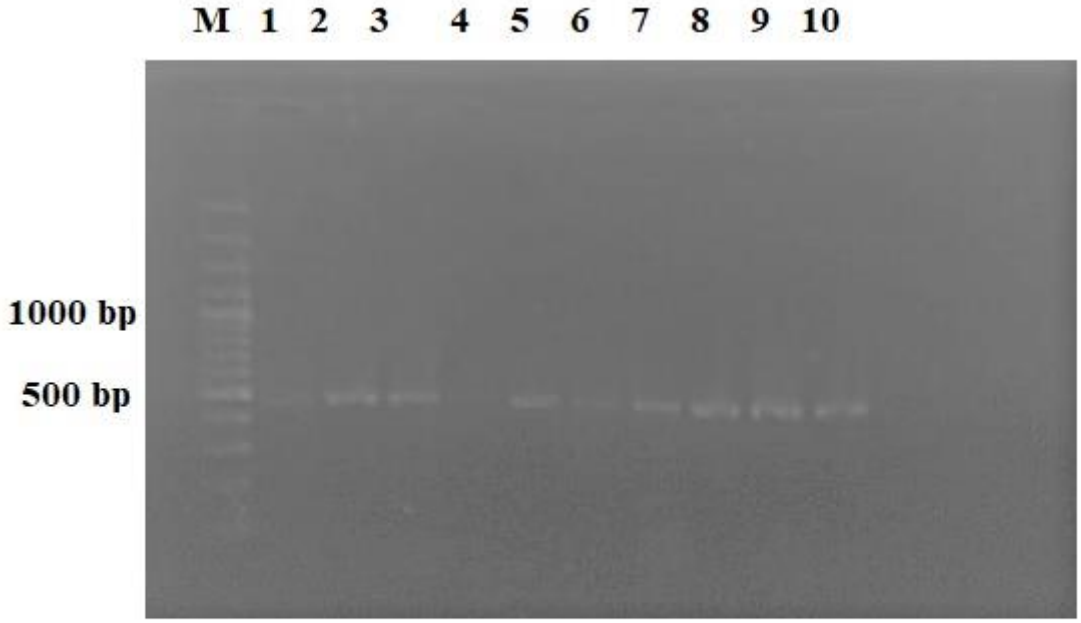


Fig. 4A PCR Amplification product of *C. perfringens* specific *16S rRNA*

M: 100bp DNA ladder

L1 to L10: 481bp amplicon of *C. perfringens* at annealing 54.5°C

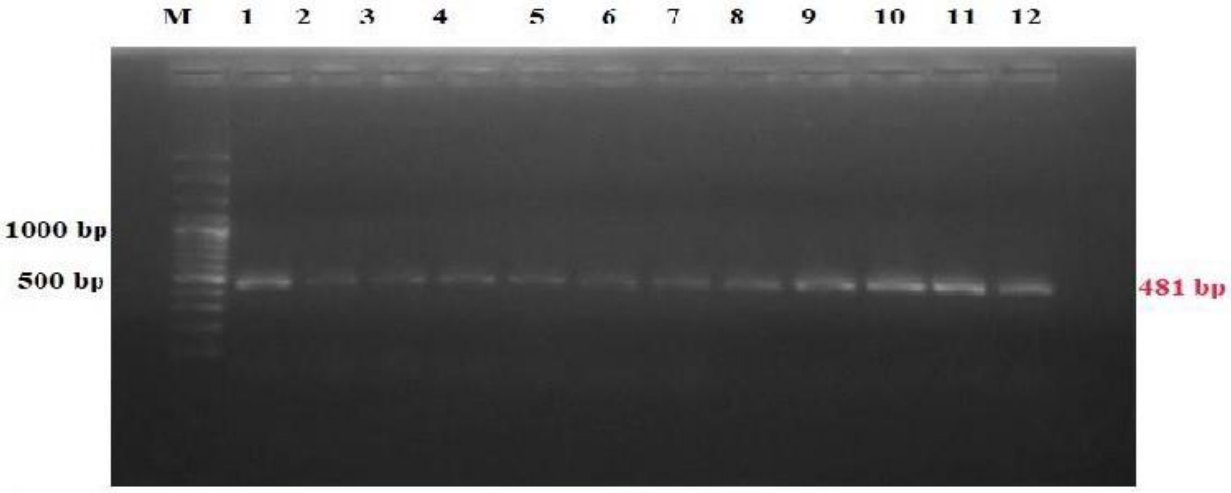


Fig 4B PCR Amplification of *C. perfringens* specific *16S rRNA*

Mw: 100bp ladder

L1 to L11: Positive for *C. perfringens* specific *16S rRNA* (481bp)

