Anaerobic Pathogens in Seafood

K.V. Lalitha
Central Institute of Fisheries Technology
Cochin 682 029.

Food-borne diseases are the major causes of illness and death through out the world. Bacteria, parasites and virus are incriminated in food-borne outbreaks. Food- borne diseases due to bacterial pathogens fall into two groups: food-borne infections and intoxications. Foods may serve as a vehicle for the introduction of infectious microorganisms into the gastrointestinal tract. These infectious microorganisms either cause disease by invasion of the body of the host or by the release of toxic substances (toxins) as a result of growth in the intestinal tract or some other organ in the body of the victim. Diseases of this type are generally called "food-borne infections". Multiplication and metabolism of certain infectious microorganisms in foods prior to consumption may result in production of toxins and diseases result from the absorption via the intestinal tract of these preformed toxins. These are generally called "food-borne intoxications".

Among anaerobic pathogens, two species are incriminated in seafood-borne disease outbreaks. *Clostridium perfringens* is responsible for food-borne infections in man. *Clostridium botulinum* is associated with food-borne intoxications.

Clostridium perfringens

Clostridium perfringens is probably the most extensively studied anaerobic bacterium pathogenic for humans. *C. perfringens,* formerly *welchii*, has been known as a cause of the serious wound infection, gas gangrene, since 1892 when it was first described by the American bacteriologist Welch. The first indication of an association with food poisoning, however, came in the 1940s from Knox and Mac Donald in England and Mc Clung in the U.S.

The organism and its characteristics

C. perfringens is a Gram-positive, non-motile rod shaped anaerobe which forms oval subterminal spores. The species is classified into five types designated A to E, based on the production of four major lethal extracellular toxins/exotoxins (alpha a, beta b, epsilon e and iota i) and eight minor ones. *C. perfringens* type A produces only the a major toxin which has lecithinase (phospholiopase C) activity and is responsible for food poisoning. Type C which produces α and β toxins causes enteritis necroticans, a more severe, but far more rare, enteric disease in which theb toxin damages the intestinal mucosa causing necrosis.

Tolerance to preservation methods

It has become well established that the growth of *C. perfringens* in foods is influenced by such factors as temperature, Oxidation–reduction potential (E_h), pH and water activity (a_w) levels.

The heat resistance of spores can clearly contribute to the organism's ability to cause food poisoning by allowing it to survive in foods that are not completely cooked. The vegetative cells of *C. perfringens* have a relatively high optimal growth temperature (43 to 45°C). Growth occurs on the temperature range 12 to 50°C. The vegetative cells of *C. perfringens* are sensitive to low temperature storage. Stationary phase cells are more resistant to the effect of cold shock than logarithmic phase cells. The vegetative cells are sensitive to frozen storage. The heat resistance of vegetative cells is comparable to that of non-spore forming bacteria. D values of spores at 100°C show wide inter-strain

variation with recorded values from 0.31 min to more than 38 min.

C. perfringens does not require an extremely reduced environment for its growth. The limiting Eh reported is —125 to +350mV. The lowest a_w supporting growth is reported to be 0.93 to 0.97. 7-8% sodium chloride is needed to prevent growth of most strains.The vegetative cells of *C. perfringens* are sensitive to pH extremes with optimal growth occurring at pH 6 to 7.

One of the important roles that food preservation factors play in controlling *C. perfringens* levels in foods is to inhibit the germination and outgrowth of *C. perfringens* spores. However, ungerminated spores in foods may remain viable and could undergo germination and outgrowth when these growth limiting factors are removed during food preparation.

Reservoirs

Occurrence of C. perfringens in fresh fish/ shellfish and in the environment.

The wide spread natural distribution of *C .perfringens type* A in soil, aquatic environment and the gastrointestinal tract of humans and other animals provides this bacterium with ample opportunities to contaminate foods. Types B,C,D and E are obligate parasites, mostly of domestic animals and do not persist in soils.

