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Occurrence of *Escherichia coli* O157: H7 in river water determined by flow cytometry

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Flow cytometry with a fluorescent antibody technique was applied to detect *Escherichia coli* O157: H7. Samples from the Neya River in Osaka Prefecture, Japan, were analyzed by flow cytometry to determine the number of *E. coli* O157: H7 in river water. The numbers of *E. coli* O157: H7 cells in the river water samples ranged from 5×10^3 cells/ml to 6×10^4 cells/ml. These antibody-positive cells grew during cultivation in modified EC medium. These results indicate that viable *E. coli* O157: H7 occurs in river water.

Introduction

Outbreaks of *Escherichia coli* O157: H7 have been reported with increasing frequency[1,3,17,18,21,22]. Prevention of outbreaks requires a rapid and sensitive method for detecting the pathogenic bacteria from freshwater samples.

Culture methods with selective media[4,7,27] have been used to detect and enumerate *E. coli* O157: H7 in the samples. Although these methods enable us to isolate the target bacteria, they usually require an incubation time of at least one night and yield only a small percentage of the actual populations from environmental samples[9].

Analysis at a single cell level with a fluorescent dye is also available instead of culture methods, and a fluorescent antibody staining is commonly used to detect specific bacteria. Epifluorescence microscopy is the popular apparatus for the detection[5,24]. However, this approach is time and labor consuming for the analysis of a large number of samples. Flow cytometric analysis has the advantage of analyzing a large number of cells and their fluorescent intensity rapidly and quantitatively for single cell detection compared with epifluorescence microscopic observation.

In this study, we applied flow cytometry along with the antibody staining technique for the rapid detection of *E. coli* O157: H7 and determined their abundance in natural river water.

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Materials and Methods

Bacterial strains

E. coli O157: H7 (a clinical isolate) was provided by Dr. T. Honda, Research Institute for Microbial Diseases, Osaka University. *E. coli* O157: H7 and *E. coli* K-12 W3110 were cultured at 37°C on LB agar plate (1% Bacto Tryptone (Difco), 0.5% yeast extract (Nacalai Tesque), 1% NaCl, 1.5% agar) for 18 h. After incubation, they were suspended in cold phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2) and washed with cold PBS twice. Cell fixation was carried out by adding 1/2 volume of 12% paraformaldehyde (dissolved in PBS). Fixed cells were washed with cold PBS twice and resuspended in PBS.

River water samples

River water was collected at two sites (Taima and Kitahashi) on the Neya River in Osaka Prefecture, Japan, on September 13, 1997 (Fig. 1). The Neya River contains effluent from the Yodo River. Taima is located at the junction of the Yodo River and the Neya River, and the riverbank is wide at this point. Kitahashi, downstream from Taima, is located in a commercial area and is highly polluted[23,25]. Domestic water flows into the Neya River upstream. Water samples were collected from the surface at each site. Water temperature (W. T.), pH, dissolved oxygen (DO), and electric conductivity (EC) were measured on site.

Direct viable count

The direct viable count (DVC) method was carried out as described by Kogure and Ikemoto[12] to enlarge viable cells. Each river water sample was enriched with 1 mg/ml yeast extract and 40 μg/ml nalidixic acid (SIGMA), and incubated at 37°C for 5 h under dark conditions.

Enrichment of E.coli O157: H7 in river water

Twenty-five ml of river water were inoculated into 225 ml of the modified *Escherichia coli* (mEC) medium[14] (2% Bacto Tryptone, 0.112% bile salts (SIGMA), 0.5% lactose, 4% K_2HPO_4, 0.15% KH_2PO_4, 0.5% NaCl, 20 μg/ml novobiocin sodium salt; SIGMA), and incubated with shaking at 37°C for 24 h.

