Original Article

Distribution of Enterotoxin Gene-positive Clostridium perfringens Spores among Human and Livestock Samples and its Potential as a Human Fecal Source Tracking Indicator

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ABSTRACT

In this study, to evaluate whether Clostridium perfringens could be a useful fecal indicator in aquatic environments and could be employed as a potential source-tracking indicator, the distribution of C. perfringens spores and their toxin types in sewage and livestock samples were analyzed. A total of 804 C. perfringens spore isolates (366 from human-related sewage and effluents, 128 from cattle, 129 from pigs, 72 from chicken, and 109 from abattoir wastewaters) were analyzed using multiplex polymerase chain reaction (PCR) to detect six C. perfringens toxin genes. On the basis of the presence of toxin genes, most of the isolates from both human sewage and livestock samples were determined as C. perfringens type A and they expressed cpa alone or cpa and C. perfringens enterotoxin (cpe) with or without cpb2. Moreover, cpe-positive C. perfringens was detected with frequencies of 29% and 32% in human sewage and effluents, respectively. However, only one isolate (from cattle feces) was cpe-positive among all the livestock samples tested. Thus, the distribution of cpe-positive C. perfringens should be considered an important source tracking indicator for human fecal pollution. Furthermore, we conclude that sewage effluents are a significant source of cpe-positive C. perfringens pollution.

Keywords: Clostridium perfringens, Clostridium perfringens enterotoxin, fecal indicators, source tracking

INTRODUCTION

Sulfite-reducing clostridia, including Clostridium perfringens and its spores, are ubiquitous in the feces of warm-blooded animals. Highly resistance of C. perfringens to various environmental stresses and disinfectants were reported. Therefore, these bacteria are considered to be suitable indicators for detecting fecal pollution in aquatic environments [1–7].

Compared with current traditional fecal indicators such as Escherichia coli, C. perfringens and its spores survive better in various aquatic environments. It has been shown that the concentration of E. coli was higher than that of C. perfringens at the point nearest to a source of contamination, but the E. coli population decreased rapidly and the concentrations declined with increasing distance from the point source [4]. In streams, the concentration of C. perfringens spores more consistently represents the influence of a human point source compared with fecal streptococci and coliform bacteria [5]. Furthermore, analysis of C. perfringens can yield more meaningful results than that of fecal streptococci and total coliforms in the context of fecal indicators in water environments in tropical countries [8]. Compared with other fecal indicators such as E. coli, fecal streptococci, and other coliform bacteria, C. perfringens is more abundant and resilient in marine and freshwater sediments [9]. Because of their robust viability in water environments, anaerobic clostridia are recommended for use as indicators
of Cryptosporidium and Giardia in Japanese regulations related to drinking water sources [10]. The importance of clostridia as a removal indicator for both Cryptosporidium and Giardia in drinking water treatment using rapid sand filtration [11] and in sewage treatment using activated sludge [12] has also been reported. Furthermore, since C. perfringens is resistant to chlorination and can be used as a surrogate indicator of water treatment effectiveness for protozoa and other chlorine-resistant pathogens [4,11,13,14].

Subtypes of C. perfringens is classified into five types (A to E) based on the combination of toxins that the species produces, and the permutations are derived from four toxins (α, β, ε, and ι) [15]. All C. perfringens produce α toxin (cpa, phospholipase A), and some type A C. perfringens strains produce enterotoxin (cpe), which causes C. perfringens food poisoning in humans [16,17]. Traditionally, C. perfringens has been used as a fecal indicator and it is also one of the major pathogens responsible for toxic food poisoning [16,17].

The objective of this study was to evaluate the suitability of C. perfringens as an indicator of fecal pollution as well as to identify the C. perfringens spores and their toxin gene types, which can be used as possible indicators for human fecal source tracking. Based on our survey of human-related sewage and livestock fecal samples, the main finding of this study is that the distribution of cpe-positive C. perfringens demonstrates their potential as a microbial source tracking indicator. Furthermore, we evaluated the efficiency of the relative ratio of the E. coli and C. perfringens spore concentrations (EC/CP ratio) for fecal source tracking.