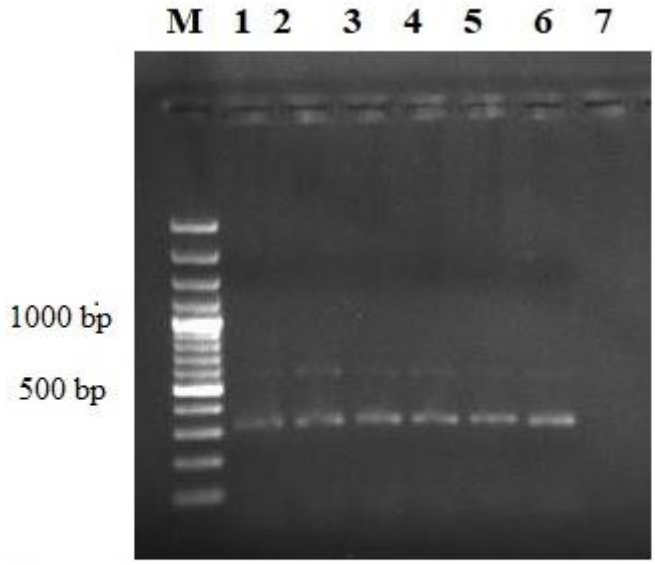


Fig. 5A PCR Amplification product of *cpa* gene of *C. perfringens*

M: 100bp DNA ladder

L1 to L6: 324bp amplicon of *C. perfringens* at annealing 50.2°C

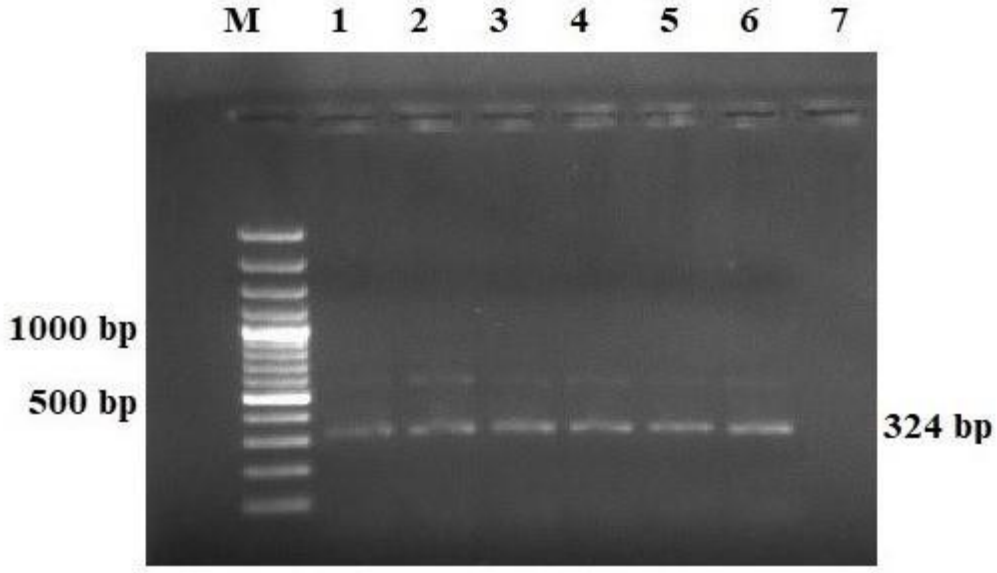


Fig 5 (B): PCR amplification of *cpa* gene of *C. perfringens*

MW :100bp DNA ladder

L1 to L6 : Positive for *cpa* gene

L7 : Control negative for *cpa gene*

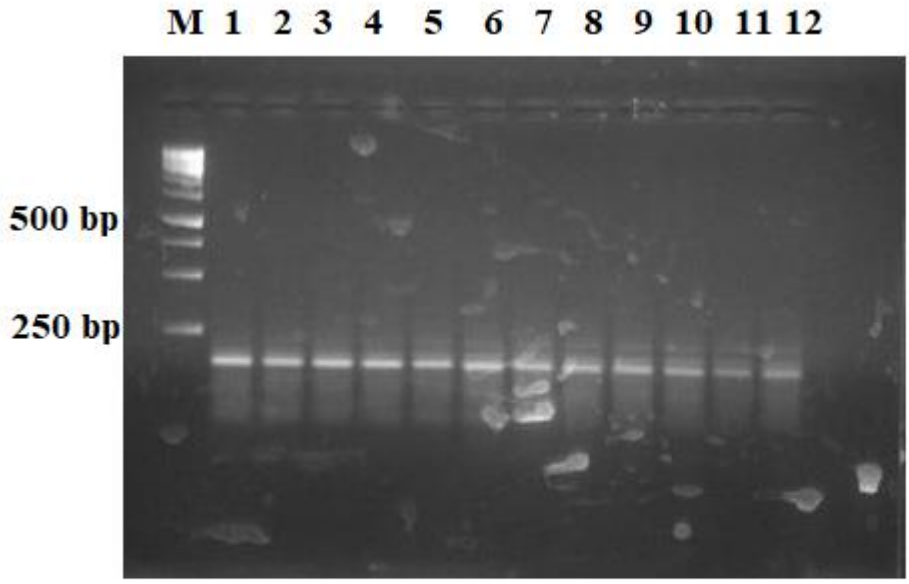


Fig. 6A PCR Amplification product of *cpb* gene of *C. perfringens*

M: 50bp DNA ladder

L1 to L12: 196bp amplicon of *C. perfringens* at annealing 55.7°C

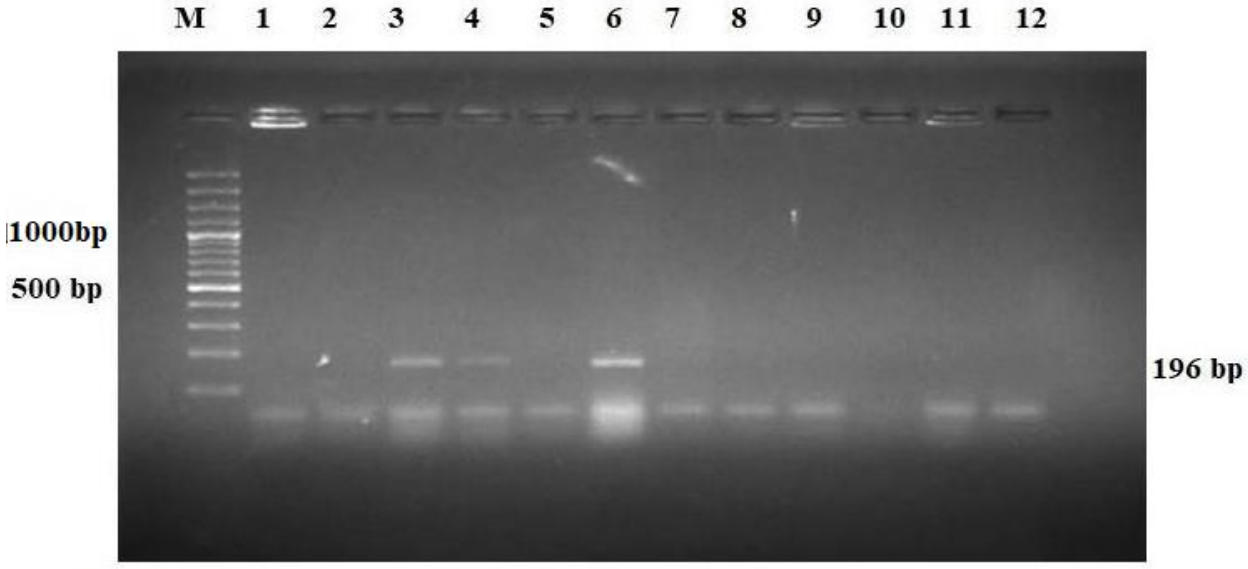


Fig 6B PCR Amplification of *cpb* gene of *C. perfringens*

Mw: 100bp ladder

L3, L4 & L6: 196bp amplicon of *cpb* gene of *C. perfringens*

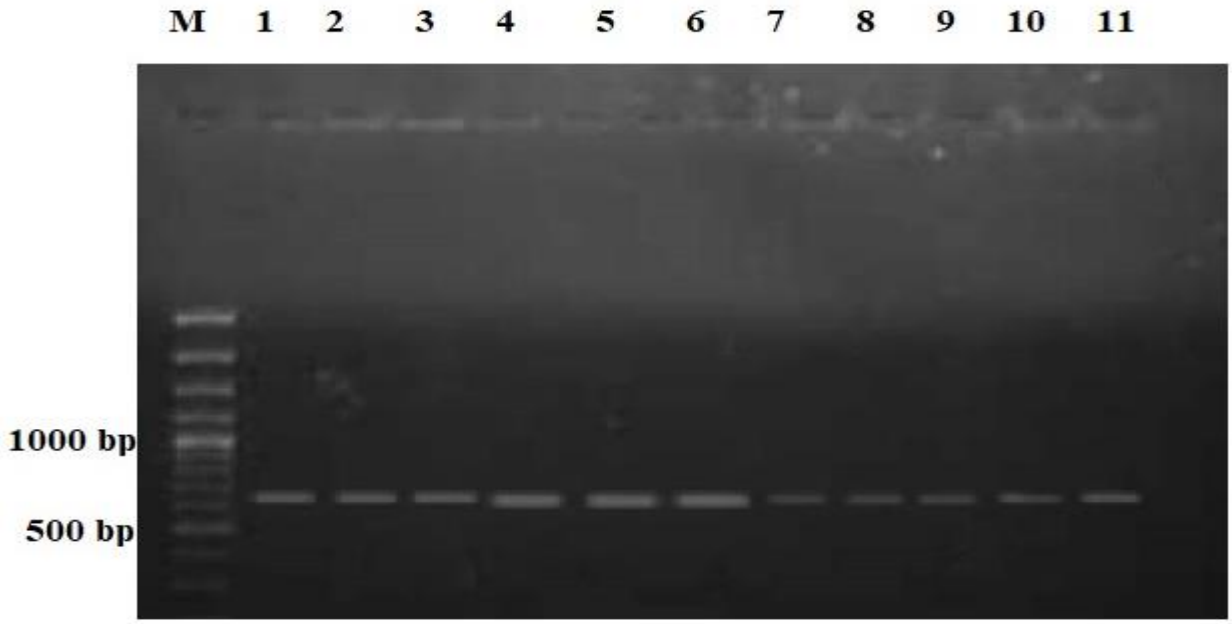


Fig. 7A PCR Amplification product of *etx* gene of *C. perfringens*

M: 100bp DNA ladder

L1 to L6: 655bp amplicon of *C. perfringens* at annealing 55.1°C

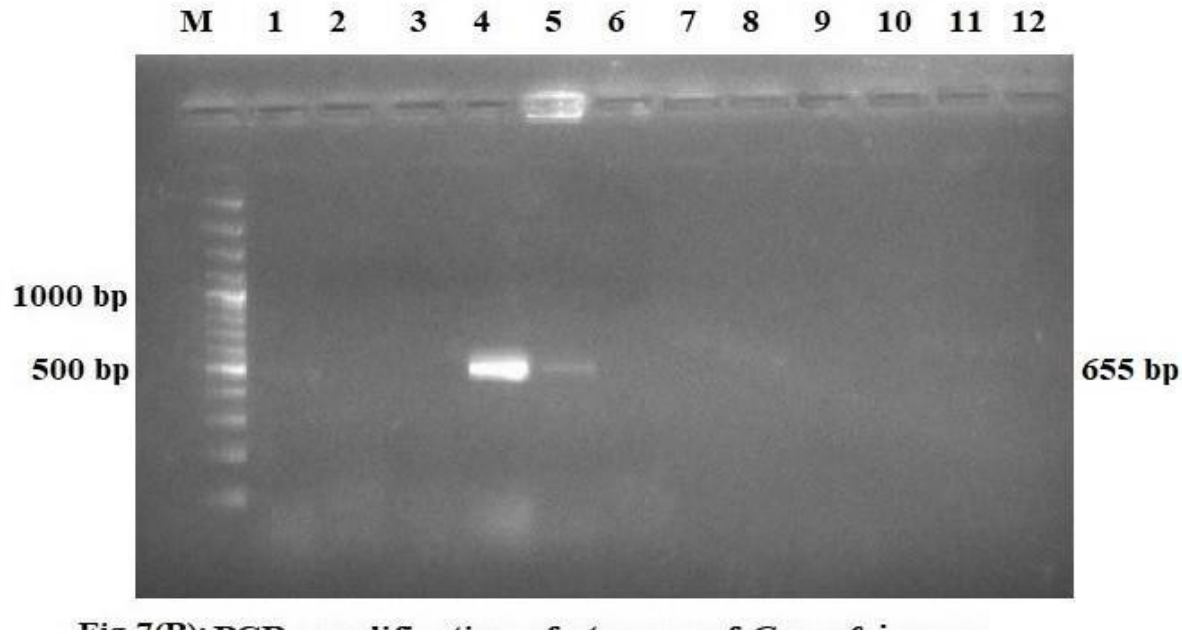


Fig 7B PCR Amplification of *etx* gene of *C. perfringens*

Mw: 100bp ladder

