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ORIGINAL PAPER

Long-term and short-term variations of *Escherichia coli* population structure in tropical coastal waters

K. W. Tan · C. W. Lee

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Abstract In this study, monthly and daily samplings were carried out at Klang, an eutrophic estuary, and at Port Dickson, an oligotrophic coastal water system. *Escherichia coli* concentration was measured via culture method, and the phylogenetic structure of *E. coli* population was via Clermont typing. Average *E. coli* concentration at Klang was higher than Port Dickson ($t = 2.97$, $df = 10$, $p < 0.05$), and daily sampling did not show any apparent temporal variation at both sites. At Klang, salinity was inversely correlated with coliform ($R^2 = 0.216$, $df = 25$, $p < 0.05$), suggesting that river flow was a mode of transport for coliform. Although *E. coli* concentration was higher at the eutrophic site, *E. coli* population structure at both Klang and Port Dickson were similar and showed neither long-term nor short-term variations. This study showed the predominance of commensal groups A and B1 in tropical coastal waters of Peninsular Malaysia.

Keywords Faecal pollution · Clermont typing · *E. coli* loading rate · Straits of Malacca

Introduction

Escherichia coli (*E. coli*) is a commensal bacterium in the large intestine of human and other warm blooded animals. The presence of *E. coli* is widely used as faecal pollution indicator in water quality assessment (Cho et al. 2010). Faecal pollution has been reported for different water bodies, e.g. fresh water (river or lake) (Ferguson et al. 2003) and salt water (seawater, coastal water and estuarine water) (Gabutti et al. 2004; Orsi et al. 2008) and is a serious problem since it represents a health risk to both animals and humans (Wilkinson et al. 2006; Orsi et al. 2008). Faecal pollution can be introduced from multiple sources, e.g. agricultural runoff, industrial waste, inadequately treated sewage from urban areas and other anthropogenic input (Geldreich 1996; Ferguson et al. 2003).

At present, the population structure of *E. coli* is characterised into four main phylogenetic groups (A, B1, B2 and D) (Herzer et al. 1990; Gordon et al. 2008). Both groups A and B1 are commensals where group A is predominant in humans, and group B1 is predominant in animals (Tenaillon et al. 2010), whereas groups B2 and D are usually pathogenic and can cause extraintestinal infections (Picard et al. 1999; Clermont et al. 2000). The distribution of *E. coli* phylogenetic groups has been attributed to environmental factors such as dietary, climatic conditions and geographic (Duriez et al. 2001; Unno et al. 2009). As the population structure of *E. coli* in humans in tropical countries are mainly groups A and B1 (Escobar-Páramo et al. 2004), it is essential to investigate whether the *E. coli* population structure in tropical coastal waters reflected that found in humans. Moreover, *E. coli* groups A and B1 are now emerging as intestinal pathogenic strains (Escobar-Páramo et al. 2004; Li et al. 2010). At present much remains unknown of the *E. coli* population structure in tropical coastal waters, and only Orsi et al. (2008) have reported *E. coli* population structure albeit in a tropical river.

K. W. Tan · C. W. Lee (✉)
Laboratory of Microbial Ecology, Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia
e-mail: lee@um.edu.my

C. W. Lee
Institute of Ocean and Earth Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia

Therefore, the objective of this study is to determine the *E. coli* population structure in tropical coastal waters. As coastal waters are dynamic ecosystems, both long-term (monthly) and short-term (daily) samplings were carried out to detect for any temporal variation as hydrological processes can change *E. coli* population structure (Ratajczak et al. 2010). A simple and rapid triplex PCR method was used to detect the different phylogenetic groups of *E. coli* (Clermont et al. 2000). This method is suitable for large-scale strain screening that can quickly detect virulent strains, and its utility is well documented (Higgins et al. 2007; Gordon et al. 2008; Li et al. 2010). In this study, *E. coli* concentration reflected the trophic status of the seawater, but the population structure remains essentially similar. Although a relatively higher percentage of *E. coli* group A was observed, further work is required to determine whether this is a specific attribute for tropical waters. This work was conducted from September 2007 until July 2009 at the Laboratory of Microbial Ecology, Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia.