Fluorescent antibody staining

Fluorescent antibody staining was carried out as described in *Antibodies: a laboratory manual*[8]. Fluorescein isothiocyanate (FITC) labeled *E. coli* O157: H7 antibody (Kirkegaard & Perry Laboratories, Inc.) was

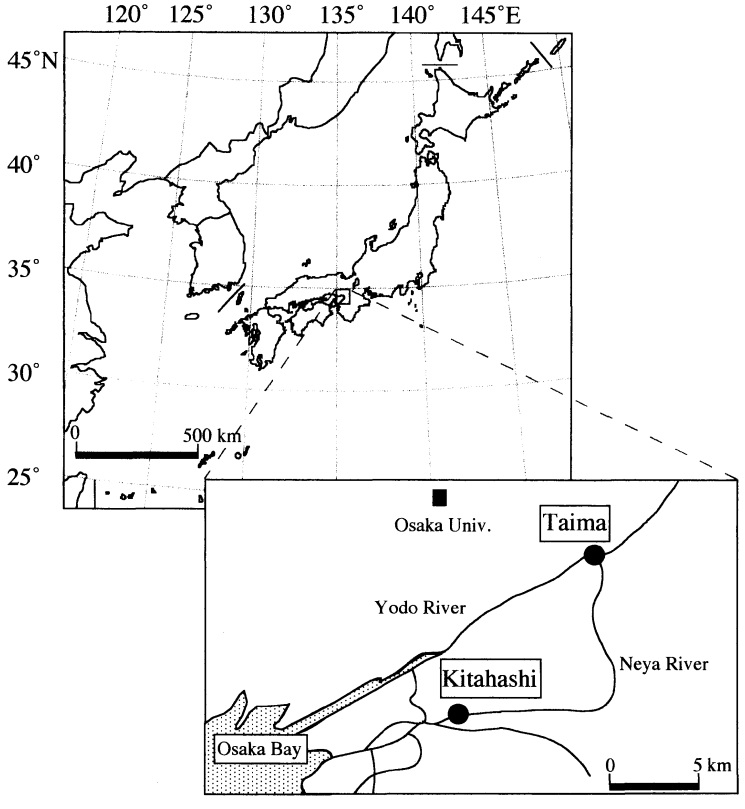

Fig. 1. Sampling stations.

used for *E. coli* O157: H7 detection. A total of 10^6 cultured cells were incubated in 10 μl of staining buffer (3% bovine serum albumin (BSA) in PBS) with *E. coli* O157: H7 antibody (2, 20 or 200 ng) at room temperature for 30 min. After incubation, 1 ml of cold PBS was added and the sample was kept on ice under dark conditions. Two hundred μl of the river water samples or 10 μl of the mEC culture were stained by adding 1/2 volume (100 μl for river water; 5 μl for mEC culture) of staining buffer (9% BSA in PBS with 200 ng of antibody).

Epifluorescence microscopy and enumeration

For the enumeration of the total direct counts (TDC) and observation of antibody-stained samples, cells were stained with 1 μg/ml of 4', 6-diamidino 2-phenylindole (DAPI; SIGMA) and filtered through a black polycarbonate membrane (Isopore Track-Etched Membrane Filter; pore size: 0.2 μm, Millipore). The membrane was observed using a BHS-RFK epifluorescence microscope (Olympus Co.) equipped with a 100 W mercury burner. The bacterial number was adjusted to approximately 60 cells per field, and 30 fields per sample were counted. The filter combination for viewing DAPI stained cells consisted of an excitation filter (UG1), a dichroic mirror (DM400) and an absorption filter (L420). Antibody-positive bacteria were viewed with an excitation filter (BP490+EY455), a dichroic mirror (DM500), and an absorption filter (O515).

Colony forming unit

The number of colony forming units (CFU) was determined by spreading diluted river water samples on R2A agar plates[16] and incubating them at 25°C for one week.

