RESEARCH NOTE

CLOSTRIDIUM PERFRINGENS AND SULPHITE REDUCING CLOSTRIDIA DENSITIES IN SELECTED TROPICAL MALAYSIAN RIVERS

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Abstract. *Clostridium perfringens* (CP) and sulphite reducing clostridia (SRC) densities in the Selangor River, Bernam River and Tengi River Canal were examined between April 2007 and January 2008. Water samples were taken from two or three locations along each river, using either depth-integration or grab sampling methods. The downstream sampling site of the Selangor River, Rantau Panjang, reported the highest arithmetic mean of CP and SRC densities (583.45 and 8,120.08 cfu/100 ml, respectively). Both CP and SRC densities in the Selangor River increased further downstream, but the reverse was true in the Bernam River. The SRC densities in these rivers were significantly different from each other ($p<0.05$) when comparing upstream and downstream results, but CP densities were not significantly different ($p>0.05$). SRC densities were significantly correlated ($p<0.05$) in different locations along the Selangor River and the Bernam River. The CP densities did not show such pattern ($p>0.05$). River discharge had no significant correlation with SRC or CP densities by study site ($p>0.05$). Since the Selangor River has a denser human population along its banks, this study confirms CP as a suitable indicator of human fecal contamination. However, tracing CP distribution along the river is more difficult than SRC. To our knowledge, this is the first study of CP and SRC densities from Malaysian rivers. CP densities found in this study were within the range of general water bodies reported from other countries.

Keywords: *Clostridium perfringens*, sulphite reducing clostridia, river, water, tropical, depth-integration method

INTRODUCTION

Clostridium perfringens (CP), is a universal gastrointestinal anaerobe that belongs to the clostridia family. CP and the majority of the clostridia members are also called sulphite reducing clostridia (SRC) since they produce sulphide as a metabolite. SRC are widely distributed in the environment because they form spores that can withstand harsh environmental conditions, and are used to indicate fecal contamination (Hill *et al*, 1993; Robles *et al*,

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2000). CP does not replicate in the natural environment, and does not overestimate the severity of fecal pollution (Fujioka, 2001; Desmarais *et al*, 2002). The hardiness of CP spores makes it useful to indicate recent fecal contamination compounded by chemical pollutants, where other fecal pollution indicators have been destroyed (Horan, 2003).

According to Council Directive 98/83/EC of the European Union (1998), water intended for human consumption (either in its original state or after treatment) which is acquired from or impacted by surface water has to be free of CP. Although CP and SRC are not compulsory microbiological indicators of water quality in the United States, the USEPA (2007) has emphasized the need to validate the robustness of CP as an indicator in various environmental samples and its correlation with health effects. This study was conducted in view of the emerging use of CP as an indicator of fecal contamination in water quality regulations. CP and SRC related water quality data in Malaysia is lacking; therefore, in this study we evaluated the presence of CP and SRC in Malaysia.

MATERIALS AND METHODS

Study sites

Three rivers within the state of Selangor were selected for this study, the Bernam River, Selangor River and Tengi River Canal. The Selangor River has a denser human population further downstream than the Bernam River; the Tengi River Canal was created for agricultural irrigation. Three study sites were selected along the Selangor River and two along the Bernam River: upstream Selangor River at Ampang Pecah (03° 32′ 25″ N, 101° 39′ 52″ E), midstream Selangor River at Kampung Timah (03° 29′ 08″ N, 101° 32′ 21″ E), and downstream Selangor River at Rantau Panjang (03° 24′ 10″ N, 101° 26′ 35″ E); and upstream Bernam River at Tanjung Malim (03° 40′ 45″ N, 101° 31′ 20″ E) and downstream Bernam River at Jambatan SKC (03° 48′ 15″ N, 101° 21′ 50″ E). The study sites on Tengi River Canal were: Ibu Bekalan Sungai Bernam (03° 41′ 33″ N, 101° 20′ 32″ E) and Jambatan Mergastua (03° 40′ 03″ N, 101° 20′ 49″ E); they are 2.8 km apart and the surrounding areas have the same type of land use. The samples from the two sites were considered as site replicates on the Tengi River Canal. Land use around the study sites were noted, and the human population density were estimated based on the types of housing.

Water sampling

Water sampling was carried out from April 2007 to January 2008, assisted by Selangor Department of Irrigation and Drainage (Selangor DID) personnel. In Ampang Pecah, Rantau Panjang, Tanjung Malim and Jambatan SKC, depth-integration water sampling (Moody and Troutman, 1992) was performed at the first, middle and third quarter points across the river. This yielded a total of three replicates per site that provided a better picture of microbial variation in the river. A simple surface grab sampling method was applied at the remaining three sites due to limited facilities. River discharge measurements (m^3/s) using a current meter method were conducted concurrently by the Selangor DID during water sampling events. The water temperature of the study sites during sampling ranged from 20 to 25°C; conductivity ranged from 17 to 60 S/cm and pH varied between 6.8 and 7.5.

