Original Article
Evaluation of *C. perfringens* cpe-positive Strain as a Source Tracking Indicator of Human Contamination in Freshwater Environments

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ABSTRACT
In this study, a field survey of fecal contamination indicators and *Clostridium perfringens* cpe(+) in river environments was conducted to verify the effectiveness of *C. perfringens* cpe(+) as a microbial source tracking indicator in public water bodies. In the Saijo River, which serves as a model of point source contamination from human sewage effluents, the concentrations of *Escherichia coli*, enterococci and *C. perfringens* increased after the inflow of sewage effluents, with the cpe-positive prevalence rate of *C. perfringens* isolates increasing from 13.3 to 21.5%. In the Nagaike River, contaminated with household sewage effluents from non-point sources, high concentrations of *E. coli*, enterococci, and *C. perfringens cpe(+) were detected, with high cpe-positive prevalence rates of 27.6 and 26.4%. Contrarily, in the Koayu River, which is contaminated with treated wastewater from a large swine farm, an increase in the concentration of fecal contamination indicators and *C. perfringens* was observed; however, the cpe-positive prevalence rate of *C. perfringens* isolates remained unchanged, ranging as low as 3.6 and 3.9%. Altogether, our results revealed that the concentration and pollution load of *C. perfringens cpe(+) is an effective microbial source tracking indicator of human fecal contamination in freshwater environments.

Keywords: microbial source tracking indicator, water and food-born disease, fecal indicator, wastewater treatment

INTRODUCTION
A variety of bacteria, viruses, and protozoa frequently cause waterborne infections in humans. The World Health Organization reports that more than 3 million people die each year of waterborne infections, including many children from developing countries [1]. It is vital to control the quality of raw water to ensure the safety of drinking water. Human and animal feces contain various pathogenic microorganisms [2], which can contaminate raw water and recreational water bodies [2,3]. Consequently, the detection of fecal contamination in water environments is essential to prevent waterborne disease transmission through drinking water and recreational water.

Intestinal bacteria, such as *Escherichia coli* and enterococci, have been used as fecal contamination indicators (FIBs) for a long time. However, recent studies have shown that *E. coli* can survive for long periods outside the intestinal tract and can regrow in soil, sand, and sediments in tropical, subtropical, and temperate climates [4]. The behavior of these bacteria in the environment also differs from that of viruses and protozoa [5]. Therefore, FIBs are not sufficient to detect contamination by pathogenic microorganisms. Besides, the source of contamination is difficult to identify because FIBs are present in the digestive tracts of many different animals [6]. At the same time, it is crucial to establish appropriate countermeasures against potential sources of pathogenic microorganism pollution.

For decades, several microbiological, genetic, and chemical methods have been proposed to track the origin of fecal contamination, including microbial source tracking (MST) [7–9]. Although the viability of *Streptococcus faecalis* can
Bacteroides and the use of the 16S rRNA gene fragment, HF183, of contamination from host-specific genes have been proposed, Enterococcus faecalis and E. faecium are frequently found in human feces, enterococci are widely used as human fecal contamination indicator bacteria; however, a considerable amount of these bacteria regrow in the environment [11]. Even though Bifidobacterium spp. is present in large amounts in human feces, it is hardly detected in animal intestinal tracts [12]. As a result, it has been proposed as a human MST marker. Nevertheless, it is believed to be significantly reduced in the environment after a short period [13]. In recent years, techniques for tracking the source of contamination from host-specific genes have been proposed, and the use of the 16S rRNA gene fragment, HF183, of Bacteroides is particularly promising [14,15]. However, the quantification of this marker is affected by rainfall, insolation, and water quality [16], whereas no significant increase is observed in it in non-point pollution-rich situations [17].

Coliphage has long been proposed as a virus-based MST method; however, the amount of phages present in the environment is often lower than the amount of bacteria. Therefore, highly sensitive test methods and enrichment are required [18]. Among the Bacteroides fragilis bacteriophages, the HSP40 strain is regarded as an excellent MST indicator because of its human host specificity [19]. Nevertheless, its distribution differs depending on the region, and the complexity of its culture adds to its disadvantage [20]. Alternatively, Pepper mild mottle virus (PMMoV) has been proposed as a human MST indicator [21]. However, PMMoV assessment requires expensive real-time quantitative PCR (qPCR) reagents and the plotting of a calibration curve. In any case, even if the virus is used as an MST indicator and genetic quantification is performed, the viability and infectivity of the virus cannot be confirmed.