MATERIALS AND METHODS

Samples and sampling process

Grab samples of influent sewage and chlorinated effluents from three wastewater treatment plants were tested in this study. Effluent samples were dechlorinated immediately using sodium thiosulfate. The S and M plants are small-scale plants that treat domestic wastewater from rural areas (with populations of about 10,000) in Hiroshima Prefecture, Japan. The H plant is a large-scale facility located in an urban area in Hiroshima Prefecture, Japan. The maximum amount of water treated is 200,000 m³/d. In total, 13 water samples were collected from the S plant over 1 year, whereas two water samples were collected from the H plant and three from the M plant.

Samples from livestock were collected from two cattle farms and one pig farm in Hiroshima Prefecture, Japan over the course of 1 year. Samples were also collected twice from each of the two chicken farms. In order to evaluate a wider area with larger sample sizes, influents and effluents from an abattoir wastewater treatment plant were tested. Sampling was performed for two days at the abattoir, which had processed 10 cattle and 86 pigs on the first sampling day and 125 cattle and 258 pigs on the second sampling day.

One-liter water samples were collected from the S, M, and H wastewater treatment plants and the wastewater treatment plants at the pig farm and abattoir. Fresh composite fecal samples were collected from storage areas at the cattle and chicken farms. In order to compare fresh samples, chicken samples collected over the course of two days were also used in this study. Fecal samples resuspended in phosphate-buffered saline (PBS) solution were used in the bacterial analyses.

Detection and enumeration of C. perfringens spores

To inactivate vegetative cells, 10 mL wastewater samples and livestock fecal samples suspended in PBS were heated in a water bath at 75°C for 20 min in a water bath and then cooled immediately in an ice bath. Spores of C. perfringens were enumerated using a triple-layered Handford modified agar method [10,18], where 5 mL of autoclaved Handford agar was placed on a plate until the medium solidified. The second layer comprised 1 mL of ten-fold serially diluted sample. Then, 10 mL of the medium was poured on top. After solidification, 5 mL of the medium was then poured onto the plate as a third layer. The plates were incubated at 44°C for 24 h in anaerobic jars. Sulfite-reduced black colonies were counted and several colonies were randomly isolated with an inoculating needle. The isolated colonies were smeared on Columbia agar with 5% sheep blood as an enrichment culture.

Multiplex polymerase chain reaction (PCR) for C. perfringens toxin genes

Pure culture colonies from the sheep blood agar were suspended in Tris-EDTA buffer and stored at −20°C. To prepare the DNA templates, 5 μL of the stored melted samples, 1 μL of 10% Triton-X 100, and 4 μL of double distilled water (DDW) (Milli-Q, Merck-Millipore, Darmstadt, Germany) were dispensed into PCR tubes, which were then placed in a thermal cycler at 95°C for 5 min. Multiple primers were used for six C. perfringens toxin genes (cpa, cph, cph2, etx, iap, and cpe), which have been described previously [19]. The final 50 μL volume contained 1 μL of template DNA, 0.4 μM cph2 primer, 0.2 μM of each of the other five primer pairs, and 25 μL of 2 × premix PCR solution (Takara-Bio,
Kusatsu, Japan). All PCR was performed under the following conditions: one cycle at 94°C for 15 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 90 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. *Clostridium perfringens* NCTC 4964 and NCTC 8084 (*cpa*, *cpb*, *etx*, *iap*), and *C. perfringens* isolated from sewage (*cpa*, *cpe*, *cpb*2), were utilized as positive controls. Double distilled water was used as a negative control. PCR-amplified products were analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide; they were visualized under UV light. The six target toxin genes yield specific products of 324 (*cpa*), 198 (*cpb*), 548 (*cpb*2), 376 (*etx*), 272 (iap) and 485 (cpe) bp.

