

CHAPTER TWO

LITERATURE REVIEW

2.1 Advantages of *Clostridium perfringens* as an indicator

Clostridium perfringens (CP) is a prevailing gastrointestinal inhabitant among humans, farm animals such as cattle, piglets, poultry, fish and also exotic animals (Gurjar *et al.*, 2008; Harmsen *et al.*, 1999; Yoo *et al.*, 1997; Gkiourtzidis *et al.*, 2001; Sipos *et al.*, 2003; Aschfalk and Müller, 2002a). CP forms spores under stressed conditions, yet the spores grow easily into vegetative cells on laboratory media, thus defining the bacterium as viable and culturable. Very often CP densities among animals are comparable to the densities in humans (Lisle *et al.*, 2004). There are growing findings regarding the fact that different CP strains are typically found in certain animals (Augustynowicz *et al.*, 2000; Songer and Uzal, 2005; Gholamiandekhordi *et al.*, 2006). For study areas with known faecal pollution sources and gradient, CP spores can be used together with *E. coli* to discriminate the clustered pollution areas (Byamukama *et al.*, 2005).

It was understood that spore-forming promoting factors in CP are acid stable until the extent of pH 1.8 (Shih and Labbe, 1996). Other tests using hydrogen peroxide, formaldehyde, and nitrous acid also confirmed the chemical resistance of CP spores. Furthermore, CP spores can also withstand moist heat and UV radiation. These are all made possible with the expression of certain small acid soluble proteins from CP that

modulates water content in the spore's core and the peptidoglycan structure of the spore's cortex (Paredes-Sabja *et al.*, 2008b).

Heat resistance in CP is generally well above the experimental sublethal heat shock temperature of 75 °C. Although different CP strain has significantly varying heat resistance, acquired thermotolerance can be induced following sublethal heat shock (Juneja *et al.*, 2003). Meanwhile, sulfite reducing Clostridia as a whole are more heat resistant than *E. coli* and *Bacteroides fragilis* in terms of thermal treatment at 80 °C (Mocé-Llivina *et al.*, 2003).

Consistent with its UV and heat resistances, CP has also superior solar resistance and thus survives better in exposed environments. Comparison among male-specific bacteriophage (MSB), *E. coli* and CP in terms of inactivation by sunlight revealed that CP and MSB coped better compared to *E. coli*, with 84, 83 and 99% of density reduction respectively (Burkhardt III *et al.*, 2000). In another similar research by Pourcher *et al* (2007), the concentrations of CP, *E. coli*, enterococci and enterovirus of sludge origins were monitored for two months after land-spreading. Results showed that CP concentration remained stable while others had decreased 1.2 to 1.8 logarithmic units.

Some other faecal pollution indicators were found to be multiplying under favourable environmental conditions. This disadvantage was not observed in CP. Desmarais *et al* (2002) performed experimental studies that compared CP, enterococci and *E. coli* counts under tide stimulations at 28 °C for 5 days. CP counts were shown to be remained while there were significant re-growth of enterococci and *E. coli*. Meanwhile, Fujioka (2001) implied that *E. coli* and enterococci of soil origins (instead of intestinal

origins) in Hawaiian stream waters had higher densities as a result of multiplication as compared to the low densities of CP (assumed to be of intestinal origins) which did not multiply.

In short, CP is able to form resistant endospores that make it survives in natural and anthropogenic environmental stress such as chemical, heat, UV, and solar radiation. Their survival enables faecal pollution identification in environments which is also affected by chemical pollution whereby other faecal pollution indicators had most probably died off. At the same time, the fact that it does not multiply under favourable condition is important to avoid exaggerating the original extent of faecal pollution. Since CP is universally found in gastrointestinal tract of human and animals and has many mentioned advantageous characteristics, it stands as good indicator for faecal pollution, especially past faecal pollution.

