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Clostridium perfringens food poisoning: An update on toxins, occurrence, diseases and detection techniques

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Abstract
Clostridium perfringens is an anaerobe, gram positive, sporogenic, non-motile and encapsulated bacillus. The organism is ubiquitous nature i.e. presence in soil, dust and the gastrointestinal tracts of animals and man, gives ample opportunity to contaminate foods. *Clostridium perfringens* can exist as a heat-resistant spore, so it may survive cooking and grow to large numbers if the cooked food is held between 4 ^{0}C and 60 ^{0}C for an extensive time period. The organism produces 17 different types of extracellular toxins, which produces different forms of enterotoxaemia in domestic and wild animals in several corners of world. Besides food poisoning, *Clostridium perfringens* also gives rise to sporadic diarrhoea, antibiotic-associated diarrhoea, gas gangrene and necrotic enteritis in humans. These diseases have high economic impact due loss of productivity and death of animals. Today, various methods have been developed *viz*. cultural method, bioassay, immunological assay and molecular techniques to detect, identify and characterize the organism. The cultural isolation is believed to be the most authentic method for the qualitative and quantitative detection and estimation of *Clostridium perfringens*. Since no single media alone is found to be the best suitable to enumerate the true presence of different strains of *Clostridium perfringens*. Therefore, multimedia study is required to show the true status of *Clostridium perfringens* in foods and also to compare the efficacy of various media in the detection and identification. Among the current technique, immunoassay and molecular methods are extensively used in the food microbiology for diagnostic purpose and epidemiological study of toxigenic strains of the pathogen. These techniques are powerful, specific and sensitive.

Keywords: Clostridium perfringens, ubiquitous, enterotoxin, food poisoning, diseases, detection

1. Introduction
Clostridium perfringens is recognized as the classical agent of food poisoning outbreaks causing many different histotoxic and enterotoxic diseases in humans and animals as a result of its ability to produce potent protein toxins. It is one of the most pathogenic bacteria among clostridia and this may be due to (1) ubiquitous presence makes its reporting in various foods inevitable, (2) very short doubling time (< 10 min) and relative aerotolerance permits rapid growth in foods, (3) the heat tolerance of vegetative cells (optimal growth temperature 43-45 °C), (4) heat resistance spores of *Clostridium perfringens*, which survive the cooking temperature and germinate into viable cells and reach the desired numbers to cause food poisoning (McClane *et al.,* 2006) [1]. *Clostridium perfringens* food poisoning is caused by an enterotoxin, a 35 kDa protein. The distribution of enterotoxin gene in *Clostridium perfringens* population is variable between 1-5% (Wen *et al*., 2003) [2]. Workers have observed difference in occurrence of enter toxigenic *Clostridium perfringens* strains (1-75%) in different species of animals ((Das *et al.,* 2009) [3]. The enterotoxin is mainly released during sporulation of the bacteria and hence it is considered to be sporulation specific product. The peak for toxin production is just before lysis of the cell's sporangium, and the enterotoxin is released along with the spores.
The earlier classification of clostridial isolates was based on their ability to produce a combination of four typing toxins - α-toxin, β-toxin, ε-toxin and ι-toxin to divide *C. perfringens* strains into toxino types A to E; based upon the variable production extracellular toxins, most of whose genes are carried on large conjugative plasmids.

However, this scheme is now outdated since it does not take into account the discovery of other toxins that have been shown to be required for specific *C. perfringens*-mediated diseases. The principles for the expansion of the typing system are described, as is a mechanism by which new toxinotypes can be proposed and subsequently approved. Based on these criteria two new toxinotypes have been established *i. e*. F and G. However, there are as many as seventeen exotoxins produced by *Clostridium perfringens* and are regarded as one of the most prolific bacteria in toxins production (Rana *et al.*, 2023) [4].