L4 to L5: 655bp amplicon of *etx* gene of *C. perfringens*

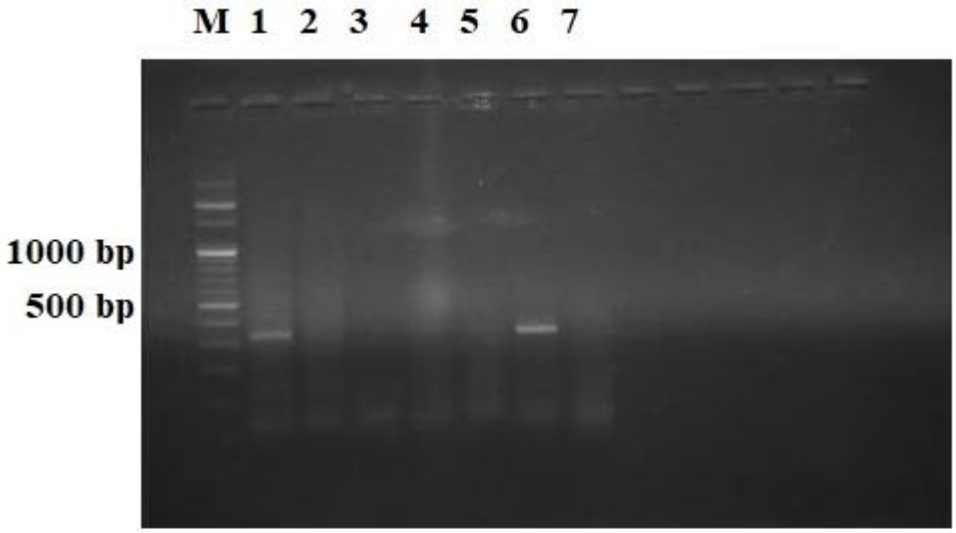


Fig. 8A PCR Amplification product of *cpe* gene of *C. perfringens*

M: 100bp DNA ladder

L1 to L6: 233bp amplicon of *C. perfringens* at annealing 50.5°C

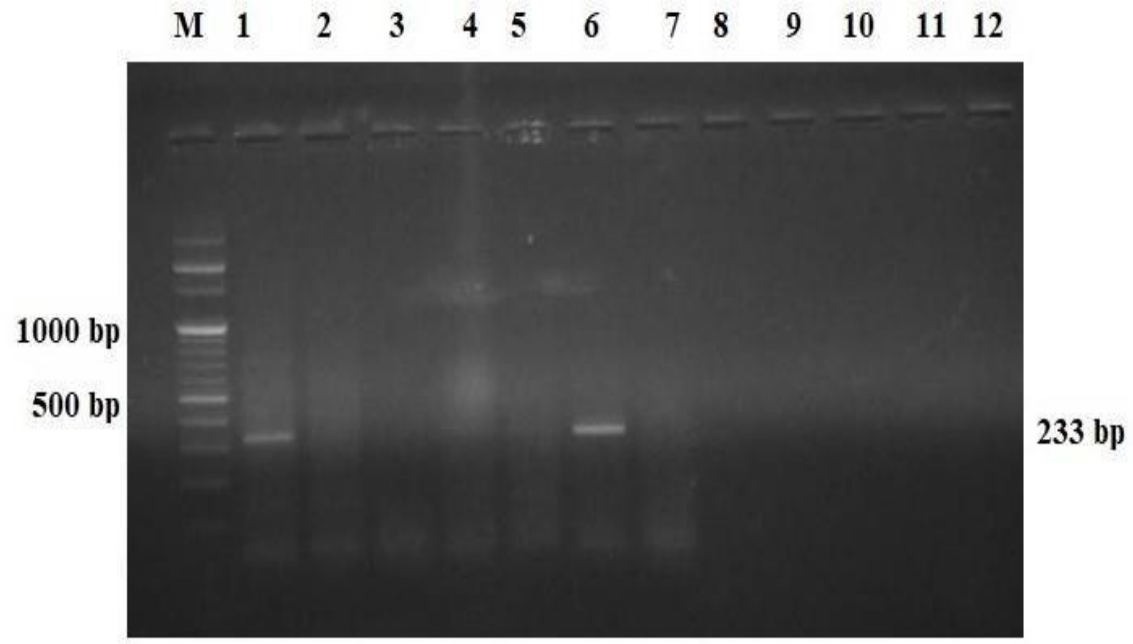


Fig 8B PCR Amplification of *cpe* gene of *C. perfringens*

Mw: 100bp ladder

L2 to L6: 233bp amplicon of *cpe* gene of *C. perfringens*

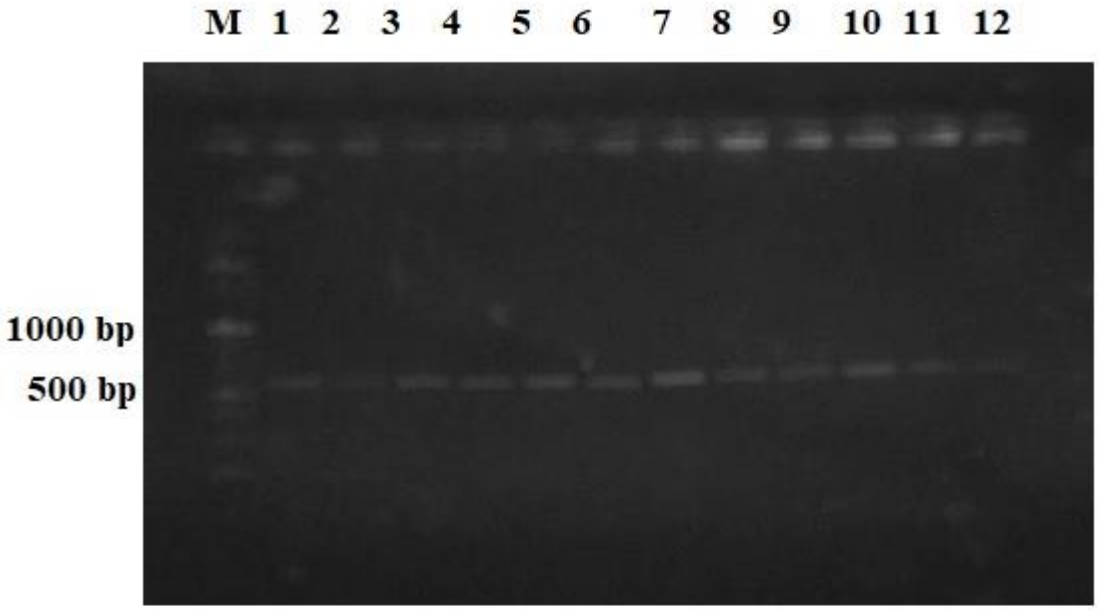


Fig. 9A PCR Amplification product of *cpb2* gene of *C. perfringens*

M: 100bp DNA ladder

L1 to L12: 567bp amplicon of *C. perfringens* at annealing 54.5°C

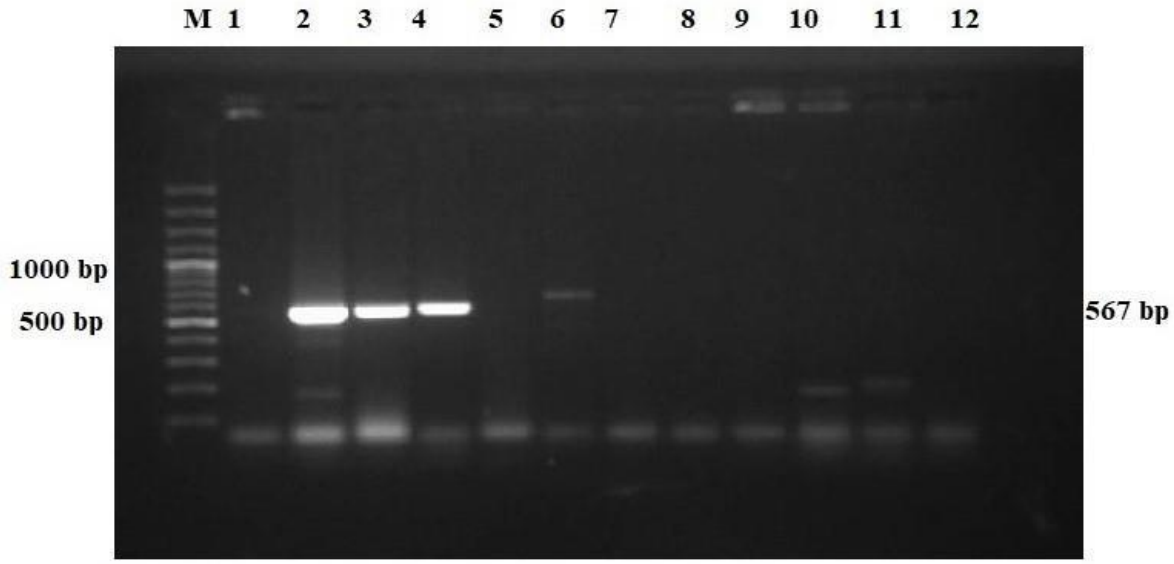


Fig 9B PCR Amplification of *cpb2* gene of *C. perfringens*

Mw: 100bp ladder

L2 to L4: 567bp amplicon of *cpb2* gene of *C. perfringens*

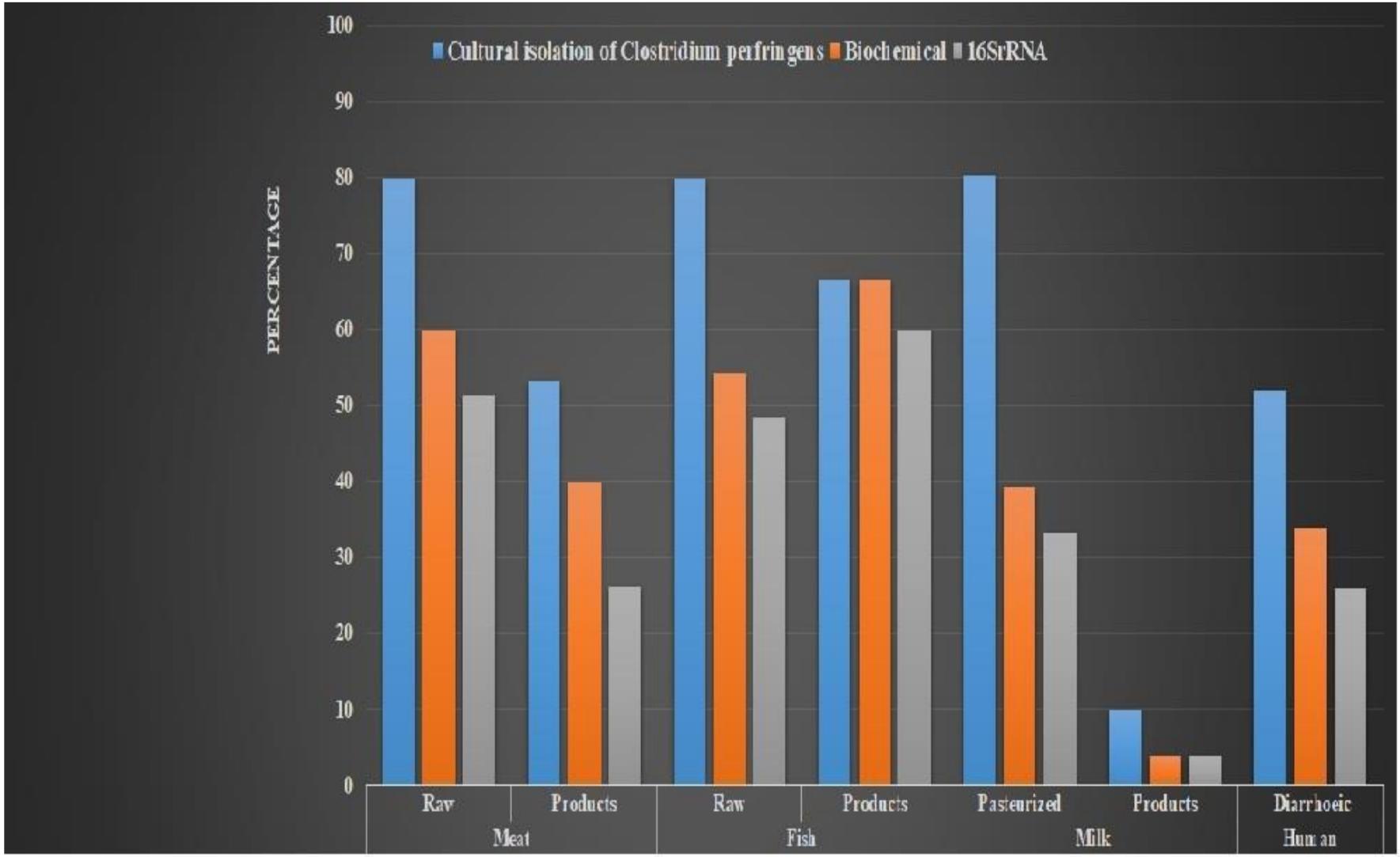


Fig 3: Isolation, Biochemical and PCR confirmation of *C. perfringens* from different food Samples

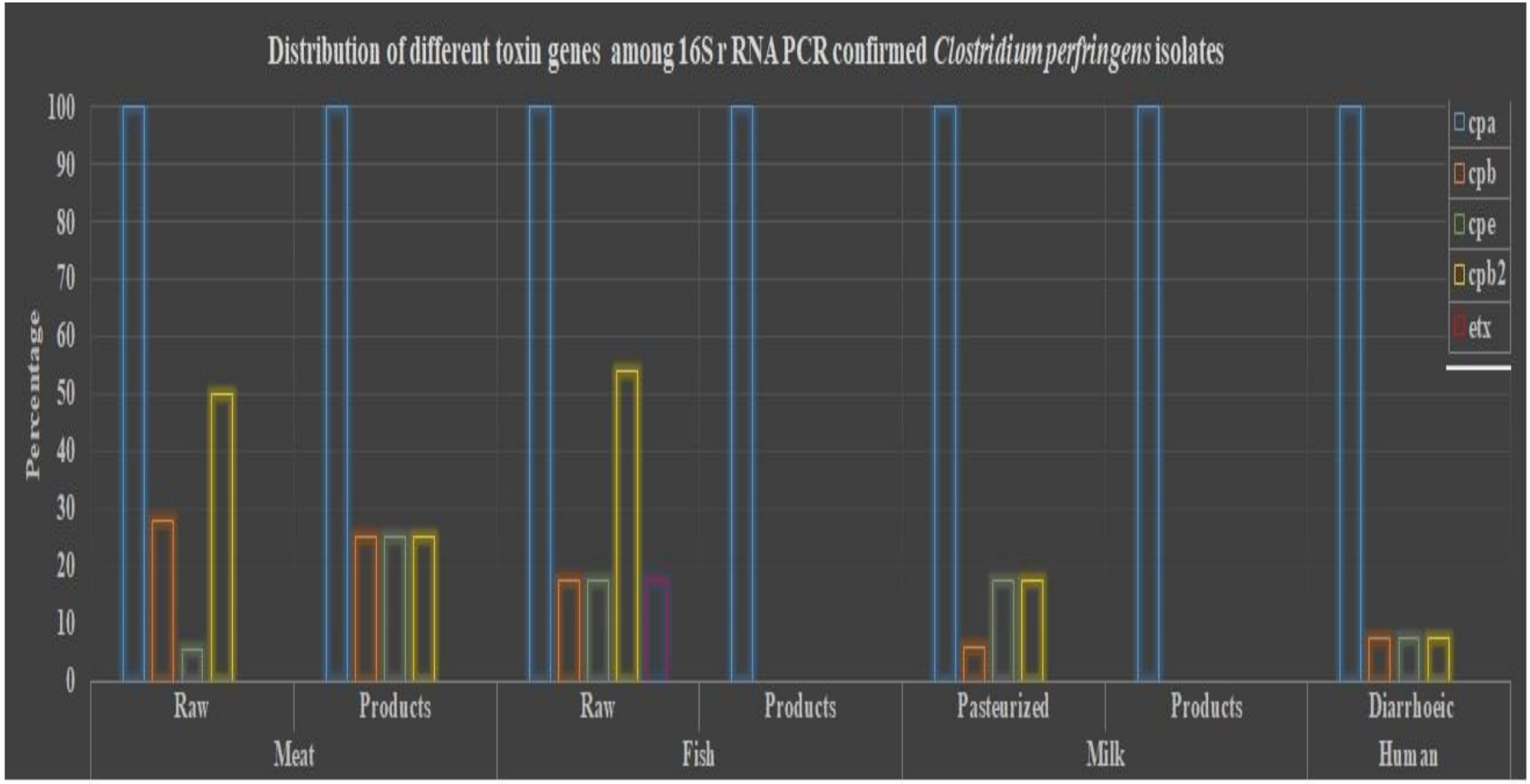


Fig 10: Distribution of toxins present among confirmed *C. perfringens* isolates of each sample