2.2 *Clostridium perfringens* In Terrestrial and Marine Environment

CP is abundant in terrestrial soil. An investigation on Costa Rican soil samples showed that it accounted for 38% of the *Clostridium* species while *Clostridium subterminale*, *Clostridium oceanicum*, *Clostridium bifermentans*, *Clostridium glycolicum*, *Clostridium sporogenes* and *Clostridium sordellii* were detected at the rate of 56%, 51%, 50%, 50%, 49% and 42% respectively (Mar Gamboa *et al.*, 2005). In sediments, CP survives mostly in spore form rather than vegetative form. Survival of the spores was also found to be independent from the presence of protozoan predators (Davies *et al.*, 1995). Compared to marine sediment, terrestrial sediment usually had higher CP concentration consequently from animal and human habitation. For examples, sediment core from high mountain

recreational lake in Spain harboured sulfite reducing Clostridia spores of $< 731 \times 10^3$ cfu/g of dry sediment (Robles *et al.*, 2000). In constructed wetlands and water pollution control ponds, CP spores were detected at the range from < 1 to 40 per 100 ml in the water column while dried sediment reported 10^4 to 10^7 per 100 gram (Davies and Bavor, 2000).

Microbiological contamination in marine environment is more likely to be associated with sediment rather than water (Coelho *et al.*, 1999). From water and sediment samples acquired from beach bathing zones in the Iberian Peninsula, Garrido-Pérez *et al.* (2007) noted that CP concentrations in sediment samples were independent from the overlying or nearby water samples. Even when CP concentrations in the water samples were low (< 5 cfu/100ml), the related sediments were found to have high CP concentration (< 810 MPN /100 g dry sediment). Meanwhile, CP concentrations were higher than faecal coliforms in water (0 to 363 cfu/100ml) and sediment (17 to 6596 MPN/100 g dry sediment) throughout their study. They therefore postulated that CP would be a good long term faecal contamination indicator.

2.3 *Clostridium perfringens* As Faecal Pollution Indicator and Surrogate Indicator In the Environment

Fujioka and Shizumura (1985) suggested CP densities of more than 50 cfu/100ml to indicate human faecal pollution (Lipp *et al.*, 2001; Barbour *et al.*, 2004). Later Fujioka (2001) recommended CP densities of 50 cfu/100ml and 5 cfu/100ml respectively as standards for marine recreational water and fresh water quality in Hawaii, which are also supposedly applicable in warm climate countries.

CP spores are of great value as microbial indicator in deep ocean sewage sludge disposal assessment. The spores were reportedly resistant to the extreme deep ocean environment of 2 °C at 250 atm of pressure and remained culturable upon recovery. Under such extreme condition, no other bacterial indicators are expected to be viable although this assumption still needs validation (Hill *et al.*, 1993). Marine sediment collected from near-shore or off-shore sewage sludge disposal sites usually had high CP densities. In the famous Massachusetts Bay off-shore sewage outfall area, CP abundance was reported in the range of 1000 to 73,000 spores per gram of marine sediments. Environmental consequences of the secondarily treated sewage outfall were evaluated. Findings showed that after adjusted with fine sediment percentages and sediment grain sizes, the CP dispersion pattern followed the sewage outfalls' dispersion pattern. The CP concentration was highest in the sewage outfall, highly variable in intersection areas and less in the far field with a detectable 20 to 25% concentration decreases. The impact was quantifiable, and also agreed with the field modelling. This study also demonstrated the non-replicating characteristic in CP (Dahlen *et al.*, 2006). Another example would be New Jersey's off-shore deep ocean dumping site. The top 1 cm sediment core from its marine sewage sludge recorded CP counts of more than 9×10^3 cfu /g of dry sediment (Hill *et al.*, 1993). Together these findings reinforced the postulation of Emerson and Cabelli (1982) that CP spores collected from marine bottom sediment cores would be very sensitive in reflecting the consequences of sewage and faecal disposition into the marine environment.

CP spores have been proposed as surrogate indicator for *Cryptosporidium* (pathogenic protozoa) oocysts. The two parameters could be sharing similar hydro dynamics, especially their ability in attaching to particulates. In estuary area where turbidity is high with particulates, their presences were found to be correlated (Touron *et al.*, 2007).