Flow cytometric analysis

Flow cytometric analyses were performed with a Bryte-HS (Bio-Rad Co.). Excitation was carried out with a xenon lamp and a band pass filter block (FITC filter block; Bio-Rad, wavelength 470 to 490 nm). All measurements were discriminated from the background by the forward scatter signal and the side scatter signal. Cellular fluorescence intensity was measured by using a GR1 filter block (Bio-Rad Co.; detection: 515 to 545 nm) as the green fluorescence for FITC. Data were collected with 25 μl of the samples and analyzed by the Win-Bryte software package (version 2.01) supplied with the instrument. The regions of "fluorescent antibody-positive cells" (Fig. 2; in region "FA(+)") were defined by their FITC fluorescence (Fig. 2B, D). The detection limit of the flow cytometry was determined by analyzing a mixture of the antibody-stained *E. coli* K-12 and *E. coli* O157: H7 in known proportions (K-12: O157 = 10^6 cells/ml: 10^1 to 10^6 cells/ml).

Results and Discussion

The antigen-antibody reaction was optimized by adding different amounts (2, 20 and 200 ng) of fluorescent antibody to 10^6 of *E. coli* O157: H7 and *E. coli* K-12 for the flow cytometric analysis of *E. coli* O157: H7. Fig. 2 shows the FITC fluorescence intensity of *E. coli* K-12 and O157: H7. Compared with *E. coli* K-12 (Fig. 2D; with 20 ng of antibody to 10^6 of cells), the FITC fluorescence intensity of *E. coli* O157: H7 was significantly higher (Fig. 2A, B; with 200 ng (A) and 20 ng (B) of antibody). However, 2 ng of antibody addition could not provide enough fluorescence to distinguish between *E. coli* O157: H7 (Fig. 2C) and *E. coli* K-12 (Fig. 2D). Consequently, 10 μl of cell suspension (containing approximately 10^6 cells) was stained with 20 ng of fluorescent antibody in the subsequent experiments, and 100 to 200 μl of each river water sample was stained with 200 ng of the antibody. Regions "FA(+)" in Fig. 2B and Fig. 2D define the antibody-positive bacteria in the samples.

The detection limit of flow cytometry was examined by analyzing mixed samples of *E. coli* K-12 (10^6 cells/ml) and *E. coli* O157: H7 (10^1 to 10^6 cells/ml). The ratios of antibody-positive cells to total bacterial cells (Fig. 2B; in region "FA(+)") were enumerated and compared with the ratios determined by direct counting (Fig. 3). With the flow cytometric analysis, the ratio of *E. coli* O157: H7 to total bacteria was considered correlative if the concentration of *E. coli* O157: H7 was more than 10^3 cells/ml. This sample contained *E. coli* O157: H7 at a ratio of 1/1000 of all