1.1 Alpha toxin
The toxin is produced by nearly all isolates but most abundantly by type a strains. This is the most important toxin biologically and is lethal, hemolytic and necrotizing type. It is also responsible for hydrolysis of phospholipids, platelets, leucocytes, endothelial and muscle cells resulting into lysis and cytotoxicity. It produces zone of opalescence on egg yolk agar due to lecithinase activity (Ochi *et al.*, 2002) [5]. The hemolytic activity occurs on most species except horse and goat.

1.2 Beta toxins
The toxin is highly trypsin sensitive protein and is responsible for mucosal necrosis (Jolivet-Renaud and Popoff, 1986) [6]. It is also associated for CNS signs in *Clostridium perfringens* induced disease in domestic animals. Analysis with different serological and molecular diagnostic techniques revealed that beta toxin gene (Cpb gene) is present in type B and type C strain of *Clostridium perfringens*.

1.3 Epsilon toxin
It possess lethal and necrotizing properties and is found only in strains of type B and type D of *Clostridium perfringens*. It is produced as minimally toxic epsilon proto-toxin which is converted to greater than 1000 fold more toxic form on proteolysis at 14-N terminal amino acids (Finnie *et al.* 2022) [7].

1.4 Iota toxin
It is two types- IA and IB. It is found in type E strains of *Clostridium perfringens* and is responsible for increase in vascular permeability. It is also dermonecrotic and lethal.

1.4.1 *C. perfringens* type F *enterotoxin*
C. perfringens type F consists of isolates that produce *C. perfringens enterotoxin* (CPE), but not β-toxin, ε-toxin or ι-toxin. *Clostridium perfringens* enterotoxin (CPE) is responsible for causing the gastrointestinal symptoms of several *C. perfringens* food and nonfood-borne human gastrointestinal diseases (Mi *et al.* 2018) [8]. The enterotoxin gene (*cpe*) is located on either the chromosome or large conjugative plasmids.

1.4.2 *C. perfringens* type G toxins
C. perfringens type G strains, which produce alpha-toxin (CPA) and the necrotic enteritis B-like (NetB) pore-forming toxin, are responsible for causing NE, an economically important disease of poultry. NetB is encoded on an ~ 42-kb plasmid-borne pathogenicity locus and has been shown to be essential for the development of NE, although other virulence or virulence-associated factors are also required. The pathogenesis of NE is complex and not fully understood, involving out-competition with the existing microbiota and rapid proliferation of type G strains in the small intestine, along with production of NetB, which is regulated by the Agr-like quorum sensing system, to ultimately produce the characteristic intestinal lesions (Van *et al.* 2022) [9].

2. Occurrence in foods
2.1 Meat and meat products
Most of the food poisoning outbreaks of *Clostridium perfringens* (60-90%) is associated with meat and meat products. *Clostridium perfringens* has been shown to be isolated from various raw, semi-processed as well as cooked meat products. The incidence of bacteria varies in different raw meats, beef and veal (50-73.1%), pork (66-68.8%) and in lamb and poultry (85%) (Cohen *et al.*, 2007) [10]. In cooked meat of different species, the prevalence rate was 16 to 33% in beef, 10-30% in pork, and 30-85% in poultry, reported for the organism. Significant differences were observed in prevalence of enterotoxigenic strains of *Clostridium perfringens* in various meat and meat products. In India, Singh *et al.* (2005) [11] have shown 9.3%, 30.2% and 15.5% enterotoxigenic strains in beef, goat and poultry respectively.

2.2 Milk and milk products
Reports have also indicated that *Clostridium perfringens* have been associated with mastitic cases (Osman *et al.*, 2009) [12]. *Clostridium perfringens* had shown significant presence in milk and milk products *viz*. raw milk (72.7%), butter (50%), cheese (32%), ice cream (30%), and yoghurt (10%) (Bassiony and Bassiony, 1980) [13]. Voidarou *et al.* (2005) [14] observed the hygienic quality of infant formula milk and showed that 1% had very low *Clostridium perfringens* spores. The count of bacteria is less in number than the bacteria associated with foodborne disease.