Water sample analysis

Water samples were analyzed within

three days of acquisition. Ten milliliters was taken from each sample and was subjected to 1/10 serial dilution followed by membrane filtration (USEPA, 2006). Membrane filtration was performed twice for each water sample. The filtered membranes were plated on freshly prepared tryptose sulphite cycloserine (TSC) medium plates (Oxoid, Hampshire, UK), which were then incubated anaerobically at 37°C for two days.

SRC and CP density calculation

The number of colony forming unit of SRC (black or grey colonies) were counted. Well-developed SRC colonies were selected for subculturing on TSC medium. The enumeration and subculturing steps were completed within three hours after removal of the plates from the anaerobic jars. After two days of incubation, the isolates were subjected to lactose, gelatin, nitrate and motility tests (Amyl medium) to confirm their identity as CP (USEPA, 2006). The number of confirmed CP divided by the number of SRC colonies selected for the biochemical confirmation test was defined as the CP isolation rate. SRC and CP densities were calculated in cfu/100 ml (Health Canada, 2001).

Data analysis

Statistical analysis was conducted using SPSS 11.5 software (SPSS, Chicago, IL). The normality of densities was checked with the Shapiro-Wilk test. Differential tests of densities between water sample duplicates, among site replicates, and between rivers were performed with the Wilcoxon signed-rank test and Friedman test. The Spearman Rho's test was used to evaluate correlation of densities along the rivers. Correlations between river discharge and SRC densities, between river discharge and CP densities, and between SRC and CP densities at the respective sites were also examined with Spearman Rho's test.

RESULTS

The Shapiro-Wilk normality test showed, except for the two upstream sampling sites at Ampang Pecah and Tanjung Malim, SRC densities were normally distributed at the other four study sites ($p>0.05$). The CP at the study sites along the Bernam River and the Tengi River Canal were normally distributed ($p>0.05$), but not along the Selangor River. There were no significant differences between SRC densities of water sample duplicates ($p>0.05$). The SRC densities of the site replicates for each study site were not significantly different ($p>0.05$) except at Tanjung Malim, which had sewage outlets on both sides of the river bank ($p<0.05$). The same statistical finding was also seen for CP densities at all study sites, including Tanjung Malim ($p>0.05$). These differential results suggest the SRC and CP densities in individual water samples, and also along the cross sectional line of the river was probably homogenous unless there is point-source pollution in the immediate vicinity of the sampling site.

Arithmetic means of CP and SRC densities are shown in Table 1. The SRC densities among the rivers were significantly different ($p<0.05$) when comparing upstream or downstream samples, but the CP densities were not significantly different ($p>0.05$) (data not shown). Both SRC and CP densities increased further downstream in the Selangor River, but decreased further downstream in the Bernam River. The CP density fluctuations in Selangor River varied more widely than the other rivers.

The SRC density correlations along the

Table 1
Arithmetic means of *Clostridium perfringens* (CP) and sulphite reducing clostridia (SRC) densities in the Selangor River, Bernam River and Tengi River Canal from April 2007 to January 2008.

Site (sampling frequency, *n*)	CP densities in cfu/100 ml (± SD) (min, max)	SRC densities in cfu/100 ml (± SD) (min, max)	CP isolation rate (%)
Selangor River			
Ampang Pecah (upstream, *n* = 12)	3.82 (± 7.41) (<1, 23)	902.92 (± 1,028.49) (183, 3,465)	0 - 6.7
Kampung Timah (midstream, *n* = 13)	109.45 (± 254.85) (<1, 770)	6,265.77 (± 3,206.67) (2,365, 12,540)	0 - 14.3
Rantau Panjang (downstream, *n* = 11)	583.45 (± 927.75) (<1, 2,694)	8,120.08 (± 3,896.88) (2,750, 17,435)	0 - 25.0
Bernam River			
Tanjung Malim (upstream, *n* = 12)	279.73 (± 233.80) (20, 763)	3,240.33 (± 3,072.49) (642, 10,303)	1.8 - 35.0
Jambatan SKC (downstream *n* = 10)	159.00 (± 137.50) (<1, 440)	2,758.40 (± 1,236.29 (1,128, 4,803)	0 - 20.5
Tengi River Canal (*n* = 12)	104.82 (± 64.34) (<1, 212)	4,203.00 (± 2,713.56) (1,027, 8,983)	0 - 10.3

Selangor River were significant between the upstream and midstream (r = 0.743, p<0.01), and between midstream and downstream (r = 0.746, p<0.01). The same was also true between upstream and downstream samples from the Bernam River (r = 0.745, p<0.05). A similar assessment of CP densities, showed no significant correlation along the two rivers. CP densities in the Selangor River were negatively correlated (r = -0.461, p>0.05; r = -0.157, p>0.05) and in the Bernam River were positively correlated (r = 0.510, p>0.05).

A significant correlation between river discharge and SRC densities was found only in Kampung Timah (r = 0.681, p<0.05), but not at the other study sites. No significant correlations were reported between river discharge and CP density at any of the study sites. Factors other than river discharge (*eg*, adjacent land use) would probably be more important in determining the densities of SRC and CP. This assessment was limited by the relatively short study period (April 2007 to January 2008) and the lack of data from storm event samplings, which may be important (Kistemann *et al*, 2002).