Contamination of fish and shellfish by *C. perfringens* is well known. Raw fish and seafood products from retail shops harboured *C. perfringens. C. perfringens* levels were higher in shellfish than finfish. Shellfish may be reservoirs for enterotoxigenic *C. perfringens.*

Food-borne outbreaks

C. perfringens type A food poisoning annually ranks among the most common food-borne diseases in the united States and Europe. In terms of number of cases of bacterial food-borne illness reported each year, *C. perfringens* type A ranks second to Salmonella as a cause of bacterial food poisoning in the United Kingdom and Canada and third in the United States. This organism is of concern to the seafood industry because of its involvement in several human food-borne illnesses.

Seafoods were implicated as vehicles in approximately 1.3% of food-borne disease outbreaks due to *C.perfringens* reported in the United States during the years 1999-2001. Both raw and processed seafoods were implicated in *C.perfringens* food-poisoning outbreaks in the United States and Japan. In Japan, fish dishes made from heated and processed fish products had contributed to *C.perfringens* food poisoning. Major contributory factors are the temperature abuse, low redox potential, poor personal hygiene, contaminated equipment, mode of preparation and consumption which can give *C.perfringens* the opportunity to multiply in dangerous levels.

Pathogenesis and clinical features

In contrast to many cases of foodborne botulism, *C. perfringens* type A food poisoning is rarely, or never, an intoxication. Instead, the pathogenesis of *C. perfringens* type A food poisoning involves the in vivo production of *C. perfringens* enterotoxin (CPE). Food poisoning due to *C. perfringens* usually occurs 8-24 hours after ingestion of heavily contaminated food ($>10^6$ to10^7 *C. perfringens* vegetative cells/g). The illness is caused by sporulation of the bacterial cells in the intestine, accompanied by production of an intracellular enterotoxin. Toxin is released into the intestinal lumen on lysis of the sporangia. Sporulation and enterotoxin production may also occur in foods. Diarrhoea and severe abdominal pain are the usual symptoms. Nausea is less common, and fever and vomiting are unusual. Death is uncommon.

One must ingest 8- 10 mg of *C. perfringens* enterotoxin (CPE) to induce symptoms of gastroenteritis. CPE is classified as an enterotoxin since it causes fluid and electrolyte (sodium and chloride) losses from the gastrointestinal tracts of a number of mammalian species. Enterotoxin causes fluid accumulation in ligated intestinal loops. Glucose absorption is inhibited whereas potassium and bicarbonate absorption is unaffected. The brush borders of villous tip epithelial cells is the primary site of action of the enterotoxin. In contrast to the effects of cholera and *E.coli* enterotoxins, this enterotoxin does not increase levels of cyclic AMP in intestinal mucosa that is actively secreting fluid.

The molecular weight of purified type A enterotoxin is approximately 35000 Da. The enterotoxin consists of a single poly peptide of 319 aminoacids. The enterotoxin is inactivated by heating for 5 min at 60^0C.

Diagnosis of *C.perfringens* food poisoning is normally based on a number of factors such as Case history and symptoms, large numbers ($>10^6g^{-1}$) of *C.perfringens* spores in the patients faeces, large numbers of vegetative cells of the same serotype in the incriminated food ($>10^6g^{-1}$) and presence of enterotoxin in faeces

Isolation and Identification

In the investigation of outbreaks, enrichment culture is rarely necessary since C. perfringens will invariably be present in high numbers in implicated foods or clinical samples.The most commonly used selective media for the enumeration of *C.perfringens* employ sulphite reduction to produce black colonies as the differential reaction.The most popular media are Tryptose Sulphite cycloserine Agar (TSC), Oleandomycin /polymyxin / sulphadiazine/ perfringens (OPSP) medium incubated anaerobically for 24 h at 37^0C using Pour Plate method. Most Probable Number (MPN) Technique is employed to enumerate low numbers of viable bacteria in food and water.

Serotyping based on capsular antigens is employed for epidemiological purposes.

A number of methods are available for enterotoxin assay. Traditional biological tests such as the Rabbit illeal loop (RIL) and mouse challenge have been superseded by more sensitive, rapid and convenient immunological methods. Several serological (ELISA, RPLA) and gene detection assays have been developed for the detection of CPE.