2.3 Sea food
In a survey mackerel, salmons, oysters and squid were found to be positive for enterotoxigenic *Clostridium perfringens* (Oka *et al.*, 1999) [155].

2.4 Other food products
Clostridium perfringens have been reported from salads, vegetable, herbs, and spices which often get contaminated with soil. In a report in Argentina, Aguilera (2005) [16] found that out of 115 samples of spices, 14 (12.17%) samples were positive for *Clostridium perfringens* strains. Four of which (28.60%) turned out to be enterotoxigenic by RPLA and PCR. Further study with PCR revealed that all the 14 strains belonged to type A *Clostridium perfringens.*

3. Diseases caused by *Clostridium perfringens*
3.1 Domestic Animals
The *Clostridium perfringens* is leader in disease causation among clostridia in animals. It has immense economic impact due loss of productivity and death of animals. It affects both domestic and wild species.

3.2 Sheep and Goat
The bacteria produce enterotoxemia in sheep worldwide. Type A produces hemolytic disease in Australia and yellow lamb disease in California wherein jaundice, hemoglobinuria, severe depression and high fever (106 °F) are characteristic signs (Uzal *et al.* 2022) [17]. The major toxin associated with the disease is alpha toxin. Type B, C and D strains are also causing lamb dysentery, struck and pulpy kidney disease respectively. Type D enterotoxemia (pulpy kidney disease)

found worldwide, occurs mainly in lambs fed with high nutritious diet. In goats, *Clostridium perfringens* is the main etiological agent of enterotoxemia with or without hemorrhagic enteritis.

3.3 Cattle
Type A strains causes hemorrhagic enteritis in calves leading to calf enterotoxemia with death in 7-10 days old calves by type B and C. Type D affected 1-4-month veal calves and also causes death in feeder cattle (Zafar *et al.* 2022) [18].

3.4 Pig
Type A and C strains of *Clostridium perfringens* lead to diarrhea and necrotic enteritis in 1-2 weeks old pigs (Uzal *et al.* 2023) [19]. The case fatality rate in necrotic enteritis is high and in severe outbreak, 80% of piglets at risk may die. Necrotic enteritis is endemic in swine units.

3.5 Horse
In equine, intestinal clostridiosis is caused by type A strain, there is acute profuse diarrhea with high mortality. Type B and C produce enterotoxemia in foals. The affected foals are few days old and these foals collapse with the sign of bloody feces, subnormal temperature, fast pulse and respiratory rate. Type D enterotoxemia is rare in horses (Caroll *et al*., 1987) [20].

3.6 Poultry
Type A and C mainly causes necrotic enteritis in domestic chickens worldwide. Mucosal necrosis of both jejunum and ileum occurs throughout the length. In mild form, there is decrease in body weight gain. High fiber litter damages the intestinal mucosa and predisposes mucosa for concurrent infection of *Clostridium perfringens* with coccidia. It has been reported that ban imposed on antimicrobials as growth promoter due to fear of antibiotic resistance have tremendously increased the cases of necrotic enteritis (Cooper and Songer, 2009). It has been suggested that butyric acid, lauric acid, essential oils (thymol, cinnamaldehyde, essential oil of eucalyptus) and use as feed additive may contribute to the prevention of necrotic enteritis in broilers (Thanissery *et al*., 2010) [21].

3.7 Wild animals
The *clostridium perfringens* produces disease in several species of wild animals. It produces septicemia and enterotoxemia with hemorrhagic enteritis in elephants, pygmi hog, hippopotamus and bear (black, polar and arctic) in different parts of world (Das *et al*., 2009; Costa *et al.*, 2022) [22, 23].