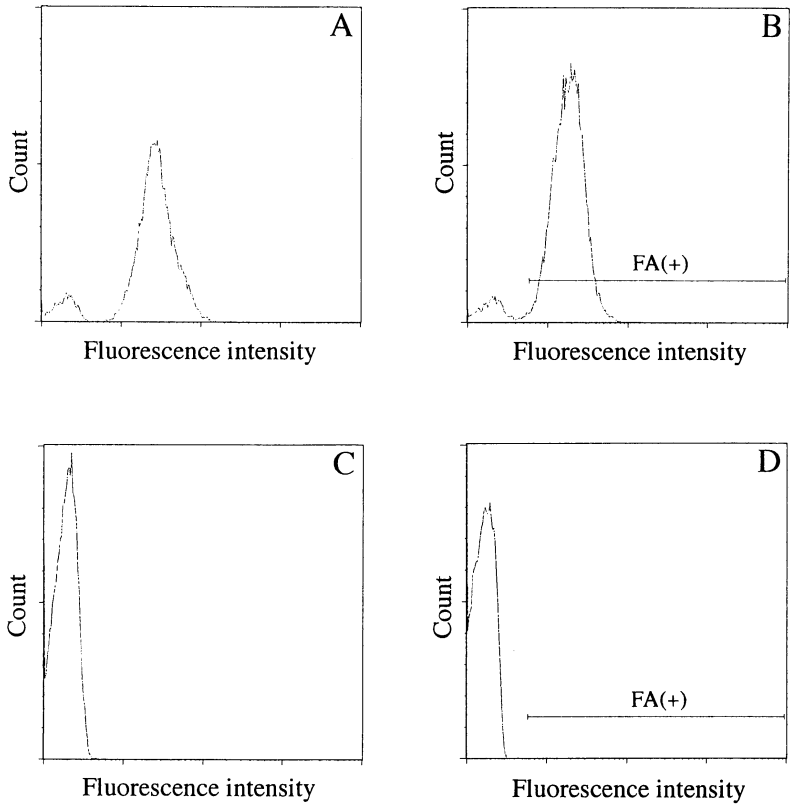

Fig. 2. Flow cytometric analysis of *E. coli* O157: H7 (A, B and C) and *E. coli* K-12 (D). 10^6 of cells were stained with 200 ng (A), 20 ng (B and D) and 2 ng (C) of FITC labeled *E.coli* O157: H7 antibody. FA(+): the region for fluorescent antibody-positive cells.

bacterial cells, while usual detection by epifluorescence microscopy has difficulty when the number of target cells is at such a low ratio. Flow cytometric analysis with sensitive fluorescent antibody staining is expected to detect specific bacterial cells even when their proportion is low. This method should be also suitable to detect specific bacteria routinely and quickly for food and water hygiene.

The abundance of *E. coli* O157: H7 in river water was determined by flow cytometry and epifluorescence microscopy. River water samples were collected at two sites (Taima and Kitahashi) on the Neya River in Osaka Prefecture (Fig. 1). Table 1 shows the physicochemical water quality data and bacterial numbers for the river water samples. TDC and CFU values were higher at Kitahashi. These data suggest that Kitahashi has heavier pollution than Taima. The numbers of antibody-stained bacterial cells are shown in Table 2. When the unstained river water sample was analyzed by flow cytometry, cells with a high fluorescence intensity (Fig. 2B in region "FA(+)") were not detected (data not shown). The numbers of antibody-positive bacteria were 5.0×10^3 cells/ml at Taima and 6.0×10^4 cells/ml at Kitahashi by flow cytometry, while there were 5.2×10^3 cells/ml at Taima and 3.7×10^4 cells/ml at Kitahashi by microscopy. Flow cytometry enabled rapid analysis compared with epifluorescence microscopy, and 10^4 cells could be analyzed within 5 minutes. Observation of many samples by epifluorescence microscopy requires much time and labor, but using a flow cytometer can expedite the process remarkably.

Kogure and Ikemoto reported the occurrence of *E. coli* O157 in river water by the DVC method with epifluorescence microscopy[12]. They applied the method to high sensitive detection of *E. coli* O157 in

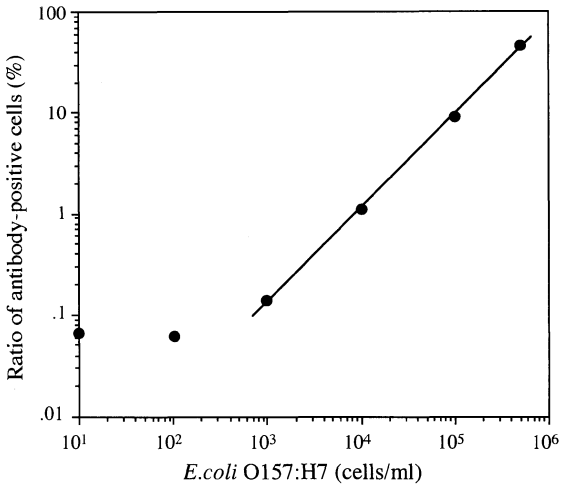

Fig. 3. The detection limit of flow cytometric analysis. *E. coli* K-12 (10^6 cells/ml) and *E. coli* O157: H7 (10^1 to 10^6 cells/ml) were stained with FITC-labeled *E. coli* O157: H7 antibody. The ratios of antibody-positive cells to total bacterial cells were determined from the histograms of FITC fluorescence in the region "FA(+)" (Fig. 2).

Table 1. Physicochemical characteristics of river water.

