Detection of *Escherichia coli* in the sewage influent by fluorescent labeled T4 phage

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Abstract

For a precise estimation of sanitary condition, *Escherichia coli* detection system using *E. coli*-specific bacteriophage T4 was constructed. To facilitate *E. coli* detection, T4e− phage which did not produce the lysozyme was constructed and green fluorescent protein (GFP) was displayed on T4e− small outer capsid (SOC) protein. This T4e−/GFP can detect *E. coli* K12 without cell lysis. In this study, we applied T4e−/GFP to the detection of *E. coli* in the sewage influent. Chromocult® coliform (CC) agar plates are generally used for simultaneous detection of total coliforms and *E. coli*. We investigated the number of coliforms and *E. coli* in the municipal sewage influent by using CC agar plates for 1 year. There were 10^5–10^6 CFU/ml of total coliforms and 10^4–10^5 CFU/ml of *E. coli* throughout the year. More than 20 strains of *E. coli* selected from CC agar were infected by T4e−/GFP. The ratio of clear plaque forming *E. coli* was different every month and very low (annual average 8%). Most of the *E. coli* showed turbid plaque or no plaque. *E. coli* formed the turbid plaque showed no-fluorescence, fluorescence only on cell surface due to phage adsorption, or heterogeneous fluorescence among the cells, while *E. coli* formed clear plaque could be detected because of T4e−/GFP amplification in the cell.

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1. Introduction

Pathogenic bacteria which cause waterborne infectious diseases are released into the water system via feces of warm-blooded animals mainly human beings. To estimate sanitary condition in the water system, fecal coliforms have been commonly used as an indicator of fecal pollution. These coliforms are Gram-negative, oxidase-negative bacilli, not sporulated bacteria, able to ferment lactose with acid and gas production at 44±0.5°C. As standard methods in the coliform detection, cultivation on the deoxycholate agar plate and most probable number method by brilliant-green lactose bile (BGLB)-lactose broth (LB) have been used. However, the coliforms cannot reflect all fecal bacteria. There is a possibility that the previous method is to misidentify non-fecal bacteria and/or soil bacteria as the coliforms. Moreover, it takes a long culture period (more than 24 h) to detect coliforms in these methods. For a precise estimation of sanitary condition, it is necessary to detect *Escherichia coli* which is one of the main fecal bacteria and does not appear spontaneously in the water environment. In Japan, “*E. coli*” was substituted for “coliforms” in water quality standard on 1 April 2004. Therefore, the rapid and accurate detection of *E. coli* in the water system has been expected.

For this end, we constructed the *E. coli* detection system by using *E. coli*-specific bacteriophage T4. Hitherto, the detection of *E. coli* by using phage with fluorescent labeled DNA has been reported[1,2]. These labeled fluorescent dyes are mostly DNA intercalators. Therefore, these fluorescent dyes are high cost and harmful chemicals to apply to detection of environmental *E. coli*. Moreover, it is difficult
to use virulent phages in the detection of specific bacteria because of their cell lytic activities. In our previous study, to facilitate E. coli detection, firstly, T4e* phage which did not produce the lysosome to lyse its host cell was constructed [3]. In addition, green fluorescent protein (GFP) as an easily detectable marker was displayed on T4e* small outer capsule (SOC) protein. This T4e*/GFP can detect E. coli K12 without cell lysis. In this study, we applied T4e*/GFP to the detection of E. coli in the sewage influent. Chromocoll® coliform agar plates are generally used for simultaneous detection of total coliforms and E. coli. They contain two chromogenic substrates, Salmon-GAL (6-chloro-3-indolyl-β-D-galactopyranoside) and X-glucuronide (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) for the detection of total coliforms measuring β-galactosidase production and the detection of E. coli through β-glucuronidase production, respectively [4–8]. We investigated the number of coliforms and E. coli in the sewage influent of the municipal wastewater treatment plant by using CC agar plates for 1 year. Moreover, more than 20 strains of E. coli selected monthly from CC agar at random were infected by T4e*/GFP. In the spot test by using T4wt/GFP, the ratio of clear plaque or turbid plaque formed E. coli was also investigated every month. To clarify the plaque turbidity, phage adsorption assay was carried out by using various purified E. coli.

2. Material and methods

2.1. Bacteriophages

T4 phage with GFP (T4wt/GFP) and its mutant (T4e*/GFP) that cannot produce the lysosome (Gpe) that induces the lysis of E. coli K12 were used. The construction of T4e*/GFP was previously described in detail [3]. T4e*/GFP can produce Gpe in E. coli CR63 amber suppressor mutant. Therefore, E. coli CR63 was used as a host cell for propagation of T4e*/GFP. As a typical T4 phage host, E. coli K12 was investigated. In batch cultures, E. coli K12 was cultivated overnight in 2 ml of Luria-Bertani (LB) medium at 37°C on a rotary shaker at 120 rpm. Cell concentrations were determined by measuring the optical density of the medium at 600 nm (OD600) using a spectrophotometer (BACT-550, Nissho Electronics Co., Tokyo, Japan).

