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Title: Seasonal change and fate of coliphages infected to *Escherichia coli* O157:H7 in a wastewater treatment plant

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**Abstract-** Seasonal change of virulent phage infected to two *Escherichia coli* O157:H7 strains (O:157-phage) in the influent of a domestic wastewater treatment plant in the central part of Japan and fate of O:157-phage in the plant were monitored almost monthly from March 2001 to February 2002. Coliphage infected to nonpathogenic *E. coli* O157:H7 ATCC43888 (43888-phage) was detected for one year. On the other hand, phage infected to pathogenic *E. coli* O157:H7 EDL933 (EDL-phage) was detected intermittently. Concentration of EDL-phage was almost one-tenth of that of 43888-phage. The progressive decrease in phage concentration with the treatment steps was observed. No phage was detected in the supernatant from the secondary settling tank and effluent. PCR amplification of the Stx 2 gene that encodes Shiga toxin (Stx) was observed when O:157-phage concentration in the influent was high  $\times 10^3$  PFU/ml order. Concentration and percentage of suspended O:157-phage decreased with the progress of the wastewater treatment. 933W phage, which encodes Stx 2 gene, was more fragile and sensitive to chlorination than T4 phage. However, addition of 0.02 mg/l chlorine, in conformance with the required concentration of the plant, did not affect the viability of T4 and 933W phages. On the other hand, 1 mg/l chlorine inactivated the 933W phage significantly.

## INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) of serogroup O157:H7 has been found to cause bloody diarrhea and hemolytic uremic syndrome (HUS) in human. EHEC produces two toxins known as Shiga toxins 1 and 2 (Stx 1 and Stx 2). The production of Stx 1 and Stx 2 in *E. coli* is conferred by toxin-converting lysogenic bacteriophages [1, 2, 3, 4]. The natural reservoirs of EHEC are cattle and other domestic animals [5]. The presence of phages carrying the Stx 2 gene is also common in sewage from developed countries [6, 7]. High levels of phage encoding Stx 2 gene in sewage indicate an abundance of such phages circulating in the human population. It is theoretically possible that the presence of phages carrying the Stx 2 gene in water environment could transduce the Stx 2 gene into Stx-negative strains such as *Shigella sonnei* and *E. coli* K-12 [8].

In addition to their role as a mediator of horizontal gene transfer, bacteriophages function to control bacterial populations by infecting and killing their specific host cells. Phage specificity of infection is determined by the interaction between receptors present on the host cell outer membrane and phage host recognition proteins [9]. The host specificity of each phage relies on its ability to recognize different receptors. Therefore, the existence of the phage in the environment suggests the coexistence of its host strains in the same environment.

Activated sludge treatment is known to reduce the concentration of enteric pathogens. It was suggested that virus loss during this treatment is due to association with wastewater particulates, which subsequently settle and become components of sludge [10]. Other evidence indicates that mixed liquor suspended solids (MLSS) of activate sludge inactivate and remove virucidal agents [9]. Most studies on bacteriophages in wastewater treatment plants are based on the characterization of pure phages seeded to the plant or model environments. However, there are large differences between the removal and/or inactivation of seeded bacteriophages and that of naturally occurring

bacteriophages. In this study, seasonal change of coliphages infected to two *Escherichia coli* O157:H7 strains in the influent of a certain domestic wastewater treatment plant and fate of phages in the plant were monitored for one year to determine the extent and frequency of sewage contamination by O:157-phage and evaluate the effectiveness of wastewater treatment used for the removal of this phage.