In recent studies, Clostridium perfringens and its spores have been categorized as candidates for bacterial MST [22–24]. As C. perfringens is widely detected in human and animal feces, it is a suitable indicator that can be easily quantified using culture-based methods to assess fecal contamination [25]. C. perfringens is ubiquitously distributed in human and non-herbivore animal feces and, therefore, it can be an excellent indicator of point contamination in rivers and other water systems [22]. In addition, the presence of C. perfringens is correlated with that of human enteric viruses, cysts, or oocysts of protozoa [23]. Due to its high survivability, it is regarded as an indicator of fecal contamination even if the source of contamination is remote [26].

The most important finding is the uneven distribution of C. perfringens with specific toxin genes in humans and animals. Although C. perfringens is widely present in cattle, pigs, poultry, and other livestock feces, its enterotoxin gene (cpe) positive strain, C. perfringens cpe(+), which causes food poisoning, has only been detected in human fecal samples [24]. Beyond that, Saito reported that no cpe-positive were detected in stool samples from 76 chickens, 131 pigs, and 51 cattle, and only 2 of 106 dog samples were positive [27]. As another approach, in retail meat and food samples, C. perfringens was detected in 142 of 200 samples (beef, chicken, and pork), only two from beef and one from chicken were cpe-positive [28]. Moreover, C. perfringens cpe(+) has high detection rates, of about 30%, in influents and effluents of sewage treatment plants [24]. In a recent study, human sewage influents and suspended matter in urban river basins are reportedly described as C. perfringens cpe(+) reservoirs [29].

In addition to its usefulness in MST, C. perfringens cpe(+) is an important food poisoning pathogen. A large amount of food poisoning cases caused by C. perfringens cpe(+) occur worldwide, with an estimated 1 million cases in the US, similar to the number of food poisoning cases caused by Salmonella spp. [30]. Moreover, in the EU, the number of food poisoning illnesses caused by C. perfringens cpe(+) is estimated to be about 5 million [31]. In Japan, C. perfringens cpe(+) is recognized as a food poisoning bacterium with an incidence of 30%, and in the past three years (2016–2018), both the number of patients per year (1,220–2,319) and the number of patients per case (45–72) are always the highest or second when compared to other bacteria [32]. It is suspected that the foods causing food poisoning include curry, stew, and other high-viscosity stewed dishes that can be easily prepared in an anaerobic state and are consumed at social events. Although C. perfringens cpe(+) is often found in boiled foods and large-scale cooked meat, seafood, and vegetables, the causative factors responsible for its food poisoning role and their contamination routes remain unclear [28].

Therefore, based on the presence of C. perfringens cpe(+) in human feces, its long-term viability in the environment, uneven distribution in human feces, and effectiveness as a pollution indicator, we assumed that the assessment of C. perfringens cpe(+) may provide the best MST method for detecting human fecal contamination. However, to date, its effectiveness as an MST indicator in river basins has not been demonstrated. The objectives of our study were to verify the effectiveness of C. perfringens cpe(+) as an MST indicator in public water bodies and to evaluate the relationship between its distribution in river environments and the characteristics
of the river basins. Furthermore, the association between human-derived contamination sources and *C. perfringens cpe* (+) is also discussed. We conducted field surveys on the concentrations of *E. coli*, fecal enterococci, and *C. perfringens* and basin utilization in two river basins to evaluate these objectives.

**MATERIALS AND METHODS**

**Sampling points**

**Sampling points and basin characteristics of the Saijo River water system**

A field survey was conducted in the Saijo River water system, Shobara city, Hiroshima prefecture, Japan, chosen as a model for a point source of human fecal contamination (Fig. 1). The survey area was approximately 500 m in length, and water samples were collected upstream point (SP1), two point sources, namely the Shobara wastewater treatment plant effluent (SP2) and the Togo River—a small tributary that flows in from the city area (SP3), and downstream point after inflows of SP2 and SP3 (SP4). An estimated population of 8,700 people (obtained from demographic information of local government) connected to the sewage treatment plant in this area. Sewage is treated using the oxidation ditch (OD) method, and then chlorine is added for disinfection prior to its release into the Saijo River. The Togo River (SP3) flows down the city and receives human fecal wastewater from septic tanks installed in each household. Approximately more than 300 people use septic tanks in this area (obtained from demographic information of local government). Human waste and household wastewater are treated with activated sludge, and chlorine is added for disinfection prior to being discharged into small discharge channels leading to the Togo River.