Control of C. perfringens in fish and fishery products

The most important attributes of *C.perfringens* are its ability to grow at elevated temperature and its ability to form spores. Control measures must consider both of these. The best way to prevent and control *C. perfringens* food poisoning is, first, to cook foods thoroughly. Another step for controlling *C. perfringens* food poisoning is to quickly cool cooked food and then store or serve the food at conditions non-permissive for growth of *C. perfringens.*

Clostridium botulinum

Clostridium botulinum, the aetiological agent of botulism, was first isolated from inadequately home cured ham in 1896 by van Ermengem. The word botulism comes from the Latin word for sausage "botulus". Botulism is a severe form of food poisoning which results from ingestion of food containing botulinum toxin produced during the growth of this organism in food.

Three forms of botulism are recognized —a. the "Classic"form is food-borne, caused by preformed toxin. b. wound botulism-a rare disease that develops from growth and toxigenesis in situ. C. infant botulism—results from ingestion of botulinal

spores and subsequent germination, growth and toxigenesis in the intestinal tracts of infants under one year of age.

Characteristics of the organism

Clostridium botulinum is the taxonomic designation given to a group of gram- positive, anaerobic, rod shaped, and sporeforming bacteria that produce any of the characteristic botulinal neurotoxins. The species *Clostridium botulinum* is classified into 7 types A to G, depending on the serologic specificity of the neurotoxins. *C. botulinum* types A, B, E and F are the common types involved in human botulism . Types C and D are pathogenic to animals. Type G has not yet been recognized as a cause of illness.

The species is also divided into four groups based on physiological differences. Group I includes all type A strains and proteolytic type B and F that do not grow at or below 10°C. These strains produce the most heat-resistant spores and can pose a problem when foods that depend upon a heating step for their stability and safety are under processed. To inhibit growth, the pH must be below 4.6, the salt concentration above 10% or the a_w below 0.94. In contrast, Group II strains represent a greater potential hazard in chilled foods. Group. II includes all type E and non-proteolytic type B and F that can grow at 3.5°C. They produce spores with a low resistance to heat. These strains are inhibited by pH below 5.0, salt concentration above 5%, or a_w below 0.97. The toxicity of these strains is usually activated by trypsin.

Types C and D that do not grow below 15°C are included in Group III. Organisms in this group grow optimally at 40°C and have an intermediate spore resistance to heat. Group IV includes type G that is asaccharolytic and does not produce lipase.

These four groups, plus neurotoxin producing strains of *C. barati* and *C. butyricum,* make a total of six distinct genomic groups, which produce botulinum neurotoxin (BoNT).

Tolerance to preservation methods

The main factors controlling growth of *C. botulinum* in foods are temperature, pH, water activity (a_w), redox potential (E_h), added preservatives and the presence of other microorganisms.

Refrigerated storage is used to prevent or inhibit growth of *C. botulinum.* For group I strains, temperature limit for growth is approximately 10°C. For group II, it is 3.5°C. Upper growth limits for strains of groups I and II are 45 -50°C and 40 -45°C respectively. Refrigeration above 3..3°C may not be a complete safeguard against botulism for foods containing non-proteolytic strains of *C. botulinum* .

Thermal processing is used to inactivate spores of *C. botulinum. C. botulinum* spores of group I, which are very heat resistant, are of particular concern in the sterilization of canned low-acid foods. The canning industry has adopted a D value of 0.25 min at 121°C as a standard for calculating thermal processes. The "botulinum cook" has been defined as equivalent to 3 min at 121°C. This value is also called the F_0 value or the process value. The F_0 value required is equivalent to 12 decimal deductions of *C. botulinum* spores (12x0.25=3). This is the so called 12D- concept designed to reduce the bacterial load of one billion spores in each of 1000 cans to one spore in a thousand cans. Strains of group II are considerably less heat resistant ($D_{100°C}$,0.1min) than those of group I($D_{121°C}$ 0.1-0.2 min).

The minimum pH allowing growth of *C. botulinum* group I is 4.6, for group II, it is about 5.0. Acid tolerant yeasts and molds in foods raise the pH in their immediate vicinity to a level that allows growth of *C. botulinum.* The toxin is unstable at alkaline pH values. It may be a significant factor in fermented fish products associated with botulism.

The growth limiting brine concentrations are about 10% (a_w 0.94) for group I and 5% (a_w 0.97) for

group II. Other solutes used to reduce water activity are sugars(glucose, sucrose, glycerol), organic acids and lipids.