Sampling stations	W.T. (°C)	pH	EC (μS/cm)	DO (mg/l)	TDC (cells/ml)	CFU (cells/ml)
Taima	28.1	7.7	245	6.9	4.2×10^6	5.7×10^5
Kitahashi	28.6	7.0	761	1.5	1.2×10^7	2.0×10^6

W.T.: water temperature. EC: electric conductivity. DO: dissolved oxygen. TDC: total direct counts. CFU: colony forming units on R2A agar plate.

river water. We also used the same technique and estimated the numbers of *E. coli* O157: H7 by both epifluorescence microscopy and flow cytometry. The numbers of antibody-stained bacteria were found to be 8.5×10^3 cells/ml at Taima and 5.3×10^4 cells/ml at Kitahashi by flow cytometry, while the number of antibody-stained bacteria was 3.4×10^4 cells/ml at Kitahashi by epifluorescence microscopy. However, no stained cells were observed at Taima (Table 2). Although the results of flow cytometry were similar to those of epifluorescence microscopy, we were able to analyze more rapidly by flow cytometry than by epifluorescence microscopy. Though the DVC method was applied to the high sensitive detection of *E. coli* O157 in river water, the numbers of antibody-positive cells were not significantly increased in this study.

River water samples were enriched in the mEC medium to increase the number of *E. coli* O157: H7[2]. The numbers of antibody-positive cells were less than 1×10^3 cells/ml (under detection limit) at Taima and 1.6×10^7 cells/ml at Kitahashi by flow cytometry. When using epifluorescence microscopy, those cells were not observed at Taima but 4.2×10^6 cells/ml were measured at Kitahashi. These results indicate that the viable *E. coli* O157: H7 in the river water from Kitahashi grew during incubation.

As a result, *E. coli* O157: H7 antibody-positive cells may be distributed commonly in natural river water and moreover, they were viable at Kitahashi.

In this study, we were able to rapidly determine and show a high abundance of *E. coli* O157: H7 in river

Table 2. Number of *E. coli* O157: H7 antibody-positive cells in river water estimated by a flow cytometer and epifluorescence microscope.

Analysis	Sampling stations	Number of antibody-positive cells (cells/ml)		
		Natural river water	Incubated by DVC method	Cultivation in mEC medium
FCM[a]	Taima	$(5.0\pm0.0^b)\times10^3$	$(1.7\pm0.5^b)\times10^3$	N.D.
	Kitahashi	$(6.0\pm0.8^b)\times10^4$	$(5.3\pm0.2^b)\times10^4$	$(1.6\pm0.1^b)\times10^7$
FMS	Taima	5.2×10^3	N.D.	N.D.
	Kitahashi	3.7×10^4	3.3×10^4	4.2×10^6

FCM: Flow cytometry. FMS: Epifluorescence microscopy. N.D.: Antibody-positive cell was not detected.

[a]: The number of antibody-positive cells were estimated from FITC fluorescence signal in region "FA (+)" (Fig. 2).

[b]: Standard deviations (n=3).

water by flow cytometry. Kogure and Ikemoto[12] previously reported that *E. coli* O157 is distributed in freshwater environments as a normal inhabitant, and *E. coli* O157 falls into a viable but nonculturable (VBNC) state. Our results confirm that *E. coli* O157 commonly occur in natural environments. Roszak and Colwell[20] showed that enterotoxigenic *E. coli* in a VBNC state were culturable after inoculation into rabbit ireal loops. Amplification of the verotoxin gene by polymerase chain reaction (PCR)[11] or in situ PCR[13] may confirm the occurrence of enterohemorrhagic *E. coli* in river water as other verotoxin-producing bacteria (e.g. E. coli O26, *E. coli* O111) are detectable by these methods. Further recognizing the distribution, physiological activity and infectivity of *E. coli* O157: H7 in natural environments yields useful information pertinent to public health. A combination of a fluorescent staining method that can estimate activity[26] (e.g. esterase activity[6], respiratory activity[19]) and cell sorting[10,15], another technique supplied with the flow cytometer, will enable us to collect physiologically active bacteria from natural river water followed by detection of specific bacteria with physiological activity.

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