However, there were also findings that showed the lack of correlation between *Cryptosporidium* oocysts and CP spores. Variations like environmental abiotic and biotic interactions, water volume that were being investigated and investigation methods as such could all resulted in different detection efficiencies among the oocysts and CP spores. In groundwater samples, absence of CP is usually accompanied by the absence of pathogenic protozoa but in wastewater, fresh water and treated water, such parallel association might not always exist (Briancesco and Bonadonna, 2005).

CP is a good faecal contamination indicator in non point source contamination such as flooding. Agricultural soil in North Carolina was examined for faecal contamination indicators after 1999's Hurricane Floyd. Faecal coliforms, *E. coli*, and coliphage were recovered from the effected soils, but somehow in a lower density than expected. In comparison, pre and post flood CP spores in the area were reported to be significantly different at 4.7 and 6.6 log₁₀ MPN/100 g of soil respectively (Casteel *et al.*, 2006). This has also highlighted again the importance of microbiological sampling during extreme rainfall and runoff on top of routine monitoring in order to achieve meaningful environmental monitoring purposes, as discussed by Kistemann *et al* (2002).

2.4 *Clostridium perfringens* As Surrogate and Real Indicator In Drinking Water and Waste Water Treatment Plants

In a study on conventionally treated (flocculation with alum) drinking water of more than 1000 litre, Clostridia and coliphages were detected in very low concentration in some of the treated drinking water while human enteric viruses were absent (Payment, 1991). Payment and Franco (1993) then further investigated the efficacy of water treatment processes, and

found that CP was statistically correlated to human enteric viruses, parasitic cysts and oocysts throughout the treatment process. Its correlation values to the pathogens were better than somatic and male specific bacteriophages (*E. coli* CN13 and Salmonella WG45 respectively). With the results they proposed CP to be surrogate for the presence of virus in water treatment plants.

On the other hand, Shannon *et al.* (2007) interestingly pointed out that CP was undetected in aeration tank of water treatment plant, but was present at all other treatment stages. Therefore they suggested that CP could be forming spores that were able to withstand aerobic condition and then thrived again under anaerobic surrounding. In contrast to Payment and Franco's (1993) findings of throughout-the-process-detection, the absence of CP in the aeration tank could probably due to Shannon *et al.*'s quantitative real-time PCR detection that could only detect vegetative CP and not CP spore. In their method, DNA was extracted from biomass of different treatment stages.

Studies thus far have been showing that conventional water treatment processes can not eliminate CP totally in the final product. For example, Payment *et al.* in 2001 studied a large physico-chemical primary treatment plant that processed about 7.6 million cubic metres of wastewater per day. The subsequent removal rate for CP was 51%, 20% for faecal streptococci, 12% for *E. coli* and 76% for *Cryptosporidium* oocysts. This could mean that faecal streptococci and *E. coli* were susceptible to the treatment processes yet were somehow able to multiply during the processes. In comparison, CP was less susceptible to the treatment processes, but did not multiply.

In another evaluation on disinfection efficiencies in conventional water treatment plant characterized by end point chlorination, only a two magnitude order of density reduction for CP was found while reduction up to ten magnitude order were achieved for *E. coli* and coliform (Briancesco and Bonadonna, 2005). This showed the resistance of CP against chlorination in water treatment.

In addition to chlorine resistance, there were also reports on CP's resistance to UV and ozone treatment after enhanced primary treatment of municipal wastewater. CP was more resistant to UV (over 10mJ/cm²) and ozone (over 40 mg/L) per log of inactivation compared to fecal coliforms (Gehr *et al.*, 2003).

The suitability of CP to indicate virus and parasitic cysts occurrence is more uncertain in wastewater treatment compared to drinking water treatment. CP densities might not correlate well with occurrences of enterovirus in treated wastewater, but their removal rates did correlate with enterovirus removal rate, which was slightly higher than those of *E. coli* and enterococci (Ottoson *et al.*, 2006). Meanwhile in a sewage sludge treatment (sludge hygienization processes) study that evaluated correlations between CP, *E. coli* and enterococci counts to *Giardia* cysts and *Cryptosporidium* oocysts occurrence, no statistical correlations were even found (Rimhanen-Finne *et al.*, 2004).

