

Research Note

Determination of toxinotypes of environmental *Clostridium perfringens* by Polymerase Chain Reaction

Florence, L.C.H.^{1*}, Hakim, S.L.², Kamaluddin, M.A.¹ and Thong, K.L.³

¹ Environmental Health Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

² Infectious Disease Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

³ Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

* Corresponding author email: florencelee@imr.gov.my

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Abstract. Toxinotype of *Clostridium perfringens* (CP) isolates collected from the Bernam River, Selangor River and Tenggi Canal between April 2007 and January 2008 were determined by Polymerase Chain Reaction (PCR) using published primers. All the 147 isolates were toxinotype Type A, harbouring the alpha toxin gene. In addition, 5 of the isolates also had the enterotoxin (CPE) gene.

Clostridium perfringens (CP), a gram positive, spore-forming anaerobe is a common gastrointestinal inhabitant that survives well in various environments. Its universal presence is attributed by the ability to withstand stressed conditions like low pH, solar radiation, and high atmospheric pressure. Studies also showed that CP does not replicate in natural settings such as unpolluted streams. These characteristics have promoted CP as an alternative faecal pollution indicator in recent years. It is particularly useful in assessing past pollution whereby other traditional faecal pollution indicators like *Escherichia coli* had died off.

Besides serving as an emerging faecal pollution indicator, CP itself is a pathogen that produces an array of toxins which can cause acute life threatening clinical and veterinary diseases. Among the toxins, the alpha, beta, epsilon and iota toxin are used to categorize CP into five different toxinotype, type A to type E (Hatheway,

1990). Alpha, beta and epsilon toxins are the main causes of gas gangrene, necrotic enteritis and enterotoxemia respectively (Titball *et al.*, 1999; Uzal & Kelly, 1998; Popoff, 2004). Another well characterized toxin of CP, the enterotoxin (CPE), causes food poisoning and antibiotic-associated diarrhea (McClane, 1996), and is usually found among Type A CP (Brynstad & Granum, 2002; Nakamura *et al.*, 2004).

In Malaysia, there is a lack of data on CP toxinotype from both clinical and environmental isolates. Hence, this study was carried out to determine CP toxinotype from river water samples, so as to provide some background information of the prevalence of CP toxinotypes in Malaysia. There is also a paucity of reports about environmental CP toxinotype from other countries (Johansson *et al.*, 2006). Polymerase Chain Reaction (PCR) method was used to investigate CP toxinotype in this study instead of the tedious conventional latex agglutination or toxicity

assay that depends on expression of the toxins. Furthermore, CP isolates do not always express the toxins although the toxin genes are present (Petit *et al.*, 1999).

Toxinotype of a total of 147 environmental CP isolates were determined using monoplex PCR. They were isolated from 69 river water samples taken from three selected Malaysian rivers (i.e., the Bernam River, Selangor River and Tenggi River Canal) in the state of Selangor between April 2007 and January 2008. The water samples were serially diluted, followed by membrane filtration with cellulose nitrate membranes (0.45 µm) and anaerobic incubation on Tryptose Sulphite Cycloserine (TSC) selective agar at 37°C for two days. Presumptive CP isolates on TSC agar were confirmed as CP isolates with lactose gelatin and nitrate motility biochemical tests (Health Canada, 2001). DNA of confirmed CP isolates was extracted using a commercial DNA extraction kit (Bio-Basic). The DNA concentration was measured using low mass DNA ladder (Invitrogen) and 1 µl of DNA extract was equivalent to 1 ng of DNA materials. Alpha, beta, epsilon and iota toxin genes were detected using primers published by Yoo *et al.* (1997), with expected PCR product sizes of 402 bp, 236 bp, 541 bp and 317 bp, respectively. Primers for the enterotoxin (CPE) gene detection were adapted from Meer & Songer (1997), and would yield a PCR product of 705 bp.

PCR amplification of alpha toxin gene was performed with initial DNA denaturation at 94°C for 5 min, followed by 24 cycles of 20 s at 94°C, 60°C and 72°C each, and a final extension for 5 min at 72°C. ATCC 13124, which is a Type A CP strain, and water was used as positive and negative control respectively. Amplifications for beta, epsilon, iota and CPE gene were performed with initial DNA denaturation at 94°C for 5 min, followed by 44 cycles of 30 s at 94°C, 60°C and 72°C each, and final extension for 7 min at 72°C. PCR products were resolved on 2% agarose gels (Pronadisa) at 120 V with 1x TBE buffer. Representative amplicons were

excised, purified and sequenced for both forward and reverse strands.

Our results showed that all 147 CP isolates belonged to Type A, harbouring the alpha toxin gene. Beta, epsilon and iota toxin genes were not detected. Five of the isolates also had the CPE toxin gene (3.4%). Nucleotide sequence analysis of the representative amplicons of alpha and CPE toxin gene (sequence length ranged from 372 to 600 bp) showed above 98% matching to the respective genes in GenBank. The alpha toxin was compared to gene sequence no. AY277724.1 and X17300.1; and CPE toxin gene to M31795.1 and Y16009.1. Although this study has no positive control strains for CP toxinotypes other than Type A, the non-detection of beta, epsilon and iota toxin genes were assumed as true results since the same set of primers used were reportedly working well in several studies (Engström *et al.*, 2003; Chalmers *et al.*, 2008). CPE gene had been detected at 7% among faeces of healthy population (Vela *et al.*, 1999), and 4.8% among faeces of diarrheal patients (Tansuphasiri *et al.*, 2002). Therefore, our CPE toxin gene detection rate of 3.4% is parallel with the related findings.

Alpha toxin gene is chromosomal while beta, epsilon and iota toxin genes are plasmid-borne (Petit *et al.*, 1999). The absence of beta, epsilon and iota toxin genes in this study is probably because the plasmidic genes were lost through the harsh environment. CP toxinotype from water sample has yet to be reported. If Type A CP is truly the prevalent toxinotype in the environment, then beta, epsilon and iota toxin genes may have developed later in specific host that manifest the diseases (Gkiourtzidis *et al.*, 2001), which are probably involved in mechanisms like insertion of mobile genetic elements (Li *et al.*, 2007; Miyamoto *et al.*, 2008). Nevertheless, Type A CP was found to be the prevalent toxinotype (>95%) in apparently healthy humans (Heikinheimo *et al.*, 2006) and also food sources, such as fish (Aschfalk & Müller, 2002), sheep (Kalender *et al.*, 2005), and poultry (Engström *et al.*, 2003). It has also been

reported that CP would accumulate in livestock and fish (Hang'ombe *et al.*, 2000; Barbour *et al.*, 2004). CP replicates rapidly if temperature abuse occur during cooking or storage (Lin & Labbe, 2003). Therefore managing the distribution of CP in the environment is important especially when the water resource or land is being used for food production and human consumption.

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