**Quantification of E. coli**

One pack of Colilert medium (Idexx Laboratories, Westbrook, United States) was suspended in each sample or diluted sample (100 mL). To estimate the most probable number (MPN) concentration, the samples were poured into Quanti-trays (Idexx Laboratories, United States) and sealed using a Quanti-tray sealer. The trays were incubated at 37°C for 24 h. After incubation, the number of blue fluorescent cells was counted under UV and the MPN was estimated using Idexx Software.

**RESULTS**

**Distribution of C. perfringens toxin types in sewage and livestock samples**

In total, 366 isolates from three sewage treatment plants were tested by PCR to identify the major *C. perfringens* toxin genes (Table 1). Most of the isolates (360 of 366, 98%) were positive for the *C. perfringens* alpha toxin gene (*cpa*), and 359 of 360 isolates were classified as *C. perfringens* type A. In the type A *C. perfringens* group, 222 isolates had both *cpa* and *cpb*2 with or without the *C. perfringens* enterotoxin gene (*cpe*), whereas 104 isolates only had *cpa*. In total, 109 isolates were *cpe*-positive (33 isolates had *cpa* and *cpe*, and 76 isolates had *cpa*, *cpe*, and *cpb*2). Only one isolate was classified as type B; this had the *cpa*, *cpb*, and *etx* genes.

Most of the isolates from livestock samples were *cpa*-positive and they were classified as type A (378 of 438, 86%), which was similar to the samples from human-related sewage. Type A isolates with the *cpb*2 gene were dominant in the pig and chicken fecal samples. In the cattle fecal samples and sewage from the abattoir that processed beef cattle, *cpa*

<p>| Table 1 Distribution of the toxin types of <em>C. perfringens</em> spores. |
|----------------|----------|----------|----------|----------|----------|----------|</p>
<table>
<thead>
<tr>
<th>Detected toxin gene</th>
<th>Type A</th>
<th>Type B</th>
<th>Type C</th>
<th>Type E</th>
<th>Toxin gene</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpa alone</td>
<td>75</td>
<td>85</td>
<td>25</td>
<td>41</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cpa + cpb2</td>
<td>29</td>
<td>61</td>
<td>8</td>
<td>35</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total human-related samples</td>
<td>104</td>
<td>146</td>
<td>33</td>
<td>76</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Influent sewage at a pig farm</td>
<td>36</td>
<td>82</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Cattle feces</td>
<td>76</td>
<td>50</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Chicken feces</td>
<td>1</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Influent from the wastewater treatment plant at an abattoir</td>
<td>29</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Effluent from the wastewater treatment plant at an abattoir</td>
<td>59</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total livestock-related samples</td>
<td>201</td>
<td>169</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Total samples tested</td>
<td>305</td>
<td>315</td>
<td>34</td>
<td>76</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>
alone was the dominant type. We detected only five type C isolates from pigs and cattle and two type E isolates from pigs. Type D was not detected in any of these samples.

There was a marked difference in the distribution of cpe-positive C. perfringens among the human-related sewage and the livestock samples (Table 2). We found that 30% (109 of 366) of the C. perfringens isolates had the cpe gene in human-related sewage, whereas among the 438 isolates from livestock samples, only one isolate from cattle feces was cpe-positive. Seasonal trends and considerable differences in the percentage of cpe-positive C. perfringens among the tested plants were not observed.

Concentrations of C. perfringens spores and E. coli in sewage- and livestock-related samples

The concentrations of C. perfringens spores and E. coli were measured in sewage and livestock samples (Table 3). The average concentrations of C. perfringens spores were $4.1 \times 10^3$ cfu/mL in influent sewage and 20 cfu/mL in chlorinated effluents. The E. coli concentration in influent sewage was $8.8 \times 10^8$ MPN/mL, which is ten times the amount of C. perfringens spores. In effluent samples, the average concentration was 7 MPN/mL. Concentrations per dry weight of both C. perfringens and E. coli in partial influent sewage samples (n = 7) were estimated, with average concentrations of $2.2 \times 10^7$ (Standard Deviation: SD = $3.2 \times 10^7$) cfu/g and $4.5 \times 10^8$ (SD = $3.0 \times 10^8$) MPN/g, respectively.