2.5 *Clostridium perfringens* In Healthy Individuals

As a normal flora of intestinal tract, CP can be found in stool of healthy individuals. CP spores densities from a Finnish population ranged from 2.3×10^2 to 2.1×10^6 per gram of stool (Heikinheimo *et al.*, 2004). Meanwhile, an average count of 7.4×10^3 spores per gram was reported by Vela *et al* (1999) from a healthy Mexican population, with the elderly reporting the highest concentration. *Clostridium* species were detectable even in faeces of infants about 30 days old, with the prevalence of 18.2% and 1×10^5 to 3×10^7 cells per gram of wet faeces. Interestingly *Clostridium* sp. densities among bottle-fed infants were significantly higher than breast-fed infants (Tonooka *et al.*, 2005).

2.6 *Clostridium perfringens* In Food Poisoning and Antibiotic Associated Diarrhea

In the United States, CP was reported as the second or third most common cause of food poisoning (Lin and Labbe, 2003). In England and Wales, CP, *E. coli* 0157, and *Campylobacter* came in after *Salmonella* species which caused over half of foodborne outbreaks. Red meat and poultry were the most commonly reported vehicle for CP food poisoning (Hughes *et al.*, 2007). Hauschild *et al.* (1975) had recommended CP spores dosage of $>10^5$ vegetative cells per gram of food or $> 10^6$ spores per gram of stool to implicate food poisoning (Maslanka *et al.*, 1999).

Apart from food poisoning, CP is also the etiologic agent for antibiotic-associated diarrhea (AAD), besides *Clostridium difficile* and *Staphylococcus aureus* (Ackermann *et al.*, 2005; Abrahao *et al.*, 2001). AAD is defined as having more than three liquid stools within 48 hours, taking antibiotics recently and warded for more than 5 days (Pituch *et al.*,

2007). Type A CP is the most commonly reported toxigenic type associated to food poisoning, AAD and also some veterinary diarrhea (Wen *et al.*, 2003; Brynestad and Granum, 2002).

2.7 *Clostridium perfringens* Pathology

Fatal necrotic enteritis is the most frequent pathology caused by CP. Autopsy often shows dilated small intestines along with lots of gas. The necrotized intestinal walls would be thickened with multifocal haemorrhages. Inflammatory cell infiltration happens too, whereby pseudo-membrane formation and adhesive peritonitis can be observed (Asaoka *et al.*, 2004).

There is no clear agreement about which CP toxin causes which typical type of pathology. Usually the observed pathology was the result of arrays of CP toxins. For fatal necrotic enteritis, different dominant CP type has been isolated among different hosts. Type A CP was reportedly dominant in wild crows (Asaoka *et al.*, 2004) while Type A and Type C CP were attributable for the same pathogenesis among swine (Songer and Uzal, 2005; Sipos *et al.*, 2003).

2.8 *Clostridium perfringens* Toxins

CP is categorized into Type A to Type E based on alpha, beta, epsilon and iota toxin genes. Alpha toxin gene present in all five CP toxinotypes. Beta toxin gene present in Type B and Type C; epsilon toxin gene in Type B and D; and iota toxin gene in Type E. CP can also express CPE (enterotoxin), θ toxin (perfringolysin O), μ toxin (hyaluronidase), κ toxin (collagenase), λ toxin (protease) and some other toxins (Rood and Cole, 1991).