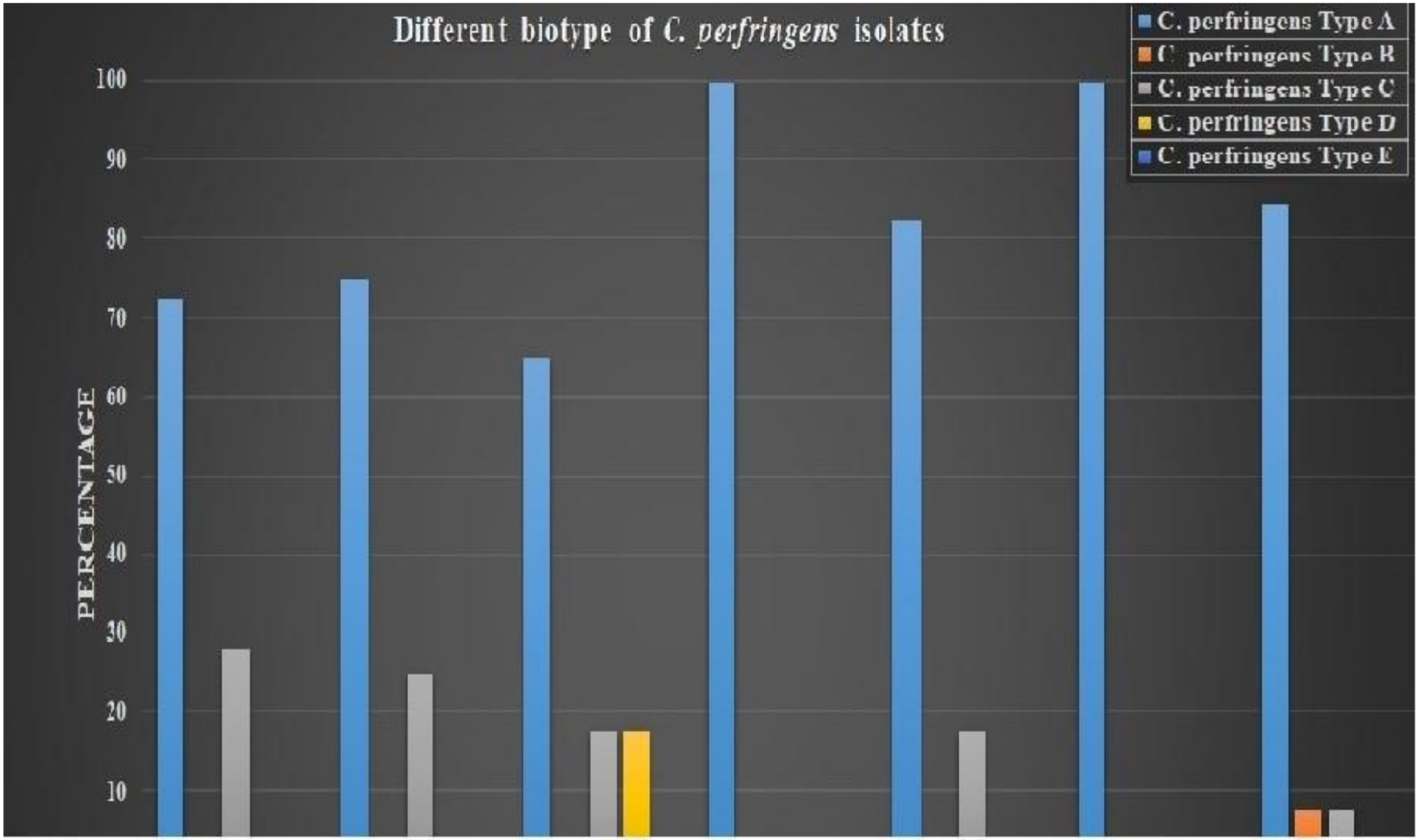


Fig 11: Distribution of different toxotypes of *C. perfringens* isolates of each sample

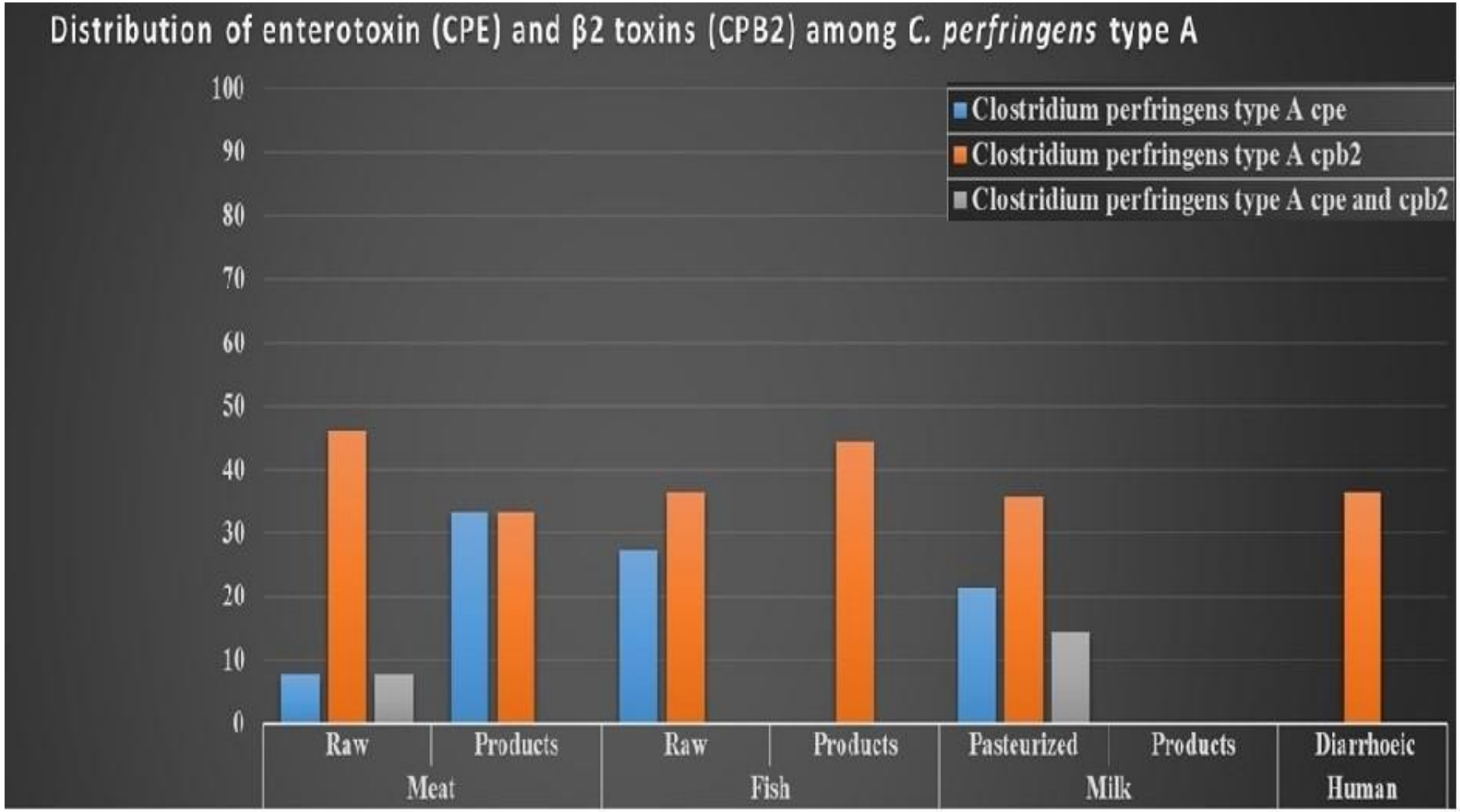


Fig 12: Distribution of *cpe* and *cpb2* or both among *C. perfringens* type A isolates of each sample

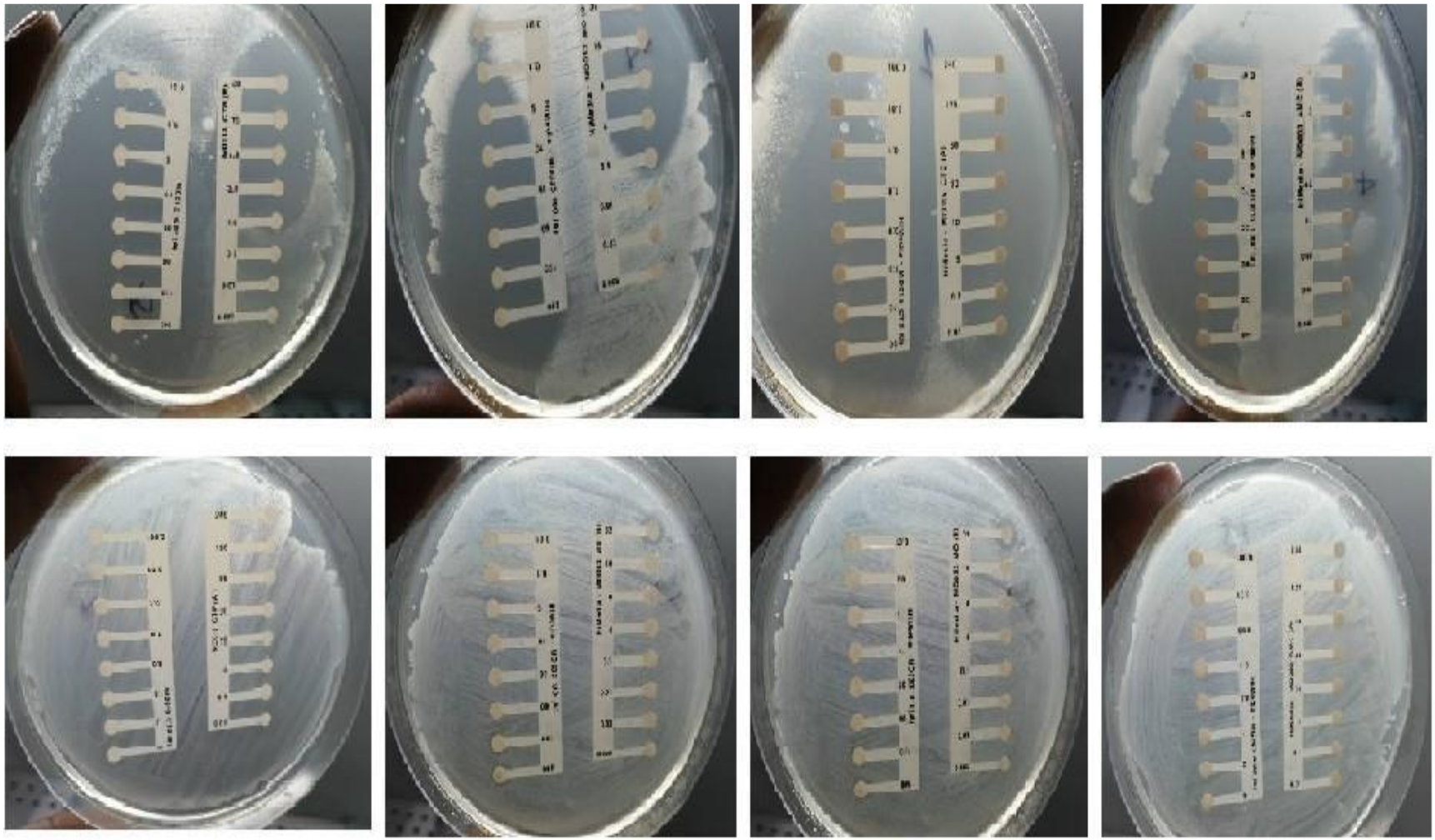
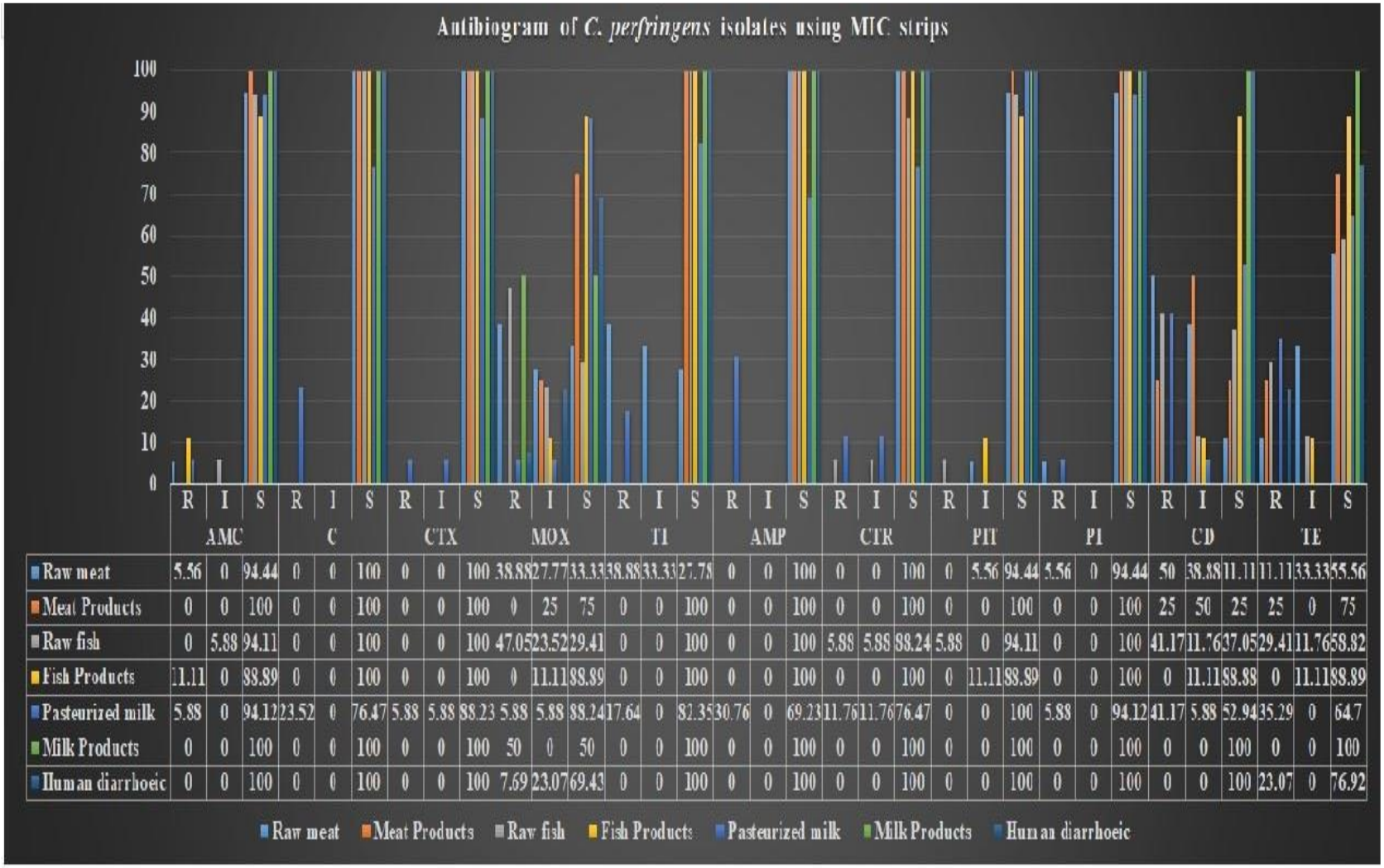


