Rapid On-Site Monitoring of Bacteria in Freshwater Environments Using a Portable Microfluidic Counting System

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Received July 3, 2019; accepted October 20, 2019

Freshwater environments and natural water parks are important as recreation areas; however, people enjoying recreation at the river- or lake-side are sometimes infected with pathogenic microbes. Microbiological monitoring is fundamental for the routine evaluation of water quality. Fluorescent staining techniques are regarded as among the most useful rapid microbiological methods; however, preparation of samples for fluorescence microscopy is often labor-intensive, and one usually has to take the samples to a laboratory for measurement, which often alters the culturability of bacteria in the samples. These factors have created demand for a rapid and simple method of bacterial quantification in freshwater that can be performed on-site. In this study, we applied our microfluidic device, which was originally designed for on-chip fluorescent staining and semi-automated counting of target microbial cells with fluorescent antibody-staining, to enumerate bacterial cells in freshwater. This was combined with a self-made portable system for rapid on-site monitoring of the bacterial cells. Numbers of both esterase-active bacteria and total bacteria in pond water samples could be successfully determined by on-chip staining with 6-carboxyfluorescein diacetate and SYBR Green II, respectively, using the portable microfluidic counting system. The counting was completed within 1 h (30 min for pre-filtration of freshwater and 30 min for on-chip staining and counting). These results indicate that rapid and accurate counting of bacterial cells in freshwater can be performed and this technique could be applied for “on-site first screening” purposes in microbial quality control of freshwater.

Key words on-site monitoring; bacteria; freshwater; microfluidic device; fluorescent staining; physiological activity

INTRODUCTION

Freshwater environments and natural water parks are important as recreation areas. The number of people who enjoy riverside/lakeside recreation and activities in freshwater environments is increasing. People directly contact water in these areas; however, they often become infected with pathogenic microbes inhabiting freshwater environments, and are sometimes infected with pathogens rarely found in freshwater environments such as Escherichia coli O157.12 The importance of microbiological quality assurance in freshwater environments and natural water parks is increasing, and microbiological monitoring is fundamental for the routine evaluation of water quality.

Culture-dependent methods are usually employed to detect and count bacterial cells in freshwater samples; however, these methods usually require a week to obtain results and cannot detect most bacterial cells because of auxotrophy among diverse bacterial species, and because damaged/injured cells are difficult to grow on conventional media but are physiologically active.5,6 Therefore, rapid quantitative methods, which do not rely on culturing, have been developed to enumerate bacterial cells in natural environments. These methods are widely used in environmental microbiological studies and are also being applied, for example, in factories for microbiological monitoring of pharmaceutical water,5 in clinical sites for rapid diagnosis, and in administrative examination such as microbiological quality assurance of regenerative medical products.6–9 Fluorescent staining techniques enable visualization of bacterial cells in samples within a few ten minutes by simple procedures and are therefore regarded as one of the most useful rapid microbiological methods. Fluorescence microscopy is widely used for detection and enumeration of fluorescently-stained bacterial cells in environmental samples. However, preparation of samples for fluorescence microscopy is often labor-intensive and one usually has to bring samples to a laboratory for measurement. Culturability of bacteria in environmental samples is often altered during long-distance transportation of the samples.10,11 In addition, in developing countries, water pollution is severe not only in large city areas but also in suburban and countryside areas,11 and WHO reports that in 2017, 144 million people collected untreated surface water for drinking.12 In developing countries, several days are often required to bring water samples to a laboratory for microbial water quality inspection, and, therefore, residents have to use water without a microbiological safety guarantee until the results of the water quality inspection are determined and announced. These factors have created demand for a simple culture-independent method of bacterial quantification in freshwater which can be performed on-site.

A microfluidic device is a small device containing micro-channels; such devices have been developed over decades of progress in microfabrication technologies. Microfluidic device-based analyses are rapid and consume less sample and reagents (<100 µL) than conventional approaches such as fluorescence microscopy or flow cytometry.13 The cost to construct such devices is low. Thus, these devices have great potential in environmental microbiology.14–17 Microfluidic devices can reduce bio- and chemical-hazard risks because cells are analyzed in a closed system and the devices are immediately
sterilized after use, making the devices suitable for application in public and environmental health microbiology settings. However, most systems using microfluidic devices have been developed to separate target microbes\(^{18,19}\) or to analyze their characteristics, rather than to determine the total number of target microbes,\(^{20}\) and there are few systems that can be used for microbiological monitoring in outdoor conditions.