3.8 Humans:
There are several diseases in humans in which *Clostridium perfringens* are found to be incriminated such as wound infection, gas gangrene, food poisoning and non-food borne gastro-intestinal diseases etc. The important ones are:

I) Food Poisoning
Such type of cases is mainly attributed to type A strain of *Clostridium perfringens* which possess enterotoxin. The food borne infections are mainly caused by heat resistance spores which could survive the cooking temperature. It occurs when large quantities of enterotoxigenic *Clostridium perfringens* (10^5-10^7 organisms) are ingested with foods (Cakmak *et al.*, 2006) [24]. After 12-24 hr of incubation, abdominal pain and diarrhoea set in with rare cases of vomiting. Diagnosis of bacteria is made by isolating heat resistant *Clostridium perfringens* type A from the food and feces.

II) Necrotizing enteritis
The disease is caused by heat resistant spores of *Clostridium perfringens* type C and type A. In infection with type C, beta toxins lead to mucosal necrosis in the intestine. The enteritis is fatal in many cases. The chances of disease occurrence increased with trypsin inhibitors like sweet potatoes. Immunisation with type C toxoid protect from the disease. Recently, an outbreak of *Jejunitis necroticans* was seen in 42 patients in Batticaloa, Sri Lanka (Mandrella, 2007) [25].

III) Non-food borne gastro-intestinal diseases
These include antibiotic associated diarrhea (AAD), sporadic diarrhoea and nosocomial diarrhea (Watanabe, 2008) [26]. About 10-15% of all cases of AAD are mainly caused either by *Clostridium perfringens* alone or in concomitant infection with *Clostridium difficile* (Vaishnavi and Kaur, 2008) [27]. In AAD, these bacteria get the upper hand to grow in GIT due to change in the microflora of the gut because of continuous use of antibiotics. *Clostridium perfringens* produced 5-20% cases of sporadic diarrhea.

IV) Gas gangrene
Clostridium perfringens is one of the most gas producing bacteria among clostridia and type A strain of *Clostridium perfringens* holds the predominant position (Gomes *et al.*, 2009) [28]. The cases of gas gangrene may also occur with other clostridia or other anaerobes or even aerobes. In adverse condition, it produces anaerobic cellulitis or gangrenous myositis.
Besides above mentioned infections in human, the organism has also been isolated from cases of gangrenous appendicitis, brain abscess, meningitis, panopthalmitis, thoracic infections, urogenital infections.

4. Detection of *Clostridium perfringens*
Detection of bacteria involves traditional cultural methods and newer techniques *viz.* immunological assay and molecular methods which are in vogue in the present era for early detection, identification and epidemiological study of the organisms.

5. Culture method
This is one of the oldest methods but most acceptable and authentic method for identification and enumeration of the organism. The detection of pathogen includes isolation and biochemical characterization for identification of bacteria. The protocol is tedious and takes longer time period. This drawback can be removed by use of fluorogenic media which takes shorter time period. The cultural method commonly includes following technique (a) Enrichment of the organism, (b) Direct plating on selective agar and (c) Enumeration directly by MPN technique.

I) Enrichment technique
It is useful when the organisms are less in number. It also enhances selectivity. Commonly used media for *Clostridium perfringens* are cooked meat media (CMM) and fluid thioglycollate media (FTM). CMM is the most widely used media for enrichment and also use as stock culture for anaerobic bacteria particularly clostridia. In a comparative study conducted by Dromigny *et al.* (1997) [29] taking five

media namely fluid thioglycollate medium (FTM), rapid perfringens medium (RPM), Columbia broth malthus, (CBM), reinforced clostridial medium (RCM) and lactose sulfite (LS) using conductance measurement with a malthus analyzer, FTM and CBM were found to be better than others.