## MATERIALS AND METHODS

### *Wastewater treatment plant*

Samples for phage enumeration were collected from an urban wastewater treatment plant in the central part of Japan almost monthly from March 2001 to February 2002. Samples were taken from seven-ports in the plant, namely influent port, primary settling tank, anaerobic tank, aerobic tank, secondary settling tank, chlorination tank, and effluent port. Part of the precipitated sludge in the secondary settling tank was returned to the anaerobic tank. The number of inhabitants serviced by this plant was about 200,000. Most of the contamination was of human origin. The plant treated no effluent from animal farms and industries. The daily volume of the influent was 28,000 m<sup>3</sup>. Hydraulic retention times in each step were 55 min in the primary settling tank, 60 min in the anaerobic tank, 6 h in the aerobic tank, and 4 h in the secondary settling tank. Polyaluminum chloride (PAC), at a final concentration of 2.5 mg-Al/l, was added in the influent to the secondary settling tank for sludge precipitation. Sodium hypochlorous acid (NaClO) was added to the chlorination tank of a final concentration of 0.02 mg-free-Cl/l.

### *Bacterial strains*

Coliphage concentration was analyzed by plaque assay using two *E. coli* strains, *E. coli* O157:H7 EDL933 and *E. coli* O157:H7 ATCC43888. *E. coli* EDL933 was obtained

from C. W. Kaspar (Food Microbiology & Toxicology, University of Wisconsin-Madison)[11]. Luria-Bertani (LB) broth was used for culture and plaque assay. *E. coli* O157:H7 ATCC43888, which produces neither Stx 1 nor 2 and does not carry the genes for these toxins, was used as the host strain in the enrichment culture. Host range of virulent phage infected to O157:H7 was analyzed using three O157:H7 *E. coli* strains, EDL933, ATCC43888 and CR-3, and nine non-O157:H7 *E. coli* strains, *E. coli* O157:H19 A2, *E. coli* O157:H37 CE273, *E. coli* K12 Hfr H, *E. coli* K12 W3110, *E. coli* C, *E. coli* C600, *E. coli* BE, *E. coli* JA300 and *E. coli* JM109. *E. coli* O157:H19 A2, *E. coli* O157:H37 CE273 were kindly provided by Muneo Nakazawa (National Institute of Animal Health, Japan).

#### *Samples and enumeration of phage*

Samples were pooled in sterilized 100 ml tubes at 4 °C and transported to the laboratory within 24 h. Fifty ml of each sample was centrifuged (1,000 g, 10 min), then the obtained supernatant was carefully transferred to a new sterilized tube, centrifuged (13,000 g, 5 min) and then subjected to enumeration of suspended phages. The solid content in a sample was defined as the gravimetric percentage of the wet pellet after the 1,000-g centrifugation of each sample. The pellet obtained after 1,000-g centrifugation was resuspended in an elution buffer (pH = 10) containing 0.5 wt% NaCl and 0.05 M boric acid, vortexed for 1 min and allowed to stand for 90 min at room temperature to detach the phages from the solid matter. The sample was centrifuged (13,000 g, 5 min) and the obtained supernatant was subjected to enumeration of phages attached to solid substances. The values for each plaque count are based on the average of triplicate enumeration.

#### *PCR DNA amplification*

In order to determine the presence of phages carrying the Stx 2 gene in sewage, a

sample was used for phage enrichment. One ml of the sample was added to a 10 ml liquid culture of the *E. coli* O157:H7 ATCC43888 strain in the logarithmic growth phase. After overnight incubation, a 10 ml aliquot was treated with 200 µl of chloroform to disrupt the *E. coli* cells and was centrifuged at 1,000 g for 30 min. The supernatant obtained was treated with DNase (10 units/ml) and RNase (0.8 units/ml) to remove free DNAs and RNAs. For phage purification, to the supernatant were added 10 % polyethylene glycol (PEG-6000) and 0.33 M NaCl to precipitate phage particles. After 15 min centrifugation at 15,000 g, the pellet was resuspended in 500 µl of SM buffer containing ( $l^{-1}$ ), 5.8 g of NaCl, 2.0 g of  $MgSO_4 \cdot 7H_2O$ , 50 ml of 1M Tris-HCl (pH=7.5), and 5 ml of 2 % gelatin. DNase and RNase treatment and phage precipitation were repeated. Purified phage was used for phage DNA extraction with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was then recovered by precipitation with ethanol. The precipitated DNA was dried and redissolved in 10 µl distilled water. One µl of the solution was used as the PCR template. PCR-DNA amplification for Stx 2 gene detection was performed using a taq DNA polymerase. The primers used for the first PCR were as follows: upper primer, 5'-GCGTTTTGACCATCTTCGT-3', and lower primer, 5'-ACAGGAGCAGTTTCAGACAG-3'. The internal primers used for nested PCR were as follows: upper primer, 5'-TAATACGGCAACAAATACT-3', and lower primer, 5'-TGATGAAACCAGTGAGTGA-3'. The conditions for amplification were as follows: one cycle at 94 °C for 3 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and finally 1 cycle at 72 °C for 10 min. Amplification products were analyzed by electrophoresis on a 2.0 % agarose gel and stained with ethidium bromide.

