Specific Detection and Isolation of Pathogenic Bacteria from Environmental Water Samples by using Flow Cytometry and Fluorescence-Activated Cell Sorting

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Introduction

Several disease incidents have occurred directly or indirectly by polluted drinking water, river water, or recreational water [1]. In recent years, pathogenic bacteria were frequently detected from vegetables at the field [2], [3]. Vegetables can be contaminated through number of routes, including surface or irrigation water, feces from livestock and wildlife, raw or poorly composted fertilizers. To assure food and water safety, we have to quantitatively monitor the presence of pathogens in water [4] and if they are present, source of contamination needs be identified and removed. In order to estimate the risks of such water- and foodborne infections, specific detection and isolation of live pathogens is desired.

Flow cytometry (FCM) and fluorescence-activated cell-sorting (FACS) has been developed as a strong tool for detection and recovery of aquatic bacteria [5]. FCM-FACS enables us to isolate single cells directly from water samples prior to incubation in a medium. This single-cell isolation reflects bacterial population diversity. This is in contrast to most of the conventional isolation procedures, in which cells are grown in a medium prior to isolation. However, FCM-FACS has not been used to isolate pathogens that are present in low concentrations in environmental samples without pre-incubation.

Therefore, my research objective was to develop FCM-FACS method to detect and isolate pathogens that are present in low concentrations in water environment.

Materials and Methods

Bacterial strains

Non-Shigatoxigenic *E. coli* serotype O157 strain LMG 21756 and *E. coli* serotype K12 strain MG1655 were used as target and control, respectively. Kanamycin-resistant strain LMG 21756 Km^R was prepared by transposon mutagenesis using pRL27 [7]. Rifampicin-resistant *E. coli* strain NS187 were provided from Dr. Kijima (National Agriculture and Food Research Organization, Japan).

E. coli strains were grown in Brilliant green bile broth (BGBB, Kanto Kagaku), Trypticase Soy Broth (TSB), LB agar plates, or Sorbitol MacConkey (SMAC) agar plates with or without antibiotics at 37ºC.

Cell densities of *E. coli* strains were determined by measuring optical density at 600 nm and direct cell counting after 4', 6-diamidino-2-phenylindole (DAPI) staining. Cells were diluted and used for the analyses described below.

Water samples for spike experiment

Environmental water samples were collected from Atsubetsu river near Atsubetsu-7-go bridge (Sapporo, Japan). *E. coli* strains MG1655 and LMG21756 were inoculated into the water at different cell concentrations (10^4, 10^5, 10^6 cells/liter). The water samples (1 L) were filtered through 0.22-μm-pore polyethersulfone membrane filters (Millipore), and bacterial cells were detached from the membrane by vigorously shaking in phosphate-buffered saline (PBS; pH 7.2) with 0.1% gelatin [8], The cells were centrifuged at 10,000 × *g* for 15 min and resuspended in 1 mL of PBS as described previously [9]. To separate cells from mineral particles, 500 μL of 60% (w/v) Nycodenz (Axis-Shield, Dundee, UK) were gently poured onto 1 mL of the concentrated samples. After centrifugation at 15,000 rpm for 60 min, low to middle density solution (1 mL) were collected to a new tube, and mixed with 9 mL of PBS. After centrifugation at 10,000 ×*g* for 60 min, cell pellets were resuspended with 1 mL of PBS.

Water samples for non-spike experiment

Miyajima-numa lake is located in Bibai, Hokkaido. In spring and autumn, thousands of migratory birds, especially wild geese and swans, visit this lake as their stopover site. Several pathogenic bacteria including *Campylobacter* spp. were frequently detected in this lake in 2012 [6]. However, it is unclear whether migratory birds contribute to the deposition of *Campylobacter* spp. Therefore, I went for sampling and tried to isolate *C. jejuni* from Miyajima-numa to identify their potential sources. Lake water was sampled by using a bucket on Apr 15, Apr 24, May 2, May 10, Jun 12, Jul 17, Sep 20, Sep 27, Oct 4, Oct 18 and Nov 7 in 2013. Bacteria were recovered by pressured filtration as described in spiked experiment. I also monitored the water quality parameters.