II) Plating media

Various media have been developed in past five decades for the identification and selective enumeration of the organisms. The well-known media include Sulfite polymyxin-sulfadiazine (SPS), Tryptose sulfite cycloserine, TSC minus egg yolk agar, Sahidi ferguson perfringens (SFP), Oleandomycin-polymyxin-sulfadiazine perfringens (OPSP), neomycin blood agar and blood free egg yolk (BCP) agar and egg yolk free bismuth sulfite cycloserine agar (BISC). Study have shown that TSC is considered to be most satisfactory for general purpose, OPSP for food containing other *Clostridia i.e. Clostridium bifermentans* and *Clostridium butyricum*, and SFP is least selective but good for stressed cells, it is further reported that freshly prepared media were superior to their commercial dried counter parts (Varnam and Evans, 1991) [30].

III) Chromogenic/Fluorogenic media

In recent years, use of chromogenic / fluorogenic media is increasing in food microbiology as it eliminates the need of tedious process like subculture and biochemical test to identify the bacteria (Manafi, 2000) [31]. In *Clostridium perfringens* identification, 4-mU-phosphate is a fluorogenic or chromogenic enzyme substrate on Florocult TSC agar. Acid phosphatase is a highly specific indicator for this bacterium and it produces light blue florescence. The antibiotics like D-cycloserine may also be added in the medium to check the growth of accompanying bacteria and it also reduces the size of the colonies of accompanying flora.

IV) Most probable number (MPN) test

MPN method is one of the best methods for presumptive identification and enumeration of the organism from food. The media in common use are lactose sulfite, rapid perfringens medium (RPM), iron milk medium (IMM) and differential reinforced clostridial medium (DRM). Antibiotics like cycloserine, neomycin and Kanamycin are now used to increase the selectivity of media. During the growth of *Clostridium perfringens* in this medium, shreds of clot stick by side of test tube and therefore, this process is known as stormy fermentation. In a study, media showed good results in meat during enumeration of presumptive enumeration of *Clostridium perfringens* (Singh *et al*., 2005) [32]. Abeyta *et al*. (2006) [33] examined IMM, TSC and SFP agar for enumeration of *Clostridium perfringens* in the samples of clams, oysters and turkey meat and observed no significant differences in recovery abilities of the three media. In 15 naturally contaminated samples, recoveries of *Clostridium perfringens* were not different in IMM compared to TSC. Thus, IMM can be used as an alternative method for routine analyses for *Clostridium perfringens* in foods.

6. Detection of *Clostridium perfringens* enterotoxin

The common media used for sporulation are Ellners medium, Duncan and Strong medium. Several modifications in these sporulating media have been made such as, replacement of starch by raffinose, increased the level of CO2 or increased culture pH to enhance the sporulation of *Clostridium perfringens* (Craven, 1988) [34]. Various techniques are applied now days to detect, identify and characterize the enterotoxinn *viz*. bioassay, cell culture, immunoassay (RPLA, ELISA etc.), flow cytometry and molecular techniques.

7. Bioassays

The commonly used bioassays are- rabbit ligated ileal loop (RLIL), Mouse ileal loop, mouse lethality test and guinea pig skin erythemal activity (Jay, 2005) [35]. Among these, RLIL is the most commonly used method. It is based on the principle that enterotoxin elicit fluid accumulation in the small intestine of rabbits. The activity of enterotoxin is shown to affect the intestinal tract in following order; ileum > jejunum > duodenum (McDonel and Duncan, 1977) [36]. Besides above mentioned bioassays, biological activity of enterotoxin can also be demonstrated by feeding enterotoxigenic culture to monkey and human volunteers. The sensitivity of these assay have been reported to be 1.84 µg of toxin for mouse lethality, 1.0 µg for mouse ileal loop; 6.25 µg for RLIL and 0.06-0.12 mg/ml for erythemal activity in Guinea pig.

8. Cell culture

A variety of cell culture systems are employed to assess the pathogenic/toxigenic potential of various pathogens. Various tissue and cell culture systems that are used for assessing *Clostridium perfringens* enterotoxin are (a) rabbit intestinal epithelial cells for binding; (b) Rat hepatocytes for membrane permeability and (c) Vero cells for binding, biological activity. Among these cell lines, Vero cells are the most widely used cell culture system for *Clostridium perfringens* enterotoxin (Horiguchi *et al.*, 1985) [37].