### *Chlorination experiments*

Chlorination experiments were performed using 100 ml pyrex Erlenmeyer flasks at 37

°C with shaking. Inactivation of phage by chlorination was conducted by adding sodium hypochlorite (14 % w/v) to the phage solution to make the final concentrations of free chlorine 0.02, 1.0 and 10 mg/l. T4 and 933W phages were used as indicators. T4 phage was propagated in *Escherichia coli* K12 W3110. 933W phage was obtained by lysogenic induction with mitomycin C of *E. coli* O157:H7 EDL933. Enumerations of 933W and T4 phages were conducted using *E. coli* K12 as the indicator host strain.

## RESULTS AND DISCUSSION

### *Seasonal change of O157:H7 coliphage in the sewage*

*E. coli* O157:H7 is difficult and time-consuming to isolate and identify. Enrichment culture and biochemical and serological tests are needed. For practical purposes such as the safety monitoring of a wastewater treatment plant, bacteriophage infected to *E. coli* O157:H7 has the potential for use as an indicator of its host cell, *E. coli* O157. Bacteriophage can be cultivated by a simple, rapid, accurate and inexpensive method. Monthly change of the O:157-phage concentration in the sewage is shown in Fig. 1. Since the phage concentration in the influents became below the identification limit of plaque assay in some cases, arithmetical mean instead of geometrical mean was used for the analysis. In sewage influents from March 2001 to February 2002, coliphage infected to *E. coli* O157:H7 ATCC43888 (43888-phage) was detected every month. On the other hand, the phage infected to *E. coli* O157:H7 EDL933 (EDL-phage) was detected intermittently. No EDL-phage was detected in six months, April, June, September, October, January and February. The concentration of EDL-phage was always lower than that of 43888-phage. In July 2001, concentrations of 43888-phage and EDL-phage were  $7.94 \times 10^3$  and  $4.78 \times 10^3$  PFU/ml, respectively. No disease outbreak due to pathogenic *E. coli* O157:H7 infection was reported during the experimental period in the central part of Japan.



EDL-phages manifested plaques that were mostly small, round and transparent. Twenty single phage plaques were selected from the algal lawns of *E. coli* O157:H7 EDL933, grown in a liquid culture of *E. coli* O157:H7 EDL933, and purified. Genomic DNA of those phages was purified and digested with the restriction enzyme *TaqI*. The restriction fragment length polymorphism (RFLP) patterns of phage DNAs were compared (data not shown). Since all 20 phages showed the RFLP pattern of the *TaqI* enzyme, their genomes were determined to be composed of double stranded DNA. Host ranges of 10 phages, which showed a distinct RFLP pattern, were analyzed (Table 1). All of the 10 phages manifested plaques on algal lawns of two other *E. coli* O157: H7 strains, ATCC43888 and CR-3, in addition to EDL933. Therefore all of the EDL-phage in the Table 1 was also 43888-phage. On the other hand, EDL-phage concentration in the influent was always less than that of 43888-phage (Fig. 1), suggested that 43888-phage was not always EDL-phage. Five phage strains, SP08, SP10, SP11, SP12 and SP20, yielded no detectable plaques on nine non-O157: H7 strains. Therefore, the existence of those O:157-phages may suggest the coexistence of their host cell, *E. coli* O157:H7 in the sewage. However, SP13 and SP15 showed a relatively broad host range and yielded clear plaques on the algal lawns of *E. coli* O157:H37 CE273, *E. coli* K12 W3110, *E. coli* C, *E. coli* BE and *E. coli* JM109. The other two phages, SP13 and SP15, can be used instead of the *E. coli* O157:H7 strains.