Clostridium botulinum grows optimally at an E_h of –350 mv, but growth initiation may occur in the range of +30 to +250 mv. The upper redox Potential (E_h) level for growth initiation from spores for both group I and II is +200mv. *C. botulinum* may grow well in air as in vacuum-packaged systems (gas flushed). Modified Atmosphere Packaging (MAP) of fish has been concern because incidence of *C. botulinum* type E spore is high in fish and *C. botulinum* can grow and produce neurotoxin in MAP fish.

Preservatives such as nitrates, sorbates, nisin, ascorbate, lactate salts, EDTA, polyphosphates, metabisulphite are active against *C.botulinum.* The use of natural and liquid smoke has a significant inhibitory effect against *C.botulinum* in fish. Antibotulinal spices such as garlic oil, onion oil, pepper etc are used to preserve food.

The competitive microflora in fish have a significant protective role by inhibition of *C.botulinum* and by spoilage that would make a toxic product less likely to be consumed. Inhibition of *C.botulinum* is observed in the presence of Lactic acid bacteria (*Lactobacillus bulgaricus, L.plantarum, Pediococcus* spp., *Streptococcus lactis etc)* which is related to drop in pH and to production of bacteriocins.

C.botulinum spores are probably the most radiation resistant spores of public health concern. Gamma irradiation-resistance of botulinal spores is extensively studied. D values for group I at –50°C to -10°C in the range of 0.2 –0.45 Mrads. For group II it is at –50°C to -10°C in the range of 0.1 –0.2 Mrads. Spores of type E are only marginally more sensitive having D values between1 and 2 kGy. *C. botulinum* spores are resistant to chlorine compounds and other antimicrobial agents.

Reservoirs

Occurrence of *C. botulinum* in the environment

Contamination of food largely depends on the incidence of *C. botulinum* in the environment.Spores of *C.botulinum is* widely distributed in soil, marine and lake sediments and the intestinal tracts of fish and animals, but their numbers and types vary depending on the location.

C.botulinum type A spores predominate in the western United States. Type B predominates in the aquatic environments of the United Kingdom. Surveys of Asia report low numbers, with the exception of a high incidence of type E spores around Caspian Sea and a high incidence of all types in Sinkiang district of China. In Japan, type E predominates in northern areas. In the tropical regions of Asia, types C and D replace type E as the predominant type in aquatic environments.

Occurrence of *C. botulinum* in fresh fish/ shellfish

The importance of the botulism hazard in seafoods is well known. Foods containing botulinum toxin in any amount is unacceptable. The predominant serotype was type E in fish from temperate countries where as types C and D were more prevalent in tropical countries.

Seafood-borne outbreaks

Several foods have been implicated in food-borne botulism outbreaks. Fish and fishery products have been implicated in 14% of all outbreaks. Home processed foods accounted for the majority of outbreaks (91%) while commercially processed foods were involved in few outbreaks(9%). Countries with the bulk of botulism outbreaks recorded located north of the tropic of cancer. In Alaska, Norway, Sweden, Japan, Iran and the former Soviet Union, the majority of the outbreaks were associated with fish and type E was involved.

Almost all type E outbreaks were due to fish, fish eggs,or sea mammals all over the world. Most of these products, however, are usually consumed directly from the container at ambient temperature with no further heating which could inactivate any toxins. Type A is the main cause of botulism in the United States, China and Argentina and increminated foods are mainly vegetables. Type B is involved in most outbreaks in continental Europe and linked with meats. *C.botulinum* type F is involved in 2 outbreaks though 1of them is not conclusively proved.

Countries having sporadic outbreaks are Britain, Sweden, Finland, Netherlands, Switzerland, Australia and countries having no recorded outbreaks are Austria, Greece, New Zealand, India.