Fig 13: Plates showing MIC for different antibiotics of *C.perfringens* isolates



	AMC			C			CTX			MOX			TI			AMP			CTR			PIT			PI			CD			TE		
Raw meat	5.56	0	94.44	0	0	100	0	0	100	38.88	27.77	33.33	38.88	33.33	27.78	0	0	100	0	0	100	0	5.56	94.44	5.56	0	94.44	50	38.88	11.11	11.11	33.33	55.56
Meat Products	0	0	100	0	0	100	0	0	100	0	25	75	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100	25	50	25	25	0	75
Raw fish	0	5.88	94.11	0	0	100	0	0	100	47.05	23.52	29.41	0	0	100	0	0	100	5.88	5.88	88.24	5.88	0	94.11	0	0	100	41.17	11.76	37.05	29.41	11.76	58.82
Fish Products	11.11	0	88.89	0	0	100	0	0	100	0	11.11	88.89	0	0	100	0	0	100	0	0	100	0	11.11	88.89	0	0	100	0	11.11	88.88	0	11.11	88.89
Pasteurized milk	5.88	0	94.12	23.52	0	76.47	5.88	5.88	88.23	5.88	5.88	88.24	17.64	0	82.35	30.76	0	69.23	11.76	11.76	76.47	0	0	100	5.88	0	94.12	41.17	5.88	52.94	35.29	0	64.7
Milk Products	0	0	100	0	0	100	0	0	100	50	0	50	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100
Human diarrhoeic	0	0	100	0	0	100	0	0	100	7.69	23.07	69.43	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100	23.07	0	76.92

Fig 14: Antibiotic susceptibility of *C. perfringens* isolates of each sample

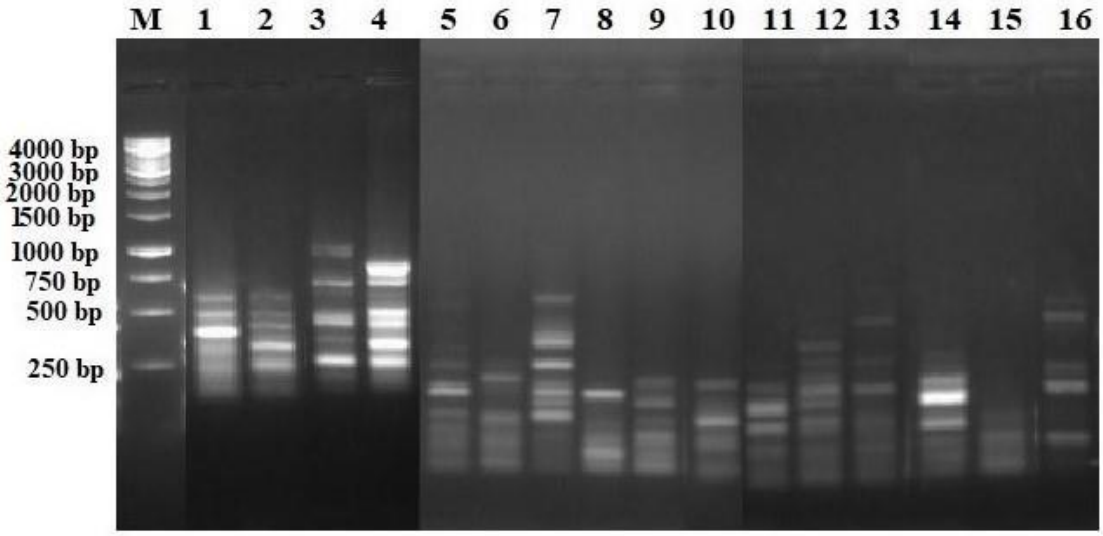


Fig 16(A): ERIC-PCR of *C. perfringens* type A isolates
MW : 1kb DNA ladder
L1 to L16: *C. perfringens* type A (16) isolates isolated from meat and meat products

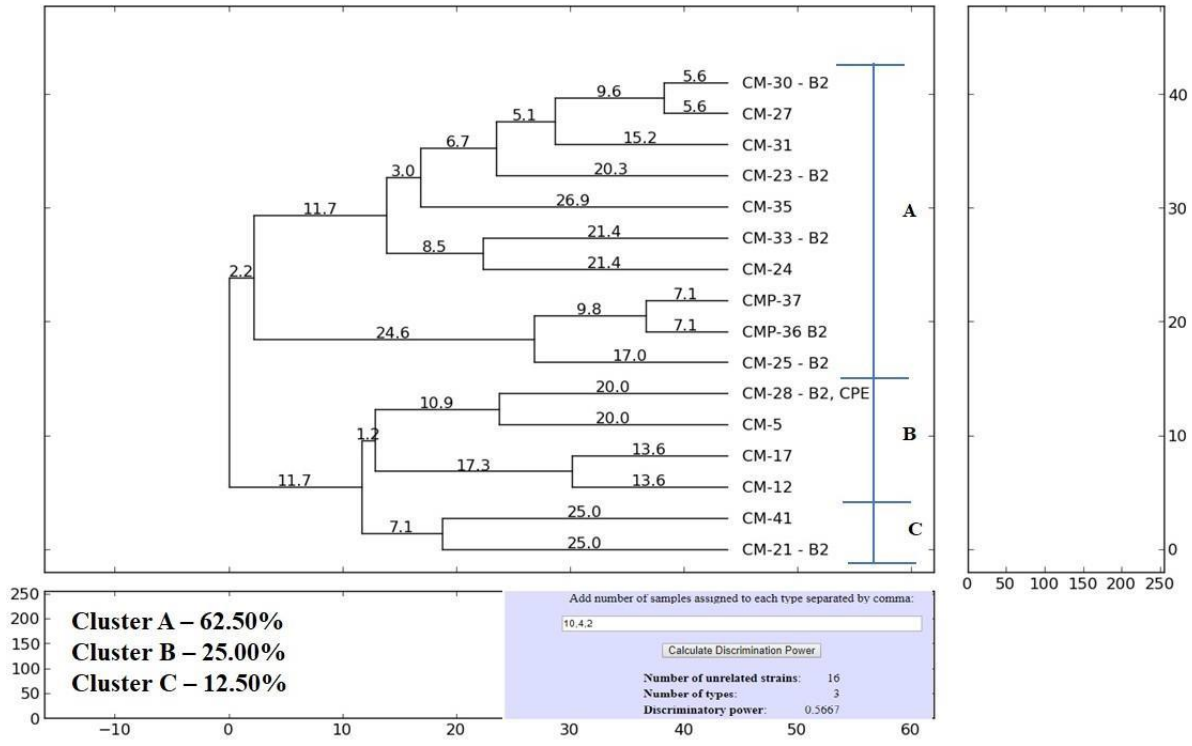


Fig 16(B):Phylogenetic (dendrogram) of ERIC-PCR profile of *C. perfringens* type A of meat and meat product isolates (A-C)

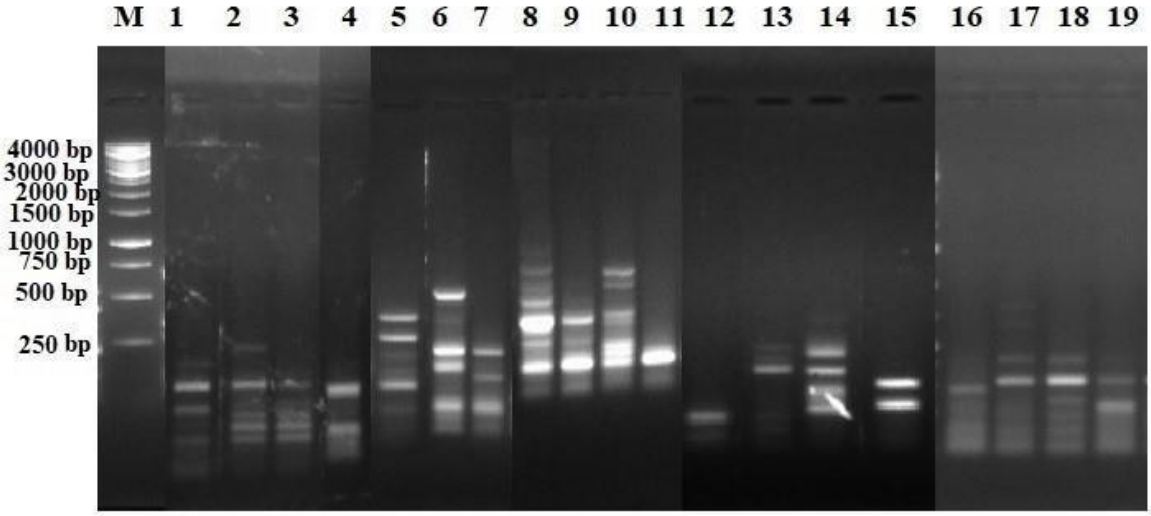


Fig 17(A): ERIC-PCR of *C. perfringens* type A isolates
MW : 1kb DNA ladder
L1 to L19: *C. perfringens* type A (19) isolates isolated from fish and fish products