In livestock samples, the average concentrations of C. perfringens spores ranged between $8.2 \times 10^2$ cfu/mL (pig) and $9.1 \times 10^7$ cfu/g (wet) (chicken feces (old)). The E. coli concentrations ranged between $7.0 \times 10^5$ MPN/g (cattle) and $3.8 \times 10^9$ MPN/g (wet) (chicken feces (fresh)) (Table 3).

Concentrations per dry weight of both C. perfringens and E. coli in partial pig samples (n = 3) were estimated, with average concentrations of $1.3 \times 10^4$ (SD $1.4 \times 10^4$) cfu/g (equal to human sewage) and $3.3 \times 10^8$ (SD $4.1 \times 10^8$) MPN/g, respectively.

Concentrations of both indicators in chicken feces were markedly different between fresh and old samples. Old fecal samples were stored in an outside stock space of the farm for at least two days. After storage, it is thought that E. coli were inactivated and decreased in concentration, whereas C. perfringens remained active and increased in relative concentration.

As an overall tendency, the concentrations of E. coli in human-related samples and livestock samples were approxi-
mately of an equal level, whereas higher *C. perfringens* spore concentrations were observed in human-related samples. However, the concentrations could not be compared directly among these samples because the form (liquefied or slurry) and components (relative proportions of feces, other debris, and water) differed markedly across these samples. Thus, the ratio of the concentration of *E. coli* relative to *C. perfringens* spores (EC/CP ratio) was calculated to allow us to compare the bacterial density in these samples (Table 3). The EC/CP ratio was 42 in the influent human-related sewage, but it was much higher in cattle feces (3,300), fresh chicken feces (50,000), and pig sewage (730,000). Thus, based on the EC/CP ratios and comparisons of the concentrations per feces (50,000), and pig sewage (730,000). Thus, based on it was much higher in cattle feces (3,300), fresh chicken 

The *H* sewage treatment plant was the largest of the three plants tested in this study. This plant treats domestic waste from an urban population of ~700,000 using standard activated sludge and chlorination processes. The plant has a capacity of 300,000 m³/d. Based on the average concentration of *C. perfringens* spores in the chlorinated effluents (6 cfu/mL), frequency of *cpe*-positive isolates (32%), and plant capacity, we estimated that the concentration of *cpe*-positive *C. perfringens* was 1.86 cfu/mL and that the daily pollution load was as high as 5.6 × 10¹¹ cfu/d.

**DISCUSSION**

Spores of *C. perfringens* are found in the feces of warm-blooded animals, and they remain viable for long periods in environmental waters [2,4,5]. Thus, *C. perfringens* and its spores are important surrogate indicators of fecal pollution and of the efficacy of water treatment [1–8,10–12,20].

The results obtained in this study suggest that *C. perfringens* spores may be a useful indicator of human fecal pollution in water environments. It is likely that the presence of *cpe*-positive *C. perfringens* in the water environment is a strong indicator of human fecal contamination. Isolates of *C. perfringens* are classified according to toxin types A to E. In the present study, most of the *C. perfringens* spores isolated from human, cattle, pig, and chicken were classified as type A. Our study showed that *cpe*-positive type A *C. perfringens* isolates were frequent in human-related samples with an isolation frequency of approximately 30% from raw sewage (66 *cpe*-positive *C. perfringens* from 230 tested sewage sample isolates).

