Rapid Monitoring of *Escherichia coli* in Southeast Asian Urban Canals by Fluorescent-Bacteriophage Assay

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The microbial water quality in canals of metropolitan Bangkok, Thailand, was assessed using the fluorescent bacteriophage assay (FBA) and fluorescence *in situ* hybridization (FISH). When FISH was used with oligonucleotide probes targeted to *Escherichia coli* (*E. coli*) and anaerobic *Bacteroides* group, < 0.1% to 7% and < 0.1% to 11%, respectively, of the total bacteria were identified. Estimates of viable *E. coli* determined by FBA with 4',6-diamidino-2-phenylindole (DAPI)-labeled T4 bacteriophage, which accounted for 1 to 8% of total cells, were significantly higher than those of fecal coliforms determined by the most probable number method. The FBA procedure can specifically detect viable *E. coli* in canal water samples that are grossly contaminated with fecal flora within 30 min, and it can quickly deliver accurate findings for risk assessment of environmental contamination in water samples.

Key words — canal, fluorescence *in situ* hybridization, fluorescent bacteriophage assay, fecal bacteria

INTRODUCTION

Contamination of municipal water with pathogenic microorganisms constitutes a serious threat to public health. The coliform count obtained using the most probable number (MPN) method is the most frequently applied indicator of recent fecal contamination in freshwater. However, at least 24 hr are required to generate results and the applicability to natural habitats is limited by low sensitivity, because many environmental bacteria resist proliferation in laboratory media.¹⁾ Thus, rapid and sensitive methods are required to detect and enumerate fecal bacteria in natural waters.

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes can provide information about the absolute abundance and size of bacterial cells with defined phylogenetic affiliations.^{1–3)} Despite some limitations, this method has potential to identify and cytometrically analyze microbes. The FISH procedure can generate results in just over 3 hr.

Fluorescent-bacteriophage assays (FBA) can identify and enumerate specific strains of bacteria and cyanobacteria in mixed microbial assemblages,⁴⁾ and determine the rates of production and removal of marine viruses.⁵⁾ Bacteriophages specific for some strains of *Escherichia coli* (*E. coli*) have been isolated,⁶⁾ and FBA has detected *E. coli* O157 : H7 in inoculated ground beef and in raw milk.^{7,8)} This technique requires less than 30 min when sample enrichment is not required.

Here, we performed fluorescence-based rapid detection of *E. coli* in aquatic samples using FBA and FISH with rRNA-targeted probes to assess microbial water quality in canals of metropolitan Bangkok, Thailand.

MATERIALS AND METHODS

Sampling Sites — Water samples were collected at 4 sites (L1, L2, L3, L4) in the Lord Canal, 2 (CP2, CP2-1) in the Chao Phraya River (Fig. 1A), and 10 in canals of metropolitan Bangkok from August 2000 to March 2001 (Fig. 1B). Figure 1C shows a wide area map of the sampling sites in Central Thailand. The Lord Canal originates in the Chao Phraya River at Phra Pin Klao Bridge, and initially flows under a

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Fig. 1. Sampling Sites in Canals and River of Metropolitan Bangkok

(A) Sampling sites around the Lord Canal. Arrows show direction of stream. Gray line represents underground stream. Map A represents magnified view of middle of map B. (B) Sampling sites in 10 canals. Map B represents magnified view of middle area in map C. (C) Map of coastal area of Thailand.

road (Fig. 1A). It appears above ground at L1, and flows for about 1.5 km before rejoining the Chao Phraya River about 400 m up from the Phra Buddha Yodfa Bridge. Domestic water flows directly into the Lord Canal, where some residents fish and wash clothes. Arrows show the direction of the stream and the gray line represents the underground stream.

Other sampling sites in canals were named as follows (parentheses contain name, width and depth of canal, respectively): BC1 (Phadung Krung Kasem, 22 m, 2.5 m), BC2 (Bang Lamphoo, 23 m, 1.3 m), BC3 (Lord, 12 m, 0.8 m), BC4 (Wat Thepthida, 5 m, 0.6 m), BC5 (Mahanak, 18 m, 2 m), BC6 (Bangkok Noi, 54 m, 7 m), BC7 (Mon, 16 m, 3 m), BC8 (Bangkok Yai, 40 m, 4 m), BC9 (Bang Nam Chon, 9 m, 1 m), BC10 (Dao Khanong, 42 m, 4 m) (Fig. 1B). All samples were taken from residential and commercial areas. Some canals are wide enough for boat running, and play important roles in daily transport and tourism. Some sewage plants have been constructed in Bangkok, but the total number remains insufficient, and drainpipe connections are sometimes incomplete. Domestic water also flows directly into some canals. Water (100 ml) sampled from the surface at each site was transported to the laboratory on ice.