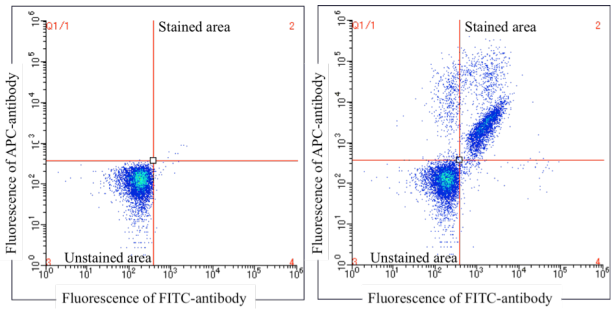

Fig.1 Cells before and after staining by fluorescein-labeled antibodies, observed by FCM.
X and Y axis show the fluorescence intensities of FITC and of APC, respectively. Unstained particles were observed in bottom-left quarter, whereas stained particles with strong fluorescence for both parameters were observed in up-right quarter. Left and right figures show the FCM results before and after staining cells with antibodies.

Preparation of antibodies

Anti-O157 and anti-*Campylobacter jejuni* monoclonal antibodies (#1061, #7721) were purchased from ViroStat (Portland, ME, USA). APC- or FITC- labeled antibodies were prepared by using allophycocyanin labeling kit-SH or Fluorescein labeling kit-NH_2 (Dojindo, Mashiki, Kumamoto, Japan), respectively, according to the manufacturer's instruction.
FITC-labeled anti-O157 monoclonal antibody (#1063) was purchased from ViroStat. Aliquots (1-10 µL) of the FITC- and APC- labeled antibodies (100 µg/mL, 500 µg/mL) were added to 1 mL of bacteria cell suspension and incubated for 20 min. Stained cells were subjected to microscopic observation or FCM-FACS analysis as described below.

Microscopic observation

1 mL of stained bacteria cell suspension was filtered under reduced pressure onto 0.25-µm-pore polycarbonate membrane filter (Millipore). Cells on the membrane were observed and counted by Axiovert fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

FCM-FACS analyses

FCM and FACS analyses were performed using a JSAN desktop cell sorter (Bay bioscience, Kobe, Japan). FCM data were analyzed using AppSan software (Bay bioscience), and target-specific area was set based on the forward scatter, side scatter, as well as FITC and APC fluorescent signals obtained when pure culture of strain LMG21756 was analyzed (Fig. 1).

For isolation of *E. coli* O157, individual cells appeared in the target-specific area by FCM were sorted into BGBB medium in 96-well plates. After overnight incubation at 37 ºC, cell cultures were stamp inoculated onto SMAC agar plates. Transparent colonies on Sorbitol MacConkey agar were considered positive for *E.coli* O157. Sorting efficiency was calculated as [i] the number of wells with O157 growth observed out of [ii] the number of wells with sorted cells. ([i] / [ii])

To isolate *Campylobacter jejuni*, cell suspension from Miyajima-numa lake samples were stained with antibodies and individually sorted into Bolton broth (Kanto kagaku) with 5% of horse blood (Cosmo Bio). After sorting, the medium was incubated at 37ºC under microaerobic condition for 48 hrs, and then stamp-transferred onto CCDA agar without antibiotics. To confirm whether the isolated bacteria are *Campylobacter* or not, *Campylobacter*-specific PCR was performed. C412F and C1288R primers were used. Discrimination from *C. jejuni* was investigated by quantitative PCR that targeted *ciaB* genes [11].

Dynabeads separation

Mixture of *E. coli* O157 strains LMG21756 Km^R and *E. coli* O157 strains NS187 Rfp^R were analyzed by FCM-FACS as describe above. The mixed samples were also used to recover *E. coli* O157 cells by using Dynabeads anti-E.coli O157 (Applied biosystems) according to the manufacturer's instruction. After Dynabeads separation, samples were diluted and spread on SMAC agar. Colonies grown on SMAC agar were picked up and inoculated to SMAC agar supplemented with kanamycin or rifampicin.