#### *Fate of coliphage in wastewater treatment plant*

Concentrations of 43888- and EDL-phages in each step of the wastewater treatment plant were analyzed to monitor the fate of O157-specific virulent phage in the plant. The arithmetic mean of the phage concentration during the test period ranging from March 2001 to February 2002 in the plant is shown in Fig. 2. A monotonic decrease of phage concentration with the progress of the wastewater treatment was observed indicating that

O:157-phage was vulnerable to the wastewater treatment. The concentration of EDL-phage was almost one-tenth of that of 43888-phage throughout the plant. Since *E. coli* O157:H7 EDL933 is a lysogenic bacterium determined using 933W phages [1, 12], the efficiency of plating (EOP) of this strain might be lower than that of nonlysogenized *E. coli* O157:H7 ATCC43888. No phage was detected in the secondary settling tank effluent.

The average percentage of suspended phage to the total phage through test period and average solid content are indicated in Fig. 3. Most of the phages in the influent and the primary settling tank were detected in the suspended forms. Since the supernatant of the primary settling tank was mixed with the returned sludge from the secondary settling tank and allowed to flow into the anaerobic tank, the solid content in the anaerobic tank reached 3.96 wt%. In spite of this, 50-90 % of the phage in the anaerobic tank was in the supernatant. On the other hand, a significant reduction in the amount of suspended phage in the aerobic tank was observed. No EDL-phage was detected in the supernatant of the aerobic tank. Activated sludge consisting of bacteria, protozoa and metazoa is important for virus removal. The virus removal process is observed to proceed in two stages [13]. In the first stage, virus was removed from the liquid phase by adsorption to the flock, whereas in the second stage, virus is removed by predation of the other microbes, i.e., protozoa or metazoa. A significant reduction in the concentration of O:157-phage in the aerobic tank suggests that the activated sludge is important for the suspended O:157-phage removal. Adsorbed phage onto the flock settled down by the addition of PAC in the secondary settling tank and remained stably in the sludge. Almost no phage was detected in the supernatant of the secondary settling tank and effluent from the process.

*Presence of phages carrying the Stx 2 gene in sewage*

In the EHEC strains, the toxins are encoded by lysogenic phages [12]. The EHEC O157:H7 strain EDL933 produces both Stx 1 and Stx 2 encoded by the temperate bacteriophage 933W. The presence of phage carrying the Stx 2 gene in sewage was examined for one year. The first round PCR amplification did not show any positive amplification by a 2.0 % agarose gel electrophoresis with ethidium bromide staining. The sole exception was July in which the O:157-phage concentration in the influent was estimated to be around  $\times 10^3$  PFU/ml order (Fig. 1) and the Stx 2 gene could be amplified DNAs of 378 bp, as expected according to the positions of the primers used for amplification of the Stx 2 gene, at the first PCR. In this sampling day, a relatively large amount of O:157-phage was observed not only in the influent but also in the primary settling tank, anaerobic tank and aerobic tank (data not shown). Therefore, the sewage in this sampling day must be contaminated with relatively a large amount of phages carrying the Stx 2 gene or Shiga-toxin-producing *E. coli*, which may spontaneously release phages carrying the Stx 2 gene. Since there is no animal farm in the vicinity of this sewage treatment plant, the origin of microbes, which carry the Stx 2 gene, could be from the inhabitants. The isolation of EHEC O157:H7 from the feces of healthy cattle was reported [14]. The origin of the microbes carrying the Stx 2 gene observed in the plant might be healthy human inhabitants. Amplified DNAs of 169 bp after nested PCR were observed in June (Fig. 4A, sample 1), October (Fig. 4D, sample 2) and November (Fig. 4E, sample 4), indicating that the occurrence and levels of phages carrying the Stx 2 gene were a result of accidental contamination of the sewage by these phages during the sampling days.