**Sampling point and basin characteristics of the Sagami River water system**

A field survey was conducted in the two tributaries of the Sagami River system, Kanagawa prefecture, Japan, which was chosen as a model for both non-point sources of human fecal contamination and point sources of livestock fecal contamination (Fig. 2). The Sagami River flows into the sea in Sagami Bay, Kanagawa prefecture, with the main stream having a basin area of 1,680 km² and a length of 113 km.

The Nagaike River, connected to the Sagami River down-stream, flows through an urbanized and populated area, and no influx of livestock fecal contamination has been confirmed in it. Using the statistical data published by the local government, there was a non-washing population of 1,705 in the basin. People in this area discharge household wastewater, including human waste, into the Nagaike River after treatment in household septic tanks installed in each household. Therefore, samples from the Nagaike River were collected using this river basin as a non-point source of human fecal contamination (SP5 and SP6).

The Koayu River (SP7 and SP8) has a small population in the river basin, and an estimated population of 10 people discharges treated-household sewage to the river upstream of SP7 and SP8. Since the sewage discharge from a large swine farm flows between SP7 and SP8, this area was used as a model of point source contamination from livestock. Information on the basin population was collected from the...
local government homepage [33], and the information on the number of pigs and drainage was collected from specific businesses in Kanagawa prefecture (unpublished data).

**Measurement of fecal contamination indicators**

**Sample collection**

Surface water samples from each sampling point were collected 10 times in the Saijo River system from November 2016 to April 2018, and six times in the Sagami River system from May 2016 to July 2018. Samples were then carried in an ice-cooler box to the laboratory, and bacterial tests were performed on the same day.

**Escherichia coli and enterococci**

*E. coli* was counted using the Colilert (IDEXX, Westbrook, USA) specific enzyme-substrate method. After adding the Colilert reagent to 100 mL of water sample and mixing, the mixture was placed in a QT tray (Quanti-Tray/2000, IDEXX), sealed, and cultured at 36°C for 24 h. Enterococci were counted using Enterolert reagent (IDEXX), and the mixture was cultured at 41°C for 24 h. The number of wells that showed a positive reaction in the QT tray were counted according to the manufacturer’s instructions. From the number of positive wells, the concentrations of *E. coli* or enterococci were calculated using the most probable number method (MPN/100 mL).

**Clostridium perfringens spores count**

To inactivate vegetative cells, water samples were incubated in a water bath at 75°C for 20 min and then cooled immediately in an ice bath. Spores of *C. perfringens* were counted using the triple-layered Handford agar method (Eiken Chemical Co., Ltd., Tokyo, Japan) [24]. In case of low concentration, filtration with a sterile cellulose acetate membrane filter (φ45 mm, pore size 0.45 µm) was performed.

**Testing for *C. perfringens* toxin gene**

From the incubated Handford plates, 166 to 442 colonies from each sample were randomly selected. Selected colonies were isolated using a sterilized loop and incubated in enrichment culture on Columbia agar with 5% sheep blood (Becton, Dickinson and Company, Franklin Lakes, USA) at 36°C for 24 h in an anaerobic jar system (Anaero Pack, Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan). Next, each isolate was tested for the presence of six types of *C. perfringens* toxin genes (cpa, cpb, cpb2, etx, iap, and cpe) using multiplex PCR, as previously described [24,35]. After that, the amplification product was confirmed by agarose gel electrophoresis, and the one in which the 485 bp band was confirmed was judged to be cpe-positive. Table 1 shows the primer sequences for the six types of *C. perfringens* toxin genes used for multiplex PCR [24,35]. No toxin gene was particularly closely related to cpe (cf. supplementary materials).

**Calculation of prevalence rate, pollution load, and the basic unit of *C. perfringens* cpe (+)**

*C. perfringens* isolates obtained from each sample was analyzed using PCR to determine the prevalence rate of *C. perfringens* cpe(+) in each sample. The concentration of *C.

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**Table 1** Primer sequences used for multiplex qPCR.