Materials and methods

Sampling

Sampling was carried out monthly at Klang (03°00.1′N, 101°23.4′E) ($n = 11$) and at Port Dickson (02°29.5′N, 101°50.3′E) ($n = 10$) from October 2007 until November 2008 (Fig. 1). These two stations are located along the west coast of Peninsular Malaysia and represent different habitats. Klang is an eutrophic estuary that receives substantial anthropogenic impacts from port and industrial activities and runoff from the most densely populated area in Malaysia, i.e. the Klang valley. In contrast, Port Dickson represents an oligotrophic coastal water system with beach recreational and tourism activities. In addition to the monthly sampling, short-term sampling, i.e. daily sampling over a one-week period was also carried out. Short-term sampling was carried out three times at both Klang (13–19 August 2008, 11–17 October 2008 and 5–11 November 2008) and Port Dickson (22–28 August 2008, 15–21 September 2008 and 18–24 October 2008). Both monthly and short-term samplings were carried out during high-tide levels. At Klang, hourly sampling (from 9:00 am until 5:00 pm) was also carried out on three occasions (28 May, 9 June and 17 June 2009).

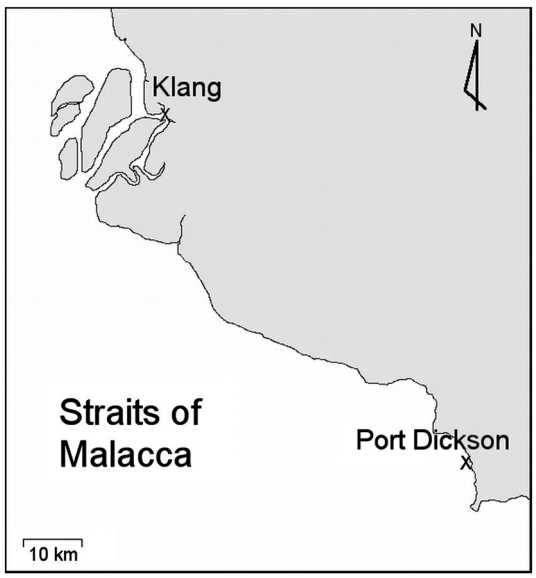

Fig. 1 Map showing the location of the sampling stations; Klang (03°00.1′N, 101°23.4′E) and Port Dickson (02°29.5′N, 101°50.3′E)

At each sampling, surface seawater temperature and salinity were measured using a conductivity meter (YSI-30, USA), whereas pH was measured with a pH meter (Thermo Scientific Orion 4 Star, USA). Dissolved oxygen (DO) concentration was measured in triplicates via the Winkler method (Grasshoff et al. 1999). For microbiology, 500 ml of surface seawater samples was collected with a sterile bottle.

Isolation and enumeration of coliform and *E. coli*

For Klang samples, the spread plating method was used. A 0.1-ml sample was plated onto MacConkey agar (Difco, USA) and then incubated overnight at 37 °C. For Port Dickson, the membrane filtration technique was used. Up to 200 ml sample was filtered onto a sterile 0.45 µm pore size nitrocellulose membrane filter (Millipore, Ireland) and placed on top of a MacConkey agar before incubation. All lactose fermenting microorganisms that appear as pink colonies with or without a zone of precipitated bile are counted as coliform (Garrity et al. 2005). The isolation procedure was replicated once, and the coefficient of variation (*CV*) for spread plating and membrane filtration method were 33 and 63 %, respectively.

All coliform colonies were purified three times via dilution streaking before Gram staining and oxidase test. The identification of Gram-negative and oxidase-negative bacteria proceeded with IMViC (Indole, Methyl Red, Voges-Proskauer and Citrate utilisation tests). Isolates that exhibited an IMViC test result of +, +, − and − were identified as *E. coli* (Garrity et al. 2005).