#### *Phage inactivation by chlorination*

Since the existence of the phage carrying the Stx 2 gene in effluent was suggested by PCR in July (Figs. 4B and C), the effect of chlorination on phage inactivation was

investigated (Fig. 5). Experiments were performed using four different concentrations, 0, 0.02, 1.0 and 10 mg/l of free chlorine. The chlorine concentration of 0.02 mg/l conformed to that of the effluent of the wastewater treatment plant in this study. The initial concentration of T4 and 933W phages was approximately  $1 \times 10^6$  PFU/ml. Six hours of shaking at 37 °C reduced the T4 phage concentration to approximately  $1 \times 10^5$  PFU/ml. Four hours of shaking at 37 °C reduced the 933W phage concentration to  $10^2$  PFU/ml order. After 6 hours of incubation without the addition of chlorine, no 933W phage was detected by plaque assay, indicating that 933W phage was more vulnerable than T4 phage. However, the addition of 0.02 mg/l chlorine, in conformance with the required concentration of the plant, did not affect the viability of T4 and 933W phages. On the other hand, addition of 1 or 10mg/l chlorine reduced the 933W phage concentration. The reason why the Stx 2 gene was amplified in the effluent in July is still unclear. Since the addition of antibiotics such as norfloxacin induces the formation of prophages of EHEC O157:H7 [15], the effect of PAC and hypochlorite addition on prophage induction of EHEC O157:H7 EDL933 was examined. Addition of PAC (2.5 mg-Al/l) or NaClO (0.02 mg-free-Cl/l) to EHEC O157:H7 EDL933 did not enhance 933W phage induction (data not shown). Detection of the phage carrying the Stx 2 gene in the effluent suggested that the sewage effluent could be a source of Stx gene in a water environment. Further accumulation of data and studies to characterize the phage carrying the Stx gene are needed to understand the role of the above-mentioned agents in the proliferation of pathogens.

## CONCLUSIONS

1. In the influent of the domestic wastewater treatment plant in the central part of Japan from March 2001 to February 2002, coliphage infected to *E. coli* O157:H7 ATCC43888 was detected for one year. On the other hand, the phage pathogenic *E.*

*coli* O157:H7 EDL933 was detected intermittently.

2. PCR amplification of the Stx 2 gene was observed in July 2001 when the O:157 phage concentration in the influent was the highest.
3. Concentration and percentage of suspended O:157 phage decreased with the progress of the wastewater treatment.
4. 933W Phage was more fragile and sensitive to chlorination than T4 phage. However, addition of 0.02 mg/l chlorine did not affect the viability of T4 and 933W phages.

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## Figure Legends

Fig. 1. Average phage concentration in influents from March 2001 to February 2002. No EDL-phage was detected in April, June, September, October, January and February.

Fig. 2. Average phage concentration in each step of the wastewater treatment plant. Numbers in the x-axis indicate the position of the sampling ports: 1-influent, 2-supernatant from the primary settling tank, 3-anaerobic tank, 4-aerobic tank, 5-returned sludge, 6-supernatant from the secondary settling tank, and 7-effluent.

Fig. 3. Percentage of suspended phage in each step of the wastewater treatment plant. Numbers in the x-axis indicate the positions of the sampling ports, which are the same as those shown in Fig.2

Fig. 4. Agarose gel electrophoresis and ethidium bromide staining of PCR products. Numbers in the figure indicate the positions of the sampling ports, which are the same as those shown in Fig.2. M, molecular weight marker; P, positive control, PCR amplified DNAs from 933W bacteriophage as a template.

Fig. 5. Inactivation of T4 and 933W phages in PBS with different concentrations of free chlorine.