2.8.1 Alpha Toxin

Alpha toxin is expressed by most, if not all of CP strains. It is a type of phospholipase C enzyme that contains zinc. It is lethally potent at less than 0.1 µg in mouse. This toxin hydrolyses the phospholipids in cell membrane through phosphatidylcholine and sphingomyelin activities and thus causes gas gangrene in human (Titball, 1999). The conventional diagnostic methods for this toxin include lechitinase and haemolytic test using egg yolk agar (Schlapp *et al.*, 1995). Alpha toxin is vigorously generated during the logarithmic growth phase (Duncan *et al.*, 1972)

2.8.2 Beta Toxin

Beta toxin is the main cause for necrotic enteritis in human (pigbel) and also animals. Beta toxin is labile and very susceptible to protease degradation. Therefore, this toxin is usually found only in circumstances whereby protease activity is low, such as in the digestive tract of newborns (Popoff, 2004). Diets rich in trypsin-inhibitory compounds such as sweet potatoes block the host's breakdown mechanisms on beta toxin and thus cause the pathogenicity (Hunter *et al.*, 1993). Beta toxin has pore forming ability that initially induces oedema in mucosal cells which then progresses to necrosis (Nagahama *et al.*, 2003).

2.8.3 Epsilon Toxin

Epsilon toxin is expressed as a prototoxin and requires activation from proteases such as lambda toxin (also expressed by CP itself), trypsin and chymotrypsin. LD50 of epsilon prototoxin treated with trypsin and chymotrypsin was 65 ng/kg of mouse body weight (Minami *et al.*, 1997). Epsilon toxin is also often fatal, causing enterotoxemia followed by “pulpy kidney” whereby kidney becomes swollen and hyperemic. In between, excessive fluid accumulates in pericardial routes, lungs and intestines with increased membrane permeability (Rood and Cole, 1991; Uzal and Kelly, 1998). Type D CP isolates are more often being reported among goats (Sipos *et al.*, 2003).

2.8.4 Iota Toxin

Iota toxin is a binary toxin consisted of an enzymatic component (Ia) and a binding component (Ib). This toxin can be expressed in active or precursor form, depending on the activation of protease responsive pathways. Proteases like α -chymotrypsin, pepsin, proteinase K, subtilisin, thermolysin and a lesser extent of trypsin are able to activate the toxin by cleaving off small peptides and a 20-kDa peptide from the inactive Ia and Ib precursors (Gilbert *et al.*, 2000). Cytotoxicity of iota toxin increases with extracellular Ca^{2+} availability which will then mediate the toxin’s entry into target cells (Kobayashi *et al.*, 2008).

2.8.5 CPE Toxin

CPE, or *Clostridium perfringens* enterotoxin, is expressed specifically during CP sporulation (Duncan *et al.*, 1972). CPE gene produces a 319-amino acid polypeptide with molecular weight of 35,317. CPE-positive CP strains express high level of the toxin during sporulation although the toxin expression itself detected in cell lysate toxin assays might not be prominent (Czeczulin *et al.*, 1993). Duncan-Strong medium was formulated to encourage sporulation among CP isolates (Duncan and Strong, 1968). A modified medium was then proposed with 0.4% of raffinose replacing soluble starch in the original composition (Labbe and Rey, 1979) which further enhances sporulation.

As one of the etiological agent of food poisoning, CPE is usually expressed in the gastrointestinal tract and causes epithelial cells destruction by disrupting the membrane permeability (McClane, 1996). CPE gene may have different prevalence in different CP type. The prevalence of CPE gene was reported to be 5% among Type A CP (Wen *et al.*, 2003) and 7% among untyped isolates recovered from faeces of healthy human subjects (Vela *et al.*, 1999). In a study on Nigerian children population below 5 years old suffered from sporadic diarrhea (not associated to antibiotic or food poisoning), 41 out of 47 type A CP isolates were CPE-positive (Efuntoye and Adetosoye, 2004). In comparison, CPE toxin gene was found to be present, although as silent gene, in most or all type E CP isolates from veterinary enteritis cases (Billington *et al.*, 1998). In healthy human stool, CPE-positive and CPE-negative CP strains co-exist. Findings showed that when the ratio of CPE-positive to total CP spores exceeded 6×10^{-5} , CPE-positive strains could be detected with the hydrophobic grid membrane filter-colony hybridization method (Heikinheimo *et al.*, 2004).