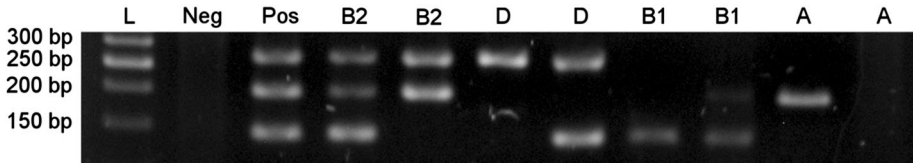

Fig. 2 Gel electrophoresis photograph of multiplex PCR assay. Lane *L* is a 50-bp DNA ladder (Norgen, Canada). Lanes *neg* and *pos* are the negative and positive controls, respectively. Expected product sizes are *ChuA* (279 bp), *YjaA* (211 bp) and TspE4.C2 (152 bp). The genotype of each *E. coli* is indicated at the *top* of its lane

Clermont typing of *E. coli*

All biochemically confirmed *E. coli* isolates were typed according to their phylogenetic groups by multiplex polymerase chain reaction (PCR). Three pairs of primers, *ChuA*.1 (5′-GACGAACCAACGGTCAGGAT-3′), *ChuA*.2 (5′-TGCCGCCAGTACCAAAGACA-3′), *YjaA*.1 (5′-TGAAGTGTCAGGAGACGCTG-3′), *YjaA*.2 (5′-ATGGAGAATGCGTTCCTCAAC-3′) and TspE4.C2.1 (5′-GAGTAATGTCGGGGCATTCA-3′), TspE4.C2.2 (5′-CGCGCCAACAAAGTATTACG-3′), which generated 279-, 211- and 152-bp fragments, respectively, were chosen for the multiplex PCR (Clermont et al. 2000). *E. coli* was suspended in sterile 5 % Triton X-100 and heated at 94 °C for 10 min. The suspension was then centrifuged at 1700 g for 5 min, and the supernatant was used as DNA template for the PCR. For a 20-µl reaction volume, 1 µM of each primer (Eurogentec Ait, Singapore), 1 × buffer (with 1.5 mM $MgCl_2$), 0.2 mM of dNTPs, 2 U of *Taq* polymerase (Finnzymes, Finland) and 5 µl of DNA template were used. PCR was performed with a thermal cycler (Applied Biosystems 2720, Singapore), and the PCR conditions were set as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s; and a final extension at 72 °C for 7 min.

The amplification products were then resolved via agarose gel electrophoresis by using a mixture of 2.25 % NuSieve agarose (Lonza, USA) and 0.75 % Seakem LE agarose (Cambrex, USA) at 7.5 V cm^{-1}. Results were interpreted according to Clermont et al. (2000): group B2 (*ChuA*+, *YjaA*+); group D (*ChuA*+, *YjaA*−); group B1 (*ChuA*−, TspE4.C2+); and group A (*ChuA*−, TspE4.C2−) (Fig. 2). One feature of the Clermont typing is that isolates with all three amplicons absent are assigned to group A (Clermont et al. 2000). Although such bacteria should be characterised further using a multi-locus sequence typing method (Gordon et al. 2008), it was not carried out. Moreover, such strains are relatively rare (Higgins et al. 2007; Walk et al. 2007; Gordon et al. 2008).

Statistical analyses

Unless mentioned otherwise, all values were reported as mean ± standard deviation (S.D.). Differences between the two sites were tested via Student's *t* test, whereas short-term and long-term temporal variations of *E. coli* counts were tested with analysis of variance (ANOVA). Correlation analysis was also carried out to determine whether coliform was contributed by river flow. Before statistical analysis, coliform and *E. coli* counts were transformed by the following equation: $\log(n + 1)$.

Results and discussion

The surface seawater temperature at both Klang and Port Dickson was similar and relatively high (28–32 °C) and is typical of tropical waters (Lee and Bong 2008). Average seawater pH was 7.4 ± 0.3 at Klang and 7.7 ± 0.5 at Port Dickson. In contrast, salinity in the estuarine waters at Klang (18.7 ± 8.2 ppt) was lower than Port Dickson (29.0 ± 0.8 ppt) (Student's *t* test: $t = 4.13$, $df = 10$, $p < 0.01$). Salinity at Klang also varied over a wider range (Coefficient of Variation, CV = 44 %) relative to Port Dickson (CV = 3 %). DO concentration at Klang (141 ± 52 µM) was also lower than Port Dickson (235 ± 42 µM) (Student's *t* test: $t = 4.58$, $df = 19$, $p < 0.001$) and reflected the eutrophic conditions at Klang (Lee and Bong 2008; Lee et al. 2009).