<table>
<thead>
<tr>
<th>Toxin gene</th>
<th>Primers</th>
<th>Sequence (5′ − 3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa(α-toxin)</td>
<td>CPAlphaF</td>
<td>GCTAATGTTACTGCCGTGTGA</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td>CPAlphaR</td>
<td>CCTCTGATACATCGTGTAAG</td>
<td></td>
</tr>
<tr>
<td>cpb(β-toxin)</td>
<td>CPBetaF3</td>
<td>GCGAATATGCTGAACTCTTA</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>CPBetaR3</td>
<td>GCAGGAACATTAGTATATCTC</td>
<td></td>
</tr>
<tr>
<td>cpb2(β2-toxin)</td>
<td>CPBeta2totalF</td>
<td>AATAATGACCTAACCACAA</td>
<td>548</td>
</tr>
<tr>
<td></td>
<td>CPBeta2totalR</td>
<td>CCAATACTYbTAATYGATGC</td>
<td></td>
</tr>
<tr>
<td>etx(ε-toxin)</td>
<td>CPEpsilonF</td>
<td>TGGGAACCTCCAGATACAGCA</td>
<td>376</td>
</tr>
<tr>
<td></td>
<td>CPEpsilonR</td>
<td>AACTGCACATAATTCCCTTTC</td>
<td></td>
</tr>
<tr>
<td>iap(ι-toxin)</td>
<td>CPIotaF</td>
<td>AATGATCTCTTTAAATATCC</td>
<td>272</td>
</tr>
<tr>
<td></td>
<td>CPIotaR</td>
<td>TTAGCAAAATGCACCTCATTT</td>
<td></td>
</tr>
<tr>
<td>cpe(enterotoxin)</td>
<td>CPEnteroF</td>
<td>TTCAGTTGGATTTACTCTTG</td>
<td>485</td>
</tr>
<tr>
<td></td>
<td>CPEnteroR</td>
<td>TGTCGAAGCTGTAATGTC</td>
<td></td>
</tr>
</tbody>
</table>
isolates was determined using the following equation (2):

\[ Q_p = C \cdot F \]

where:
- \( Q_p \): Bacterial contamination load (cfu/day)
- \( C \): Bacterial concentration (cfu/m³)
- \( F \): flow rate (m³/day)

To calculate the discharge in the Saijo River, the river water level and discharge were provided by the River Management Office. The sewage discharge water was confirmed with the City Office Sewer Section. The flow speed and inflow area of the Togo River were measured. The pollution load of \( E. coli \) and enterococci were calculated similarly.

For \( C. perfringens \), the pollution load was divided by the population of the basin or the treated water population, and the unit discharge (cfu/person/day) of \( C. perfringens \) isolates was determined using the following equation (1):

\[ Q_p = C_i / P \]

where:
- \( Q_p \): Bacterial contamination load (cfu/person/day)
- \( C_i \): Bacterial concentration (cfu/m³)
- \( P \): Population of the river basin using a septic tank or sewer system (people)

To calculate this value, a survey of the population of the river basins using a septic tank or sewage treatment facilities was carried out. For each population, statistical data published by Shobara city or Atsugi city was used. The data used for the calculation are shown in Tables 2 and 3.

RESULTS

Effect of inflow from human and livestock point source contamination on FIB concentration

Table 2 shows concentrations of bacterial indicators at each sampling point from SP1 to SP4 in the Saijo River. This sampling area has two human fecal point sources of contamination from wastewater effluents from the treatment plant (OD process) (SP2) and household septic tanks (activated sludge method) in the Togo River basin (SP3) (Fig. 1). Lower concentrations of enterococci, \( E. coli \), and \( C. perfringens \) were observed at SP1 before the inflow of the two human point sources of contamination. The concentrations of the FIBs, enterococci, and \( E. coli \) were 0.65 and 0.52 MPN/mL, respectively. The concentrations of \( C. perfringens \) and \( C. perfringens \) isolates were 0.27 and 0.0037 cfu/mL, respectively. After SP2 and SP3 inflows, the concentrations of FIBs and \( C. perfringens \) were slightly increased downstream these two inflow points (SP4): 0.81 MPN/mL of enterococci, 1.37 MPN/mL of \( E. coli \), 0.54 cfu/mL of \( C. perfringens \), and 0.1 cfu/mL of \( C. perfringens \) isolates. Comparing SP1 and SP4, the \( p \) values from the Student \( t \)-test for \( C. perfringens \), \( E. coli \), and enterococci were 0.08, 0.03, and 0.78, respectively. That is, \( E. coli \) concentration showed a significant increase, \( C. perfringens \) also had a tendency to increase, but no significant difference was observed.