Total Direct Counting and Quantitative Direct Counting — Total direct counts (TDC) in the river and canal water samples were determined using 4',6'-diamidino-2-phenylindole (DAPI) at a final concentration of $1 \mu\text{g ml}^{-1}$. Viable bacteria in the water samples were determined by quantitative direct viable counting as described by Yokomaku *et al.* (quantitative direct viable counts, qDVC).⁹⁾ This method

is based on the selective lysis of viable cells by spheroplast formation after incubating samples with antibiotics, nutrients (yeast extract) and glycine. Antibiotics specifically inhibit DNA synthesis and prevent cell division without affecting other cellular metabolic activities. Glycine induces spheroplast formation by viable cells because glycine interferes with several steps in peptidoglycan synthesis for bacterial cell wall formation. This effect leads to swollen cells with very loose cell walls. The viable cells are lysed by freeze-thaw treatment following spheroplast formation.

Plate Counting and Fecal Coliform — The number of colony forming units (CFU) was determined by spreading dilute river water samples on R2A medium,¹⁰⁾ which were incubated at 30°C for 1 week before counting. The mean and standard deviations were calculated from triplicate samples. Fecal coliforms were determined using the MPN method with EC broth.¹¹⁾

Fluorescent-Bacteriophage Assay — The T4 phage labeled with DAPI was obtained from Organo Co. (Toda, Japan) and from Fuji Electric Co. (Yokosuka, Japan). Modified Sholten's broth¹²⁾ was inoculated with the samples at a final concentration of 10% (v/v), and incubated at 30°C for 30 min. Fluorescent-bacteriophage suspension (1/16 volume of each sample) was added and the samples were incubated at 30°C for 20 min. Ethidium bromide (EtBr; 2 mg ml^{-1} in sterilized water; Sigma) and 9% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4 , 1.5 mM KH_2PO_4 , pH 7.2) were added to the samples at a final concentration of $120 \mu\text{g ml}^{-1}$ and 3% (w/

v), respectively. After a 10 min incubation, stained bacteria were trapped onto a black polycarbonate membrane (diameter, 25 mm; pore size, 0.2 μm ; ADVANTEC, Tokyo, Japan).

Oligonucleotide Probes — The following rRNA-targeting oligonucleotide probes were used for FISH: (i) EUB338 (5'-GCTGCCTCCCGTAGGAGT-3'), which is complementary to a conserved region of most bacterial 16S rRNA molecules;¹³⁾ (ii) BAC303 (5'-CCAATGTGGGGGACCTT-3'), which is complementary to a signature region of the 16S rRNA of *Bacteroides* cluster of CFB-phylum;¹⁴⁾ (iii) ES445 (5'-CTTTACTCCCTTCCTCCC-3'), which is complementary to a signature region of the 16S rRNA of *E. coli*;¹⁵⁾ (iv) NON338 (5'-ACTCCTACGGGAGGCAGC-3'), which is negative control probe.¹⁶⁾ All oligonucleotide probes were fluorescently labeled with CY3.

Fluorescence in situ Hybridization — FISH was carried out as described by Kenzaka *et al.* (2001).¹⁵⁾ 12% (w/v) paraformaldehyde in PBS was added to the collected samples in a final concentration of 3% (w/v) and cells were fixed at 4°C overnight. Cells were concentrated from water samples (0.5 to 1.0 ml) on black polycarbonate filters (diameter, 25 mm; pore size, 0.2 μm ; ADVANTEC). Hybridization solution consisted of 0.9 M NaCl (0.45 M for probe ES445), 20 mM Tris-HCl (pH 7.5), 0.1% sodium dodecyl sulfate and 50 ng of CY3-labeled oligonucleotide, and each filter section was incubated at 46°C (41°C for probe ES445) for 3 hr. The filter section was incubated in washing solution [0.9 M NaCl (80 mM for probe ES445), 20 mM Tris-HCl

(pH 7.5), 0.1% sodium dodecyl sulfate] at 48°C (41°C for ES445 probe) for 15 min. Counterstaining was carried out with DAPI solution (2 $\mu\text{g ml}^{-1}$ in sterilized water).

Microscopy — The filter sections were observed with ECLIPSE E400 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a 100-W mercury burner and specific filter sets (UV-2A for DAPI; HQ:CY3 for CY3; B-2A for EtBr). For the DAPI examination, more than 1500 cells were counted per sample. All probe-specific cell counts are presented as the percentage of cells visualized by DAPI. The mean abundances and standard deviations were calculated from the counts of 10 to 20 randomly chosen microscopic fields on each filter section. For FISH, all counts were corrected by subtracting the counts obtained with the negative control NON338. For FBA, all counts were corrected by subtracting the counts of particles with autofluorescence. All results were shown as representative data. The mean abundances and standard deviations were calculated from the counts of 10 to 20 randomly chosen fields on each filter section.