Distribution of coliform and *E. coli*

From the monthly sampling, coliform was detected at Klang in 90 % of the sampling, whereas at Port Dickson, coliform was detected in 80 % of the sampling (Fig. 3). Coliform concentration (as colony forming units per ml or cfu ml^{-1}) at Klang (380 ± 162 cfu ml^{-1}) was markedly higher than Port Dickson (1.0 ± 0.4 cfu ml^{-1}) (Student's *t* test: $t = 2.34$, $df = 10$, $p < 0.05$). The dif-

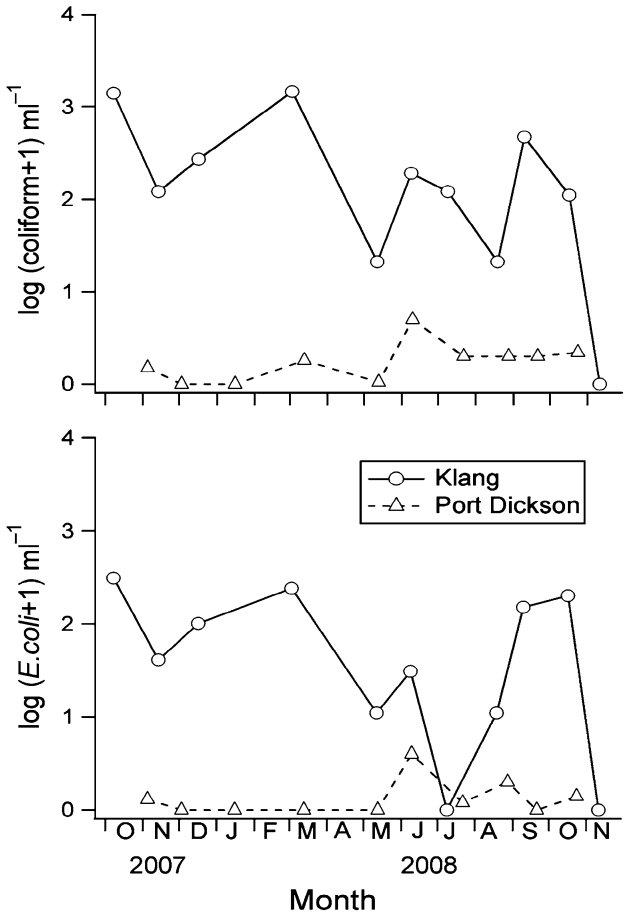

Fig. 3 Temporal variation of total coliform and *E. coli* counts at Klang and Port Dickson

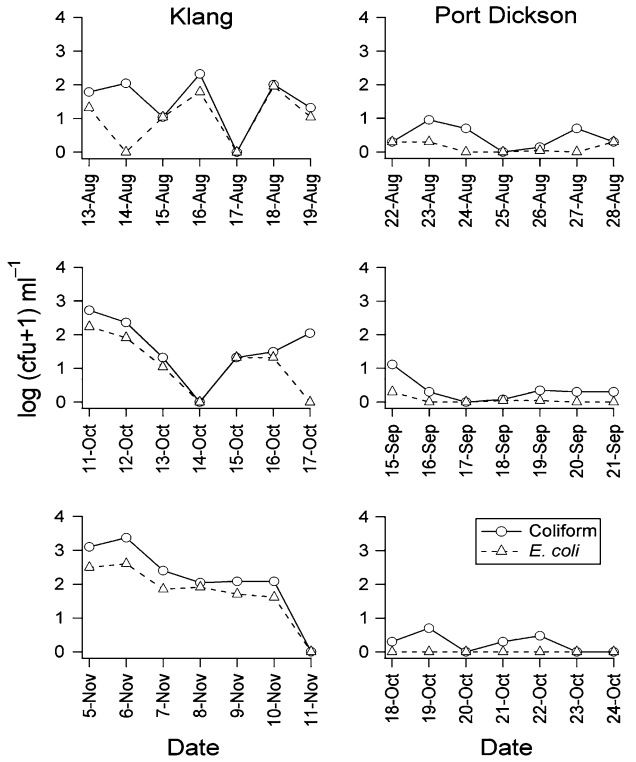

Fig. 4 Daily variation of total coliform and *E. coli* counts at Klang and Port Dickson

ferent methods used at the two different sites did not affect the results as coliform concentration at both Klang and Port Dickson differed by more than two orders. The membrane filtration method was not suitable for Klang as bacterial colonies frequently overgrew on the membrane filters, whereas the detection limit for the spread plating method (i.e. 10 cfu ml^{-1}) was not suitable for Port Dickson.