The estimated loads of FIBs and \( C. perfringens \) at SP4 and the inflow from two point sources coincided with the loads obtained from the actual measurements of FIB and \( C. perfringens \) concentrations. The measured value of enterococci was \( 5.7 \times 10^{11} \) MPN/m³/day for the estimated load of \( 5.1 \times 10^{11} \) MPN/m³/day. The measured value of \( E. coli \) was \( 7.5 \times 10^{11} \) MPN/m³/day for the estimated load of \( 5.5 \times 10^{11} \) MPN/m³/day. The measured value for \( C. perfringens \) was \( 3.2 \times 10^{11} \) cfu/m³/day for the estimated load of \( 3.3 \times 10^{11} \) cfu/m³/day. The values obtained from these point source inflows confirmed that FIBs and \( C. perfringens \) increased at SP4.

SP7 and SP8 in the Koayu River, a branch of the Sagami River water system, were located in a mountainous area with a small population. Treated wastewater effluents from a large swine farm flow into the Koayu River between SP7 and SP8. Therefore, the concentration of \( E. coli \) increased from 1.39 to 2.03 MPN/mL after the inflow, and that of \( C. perfringens \) increased from 0.1 to 1.45 cfu/mL (Table 3).

Effect of inflow from human non-point source contamination on FIB concentration

Table 3 shows the bacterial concentration at each sampling point corresponding to SP5 to SP6 in the Nagaike River in the Sagami River system (Fig. 2). Sampling points in the Nagaike River, SP5 and SP6, were located in an urban area, and high fecal contamination from non-point human sources was analyzed. At SP5 and SP6, the concentrations of \( E. coli \) were 7.24 and 12.2 MPN/mL, respectively, whereas those of enterococci were 12.23 and 6.85 MPN/mL, respectively. High concentrations of \( C. perfringens \)—1.87 and 1.99 cfu/mL—were also observed at SP5 and SP6 points, respectively. According to the comprehensive Sagami River-related information published by the Ministry of Land, Infrastructure and Transport of Japan [34], land use in the river basin is about 73% in mountainous areas, about 7% in agricultural lands such as paddy fields, and about 3% in rivers and lakesides. Residential land and other urban areas account for about 12% of the total land, and the population is concentrated in urbanized areas, such as Atsugi.
Table 2 Concentrations and pollution loads of indicators in the Saijo River (inflow point source of human contamination).

<table>
<thead>
<tr>
<th>Sampling point</th>
<th>Type of fecal contamination sources</th>
<th>Average concentrations (MPN or cfu/ml)</th>
<th>Average pollution loads (MPN or cfu/m³/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EC $(n = 10)$ ENT $(n = 8)$ CP $(n = 10)$ calculated cpe $(+)$ CP</td>
<td>EC ENT CP</td>
</tr>
<tr>
<td>SP1※1)</td>
<td>Saijo River: before inflow human point sources</td>
<td>0.52 (SD. 0.43) 0.65 (SD. 0.72) 0.27 (SD. 0.27) 0.03</td>
<td>13.3 (92/691) 4.1 × 10¹¹ 4.0 × 10¹¹ 2.3 × 10¹¹ 2.3 × 10¹⁰</td>
</tr>
<tr>
<td>SP2※2)</td>
<td>Point source: sewage effluents</td>
<td>11.32 (SD. 21.92) 0.8 (SD. 0.71) 31.4 (SD.19.26) 7.93</td>
<td>30.2 (129/427) 3.6 × 10¹⁰ 2.5 × 10⁹ 9.9 × 10¹⁰ 2.5 × 10⁹</td>
</tr>
<tr>
<td>SP3※2)</td>
<td>Point source: Togo river (Receiving effluents from domestic septic tanks in basin area)</td>
<td>4.44 (SD. 3.37) 3.25 (SD. 4.47) 0.26 (SD. 0.28) 0.05</td>
<td>20.4 (70/343) 1.0 × 10¹¹ 1.1 × 10¹¹ 9.3 × 10⁹ 1.7 × 10⁹</td>
</tr>
<tr>
<td>Estimate SP4 load</td>
<td>(total load of SP1 to 3)</td>
<td>- - - -</td>
<td>5.5 × 10¹¹ 5.1 × 10¹¹ 3.3 × 10¹¹ 5.0 × 10¹⁰</td>
</tr>
<tr>
<td>SP4</td>
<td>After inflow two human point sources</td>
<td>1.37 (SD. 1.00) 0.81 (SD. 1.33) 0.54 (SD. 0.34) 0.1</td>
<td>21.5 (95/442) 7.5 × 10¹¹ 5.7 × 10¹¹ 3.2 × 10¹¹ 4.3 × 10¹⁰</td>
</tr>
</tbody>
</table>