2.2. Measurement of concentrations E. coli and coliform in the environmental water

The sewage influent were taken from a municipal wastewater treatment plant (Tokyo, Japan) at the scheduled time every month. Within an hour, the samples were diluted by phosphate buffer saline (PBS) and 100 μl of diluted sample solution were spread on Chromocoll® coliform (CC) agar (Merck KGaA, Darmstadt, Germany) plates. The CC agar plate contains two chromogenic substrates, Salmon-GAL (6-chloro-3-indolyl-β-D-galactopyranoside) and X-glucuronide (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), which allow for the simultaneous detection of total coliforms and E. coli. These chromogenic compounds were substrates of β-galactosidase produced by coliforms and β-glucuronidase produced by E. coli, respectively. The CC agar plates were incubated at 37°C for 24 h. The violet colonies and red colonies were defined as E. coli and other coliforms. The color of coliforms’ colonies become salmon pink, because of the product, chloroindigo, under the catalysis of β-galactosidase. On the other hand, the colonies of E. coli, which possess β-glucuronidase in addition to β-galactosidase, become violet due to the colors of their products. The growth of Gram-positive bacteria and other non-enterobacteria were inhibited by Tergitol® 7.

2.3. Plaque assay of T4 phage

After selection of E. coli from the CC agar plate, more than 20 strains of E. coli were purified every month. The violet colonies on the CC agar plate were selected at random and they were suspended in the PBS. The cell suspension was spread on the CC agar plate again to purify E. coli in the environmental water sample. The violet colonies were suspended in LB medium and cultivated at 37°C for 24 h. In the spot test, 100 μl of purified E. coli suspension (ca. 10^7 CFU/ml) was mixed with the 0.5% soft agar maintained at 45°C and overlaid on LB agar plates. After solidification of soft agar, 2 μl of T4 phage suspension (ca. 10^10 plaque forming unit [PFU]/ml) was dropped on the surface of soft agar and cultivated overnight at 37°C. The plaques formed by T4 phage were categorized as clear plaque, turbid plaque or non-plaque. The clear plaques and turbid plaques indicate transparent plaques and cloudy plaques, respectively.

2.4. Adsorption assay of T4 phage to various E. coli

To investigate adsorption of T4 phage to various E. coli, adsorption assay was carried out. Purified E. coli were cultivated in LB medium overnight. The cells were inoculated into 10 ml LB medium to adjust a final concentration at 10^9 CFU/ml and incubated at 37°C. After 60 min incubation, T4 phage were infected with a final concentration of 10^7 PFU/ml. The suspension were periodically taken and centrifuged at 15,000 × g, 4°C for 1 min. The unadsorbed plaques in the supernatant were measured by the plaque assay. The plaque titer at time 0 was defined as 100%.

2.5. Fluorescent detection of E. coli by T4e*/GFP

In the spot test of purified E. coli from the sewage influent by T4 phage, some E. coli which formed clear plaques and turbid plaques were observed by using T4e*/GFP under the fluorescent microscope (IX50; Olympus Co., Tokyo, Japan) with the GFP-specific filter (U-MGFPHQ, Olympus Co.). The sensitivity and exposure time under the fluorescent observation were ISO200 and 2 s, respectively.
After inoculation of 20 µl of E. coli suspension to 2 ml of LB medium, the cell suspension was cultivated at 37°C on a rotary shaker at 120 rpm. When the cell growth was in the logarithmic growth phase where the cell concentration was about 10⁷ CFU/ml (OD₆₆₀: 0.1), same amount of phage solution (10⁶ PFU/ml) was added at a multiplicity of infection (MOI) of 10 and incubated at 37°C for an hour with shaking at 120 rpm. The non-adsorbed phages were removed by centrifugation (7700 × g, 3 min, 4°C) and the pellet was rinsed by 100 µl PBS. After centrifugation (7700 × g, 3 min, 4°C), the pellet was suspended by 10 µl PBS. The cell suspension was dropped on an agarose-gel coated slide glass. The agarose-gel coated slide glass was prepared as follows. Firstly, 2 ml of 0.5% (w/v) agarose (Agarose S; Nippon Gene Co. Ltd., Tokyo, Japan) solution was uniformly smeared over a slide glass. Secondly, after solidification of gel surface, the slide glass was transferred to dry oven and kept at 60°C for 4 h. The agarose-gel coated slide glass can facilitate observation because of a same focal length to all bacteria and a reduction of the bacterial motility.