In this study, we applied our microfluidic device\(^{21}\) to determination of the number of bacterial cells in freshwater without intensive labor; this device was originally designed for on-chip fluorescent staining and semi-automated counting of target microbial cells with fluorescent antibody-staining. In conjunction, we used a self-made portable system for rapid on-site monitoring of bacterial cells\(^{22}\) to enumerate the total or physiologically active microbial cells flowing in the micro-channel.

**MATERIALS AND METHODS**

**Freshwater Samples** Pond water samples were collected from Inukai Pond (Suita, Osaka, Japan; 34°49′21″ N, 135°31′32″ E) on May 2 and October 15, 2014 (Fig. 1a). River water samples were collected from the Yodo River (Settsu, Osaka, Japan; 34°46′38″ N, 135°35′42″ E) on November 12 and 27, 2014 (Fig. 1b). The collected samples were immediately analyzed after sampling (Figs. 1c, 1d). Before counting of bacterial cells, each sample was filtered through a 3-µm pore size filter (ADVANTEC Toyo, Tokyo, Japan) to remove floating organic matter.\(^{23}\)

**Fluorescence Microscopy** Bacterial number in each sample was determined by fluorescence microscopy in our laboratory to obtain precise counts of bacteria to compare with the values determined using the microfluidic counting system. To determine total bacterial numbers, nucleic acid-binding dyes SYTO BC (Invitrogen, Carlsbad, CA, U.S.A.; excitation 480 nm, emission 500 nm), SYBR Gold (Invitrogen; excitation 495 nm, emission 537 nm), SYBR Green I (Invitrogen; excitation 497 nm, emission 520 nm) or SYBR Green II (Invitrogen; excitation 497 nm, emission 520 nm), were respectively added to the freshwater sample (final concentration, 10000-fold dilution of supplied product). To determine numbers of esterase-active cells, 6-carboxyfluorescein diacetate (6CFDA; Sigma-Aldrich, St. Louis, MO, U.S.A.) was added to the freshwater sample (final concentration 150 µg/mL).\(^{23}\) Cells were fluorescently stained for 3–5 min at room temperature in the dark, and stained cells in the sample were filtered onto a black polycarbonate membrane filter (pore size 0.2 µm, diameter 25 mm; ADVANTEC Toyo). The stained cells were counted at \(1000\times\) magnification (objective lens Plan Fluor 100×; Nikon, Tokyo, Japan) under blue excitation (B-2A cube; Nikon) using an epifluorescence microscope (E-400; Nikon).

**Microfluidic Device Designed for “On-Chip” Staining and Counting** Polydimethylsiloxane-glass hybrid microfluidic devices (Fig. 2) were constructed using rapid prototyping and replica-molding techniques.\(^{23}\) The depth of the micro-channel was 15 µm and the width was 100 or 500 µm.

**Portable Microfluidic Counting System** A portable microfluidic counting system was used for on-site counting.
of fluorescently-stained bacterial cells flowing in the microchannel of the microfluidic device (Figs. 1c, 1d). A portable battery (NMP822AC30; NOATEK, Shizuoka, Japan) was used as the power supply. Samples and fluorescent dye were each placed in a 100-µL gastight syringe (1710LT; Hamilton, Reno, NV, U.S.A.) and sheath fluid (distilled water) was placed in 1-mL gastight syringes (1001LT; Hamilton). These fluids were injected into the microfluidic device via Teflon tubes by the syringe pumps of the system. Stained cells flowing in the microchannel were monitored through an objective lens (UP-lanApo 40×; OLYMPUS, Tokyo, Japan) under blue excitation by a diode laser (473 nm, 60 mW) and recorded as a video using a CCD camera (WAT-902H2; Watec, Yamagata, Japan) for 10–15 min per sample. A filter block for an epifluorescence microscope was used to selectively detect signals from nucleic acid-binding dye or 6CFDA (Olympus U-MNB2 cube consisting of a dichroic mirror 520IF and an absorption filter 500). The flow rate was 0.01 µL/min for the sample and staining fluid, and 0.005 µL/min for the sheath fluid. Flowing cells in the movie were processed and counted using image analysis software, which can enhance positive signals and discriminate background fluorescence by binarization. The bacterial number in each sample was calculated as cells/mL, as determined from the cell count and flow volume.