EC: E.coli, ENT: enterococci, CP: C.perfringens, (+): positive, SD: standard deviation
※1 the flow rate of the Saijo river is $5.1 \times 10^5$ m³/day
※2 percentage of sewered population is 96.3%, sewered population is $8.7 \times 10^3$ [unpublished data]
  the discharge amount of Shobara wastewater treatment plant is $3.1 \times 10^3$ m³/day [unpublished data]
※3 the flow rate of the Togo river is $2.2 \times 10^4$ m³/day [measured value]
  population of user of domestic septic tanks sewer system is $3.3 \times 10^2$ [unpublished data]
<table>
<thead>
<tr>
<th>Sampling point</th>
<th>Type of fecal source</th>
<th>Average concentrations (MPN or cfu/ml)</th>
<th>Average pollution loads (MPN or cfu/m³/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EC ((n = 2–3)) ENT ((n = 3)) CP ((n = 4–6)) calculated cpe (+) CP</td>
<td>EC ENT CP cpe (+) CP</td>
</tr>
<tr>
<td>SP5※1)</td>
<td>Nagaike river (Receiving effluents from domestic septic tanks in basin area (non point human sources))</td>
<td>7.24 (SD. 7.68) 12.23 (SD. 9.20) 1.87 0.52</td>
<td>27.6 (58/210) 2.1 × 10¹² 3.5 × 10¹² 5.4 × 10¹¹ 1.5 × 10¹¹</td>
</tr>
<tr>
<td>SP6</td>
<td>ENT ((n = 3)) CP ((n = 4–6)) calculated cpe (+) CP</td>
<td>12.2 (SD. 2.19) 6.85 (SD. 6.62) 1.99 0.48</td>
<td>26.4 (66/250) 3.5 × 10¹² 2.0 × 10¹² 5.8 × 10¹¹ 1.4 × 10¹¹</td>
</tr>
<tr>
<td>SP7※2)</td>
<td>Koayu River: before inflow points ources, very small population and human fecal pollution</td>
<td>1.39 (SD. 1.21) 4.48 (SD. 4.71) 0.1 0.003</td>
<td>3.6 (6/166)</td>
</tr>
<tr>
<td>SP8※3)</td>
<td>Koayu River: after inflow points ources (effluents of swine waste water treatment plant)</td>
<td>2.03 (SD. 0.88) 4 (SD. 2.66) 1.45 0.06</td>
<td>3.9 (11/279)</td>
</tr>
<tr>
<td>SP9</td>
<td>Sagami River (downstream of the main river)</td>
<td>1.24 (SD. 0.28) 4.49 (SD. 5.34) 1.41 0.1</td>
<td>5(13/258)</td>
</tr>
</tbody>
</table>

EC : *E.coli*, ENT : enterococci, CP : *C.perfringens*, (+) : positive, SD: standard deviation
※1 The population is 62,000 and percentage of sewered population is 97.2% [33]
The flow rate of the Nagaike river is 2.9 × 10⁵ m³/day [measured value]
※2 The population is 712 and percentage of sewered population is 97.6% [34]
※3 The number of swines is 4,900. [34]
city, in the downstream area of the basin of the Nagaike River.

**Distribution of *C. perfringens cpe(+) in point and non-point source contaminated areas**

SP4 at the Saijo River is a model of human point source inflow. Consequently, the concentration of *C. perfringens cpe(+) was one order higher, and its pollution load was two times higher as compared to those at SP1, which is upstream of SP4 (Table 2). This increase was considered to be due to the influx of fecal contamination and was similar to that of the analyzed FIBs. In areas with human fecal contamination, the *cpe*-positive prevalence rate was higher, with 30.2% at SP2 at Shobara WWTP and 20.4% at SP3 in the Togo River. After the inflow of these two human point sources, the *cpe*-positive prevalence rate increased from 13.3% (SP1) to 21.5% (SP4). This result was consistent with previous studies showing that *C. perfringens cpe(+) is ubiquitous in human feces [24]. In addition, the pollution load of *C. perfringens cpe(+) increased from 2.3 × 10^{10} cfu/m^3/day at SP1 to 4.3 × 10^{10} cfu/m^3/day at SP4. Furthermore, when the total values of SP1, SP2, and SP3 were compared with the SP4 theoretical value, the measured value was 4.3 × 10^{10} cfu/m^3/day, compared to the theoretical value of 5.0 × 10^{10} cfu/m^3/day. The estimated basic unit from the microfication model of SP2 (Shobara WWTP effluents) was 1.1 × 10^7 cfu/person/day for *C. perfringens* and 2.5 × 10^6 cfu/person/day for *C. perfringens cpe(+).