In this study, *E. coli* concentration at Klang was also higher (99 ± 33 cfu ml^{-1}) than Port Dickson (0.5 ± 0.3 cfu ml^{-1}) (Student's t test: $t = 2.97$, $df = 10$, $p < 0.05$) (Fig. 3). At Klang, *E. coli* was observed in 82 % of the sampling, whereas at Port Dickson, *E. coli* was only observed in 50 % of the sampling. According to the Malaysia Interim Marine Water Quality Standards (Department of Environment 2009), the limit for *E. coli* is 100 most probable number(MPN) in 100 ml seawater. Therefore, at Klang, *E. coli* counts exceeded the limit 36 % of the sampling, whereas *E. coli* counts at Port Dickson were within the criterion. It was clear from this study that Klang harboured more coliform and *E. coli* than Port Dickson. Although Department of Environment monitoring data showed that most coastal waters west of Peninsular Malaysia had coliform and *E. coli* counts exceeding the limit (Chua et al. 2000), this study showed that coastal waters at Port Dickson were still relatively free from faecal pollution.

Short-term sampling at both Klang and Port Dickson revealed no discernible pattern as both coliform and *E. coli* counts fluctuated daily (Fig. 4). At Klang, *E. coli* was below detection limit at least once a week and showed the importance of regular sampling to prevent biased results. However, the average coliform and *E. coli* counts from the daily sampling did support monthly observations. At Klang, coliform and *E. coli* counts were 270 ± 554 cfu ml^{-1} and 69 ± 105 cfu ml^{-1} and higher than Port Dickson (2 ± 3 cfu ml^{-1} and 0.2 ± 0.4 cfu ml^{-1}, respectively) (Student's t test: $t > 2.21$, $df = 20$, $p < 0.05$).