According to several studies on the frequency of *cpe* in human feces, animal feces, and retail meat, an uneven distribution was proposed; for example, the *cpe*-gene-positive *C. perfringens* was present in the feces of 18.4% of healthy food handlers [21]. Three isolates of *cpe*-positive *C. perfringens* were detected among 43 fecal isolates of type A *C. perfringens* in a cohort of 23 healthy people [22]. Furthermore, Saito [23] suggested that humans are a possible reservoir for outbreaks of food poisoning caused by *cpe*-positive *C. perfringens* since five of 80 samples (6%) from the feces of food handlers were *cpe*-positive. These studies did not report the percentages or quantity of *cpe*-positive *C. perfringens* in individual host feces. In addition, both the percentage and quantity of *cpe*-positive *C. perfringens* in individual host feces, and the percentage of *cpe*-positive *C. perfringens* carrier in a population, may differ based on the geographical location, age structure, and health condition of the host. However, the detection and isolation of *cpe*-positive *C. perfringens* from healthy human feces and sewage clearly showed that human feces are a very important source of *cpe*-positive *C. perfringens*.

In agreement with our results, earlier studies have reported a low frequency of *cpe*-positive *C. perfringens* in samples of animal feces and retail meat. For example, only one sample from 10 cattle fecal samples, and no samples from 10 pig fecal samples, were found to be *cpe*-positive [24]. No *cpe*-positive stool samples from 76 chickens, 131 pigs, and 51 cattle were detected, and only two of 106 dog samples were positive [23]. Furthermore, *cpe*-positive *C. perfringens* was not detected among 73 isolates from the stool samples of healthy and sick poultry, cows, and pigs [25]. A low *cpe* frequency was also reported based on a survey of retail meat and other food samples. In particular, although *C. perfringens* was detected in 142 of 200 samples (beef, chicken, and pork), only two from beef and one from chicken were *cpe*-positive [26]. In another study of 887 food samples, 278 were positive for *C. perfringens* but only 10 were also *cpe*-positive [27]. The type A strain of *C. perfringens* was detected in 22 of 180 turkey meat samples and most were *cpe*-negative [28]. Most surveys of animal feces and retail foods report low occurrence rates of *cpe*-positive *C. perfringens*. The exception was the detection of *cpe* in horses, cattle, and poultry at frequencies of 14%, 22%, and 10%, respectively, using a different detection
method (DNA hybridization) [29].

Because of the uneven distribution of cpe-positive \textit{C. perfringens} in human-related samples and livestock feces, cpe-positive \textit{C. perfringens} could be exploited as a human fecal source-tracking indicator. Previous studies of potential human fecal source-tracking indicators have suggested that viruses, such as polyomaviruses, somatic coliphage, adenoviruses, and pepper mild mottle virus, may all be suitable indicators [30–33]. Human fecal pollution-source tracking is extremely important for investigations of pathogens such as noroviruses or \textit{Cryptosporidium hominis}, which cause illnesses via person-to-person transmission. Although the biological properties and size of \textit{C. perfringens} differ from those of viruses and protozoa, we suggest that \textit{C. perfringens} may be a useful bacterial source-tracking indicator based on measurements of its concentration; thus, tracing the distribution of cpe-positive isolates may be effective in determining the intensity of human-related fecal pollution.

It is also possible that the EC/CP ratio might be employed to detect pollution sources and the relative period of time since the pollution event. The results obtained in the present study suggest that the \textit{C. perfringens} concentrations in human-related samples are higher than those in livestock samples. Higher concentrations of \textit{C. perfringens} in human feces have also been reported previously: depending on the study, the total \textit{C. perfringens} concentration in human feces ranged between $10^{3}$/g and $10^{6}$–$10^{7}$/g in 13%–35% of humans tested [20]. Spores of \textit{C. perfringens} were also detected in the feces of 27 of 43 healthy humans (63%) at a concentration of $10^{5.5} \pm 6$ cfu/g [22].

The average densities of \textit{C. perfringens} in livestock feces were reported in a previous study, where livestock were shown to be major pollution sources in surface water at relatively low concentrations, i.e., cattle (60 cfu per wet gram), sheep (70 cfu per wet gram), and horses (< 10 cfu per wet gram) [5]. Similar results were obtained by Geldreich for cattle (200 cfu per wet gram), pigs (3,980 cfu per wet gram), and especially sheep (199,000 cfu per wet gram) [20]. A more recent survey of domestic animals and wildlife showed that the concentration of \textit{C. perfringens} spores was $2.9 \times 10^{5}$ in pigs, $4.6 \times 10^{3}$ in poultry, and $3.6 \times 10^{5}$ and $3.3 \times 10^{6}$ per gram in dogs and cats. High concentrations were detected in domestic animals but rarely in wildlife [34].