In the non-point human fecal contamination models in the Nagaike River, SP5, and SP6, the concentration of *C. perfringens* was one order higher than the concentration upstream of the Saijo River (SP1), which has low human pollution. The *cpe(+) prevalence rates were 27.6% and 26.4% at SP5 and SP6 in the Nagaike River, respectively. This appears to be the result of the influx of human fecal contamination and the cause of the increased concentration of FIBs. The pollution load of *C. perfringens cpe(+) in the Nagaike River (SP5 and SP6) is 1.4–1.5 × 10^{11} cfu/m^3/day higher than that of sewage effluent from Shobara WWTP (SP2; 2.5 × 10^{10} cfu/m^3/day). The estimated basic unit from the population of the river basin at the Nagaike River was 3.4 × 10^5 cfu/person/day for *C. perfringens* and 8.2 × 10^7 cfu/person/day for *C. perfringens cpe(+)*. The septic tanks installed in each household of the Nagaike River basin are simple, on-site small treatment facilities, and have a different treatment capacity when compared to the large sewage treatment facility. Thus, it is very likely that the pollution load of the Nagaike River, after the inflow of sewage, exceeded that of effluents of WWTP (SP2). The daily load per person may vary depending on the performance of the treatment facility.

In the Koayu River, the model of point livestock contamination, the usefulness of *C. perfringens cpe(+) as a human MST indicator, was assessed. Between SP7 and SP8, the concentration of *E. coli* and *C. perfringens* increased clearly from 1.39 to 2.03 MPN/mL (since *p* = 0.0038 *t*-test, following the inflow of swine wastewater. In contrast to the areas of suspected human-derived contamination (26–27%: SP5 and SP6) and the treated-sewage effluents (30%: SP2), the low prevalence rate of *C. perfringens cpe(+) was not changed, even after the inflow of treated swine wastewater (3.6%–3.9%: SP7 and SP8). All raw data used in the calculation are shown in the supplementary materials.

**DISCUSSION**

Various microorganisms have been proposed as MST indicators. In particular, human adenovirus and human polyomavirus are promising human MST indicators because of their absolute species specificity [36]. PMMoV is a promising MST indicator as its concentration is highly correlated with human fecal contamination [21]. However, when introducing a virus as an MST indicator, many technical issues, such as the need to concentrate the virus, the use of a highly sensitive detection device, and plotting a calibration curve for qPCR analysis [18–21]. Conversely, the number of living bacteria can be confirmed by simple culture methods, and the quantification work is relatively easy compared to that with viruses. Considering the aforementioned facts, in this study, we proposed *C. perfringens cpe(+) as an MST indicator for point and non-point source contamination in rivers.

The analysis of the point source contamination in the Saijo River and the Koayu River showed that the prevalence rate of *C. perfringens cpe(+) increased only after the inflow of sewage effluents or river water containing human-derived pollution, thereby confirming that *C. perfringens cpe(+) can quantitatively assess human fecal contamination as an MST indicator in the evaluation of point sources of contamination. This can be inferred from the fact that conventional FIBs simply increased before and after the influx of fecal contamination, whereas the influx of human feces increased the prevalence rate of *C. perfringens cpe(+) and their ratio was consistent. Besides, the amount of *C. perfringens cpe(+) increased in concentration and pollution load at the influx point of human wastewater effluents, whereas it remained unaffected by swine wastewater inflow, thus providing
evidence that the analysis of C. perfringens cpe(+) is useful as a human-specific MST indicator. Even in rivers that are presumed to be highly contaminated by human fecal surface sources, conventional FIB concentrations increased, and the prevalence rate of C. perfringens cpe(+) was similar to that of the sewage treatment plant effluent, a model of point source contamination. This is consistent with previous studies showing that C. perfringens cpe(+) is ubiquitously present in human feces [24], thus suggesting that C. perfringens cpe(+) is effective as a human MST tool even in point sources of human feces contamination.