RESULTS

Figure 2 shows the number of bacteria in the Lord Canal and Chao Phraya River. Total cell counts increased in water flowing from the Chao Phraya River to the Lord Canal, and the value was the highest at L3. However, the value decreased approximately by one order of magnitude after the canal

Fig. 2. Abundance of Heterotrophic Bacteria (A) and *E. coli* (B) in Bangkok Canals and One River

(A) TDC, total direct counts (determined with DAPI); qDVC, viable cell counts (determined with quantitative direct viable counting); CFU, colony forming units (determined with R2A medium). (B) FISH, *E. coli* cell counts (determined by direct viable counting-fluorescence *in situ* hybridization); FBA, viable *E. coli* cell counts (determined with fluorescent bacteriophage assay); FC, fecal coliforms (determined with EC broth). Error bars indicate standard deviation ($n = 4$).

Table 1. Abundance of Fecal Bacteria in Canals of Metropolitan Bangkok

Sites	TDC 10 ⁶ ml ⁻¹	CFU % ^{e)}	FISH % ^{e)}	Fecal coliform ^{a)} 10 ³ ml ⁻¹ (%) ^{e)}	FBA ^{b)} 10 ³ ml ⁻¹ (%) ^{e)}	ES445 ^{c)} 10 ³ ml ⁻¹ (%) ^{e)}	BAC303 ^{d)} 10 ³ ml ⁻¹ (%) ^{e)}
BC1	4.4	32 ± 14	38 ± 7.8	1.3 (0.029)	47 ± 54 (1.1 ± 1.2)	< 4.4 (< 0.1)	28 ± 39 (0.6 ± 0.9)
BC2	4.6	28 ± 16	26 ± 12	1.3 (0.028)	39 ± 48 (0.8 ± 1.0)	29 ± 38 (0.6 ± 0.8)	4.6 (< 0.1)
BC3	10	72 ± 14	76 ± 16	13 (0.13)	90 ± 54 (0.9 ± 0.5)	410 ± 220 (4.1 ± 2.2)	1110 ± 260 (11 ± 2.6)
BC4	12	76 ± 2.9	69 ± 11	24 (0.19)	150 ± 71 (1.2 ± 0.6)	700 ± 300 (5.9 ± 2.5)	930 ± 390 (7.8 ± 3.2)
BC5	8.7	22 ± 1.2	83 ± 7.0	54 (0.62)	450 ± 280 (5.1 ± 3.2)	540 ± 220 (6.2 ± 2.5)	190 ± 150 (2.1 ± 1.7)
BC6	5.0	6.7 ± 1.3	11 ± 6.3	0.13 (0.0026)	380 ± 150 (7.6 ± 2.9)	176 ± 200 (3.5 ± 4.0)	176 ± 110 (3.5 ± 2.2)
BC7	5.8	16 ± 4.7	10 ± 3.7	0.23 (0.0040)	90 ± 48 (1.5 ± 0.8)	52 ± 57 (0.9 ± 1.0)	33 ± 35 (0.6 ± 0.6)
BC8	5.5	20 ± 5.5	22 ± 9.6	0.22 (0.0040)	89 ± 40 (1.6 ± 0.7)	140 ± 146 (2.5 ± 2.7)	180 ± 90 (3.2 ± 1.6)
BC9	13	54 ± 14	64 ± 15	2.4 (0.019)	150 ± 86 (1.1 ± 0.7)	860 ± 300 (6.6 ± 2.3)	480 ± 240 (3.7 ± 1.9)
BC10	2.2	52 ± 3.1	27 ± 10	0.33 (0.015)	70 ± 30 (3.2 ± 1.4)	140 ± 71 (6.1 ± 3.2)	140 ± 71 (6.1 ± 3.2)

a) Fecal coliform determined by MPN method. b) Viable *E. coli* determined by FBA. c) *E. coli* determined by FISH. d) *Bacteroides* group determined by FISH. e) % of total count.

joins the river. This was also true of viable cells determined by direct counting. The change in the numbers of colony forming units in water flowing between the river and canal was more appreciable.

The applicability of FBA was examined using the same water samples. Figure 2B shows the number of *E. coli* in the Lord Canal and Chao Phraya River. *Escherichia* group counts and viable *E. coli* cell counts increased in water flowing from the Chao Phraya River to the Lord Canal, and the value peaked at L3. This value also decreases after the canal joins the river. Estimates of fecal coliforms determined by the MPN method were 6 to 200 fold-lower than those by the other two methods, and the change in water flowing between the river and canal was more obvious.