9. Immunoassays

In recent years, serologial methods have been in regular use and these techniques have superseded biological assay and cell culture for determination of *Clostridium perfringens* toxin. A number of serological tests have been applied to detect *Clostridium perfringens* enterotoxin (Moustafa *et. al.* 2022) [38]. These include immunodiffusion, electro-immunodiffusion, counter immuno electrophoresis, reverse passive hemagglutination, reverse passive latex agglutination, fluoroscent antibody technique (FAT), enzyme linked immunosorbent assay (ELISA) and flow cytometry. However, ELISA and RPLA are the most commonly used and sensitive methods for detection of *Clostridium perfringens* enterotoxin. Rapid sandwich ELISA, indirect ELISA, Immunomagnetic separation ELISA (IMS-ELISA) have been reported for identification and quantification of the enterotoxin. Rapid sandwich ELISA and indirect ELISA can easily detect 1 pg/ml and 25 ng/ml of enterotoxin, respectively (McClane, *et al.*, 1984) [39], whereas, IMS-ELISA can detect 2.5 ng/ml of enterotoxin.

10. Molecular methods

Molecular methods are now days used extensively in the food microbiology. The commonly used method in the study is polymerase chain reaction (PCR) and nucleic acid probes. Different forms of pcr (single gene pcr, duplex, nested and multiplex pcr) are used in identification and epidemiological study of the pathogen (Forti *et al.*, 2020) [40]. Its reliability is due to specificity, sensitivity, reproducibility, cost effectiveness and rapid results. In duplex pcr, two genes are amplified and detected at a time. In *Clostridium perfringens* study, the two most commonly targeted genes are phospholipase (plc) and enterotoxins (cpe) genes (Lin and Labbe, 2003) [41]. Duplex pcr can detect approximate 105 *Clostridium perfringens* cells/g of stool or food sample and

detection level can reach as low as 10 cells in artificially inoculated food samples on overnight enrichment. Now a day multiplex pcr are routinely applied in genotyping of *Clostridium perfringens* in humans and animals. Duplex and multiplex pcr are used to differentiate chromosomal cpe from plasmid cpe *viz* strains associated with food poisoning outbreaks with non-food poisoning outbreaks (sporadic diarrhea and antibiotic associated diarrhea) respectively (Lahti *et al*., 2008) [42].

For detection of enterotoxigenic strains of *Clostridium perfringens* nucleic acid probes directed against enterotoxin gene (*cpe)* of *Clostridium perfringens* are most commonly used. The sensitivity of chromogenic, non-radioactive colony hybridization assay is reported to be < 10 cfu/g of raw beef for *Clostridium perfringens*. Recently a new form of PCR (Immunonagnetic separation PCR; IMS-PCR) have been developed which detect enterotoxigenic Clostridium perfringens in meat samples within 10 hour. Further it is also reported that IMS-PCR is capable of detecting as few as 10 colony forming units (cfu)/gm of Clostridium perfringens cells in the meat samples. In comparison to conventional culture method, IMS-PCR is rapid and specific method and has potential use as a screening tool for Clostridium perfringens in food samples (Yang *et al*., 2010) [43].

11. Antibiotic sensitivity pattern

Different studies have shown that antibiotic resistance against strains of *Clostridium perfringens* is transferable through conjugate R-plasmid. Gene based resistance study against tetracycline and macrolide antibiotics study revealed that resistant genes were found due to conjugal transfer of genes (Holzel *et al*., 2010) [44]. Voidarou (2005) [45] observed that *Clostridium perfringens* isolates from infant milk formula showed resistance to vancomycin, ceftiofur, ampicillin, penicillin, neomycin, tetracycline, cephalexin, amoxicillin, gentamycin and sulphamethoxazol.

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