### 3. Results and discussion

#### 3.1. Total coliforms and E. coli concentrations in the sewage influent

Seasonal changes of total coliforms and E. coli concentrations in the sewage influent are shown in Fig. 1. On the CC agar plate, most of colonies were salmon pink (coliforms) or violet (E. coli) (Fig. 2(A)). Generally, it is known that bacteria under the real environment, such as wastewater seawater and so on are situated on the verge of death or under viable but non-culturable (VBNC) state because of facing limited nutrient availability, wide variety of temperature or high osmotic pressure [9,10]. If these bacteria are transferred into a rich nutrient medium to form colonies, it is impossible to recover the whole bacteria due to generating the metabolic by-product, hydrogen peroxide. To reduce the oxidative stress, catalase, sodium pyruvate or α-ketoglutaric acid can degrade the hydrogen peroxide [11]. The CC agar contains the sodium pyruvate at an effective concentration (0.1%, w/v). Therefore, bacteria even though under VBNC state seem to be restored on the CC agar plate.

The annual average concentration of total coliforms and E. coli were 5.8 × 10⁵ and 5.4 × 10⁴ CFU/ml, respectively. From these results, there was no big seasonal variation among total coliforms concentrations and E. coli concentrations in the sewage influent. E. coli concentrations were about one-tenth of total coliforms concentrations through the year. That means almost every E. coli in the sewage influent seems to be derived from the excretory substances of human beings. Therefore, E. coli were detected in the sewage irrespective of the seasons.
3.2. T4 host range and phage adsorption

T4 host range against various *E. coli* purified from the sewage influent was investigated by the spot test. From the CC agar plate, the violet colonies were picked up at random to purify *E. coli* in the sewage influent. After purification of *E. coli*, all colonies became violet on the CC agar (Fig. 2(B)). More than 20 strains of *E. coli* were picked up at random and purified every month. In this purification method, it is possible that there was an overlap selection of the same type of *E. coli* in the sewage influent. However, different types of *E. coli* were not emphasized in this study. Because the purpose of this study is to investigate the possibility of detecting the population of *E. coli* in environmental water by the fluorescent-labeled T4 phage, the overlapped *E. coli* seems to be dominant in the real situation.

Using T4 phage, formed plaques were classified into three patterns, non-plaque, turbid plaque, and clear plaque as shown.

![Various kinds of plaques](image)

(A) non-plaque, (B) turbid plaque, (C) clear plaque. Each bar indicates 200 μm and 2 μl phage solution was dropped in the center of each image.

![Percentage of plaque](image)

Fig. 4. T4 host range against *E. coli* purified from an environmental water. Open bar, hatched bar and solid bar indicate clear plaque, turbid plaque and non-plaque, respectively.

![Phage adsorption](image)

Fig. 5. Phage adsorption to various types of *E. coli*. Solid symbols (circle and triangle) and open symbols indicate turbid plaque formed *E. coli* and clear plaque formed *E. coli*, respectively.
in Fig. 3. The typical E. coli K12 formed clear plaques. The seasonal changes of percentage of each plaque type are shown in Fig. 4. The percentage of E. coli which formed clear plaques dynamically changed and monthly mean percentage was very low (ca. 8.0%) compared with that of turbid plaque formed E. coli or non-plaque formed E. coli. The host range of T4 phage against various E. coli in the sewage influent was very restricted. The clear plaque formed E. coli is expected that it can be more easily detected by the fluorescent labeled T4 phage than other types of E. coli. Considering them, it is difficult to detect large portion of E. coli in the environmental water by only the fluorescent labeled T4 phage.

To investigate the reason for the difference of plaque turbidity, the adsorption of T4 phage to various types of E. coli was measured. The ratio of unadsorbed phage in the bulk solution is shown in Fig. 5. T4 phage adsorption to clear plaque formed E. coli was faster than that to turbid plaque formed E. coli. However, among the turbid plaque formed E. coli, there was a wide distribution of adsorption rate calculated from the data of the initial

Fig. 6. Detection of various kinds of E. coli in the sewage influent. (A and B) turbid plaque; (C) clear plaque.
15 min after phage infection. In Fig. 5, the adsorption rate constant of turbid plaque formed E. coli (solid triangles; 3.3 × 10^{-12} ml/CFU/s) was about one-tenth smaller than that of another turbid plaque formed E. coli (solid circles; 3.2 × 10^{-13} ml/CFU/s). Moreover, there was no quantitative relation between the adsorption rate constant and plaque turbidity. It seems that plaque turbidity depends on not only phage adsorption but also other factors such as its burst size.

3.3. Fluorescent detection of E. coli by T4e−/GFP

Following the result of spot test using various purified E. coli from the sewage influent, E. coli which formed the clear plaques and the turbid plaques were used in the fluorescent detection by T4e−/GFP. The optical and fluorescent images of various purified E. coli with T4e−/GFP are shown in Fig. 6. Two different types of turbid plaque formed E. coli were tested. One was E. coli with a relatively larger T4 phage adsorption rate constant, the other was E. coli with a relatively smaller adsorption rate constant. The former E. coli was heterogeneously infected and the latter E. coli was not infected by T4e−/GFP. To overcome these disadvantages, further experiments are necessary to find out other phage with wide host range against the environmental E. coli and label it with the fluorescent marker.

References