Fresh and frozen fish has never been incriminated in human botulism. Fresh fish normally spoil before being toxic. Botulism associated with commercially prepared fish products has involved canned clams, clam juice, crab, canned salmon, canned tuna, sardines, sprat. Pickled trout and carp from Argentina and pickled fish in Britain were implicated in botulism outbreaks. In the USA, commercially produced smoked fish have been involved in nine outbreaks and were due to *C. botulinum* type E. Cured fishery products, eg. cured carp, carp eggs (ashbal) are semipreserved and in most cases, they are consumed without additional preparation or with very little; hence, if they contain sufficient numbers of *C. botulinum,* food-borne disease outbreak can result. Some products, such as "Kapchunka", an ungutted partially dried whitefish, salted fish eggs, "izushi", "kirikomi" in Japan have been the cause of frequent and large scale outbreaks in Israel, Iran and Japan. In Egypt, faseikh-a traditional salted fish was implicated in botulism outbreak. .

Fermented salmon eggs, raw or parboiled meats from sea mammals , fermented meats such as urraq, or muktuk, fermented fish, fermented fish products(izhushi, kirikomi) were identified as responsible foods in the food-borne outbreaks in Canada. Vacuum-packed ciscoes and whitefish were implicated in food-borne outbreaks.

Pathogenesis and clinical features

Clostridium botulinum neurotoxins are high molecular mass (150k Da) proteins which are among the most toxic substances known (mouse $LD_{50} < 0.1$ ng/kg). BoNTs form complexes with non-toxic proteins in naturally contaminated foods to form what is referred to as progenitor toxin. At pH values greater than 7.2, the non-toxic proteins dissociate from BoNT. Three forms of progenitor neurotoxin have been distinguished and are referred to as M (medium sized), L (large), LL (extra large) toxins. M is produced by all strains producing neurotoxins except type G.

The botulinum toxins act on peripheral nervous system where they inhibit release of acetyl choline from the neuromuscular junctions resulting in general paralysis. Signs and symptoms of botulism develop within 12- 36h, after ingestion of the toxin – containing food. The symptoms include nausea, vomiting, fatigue, constipation, urine retention, paralysis of muscles, double vision, difficulty in swallowing (dysphagia), dry mouth and difficulty in speaking (dysphonia). Respiratory failure and airway obstruction are the main causes of death. The mortality rate is less than 10%.

Isolation and identification

C. botulinum will often constitute only a small proportion of the total microflora so enrichment or pre-incubation is necessary to improve the chances of isolation. After enrichment, the culture is streaked on to fresh egg yolk agar or blood agar and incubated anaerobically for 3 days. Characteristic colonies , 2-3 mm in diameter with an irregular edge and showing lipolytic activity on egg-yolk agar (type G excepted).

Several in vitro assays have been described for the detection of botulinum toxin-electroimmuno diffusion, reversed passive haemagglutination (RPHA), radioimmunoassay and enzyme linked immunosorbent assay (ELISA). DNA based methods (eg. Polymerase Chain Reaction) supplement classical methods. It allows rapid and selective identification of pathogens. They are specific and sensitive. Despite the development of a range of *in vitro* immunoassay procedures for toxin detection, the mouse neutralization test, remains the most sensitive .

Control of *C.botulinum* in fish/fishery products

Control of food-borne botulism is based almost entirely on thermal destruction of the spores or inhibition of spore germination and bacterial cell growth in foods. In most foods, *C.botulinum* is controlled by inhibition rather than destruction. The ultimate safe guard is very low heat stability of botulinum toxin (80°C 10 min) which means that normal household cooking will destroy any preformed toxin. The risk of botulisum is clearly associated with foods that do not require cooking immediately before consumption. Pasteurized, cold-smoked and fermented fish products are high risk foods and safety of these products is based on chill storage and cooking before being eaten.

The minimally processed foods packaged in modified atmospheres is of great concern. The combination of lack of a heat treatment sufficient to destroy *C. botulinum* spores, lack of heating prior to consumption, and packaging in an atmosphere with reduced or no oxygen increases the risk of botulism from these foods. Refrigeration is an important control for perishable vacuum packaged foods.

Control of *C.botulinum* in smoked fish products depends on microbial inactivation by heat plus the inhibitory effects of salt, smoke constituents and surface drying and storage at temperatures below 4°C. Low heat treatments (pasteurization) in combination with other control measures can be used to prevent growth of *C.botulinum*. Typical methods to prevent botulism include reduction of vegetative cell and /or spore contamination in the food by heat treatment to obtain commercial sterility.