Statistical Analysis Statistical significance was determined by t-test.

RESULTS AND DISCUSSION

Selection and Optimization of Fluorescent Dye for Total Cell Enumeration Several nucleic acid-binding fluorescent dyes have been developed and used in life science studies. Here, initially, suitable fluorescent dye was selected for staining bacterial cells in freshwater using the microfluidic device and portable counting system. The portable microfluidic counting system is equipped with a diode laser with blue excitation, and, therefore, four fluorescent dyes suitable for blue excitation were evaluated: SYTO BC, SYBR Gold, SYBR Green I and SYBR Green II. Bacterial cells in pond water samples (collected in May at Inukai Pond; n = 3) were stained with each fluorescent dye and counted by fluorescence microscopy and using the portable system (Table 1). Fluorescence from stained cells was weak on staining with SYTO BC or SYBR Green I, and bacterial numbers determined using the portable system were lower than those determined by fluorescence microscopy. Bacterial cells stained with SYBR Gold or SYBR Green II showed bright green fluorescence, but background after staining was somewhat lower in SYBR Green II-staining than SYBR Gold-staining. SYBR Green II was therefore selected for use in the following experiments to stain total bacterial cells.

Next, the design of the microchannel was optimized. A microfluidic device with different widths of the microchannel (width of the mixing chamber 500 µm, width of the detec-

|               | SYTO BC  | SYBR Gold | SYBR Green I | SYBR Green II |
|---------------|----------|-----------|--------------|---------------|
| Fluorescence microscopy | 4.8 ± 0.65* | 5.3 ± 0.62 | 5.0 ± 0.47 | 5.1 ± 0.55 |
| Microfluidic device    | 3.0 ± 1.5 | 4.5 ± 1.5 | 3.4 ± 1.4 | 4.6 ± 1.3 |

* Mean ± standard deviation. (×10⁶ cells/mL).
tion chamber 100 µm) (Fig. 2a) was originally developed for detection of *Legionella pneumophila* in cooling tower water because antibody-labeling of the target bacterial cells required approximately 15 min. However, SYBR Green II can stain bacterial cells within a few minutes, and, therefore, a modified microfluidic device with a short mixing chamber was evaluated (width of mixing and detection chambers both 100 µm) (Fig. 2b). Bacterial numbers in pond samples were determined using these devices (n = 3) and the counts obtained using the modified device (i.e., 100 µm width and short length of the mixing chamber) were close to those determined by fluorescence microscopy (no significant difference), while statistically significant difference was observed between the microscopic counts and the counts obtained using the original device (p < 0.05) (Fig. 3). Therefore, in the following experiments, total bacterial numbers were determined using the modified microfluidic device (Fig. 2b) and SYBR Green II-staining.

**Optimization of Conditions for Detection of Active Cells**

In microbiological monitoring of freshwater, it is often desired to determine both total numbers of bacterial cells and numbers of active bacterial cells. Increase in bacterial cells can be identified by regular monitoring of total bacterial numbers, and numbers of active bacterial cells reflect the potential for increase of the number of bacterial cells. 6CFDA is widely used to detect physiologically active bacterial cells based on their esterase activities and, therefore, conditions for on-chip 6CFDA-staining were evaluated.