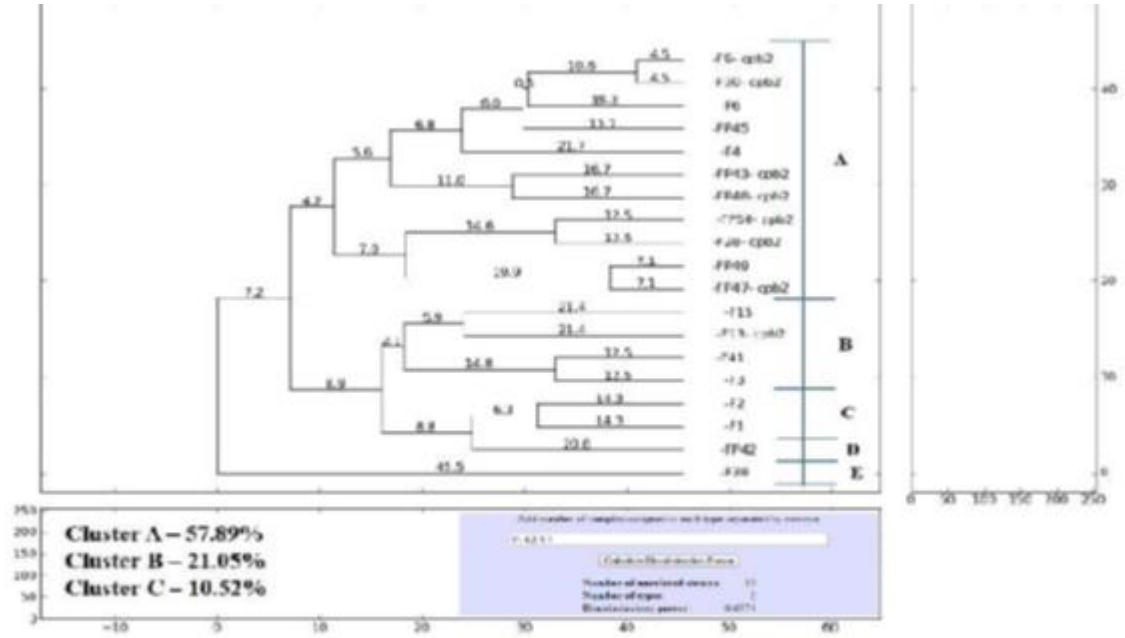


Fig 17B: Phylogenetic (dendrogram) of ERIC-PCR of *C. perfringens* type A of Fish and Fish products isolates (A to E)

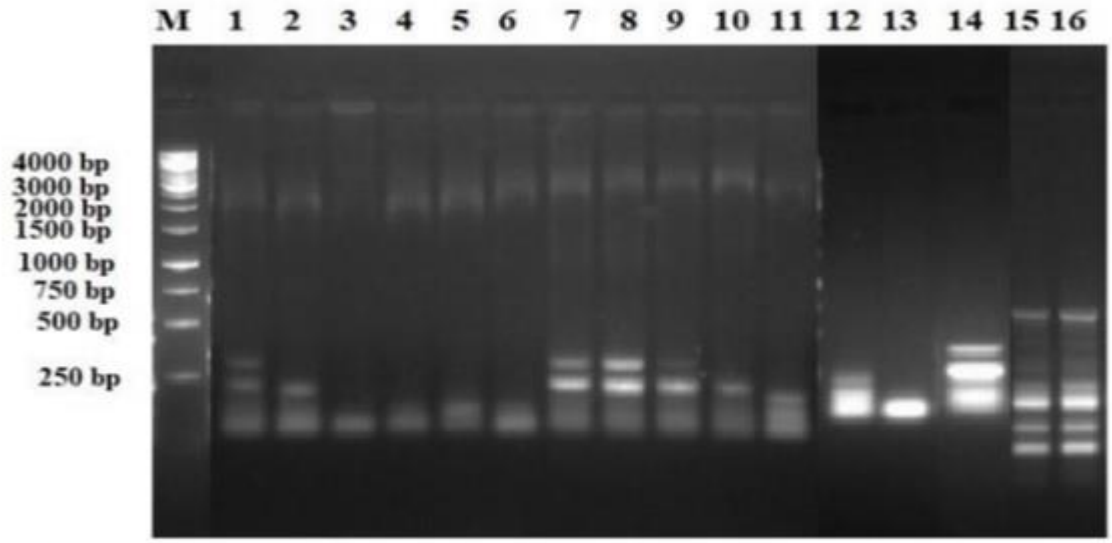


Fig 18A: ERIC-PCR of *C. perfringens* type A isolates
MW : 1kb DNA ladder
L1 to L16: *C. perfringens* type A (16) isolates isolated from milk and milk products

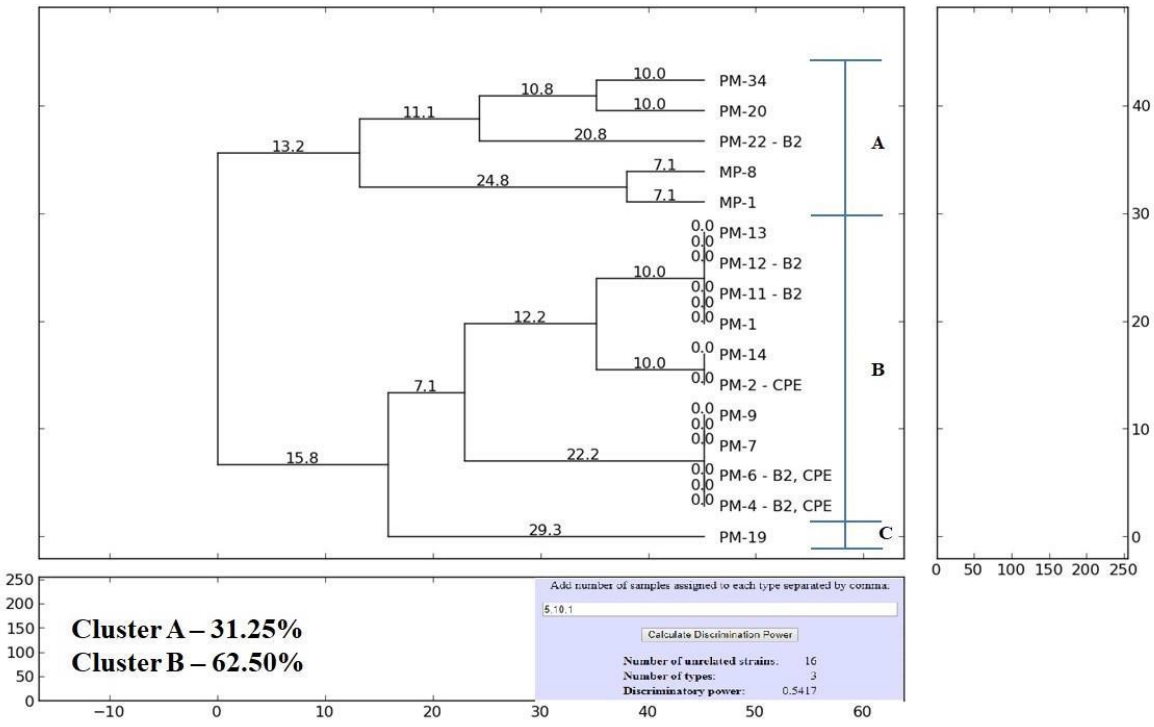


Fig 18(B):Phylogenetic (dendrogram) of ERIC-PCR profile of *C. perfringens* type A of milk and milk products isolates (A-C)

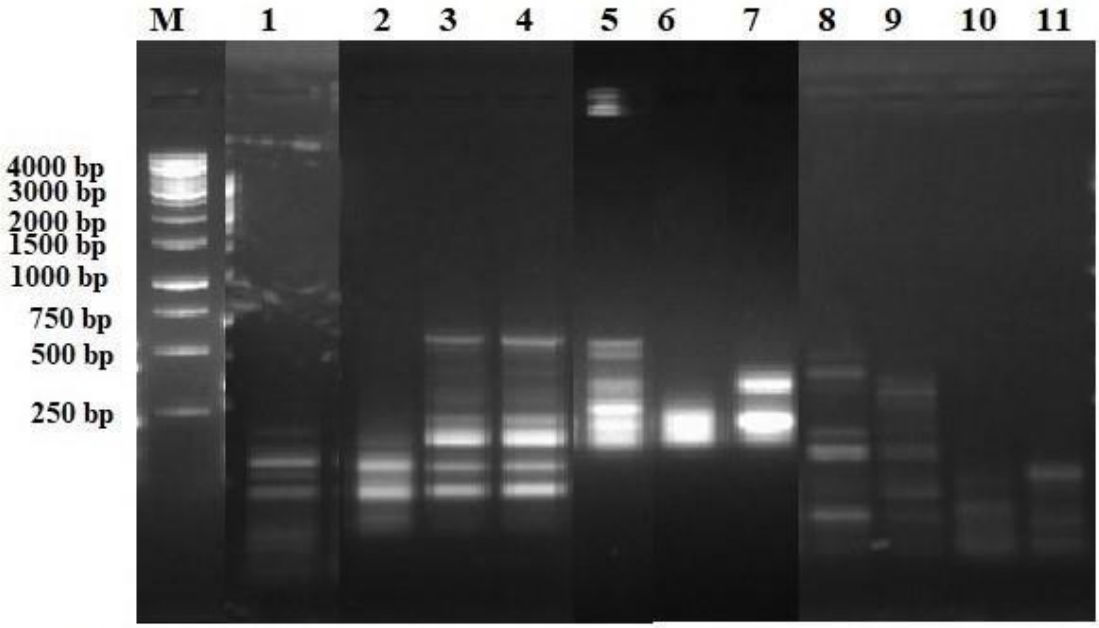


Fig 19(A): ERIC-PCR of *C. perfringens* type A isolates

MW : 1kb DNA ladder

L1 to L11: *C. perfringens* type A (11) isolates isolated from human stool

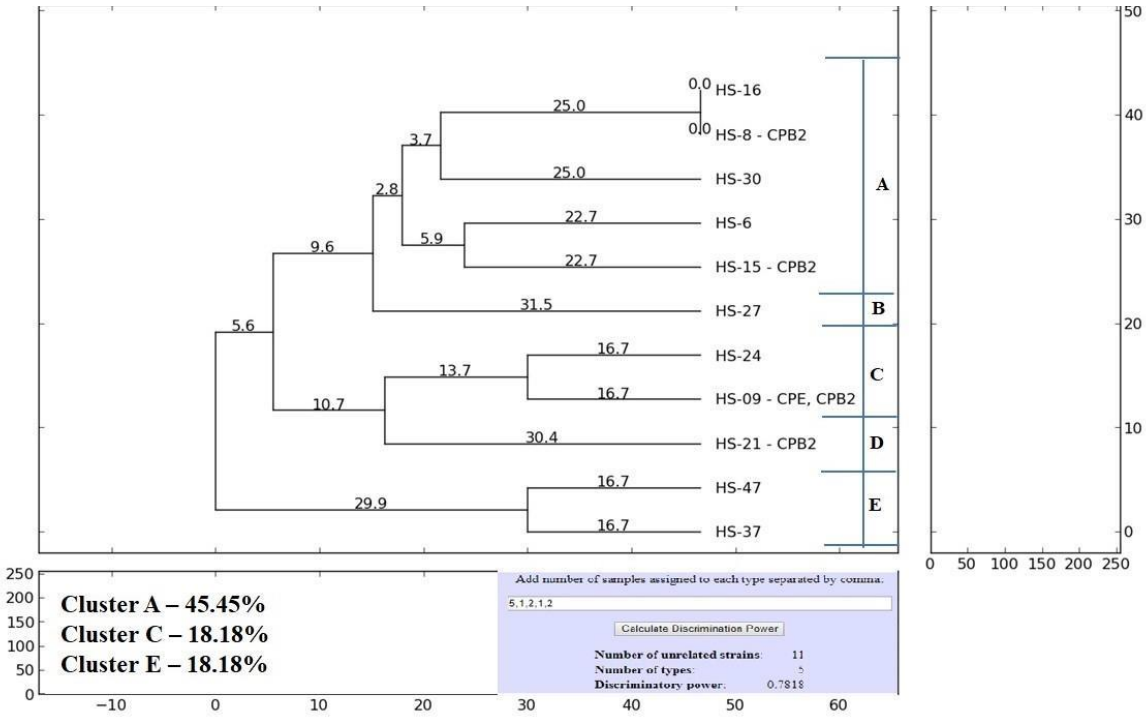


Fig 19(B) :Phylogenetic (dendrogram) of ERIC-PCR profile of *C. perfringens* type A of human stool isolates (A-E)