Sequence analysis for regions of 16S rRNA gene

Amplicon of *Campylobacter*-specific PCR [10] were sequenced to confirm their taxonomic identity. Sequencing was performed by Takara Bio (Mie, Japan). For strains that showed no amplification by *Campylobacter*-specific PCR, amplicons obtained by PCR with universal primers, 27F and 1492R [12], were used for sequence analyses. Taxonomic classification of the strains was performed according to RDP classifier database.

Results and discussions

Specific sorting from K12 and O157 mixed culture sample

Results of sorting efficiency are shown in Fig. 2. Even when 0.01% of cells were the targets, isolation was achieved at 13% of probability.

Specific sorting from spiked water sample

Results of sorting efficiency are shown in Fig. 3. Even when 10 cells/mL of O157 were present in spiked water, cells could be isolated at 25% of probability. From non-spiked sample (original river water), no O157 was recovered.

In this study, isolation of target pathogenic bacteria was successful from water samples spiked with pathogens at > 10 cells/mL. This concentration can be appeared in natural, non-spiked environmental water (e.g. urban river). In our laboratory, concentrations of *eaeA*-positive pathogenic *E. coli* were monitored in

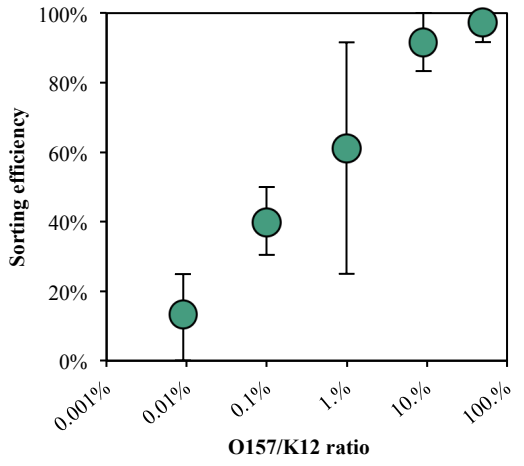

Fig.2 Sorting efficiency from mixed pure culture samples.
X-axis shows the proportion of O157 present in O157:K12 mixed samples. O157 culture were mixed with K12 culture (10^6 cells/mL) to have different O157:K12 ratio. Y-axis shows sorting efficiency. This value was defined as [i] the numbers of wells with O157 growth observed out of [ii] the numbers of wells with sorted the cells ([i] / [ii]).

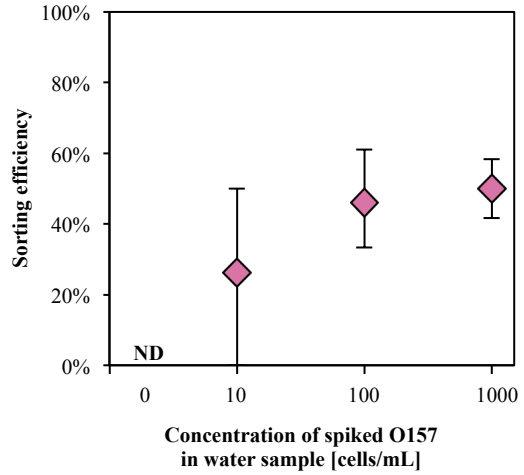

Fig. 3 Sorting efficiency from spiked water samples.
X-axis shows the concentration of O157 spiked to water samples [cells/mL]. Y-axis shows the sorting efficiency. ND means there was no colony observed after sorting and incubation.

river in Sapporo city by qPCR method during Dec 2012–Apr 2013. In clean river (little inflow of treated or untreated waste water), avg. 0.1 cells/mL of pathogenic *E. coli* (n=24) with the highest concentration of 1.4 cells/mL were observed, while at the river with WTP near upstream, avg. 1.9 cells/mL of pathogenic *E. coli* (n=32) with the highest concentration of 32.8 cells/mL were observed. Therefore, 10 cells/mL of sorting limit can be considered applicable to isolate pathogenic bacteria from polluted environmental water samples. In addition to the high sensitivity, FCM-FACS method can shorten the time required for analysis. From water sampling to isolation of the target bacteria, it needs about 6-8 hrs for sample processing, 3-4 hrs for sorting, and overnight for incubation. Incubation will depend on target bacteria, however, this method is two or more days faster than the conventional method.