Therefore, it is possible that in some individuals, the frequency is between 10- and 1000-fold higher than that in cattle and pig feces. Our results, and those obtained in previous studies, suggest that the abundance of \textit{C. perfringens} and its density of spores are higher in human feces than livestock feces. Since the composition and ratio of non-fecal debris, feces, water etc. were different between human and livestock samples in this study, it was not possible to compare the human and animal data directly. Therefore, the EC/CP ratio was estimated to compare the results obtained from the sewage and livestock samples. Based on the differences in environmental resistance and primary concentrations of the two indicators in fecal samples, the EC/CP ratio may be a useful tool for estimating fecal pollution sources as well as the relative time or distance from fecal pollution points.

Several factors, including human-related fecal pollution, water treatment, disinfection, and a long duration since discharge, can cause a low EC/CP ratio. A high EC/CP ratio strongly suggests recent pollution from a nontreated livestock discharge. Therefore, we conclude that in addition to the traditional efficacy of \textit{C. perfringens} as an indicator, determining the presence of cpe-positive \textit{C. perfringens} and the use of the EC/CP ratio may be beneficial tools for estimating fecal pollution and source tracking.

From a public health perspective, cpe-positive \textit{C. perfringens} is an important pathogen that causes food poisoning in human populations [16,17]. It is unclear whether cpe-positive \textit{C. perfringens} exhibits host specificity or habitat isolation between humans and other animals. However, our results showed clearly that approximately 30% of the \textit{C. perfringens} isolates from the three different human sewage and effluent sites possessed the cpe gene. A similar cpe gene frequency was detected in influent sewage in the USA [35]. Importantly, we detected cpe-positive \textit{C. perfringens} in chlorinated effluents from all three sewage treatment plants. It is difficult to directly link polluted water in the environment with \textit{C. perfringens} food poisoning cases, but we estimate that the daily load of cpe-positive \textit{C. perfringens} from tested sewage treatment plants is $5.6 \times 10^{11}$ cfu/d. This suggests that despite the chlorination process, sewage effluents are extremely important sources of cpe-positive \textit{C. perfringens} pollution.

**CONCLUSIONS**

\textit{Clostridium perfringens} has been used as a surrogate indicator of fecal pollution in aquatic environments. In this study, the concentrations and toxin genes of \textit{C. perfringens} were tested in human-related sewage and livestock-related samples. In 804 isolates, 738 isolates (360 from human sewage and 378 from livestock samples) were positive for the \textit{C. perfringens} alpha toxin gene (\textit{cpa}). The distribution of enterotoxin gene (cpe)-positive \textit{C. perfringens} among human-related sewage samples was 30% (109 of 366), whereas in...
the livestock samples, only one isolate from cattle feces was detected from 438 isolates.

The average concentrations of *C. perfringens* spores were $4.1 \times 10^3$ cfu/mL in influent sewage and 20 cfu/mL in chlorinated effluents. In livestock samples, the average concentrations of *C. perfringens* spores ranged between $8.2 \times 10^2$ cfu/mL (pig) and $9.1 \times 10^7$ cfu/g (wet) (chicken feces (old)). Overall, the concentrations of *E. coli* in human-related samples and livestock samples were approximately equal, whereas higher *C. perfringens* spore concentrations were observed in human-related samples.

The use of multiplex PCR for the detection of *C. perfringens* toxin genes, particularly those containing the food poison-associated *cpe* gene, may be a powerful approach for monitoring human fecal pollution and source tracking, which is important for controlling human-specific water-borne pathogens such as norovirus or *Cryptosporidium hominis*.

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