The concentration of 6CFDA for on-chip staining in the modified microfluidic device (Fig. 2b) was optimized in pond water samples (collected in May at Inukai Pond; n = 3). The numbers of esterase-active cells determined by on-chip stain-
ing with 6CFDA were 
\((1.0 \pm 0.61) \times 10^7\), 
\((1.6 \pm 0.56) \times 10^7\), 
\((1.3 \pm 0.68) \times 10^7\) and 
\((1.4 \pm 0.85) \times 10^7\) cells/mL (mean ± standard deviation) when 50, 75, 100 and 150 µg/mL 6CFDA were used, respectively. Background fluorescence after staining was not high in fluorescence microscopy (Supplementary Fig. S1) and 75 µg/mL of 6CFDA was therefore used in the following experiments to stain esterase-active cells.

**On-Site Measurement of Bacterial Cells in Pond Water**

After optimization of the system described above, numbers of esterase-active cells and total cells in the pond water collected in October were respectively determined by 6CFDA- and SYBR Green II-staining. Values determined using the portable microfluidic counting system were close to the counts obtained by fluorescence microscopy (no significant difference) (Fig. 4). Therefore, the numbers of both esterase-active bacteria and total bacteria in pond water samples could be successfully determined using on-chip staining and the portable microfluidic counting system.

**Monitoring of Bacterial Cells in River Water**

Numbers of esterase-active cells and total cells in river water samples were determined using 6CFDA- and SYBR Green II-staining. The number of total bacterial cells could be counted accurately using the portable microfluidic counting system, compared with the reference values determined by conventional fluorescence microscopy (no significant difference) (Fig. 5). However, the number of esterase-active cells counted using the portable microfluidic counting system was <60% of that determined by fluorescence microscopy \((p < 0.05)\). It was confirmed by fluorescence microscopy that bacteria in the Yodo River water samples were rather small, and fluorescence from these small bacterial cells was weak (Supplementary Fig. S2). Counting of small esterase-active bacterial cells may be improved by using a brighter excitation light source or a higher sensitivity CCD camera or Complementary Metal Oxide Semiconductor image sensor in the portable system.

The counting was completed within 1h (30 min for precipitation of the freshwater and 30 min for on-chip staining and counting using the portable system). Several culture-independent methods can be used to determine numbers of total bacteria or physiologically active bacteria in freshwater environments. Real-time PCR is sensitive and specific, and thus widely used in environmental microbiology to determine bacterial numbers in aquatic or atmospheric environments. However, this technique requires more time and labor on-chip fluorescent staining methods because of DNA extraction (30–60 min) and amplification (60–120 min) processes. Our results indicate that rapid counting of bacterial cells in freshwater can be performed and appropriate microbiological control of freshwater environments can be confirmed on-site by the combined use of the microfluidic device and the portable counting system described in this study. Furthermore, combination of this system with an electric vehicle would expand its usefulness because it would enable easy transport of the system to a monitoring site and provide a highly energetic electric power source outside of a laboratory.

**CONCLUSION**

In this study, we applied a microfluidic device and a portable counting system to address a problem of public and environmental health microbiology in the field. Our technique effectively enumerated bacterial cells without culturing and could therefore be applied for “on-site first screening” purposes in the microbial quality control of freshwater.

Our technique offers the following advantages: (i) rapid performance (1 h to complete); (ii) ease of use as a semi-automated system (for on-chip staining and counting); and (iii) portability for on-site measurement. A specific feature of this system that differs from gene-targeting techniques or electrochemical detection techniques is that it can visualize bacterial cells in the sample. By watching a video of the flow in the microchannel, which is shown on the display of the portable counting system, and recognizing the frequency of flowing bacterial cells, one can directly grasp an increase or decrease in the number of bacterial cells even without having specialist microbiological knowledge. This feature is important for the rapid examination of bacterial cells in microbiological quality assurance of freshwater.

We therefore propose that our findings contribute to technical progress in developing effective quality control systems for freshwater used for human consumption, recreation, food preparation and industry.

**Acknowledgments**

This research was supported by the JSPS KAKENHI (Grant numbers JP26670062 and 18K11688), the River Foundation, and the Lake Biwa-Yodo River Water Quality Preservation Organization. We are grateful to Yuki Iwai for technical assistance, and to Profs. Tomoaki Ichijo and Masao Nasu for contributions to management of the project. We thank James Allen, DPhil, for editing a draft of this manuscript.

**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Materials**

The online version of this article contains supplementary materials.

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