Comparison of diversity preservation capability

Theoretically, FCM-FACS can preserve original population because it isolates single cells without pre-incubation. To evaluate this, I compared the O157 populations obtained by FCM-FACS with those by Dynabeads separation method.

Population obtained by FCM-FACS method relatively well reflected original Km^R:Rfp^R ratio (Fig. 4). In contrast, O157 Km^R strains became dominant after Dynabeads separation. This change was probably caused during the incubation step, most likely due to the different cell growth rates.

Recovery of C. jejuni from Miyajima-numa

To test if the FCM-FACS method can be applicable to isolate pathogens from natural, non-spiked environmental water samples, I collected water samples from Miyajima-numa lake and performed FCM-FACS targeting *Campylobacter jejuni*. One, five, one, twenty-one and three strains (Total=31) were isolated by using FCM-FACS from Miyajima-numa water samples collected on Apr 24, May 2, Jul 17, Sep 20 and Nov 7, respectively. Among these, 20 strains (64.5%) were positive for *Campylobacter*-specific PCR; however, some of them showed multiple bands. For these isolates, sequencing analysis was done to clarify their identity.

No strain was positive for *ciaB*-targeted qPCR. The *ciaB* genes encode invasion antigen B of *C. jejuni*. Although most *C. jejuni* is reported to possess *ciaB*, in some cases, this gene is not detected by PCR probably because of the diversity present in *ciaB* sequences among *Campylobacter* species. The obtained results imply that isolated strains are not typical *C. jejuni* strains or other *Campylobacter* species.

Identifying isolated strains based on sequencing analyses of 16S-rRNA

16S-rRNA gene sequence analysis was performed to identify the taxonomic classification of the isolates. None of the isolated strains was *C. jejuni* (Table 1). The taxonomic classification of some strains could not be assigned by RDP classifier, suggesting that sequencing results obtained from C412F primer might hybridize to genes other than 16S rRNA gene of *Campylobacter* spp.

Results of identification from sequence analysis suggested there was no *Campylobacter spp.* This might be caused by unspecific binding of *Campylobacter jejuni* antibody, or by non-selective incubating condition after isolation.

Conclusions

- As a result of developing the FCM-FACS method, specific isolation was achieved even when the target was present at 0.01% of the total population in pure culture study, and when the target was present at 10 cells/mL in spiked water samples.
- As a result of comparison with conventional methods, the bacterial proportion was almost preserved by FCM-FACS method better than the result of by dynabeads separation technique.
- 31 strains were isolated from Miyajima-numa lake using with FCM-FACS method and 20 strains (64.5%) were positive for *Campylobacter*-16S rRNA-targeted PCR. But, none of 31 isolates was *Campylobacter* according to 16S rRNA sequence analysis.
- These results suggest that although FCM-FACS isolation was effective to isolate pathogens present in low concentrations in environmental water samples, successful isolation from natural, non-spiked environmental water samples strongly depend on the specificity of antibodies.

Reference

[1] K.Tani and M.Nasu, *Micobes Environ.,*vol. 14, no. 2, pp. 101-105, 1999.

[2] M. T. Jay, et al., *Emerg. Infect. Dis.,* vol. 13, no. 12, pp. 1908-11, 2007.

[3] M. Gallegos-Robles, et al., *J. Food Prot.,* vol. 71, no. 11, pp. 2217-22, 2008.

[4] S. Toze, *Water Manag.,*vol. 80, no. 1-3, pp. 147-159, 2006.

[5] J. Porter, et al., *Appl. Environ. Microbial.,*vol. 59, no. 10, 1993.

[6] T. Nakamura, B. Eng. Thesis. Hokkaido University, 2012.

[7] R. A. Larsen, et al., *Arch. Microbiol.,* vol. 178, no. 3, pp. 193-201, 2002.