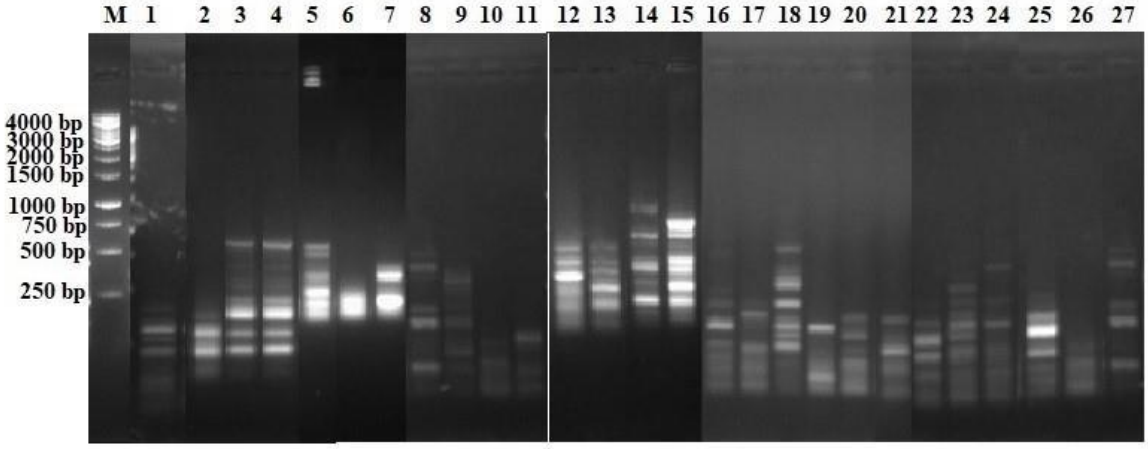


Fig 20(A): ERIC-PCR of *C. perfringens* type A isolates

MW :1kb DNA ladder

L1 to L11: *C. perfringens* type A(11) isolates isolated from human stool

L12 to L27: *C. perfringens* type A(16) isolates isolated from meat and meat products

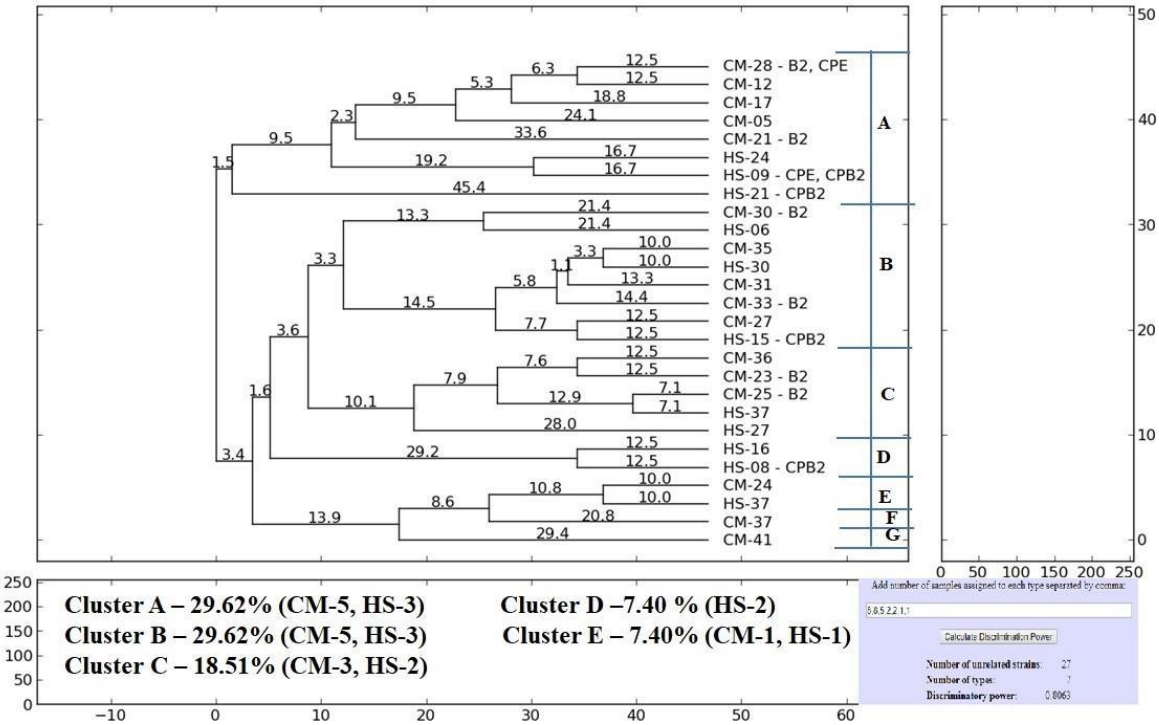


Fig 20(B): Phylogenetic (dendrogram) of ERIC-PCR profile of *C. perfringens* type A of meat and meat product and humam stool isolates (A-E)

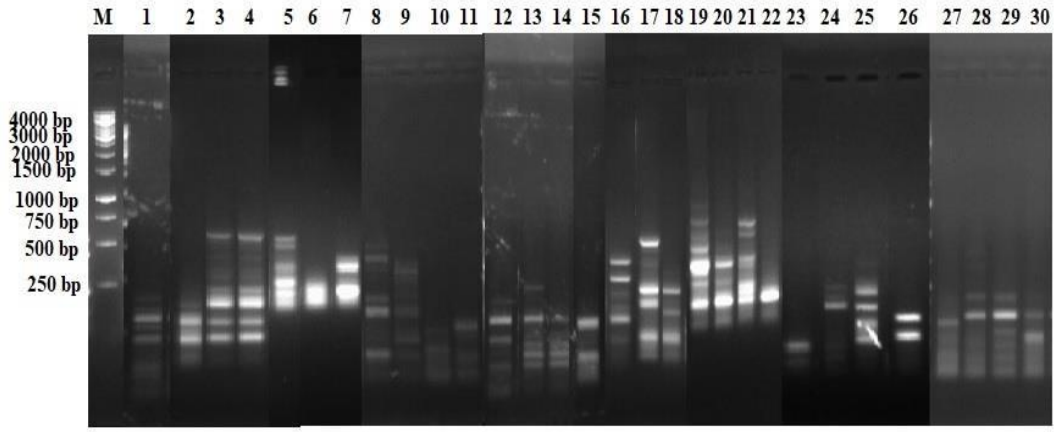


Fig 21(A):ERIC-PCR of *C. perfringens* type A isolates

MW: 1kb DNA ladder

L1 to L11: *C. perfringens* type A(11) isolates isolated from human stool

L12 to L30: *C. perfringens* type A(19) isolates isolated from fish and fish products

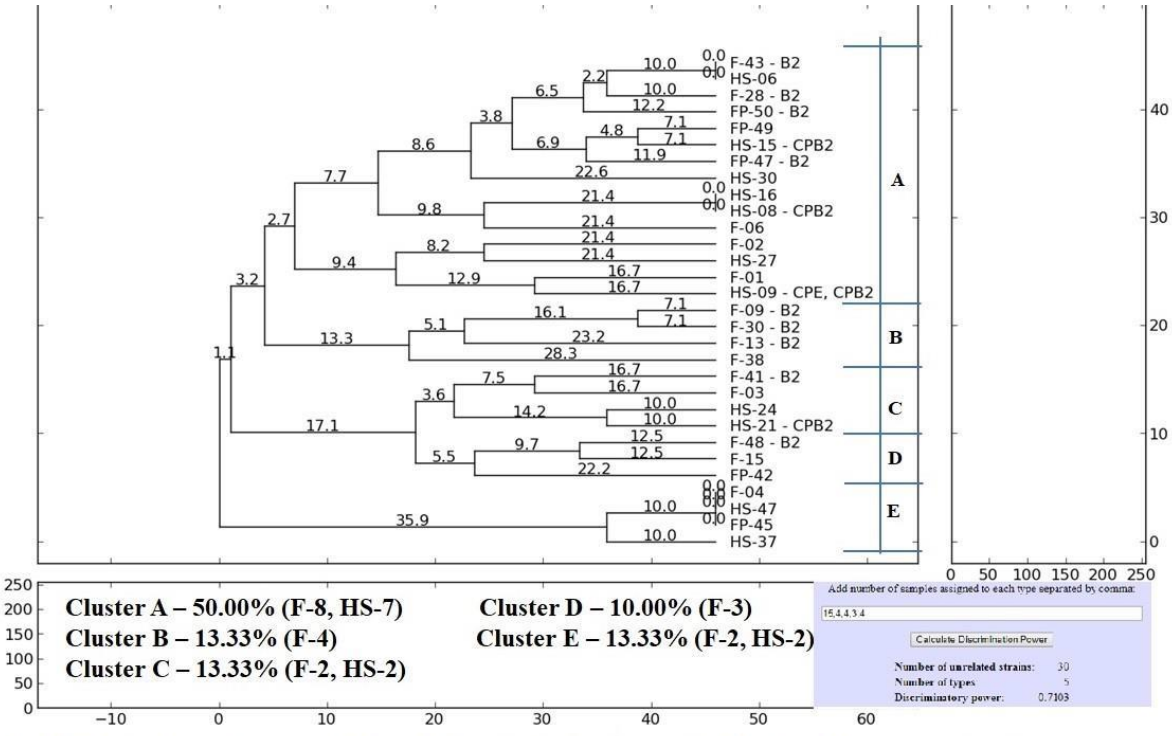


Fig 21(B):Phylogenetic (dendrogram) of ERIC-PCR profile of *C. perfringens* type A of fish and fish product and human stool isolates (A-E)

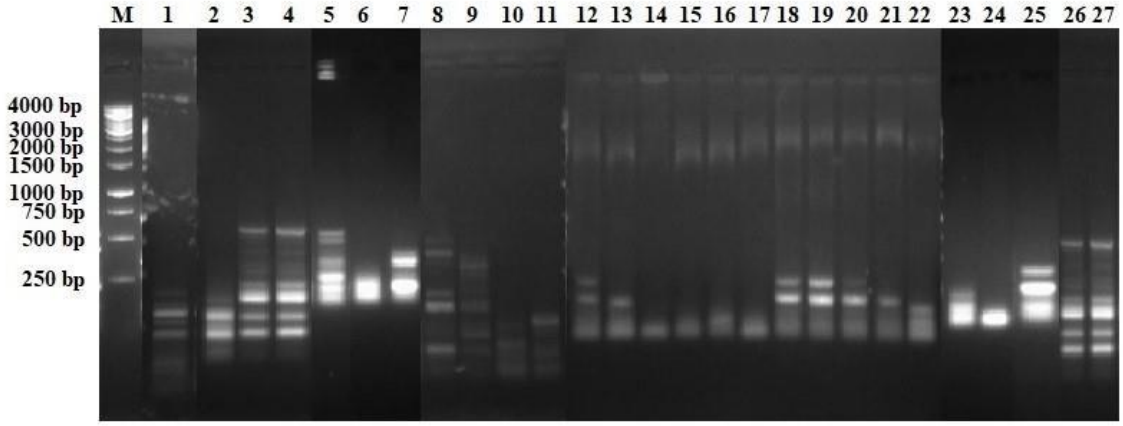


Fig 22(A): ERIC-PCR of *C. perfringens* type A isolates

MW : 1kb DNA ladder

L1 to L11: *C. perfringens* type A(11) isolates isolated from human stool

L12 to L27: *C. perfringens* type A(16) isolates isolated from milk and milk products