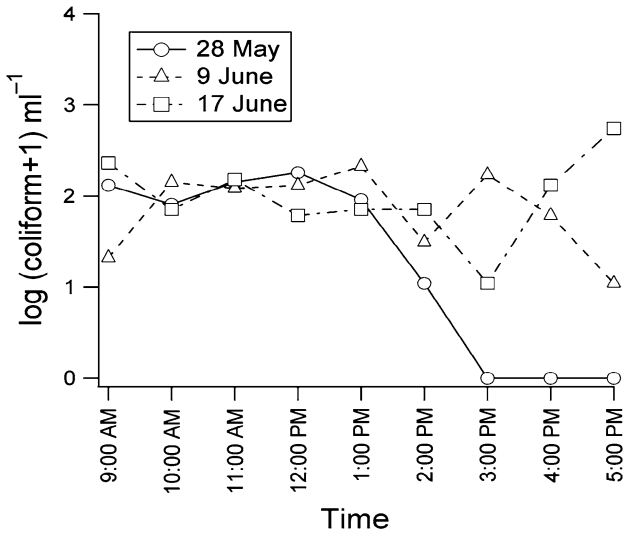

Fig. 5 Hourly variation of total coliform counts at Klang

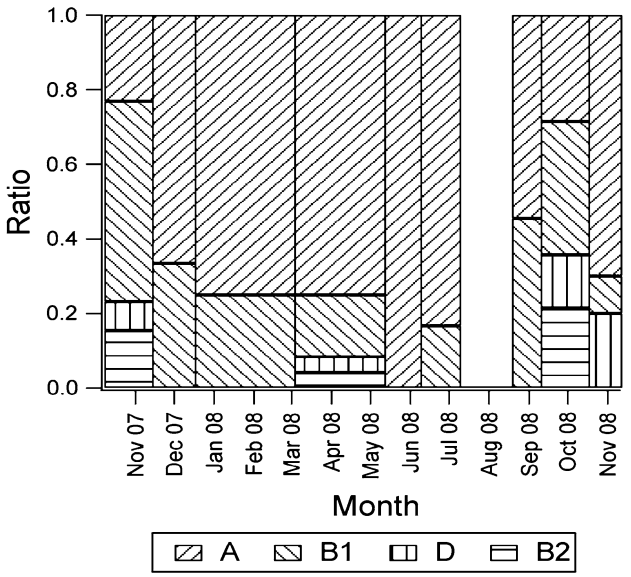

Fig. 6 Temporal cumulative distribution of *E. coli* groups at Klang

At Klang, *E. coli* decay rate is about 3.3 ± 0.8 d^{-1} (Lee et al. 2011). In this study, *E. coli* counts showed no significant daily variation over one week, hence *E. coli* input rate was inferred from decay rate on the assumption that *E. coli* input is balanced by decay. As irradiation die-off, settling and resuspension can affect *E. coli* concentration (Cho et al. 2010), the *E. coli* input rate was at best a conservative estimate. By multiplying the decay rate with *E. coli* concentration, *E. coli* input rate was estimated at about 230 cfu ml^{-1} d^{-1}. As the average Klang River flowrate is 25 m^3 s^{-1} (Lai 1983), *E. coli* loading at Klang estuary was estimated at 5.8 × 10^9 cfu s^{-1}. The high loading rate is reflective of a severely polluted aquatic system (Poté et al. 2009).

Hourly sampling was also carried out at Klang but due to logistic constraints, only coliform counts and salinity were measured. For coliform counts, spread plating of samples was carried out in situ. As the distribution of coliform correlated well with *E. coli* ($R^2 = 0.629$, $df = 30$, $p < 0.001$), the results were extrapolated to *E. coli* distribution. There was no significant temporal variation of coliform distribution, and coliform counts ranged from <10 cfu ml^{-1} to 550 cfu ml^{-1} (Fig. 5). The average coliform count throughout a day ranged from 70 ± 70 to 149 ± 163 cfu ml^{-1}. In order to ascertain whether Klang River transported coliform into the estuary, correlation analysis between coliform counts and salinity was carried out. Salinity is a conservative parameter that reflects river–seawater interaction. In this study, increasing salinity and therefore lesser river influence correlated with decreasing coliform counts ($R^2 = 0.216$, $df = 25$, $p < 0.05$).

Although causality cannot be inferred from correlation analysis, this study suggested that river flow was a significant mode of transport for faecal pollution at Klang. Faecal pollution in rivers has been reported in Malaysia (Law 1980; Kenzaka et al. 2001). The main sources of faecal contamination are poultry manure, agricultural runoff, industrial waste, poorly treated or untreated sewage from urban areas and other anthropogenic input (Geldreich 1996; Ferguson et al. 2003; Wilkinson et al. 2006). As shown by the correlation coefficient, salinity explicated about 22 % of change in coliform counts, therefore, other factors, e.g. decay, irradiation die-off, settling and resuspension also influenced coliform distribution (Cho et al. 2010).

Phylogenetic grouping of *E. coli* environmental isolates

At Klang, all four groups of *E. coli* were observed (Fig. 6). Group A was predominant and was observed in every sampling, whereas group B1 was observed on most of the samplings. In contrast, groups D and B2 were least abundant and rarely detected. Others have also reported a small percentage of groups D and B2 in aquatic systems albeit for rivers and streams (Higgins et al. 2007; Walk et al. 2007; Orsi et al. 2008; Unno