[8] M. J. Hamilton et al., *Water Res.,* vol. 44, no. 18, pp. 5463-73, 2010.

[9] A. Kobayashi et al., *Appl. Microbiol. Biotechnol.,*vol. 97, no. 20, pp. 9165-73, 2013.

[10] D. Linton, et al., *Res. Microbiol.,*vol. 147, no. 9, pp. 707-18, 1996.

[11] S. Ishii, et al., *Appl. Environ. Microbiol.,*vol. 79, no. 9, pp. 2891-8, 2013.

[12] D. J. Lane, et al., *Proc. Natl. Acad. Sci. U. S. A.,* vol. 82, no. 20, pp. 6955-9, 1985.

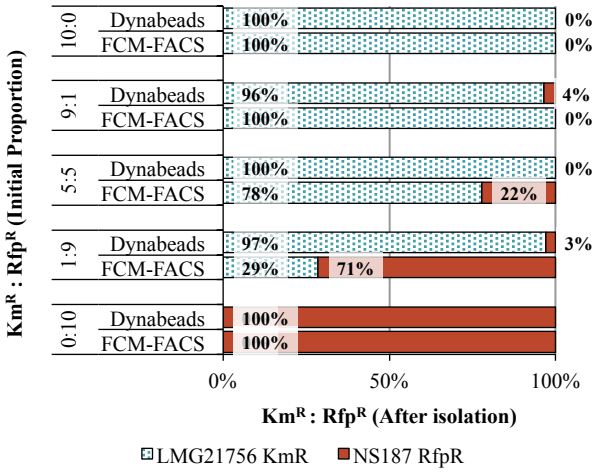

Fig.4 Diversity preservation before and after cell isolation or separation

X axis shows the relative proportion of kanamycin-resistant O157 LMG21756 Km^R and rifampicin-resistant O157 NS187 Rfp^R after isolation. Y axis shows the initial proportion before isolation, as calculated based on the OD600 measurement. FCM-FACS can directly isolate individual cells; whereas Dynabeads method needs enrichment step, which can influence Km^R:Rfp^R ratio because of the difference in cell growth rates.

Table.1 Classification of the strains obtained from Miyajima-numa water samples by FCM-FACS.

Taxonomic assignment was done based on *ca.* 630-bp 16S rRNA gene sequences and RDP classifier program.

No.	Isolation_date	Candidate_bacteria
Oz_01	2013/4/24	genus Ralstonia
Oz_02	2013/5/2	genus Burkholderia
Oz_03	2013/5/2	genus Burkholderia
Oz_04	2013/5/2	genus Burkholderia
Oz_05	2013/5/2	genus Burkholderia
Oz_06	2013/5/2	genus Burkholderia
Oz_07	2013/7/17	unclassified bacteria
Oz_08	2013/9/20	unclassified bacteria
Oz_09	2013/9/20	genus Plesiomonas
Oz_10	2013/9/20	genus Escherichia/Shigella
Oz_11	2013/9/20	genus Burkholderia
Oz_12	2013/9/20	unclassified bacteria
Oz_13	2013/9/20	unclassified bacteria
Oz_14	2013/9/20	genus Aeromonas
Oz_15	2013/9/20	unclassified bacteria
Oz_16	2013/9/20	genus Aeromonas
Oz_17	2013/9/20	unclassified bacteria
Oz_18	2013/9/20	genus Burkholderia
Oz_19	2013/9/20	genus Burkholderia
Oz_20	2013/9/20	genus Azospira
Oz_21	2013/9/20	family Enterobacteriaceae
Oz_22	2013/9/20	genus Acinetobacter
Oz_23	2013/9/20	genus Burkholderia
Oz_24	2013/9/20	genus Aeromonas
Oz_25	2013/9/20	family Enterobacteriaceae
Oz_26	2013/9/20	genus Burkholderia
Oz_27	2013/9/20	genus Burkholderia
Oz_28	2013/9/20	genus Aeromonas
Oz_29	2013/11/7	genus Burkholderia
Oz_30	2013/11/7	genus Pseudomonas
Oz_31	2013/11/7	genus Pseudomonas