This study reaffirmed the use of CP as an indicator of human fecal contamination since the highest CP density was seen at Rantau Panjang, the downstream study site in the Selangor River. Interestingly, the SRC and CP densities in Rantau Panjang had a significant positive correlation by the Spearman Rho's test, with a correlation coefficient of 0.620 (p<0.05); such correlations were not found at the other study sites (data not shown).

DISCUSSION

The Tengi River Canal is situated near wetland. SRC and CP densities at this site were both moderate, compared to the Bernam and Selangor Rivers. These findings were consistent with other reports that *Clostridium* species are prevalent in wetlands, since the habitat is rather anaerobic and carcasses usually support the growth of clostridia (Sandler *et al*, 1998). The immediate vicinity of Kampung Timah is surrounded by cattle and fish farming. The high SRC and low CP densities at this site may imply *Clostridium* species other than CP are closely associated with these two types of agricultural activities. Tanjung Malim was considered a pristine upstream study site while Tanjung Malim is situated in a town. The comparatively low SRC density at the two sites suggests the pristine water catchment area and human population are not the main contributors of SRC.

The arithmetic mean of CP density was highest in Rantau Panjang and comparably lower in Tanjung Malim (Table 1). However, the geometric mean of CP density at Tanjung Malim was higher than Rantau Panjang (176.08 cfu/100 ml and 33.52 cfu/100 ml, respectively) (data not shown). At Rantau Panjang there were five sampling with non-detection and three with high CP densities (>1,000 cfu/100 ml) out of 11 sampling events. The extreme CP densities was less markedly reflected by the geometric mean than the arithmetic mean. CP was consistently found, but at a lower density, in Tanjung Malim. Different interpretations can be made depending on whether geometric or arithmetic bacterial means are considered. Therefore, knowing both types of means would probably better facilitate suitable control measures.

A few studies have assessed the densities of CP in river water. Shehane *et al* (2005) reported CP geometrical means of less than 1.9 cfu/100 ml in the Florida River. Bezirtzoglou *et al* (1996) detected CP in 100-fold diluted river water samples at a frequency of 0 to 5.5% in northwestern Greece. Byamukama *et al* (2005) found the median density of CP spores was <2.3 log cfu/100 ml in water samples from streams, springs and lakes of Uganda. Therefore crude comparison without considering differences in climate or adjacent land use suggests CP densities in the rivers of this study were within the range of general surface water bodies.

Species identification of SRC isolates was not performed in this study. It has been reported *C. bifermentans*, *C. botulinum*, *C. baratii*, *C. absonum*, *C. sporogenes* (Labbe, 2001), *C. chauvoei*, *C. paraputrificum*, and *C. tertium* (Sartory *et al*, 1998) can grow on TSC media. Following aerobic exposure during SRC enumeration and subculture, only 85% to 90% of the subcultures grew (average by respective study sites, data not shown). Isolates that failed to grow were assumed to be strict clostridial anaerobes. CP is unlikely to be affected by aerobic exposure since it is reportedly able to withstand atmospheric oxygen up to 72 hours (Rolfe *et al*, 1978).

Since CP and SRC are spore-forming bacteria, the natural environment is expected to have an intrinsic level of clostridial densities through accumulation over time. Fujioka (2001) recommended CP densities exceeding 300 cfu/100 ml were indicative of fecal contamination of water. It is important to monitor the level of fecal contamination in source water, especially when it involves agricultural food production and domestic uses. This will help to minimize microbial contamination risk in raw food products,

thus lowering the risk of microbial food poisoning, besides reducing the burden of water treatment for domestic uses. Since SRC densities at different sampling locations along the rivers had significant correlations in this study, it is possible to model the distribution of SRC in general river water and then estimate microbial load. However, CP modelling could be more challenging if the correlations are fundamentally weak, as in this study.

In conclusion, this study provides preliminary data regarding CP and SRC densities in the Selangor River, Bernam River and Tengi River Canal. With only 10 months data, not including storm event samplings, river discharge had no significant correlation with SRC densities or CP densities. The Selangor River, which supports a higher human population, had increasing SRC and CP densities further downstream. This reinforces the use of CP as an indicator of fecal contamination. Significant correlations between SRC densities at different study sites along the Selangor and Bernam Rivers, imply SRC modeling in a river system is possible. Land use characteristics surrounding the study sites suggests identification of SRC species in addition to CP may elucidate the impact of different land use type.

ACKNOWLEDGEMENTS

We thank the Director General of Health, Malaysia and the Director General of the Department of Irrigation and Drainage for permission to publish this paper. This research was funded by the Ministry of Health Malaysia (Grant no. JPP-IMR 07-030, NMRR ID 08-212-1367) and the University of Malaya (Grant no. P0237/2007A and FR177/2007A). We sincerely thank the DID Selangor and Afftech Automation System Sdn Bhd for acquiring water samples, and the Information Unit of DID Malaysia for providing the supporting data. We also thank personnel from the various research centers in the Institute for Medical Research, and Biomedical Science & Molecular Typing Lab (A407) of the Institute of Postgraduate Studies, Malaya University for their help and facilities.

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