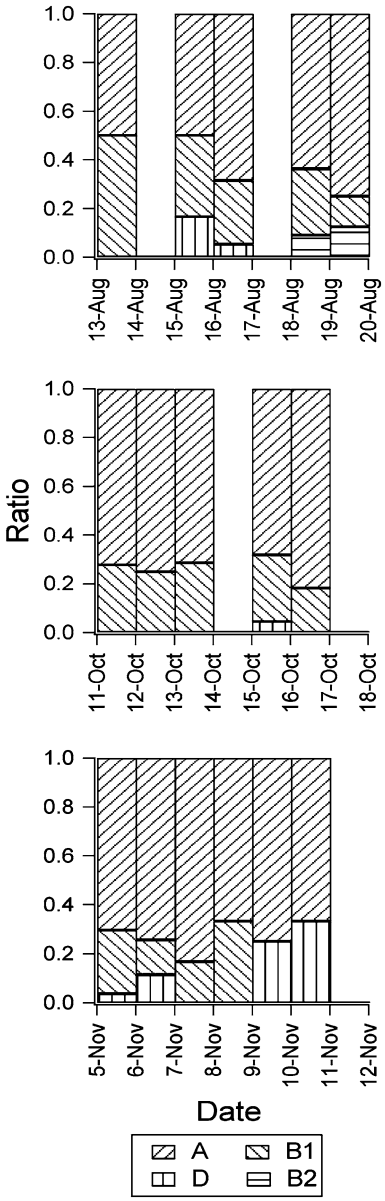

Fig. 7 Daily cumulative distribution of *E. coli* groups at Klang

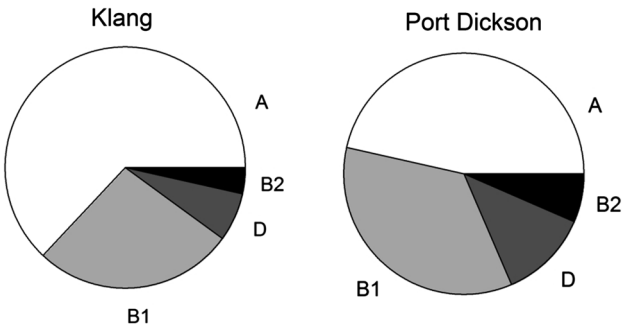

Fig. 8 The distribution of *E. coli* groups at Klang and Port Dickson

et al. 2009). In this study, there was no apparent variation of *E. coli* groups. The results in this study contrasted with Ratajczak et al. (2010) that reported temporal change in phylogenetic groups. Short-term sampling also showed that *E. coli* groups did not vary significantly and their distribution was similar to monthly samplings (Fig. 7).

Although *E. coli* isolates were seldom isolated from Port Dickson, every *E. coli* obtained was typed to provide a snapshot of the *E. coli* population structure at Port Dickson. In this study, a total of 314 strains from Klang and 221 strains from Port Dickson were screened. Group A was the dominant group at both Klang (63 %) and Port Dickson (46 %) (Fig. 8). This was followed by group B1 (27 % at Klang, 35 % at Port Dickson). In contrast, groups D and B2 were least abundant in this study. There were about 6 and 12 % of group D at Klang and Port Dickson, respectively. For group B2, there were 3 and 6 % at Klang and Port Dickson, respectively.

More than half of the *E. coli* isolated from both Klang and Port Dickson were commensal strains, i.e. groups A and B1. The virulent strains, groups D and B2, were less abundant. The predominance of groups A and B1 is similar to other studies (Higgins et al. 2007; Orsi et al. 2008; Unno et al. 2009). Even though the distribution of *E. coli* phylogenetic groups is attributed to environmental factors such as dietary, climatic conditions and geographic (Duriez et al. 2001; Unno et al. 2009), no apparent difference between an eutrophic estuary and an oligotrophic coastal water system was found. However, in this study, about 20 % of the isolates placed in group A did not show any amplicon. This percentage was relatively higher than earlier reports (7–9 %) (Higgins et al. 2007; Walk et al. 2007; Gordon et al. 2008). As Clermont typing is not suitable for group A *E. coli* that does not show any amplicons (Gordon et al. 2008; Li et al. 2010), further studies, e.g. multi-locus sequence typing are needed to understand this difference.

Although mainly commensal, *E. coli* in groups A and B1 are now emerging as intestinal pathogenic strains (Escobar-Páramo et al. 2004). Moreover, groups A and B1 are also closely associated with antibiotics resistance, e.g. ampicillin (Bukh et al. 2009; Unno et al. 2009). Therefore, the prevalence of *E. coli* phylogenetic groups A and B1 in the

coastal waters of Malaysia still presents a potential threat to human health.

Conclusion

E. coli counts at Klang were frequently above the marine water quality criterion, whereas Port Dickson was relatively free from faecal pollution.

This study showed that Klang River was a significant mode of transport for coliform and *E. coli*.

E. coli population structure showed no temporal variation, and groups A and B1 were predominant in the tropical coastal waters of Peninsular Malaysia.

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