In addition to the ubiquitous distribution of C. perfringens cpe(+) in human feces [24], the higher viability, and the higher correlation with intestinal viruses [29], C. perfringens cpe(+) is effective as a human MST indicator. By accumulating data in the future, it may be possible to semi-quantitatively assess the level of human fecal contamination in water bodies from the concentration and flow rate of C. perfringens cpe(+), to estimate the number of people potentially associated with the contamination. As a tailwind to the introduction of C. perfringens as an MST indicator, C. perfringens is easy to culture, and the cpe-positive prevalence rate can be evaluated using PCR, thus allowing for an easier analysis than when using a virus as an MST indicator. Of note, while PCR is simpler in terms of equipment and process than qPCR. Furthermore, the viral activity cannot be quantified by qPCR, which is typically used without culturing. For this reason, there is a possibility that the residue of contamination that is spatiotemporally segregated from the surveyed area of fecal contamination will be evaluated. Nevertheless, cpe(+) was confirmed to be activated or inactivated by culture; thus, it offers the advantage that the spatiotemporal information of contamination is preserved.

In addition, C. perfringens cpe(+) spores can persist in the environment for long periods and can be an indicator of long-term fecal contamination. Additionally, C. perfringens cpe(+) can be used to indicate the efficiency of chlorine disinfection of drinking water against protozoa, such as Cryptosporidium, in waterworks [23].

Strikingly, there is a 10-fold difference between effluents of the sewage treatment plant at the Saijo River and the Nagaike River in the basic unit per person of C. perfringens cpe(+) (2.5 × 10^6, 8.2 × 10^7 cfu/person/day, respectively). We believe this is due to the difference between large-scale sewage treatment plants and household septic tanks. Therefore, to use C. perfringens cpe(+) as a quantitative MST indicator, it is necessary to consider the conditions of the basin and the treatment method of the inflowing contamination. Another issue is that the detection of cpe in C. perfringens cultures is complicated, while the overarching goal is to establish a semi-quantitative detection method using qPCR that does not depend on the ability of the operator and only requires one step.

Generally, meat, such as poultry, has been suspected to be an important source of contamination of C. perfringens; however, many studies reported low detection of cpe strains among retail meat [30–32]. As shown in this study, despite sewage treatment and chlorination, high loads of C. perfringens cpe(+) were released to the environment from a sewage treatment plant (in the Saijo River: 2.5 × 10^10 cfu/m^3/day) and household septic tanks in the basin area (in the Nagaike River: 1.4 × 10^11 cfu/m^3/day). Although the contamination route of C. perfringens food poisoning is still unclear, these treated and chlorinated effluents may be important pollution sources of irrigation water, marine products, vegetables, live-stock, etc. Disinfection of sewage effluents using chlorine is widely adopted worldwide and will continue to be a priority for disinfection technology because of its broad spectrum of sterilization and outstanding cost-effectiveness. Nevertheless, various problems have been identified in recent years, such as the effects of residual chlorine and by-products, such as trihalomethane, in aquatic organisms [37], and chlorine odor. Our data show that chlorinated effluents are a major source of contamination of C. perfringens cpe(+), pointing to the need to consider alternative disinfectants, such as peracetic acid, that can be expected to affect spores.

**CONCLUSIONS**

Detection of fecal contamination in water environments is crucial to prevent waterborne disease transmission through drinking water and recreational water. In this study, we evaluated the effectiveness of Clostridium perfringens cpe-positive strain as an MST indicator in river environments. The cpe-positive prevalence rate of C. perfringens isolates increasing from 13.3 to 21.5% after the inflow of point source contamination and was high in a model river of non-point source contamination, 27.6 and 26.4%. In contrast, in the Koayu River, which was contaminated with treated wastewater from a point source of large swine farm contamination, increased FIB and C. perfringens concentrations were observed, whereas the concentration of C. perfringens cpe(+) remained unchanged 3.6 to 3.9%. Taken together, our results revealed that the assessment of the concentration and pollution load of C. perfringens cpe(+) is an effective MST indicator of human fecal contamination in freshwater environments.
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SUPPLEMENTARY MATERIALS

Supplementary Materials file for this article is available at the link below.
https://www.jstage.jst.go.jp/article/jwet/19/1/19_20-089/_supplement/_download/19_20-089_1.pdf

REFERENCES