The abundance of fecal and viable bacteria in other canals of metropolitan Bangkok was further investigated, and the results are summarized in Table 1. The total cell counts in the canals (2.2×10^6 to 1.3×10^7 cells ml⁻¹) were within the range reported for eutrophic river systems.¹⁷⁾ Estimates of culturable bacteria in the canals were extremely high, constituting 6.7 to 76% (average: 38%) of the total bacteria. Direct microscopic counts of bacteria from aquatic habitats usually exceed viable plate counts by several orders of magnitude.^{18,19)} Plate counting with R2A media has yielded more colony forming bacteria,¹⁰⁾ especially in eutrophic river samples.¹⁷⁾ Between 10 and 83% (average: 43%) of the total cells was visualized by FISH using the probe EUB338 targeted to the domain *Bacteria*.

The anaerobic *Bacteroides* genus is the most common among human intestinal microflora and it outnumbers coliforms.^{20,21)} Thus, abundance of

Bacteroides organisms in canals was also examined using FISH with rRNA-targeted probes for *Bacteroides* group (Table 1). Estimates of fecal coliforms in samples from BC3, BC4 and BC5 were much higher than those found in ambient water quality criteria,²²⁾ and *in situ* detection with BAC303 probes revealed that the *Bacteroides* group accounted for 2 to 11% of the total bacteria except for BC1, BC2 and BC7. Estimates of *E. coli* determined by FBA were higher than those of fecal coliforms obtained by MPN, and accounted for 1 to 8% of the total cells. FISH with ES445 identified < 0.1% to 7% of the total bacteria.

DISCUSSION

The standard indicator of recent fecal contamination under most conditions in temperate freshwater has historically been coliforms. Contamination levels in tropical countries have been monitored using coliforms despite reports that free-living species might be indigenous to some tropical waters.²³⁾ The present study used the conventional fecal coliform test as well as culture independent methods, namely *in situ* identification with rRNA-targeted probes for *E. coli* and fluorescently labeled T4 phage.

Figure 2 shows an obvious difference in the quantity of fecal bacteria between culture-dependent and -independent methods. The number of culturable *E. coli* is highly dependent on culture conditions as well as on the components of the media, and natural habitats are difficult to adapt to high nutrient concentrations.¹⁰⁾ On the other hand, the FBA procedure is based on direct observation under an epifluorescence microscope and should be more

rapid and sensitive. A comparison of estimates of *E. coli* determined by FBA and by FISH showed that the numbers of *E. coli* determined by FBA were generally lower than those by *in situ* hybridization with ES445 except for samples from a few sites (Table 1). Only the most physiologically active viable *E. coli* cells can be determined by FBA. The identification of microorganisms based upon bacteriophage depends on the host range of the bacteriophage. Although the T4-phage is specific for *E. coli*, some *E. coli* strains are not infected by this phage.

The results shown in Fig. 2 and in Table 1 imply that the physiological state of resident bacteria in the canal differs greatly from habitats in the river. The high abundance of *E. coli* and physiological activity in the canal are decreased in water flowing into rivers but they do survive in the Chao Phraya River. Several of the canals with a high abundance of *E. coli*, *Bacteroides* and high physiological activity are shallow and less than 20 m wide. A large amount of untreated or incompletely treated domestic waste flows directly into these shallow and narrow canals, and active cells in such sources might cause contamination. Waste water is not diluted in those canals as effectively as in wider canals. Thus, the *in situ* physiological activity of resident bacteria might generally be high in several canals. In general, physiological activity of resident bacteria in eutrophic environments is high and the difference between numbers of culturable bacteria and nonculturable bacteria in eutrophic environments were smaller than in oligotrophic environments.^{15,17)} The difference between numbers of fecal bacteria determined by the MPN method and by culture-independent methods (FBA and FISH) in highly contaminated canal samples was relatively smaller compared to river samples. Thus the difference might be affected by physiological state of resident bacteria.

The high abundance of *E. coli* determined by both culture-dependent and -independent methods indicated that the waters of some canals are grossly contaminated by fecal flora, which might constitute a considerable cell fraction. The U.S. Environmental Protection Agency (USEPA) has suggested an ambient water quality goal for *E. coli* of 126 CFU 100 ml⁻¹ in freshwater environments.²²⁾ We cannot reasonably conclude that the *E. coli* detected by our techniques corresponds to a fecal source of pollution in the canals, but the high abundance of anaerobic *Bacteroides* does support this notion because these microbes cannot survive for long in the presence of dissolved oxygen.²⁰⁾

Rivers and canals are important sources of fresh water, and outbreaks of infectious disease from unsafe water represent primary threats.^{24,25)} The present study is the first step towards assessing microbial quality in tropical fresh waters. Some researchers have isolated bacteriophages specific for pathogenic bacteria such as *E. coli* O157 : H7, *Salmonella typhimurium* and *Bacteroides fragilis*.⁶⁾ We believe that FBA can rapidly and sensitively detect viable pathogenic bacteria, and thus should be a valuable tool with which to assess threats to public health from contaminated waters.

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