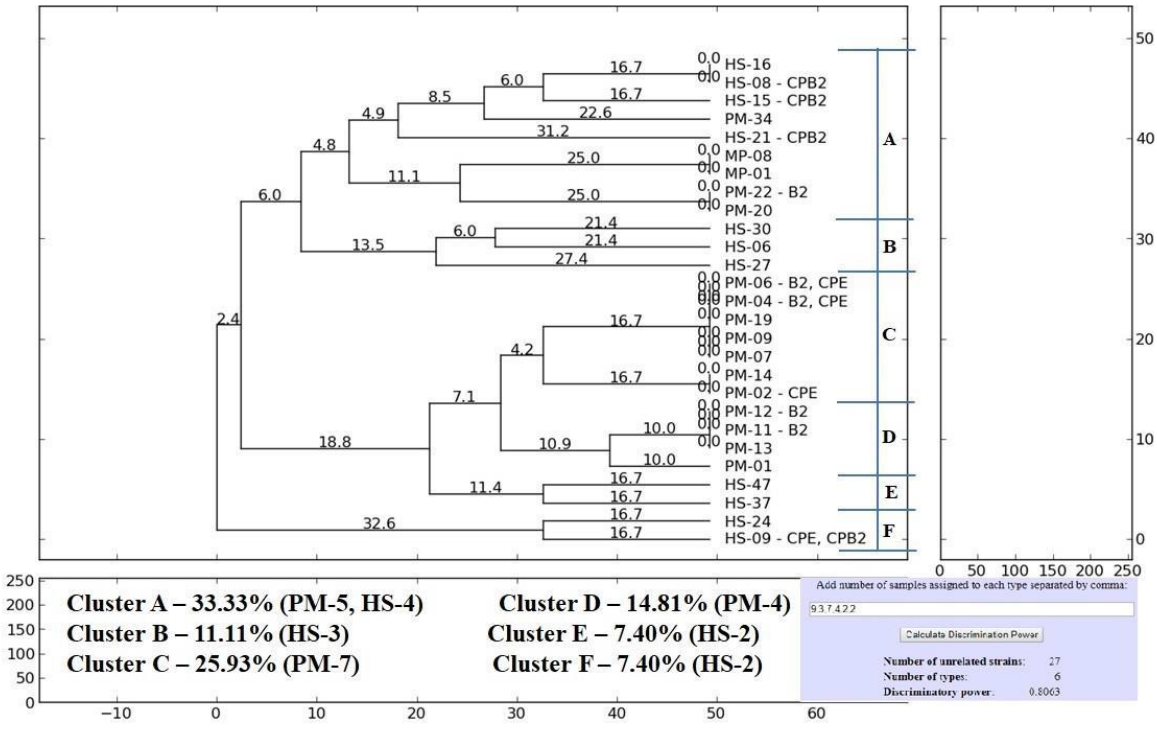
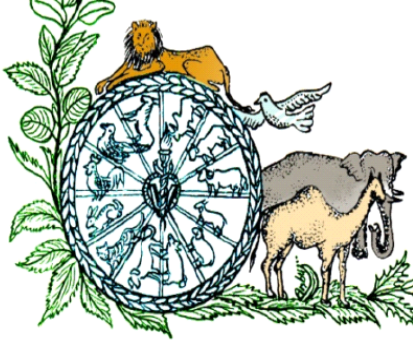


Fig 22(B):Phylogenetic (dendrogram) of ERIC-PCR profile of *C. perfringens* type A of milk and milk products and human stool isolates (A-F)

Summary and Conclusions



5 SUMMARY AND CONCLUSIONS

The present study was performed for isolation and identification of *Clostridium perfringens* by biochemical and molecular methods along with toxotyping and antimicrobial susceptibility of isolates from the samples of animal origin foods viz. meat, milk and their products along with human stools. By conventional enrichment and plating on SFP Agar, typical round, 2-3 mm in diameter, black colored colonies with opaqueness around the colonies of *C. perfringens* were produced in 58.16% (146) samples of which 65.07% (95) were able to produce characteristic biochemical reaction for *C. perfringens*. For molecular identification of *C. perfringens* a 16S *rRNA* PCR assay was standardized and by which 31.87% of samples constituted by raw and processed meat, fish and processed fish, pasteurized milk and milk products and human diarrhoeic samples were found as contaminated with *C. perfringens.* The *C. perfringens* were detected in 51.42%, 26.67%, 48.57%, 60.00%, 33.33%, 4.00% and 26.00% of raw meats, meat products, raw fish, fish products, pasteurized milk, milk products and human diarrhoeic stool samples, respectively.

Toxotyping of *C. perfringens* was performed by PCR amplification of major toxins α (*cpa*), β (*cpb*) and ε (*etx*) and according to the presence of toxins all isolates were classified as *C. perfringens* type A (α toxin), type B (α, β, and ε toxins), type C (α and β toxins) and type D (α and ε toxins). All *C. perfringens* isolates (80) were found to harboured *cpa* gene while 18.75% (15) isolates *cpb* gene and 6.25% (5) *etx* gene. Further, all isolates were also screened for enterotoxin (*cpe*) gene and β_2 toxin (*cpb2*) gene and 12.50% (10) and 40.00% (32) *C. perfringens* isolates were found to harboured *cpe and cpb2* gene, respectively. The toxino-typing profile of *C. perfringens* showed that 77.50% (62) of *C. perfringens* were type A, 2.50% (2) type B and 16.25% type C and 3.75% (3) type D. Among *C. perfringens* type A, 4.84%, and 29.03% were found to harbour enterotoxin (cpe^+) and β2 toxin (cpb2+) gene, respectively while 6.45% isolates harboured both enterotoxin & β2 toxin (cpe^+ & $cpb2^+$) genes. Among *C. perfringens* type B, 50.00% was found to harbour each of enterotoxin and β2 toxin gene separately. Among *C. perfringens* type C, 7.69% and 30.77% isolates were found to harbour enterotoxin and β2 toxin gene. However, 33.33% each isolate

of *C. perfringens* type D were found to harbour enterotoxin, β2 toxin and both enterotoxin & β2 toxin genes, respectively. The *C. perfringens* isolates from raw meat samples showed that 72.22% isolates were type A of which 38.46% harboured only β2 toxin gene, while 7.69% harboured both enterotoxin and β2 toxin genes. Further 27.78% isolates were found as type C of which 60.00% harboured only β2 toxin gene. The finding of processed meat samples showed that 75.00% isolates were type A of which 33.33% each harboured enterotoxin and β2 toxin gene while 25.00% isolates type C. The *C. perfringens* isolates raw fish showed that 64.71% isolates were type A, 5.88% type B, 17.65% type C and 11.76% type D. Among *C. perfringens* type A 62.50% isolates harboured β_2 toxin gene. *C. perfringens* type B was also found to harbour β2 toxin (cpb2 gene). Among C. *perfringens* type D isolates 50.00% each isolates were found to harboured enterotoxin gene and both enterotoxin and β2 toxin gene). The finding of processed fish samples showed that 88.89% isolates were type A and 11.11% type D. 36.36% of *C. perfringens* type A isolates were found to harboured β_2 toxin gene. The *C. perfringens* type D isolate from processed fish samples also found to harbour β2 toxin gene. The isolates of *C. perfringens* from pasteurized milk showed that 82.35% isolates were type A and 17.65% type C. Among *C. perfringens* type A 14.29% isolates harboured enterotoxin gene and β2 toxin gene, while 21.43% isolates β2 toxin gene. Among *C. perfringens* type C 33.33% each isolates harboured only enterotoxin gene and β2 toxin gene. The finding of milk product samples showed that all isolates were type A and none of the isolates harbour either enterotoxin and β2 toxin gene. The isolates of *C. perfringens* from human diarrhoeic stool samples showed that, 84.61% isolates were type A and 7.69% each of type B and type C. 18.18% of *C. perfringens* type A isolates harboured only β2 toxin gene while 9.09% harboured both enterotoxin and β2 toxin gene. The *C. perfringens* type B isolate from human diarrhoeic samples was also found to harbour enterotoxin gene.

The MIC study *C. perfringens* using HiComb^{TM} MIC Test (Hi-Media, India) using seven antibiotics group including penicillins (ampicillin, ticarcillin and piperacillin), β-Lactam (Amoxyclav and piperacillin – tazobactum) cephems (ceftriaxone and cefotaxime), tetracycline (tetracycline), fluoroquinolones (moxifloxacin), linosamides (clindamycin) and phenicols (chloramphenicol) showed a MIC of 0.1 for most of the antibiotics with different calculated MIC_{50} and MIC_{90}. The antibiotic susceptibility profile study on the basis of MIC

study showed that that 50% *C. perfringens* isolates from raw meat samples were resistance to CD followed by 39% to MOX and TI, 11% to TE and 5.6% to AMC and PI. Whereas, 25% isolates from meat products samples were resistance to CD and TE. The antibiotic susceptibility profile also depicted that 47% isolates from raw fish samples were resistance to MOX followed by 41% to CD, 29% to TE and 5.9% to CTR as well as PIT whereas 11% isolates from fish product samples were found as resistance to AMC, MOX and PIT. The antibiotic susceptibility profile of isolates from pasteurized milk samples showed that 41% isolates were resistance to CD followed by 35% to TE, 31% AMP, 24% from C, 18% TI, 12% CTR and 5.9% from AMC, CTX, MOX and PI. Whereas, the isolates from milk product samples did not showed resistance of any of the antibiotics used under study. The antibiotic susceptibility profile *C. perfringens* isolates from human diarrhoeic samples showed that 23% isolates were resistance to TE followed by 7.7% to MOX.

The phylogenetic study of *C. perfringens* type A isolates using ERIC-PCR showed that three different clones of *C. perfringens* type A isolates were circulated among meat and meat product samples constituted by 62.50%, 25.00% and 12.5% isolates. Further, 5 different clones among isolates from fish and fish product samples with highest 57.89% of isolates in a cluster. ERIC-PCR of isolates from raw and pasteurized milk samples showed the circulation of 3 three different clones constituted by 31.25%, 62.50% and6.25% of isolates. *C. perfringens* type A isolates belonged to human diarrhoeic samples isolates were comes under 5 major clusters constituted by 45.45%, and 18.18% of population. The phylogenetic analysis of *C. perfringens* type A isolates from human and food of animal origin showed that isolates belonged to meat and meat product samples and human diarrhoeic samples revealed the genetic linkage with circulation of 4 common clones constituted by ~30%, ~29.62%, ~18.51% and ~7.40% of isolates at >95% genetic similarity. The isolates belonged to fish and fish product samples and human diarrhoeic samples, suggested that three different clones constituted by ~50% and two each of~13.33% of isolates with >95% genetic similarity circulating in both fish and human population. The isolates belonged to pasteurized milk and milk products and human diarrhoeic samples, also suggested that a single common clone of ~33% isolates with 95% genetic similarity circulating in both pasteurized milk as well as human population.

From the finding of present study, it may be presumed that meat and meat products, fish and fish products as well as pasteurized milk and milk products in this area harbour the pathogenic *C. perfringens* type A and serve as reservoir/ source of this organism to human beings by entry into food chain. Alternatively, the possibility for transmission of this infection from human to animals may not be ruled out. In such occasion, human carrier may shed the pathogens into the atmosphere and the organism in placing its spore formation get the best opportunity for optimum expression of its genetic characters and they perpetuate therein with longing whenever to get entry in human health through food chain. In these circumstances, our traditional cooking practices not the fast food habit bestowed upon boon to limit probability of occurrence of diarrhoea and gastroenteritis with intake of such suspected foods of animal origin.

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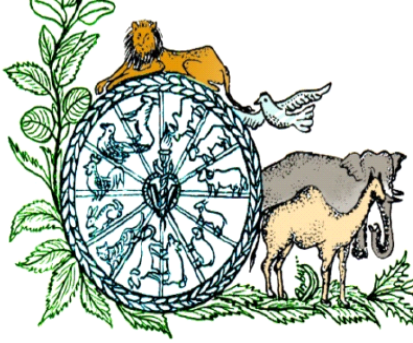

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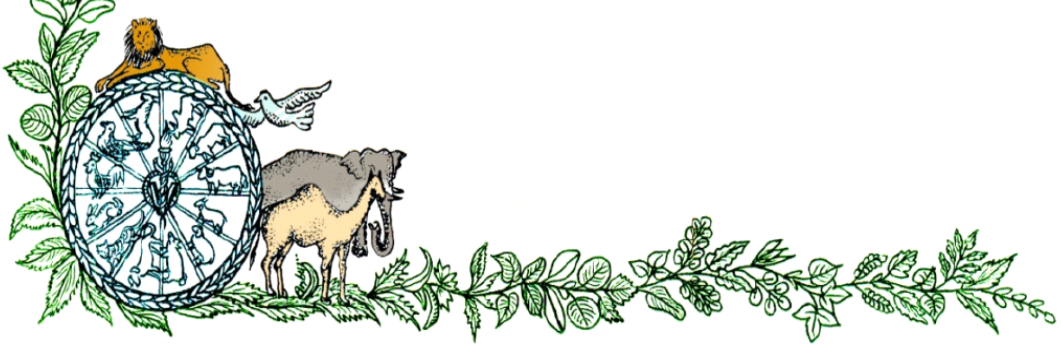
Resume



RESUME

8

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