Annual and Diurnal Profiles of Cryptosporidium and Giardia in River Water in Japan

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ABSTRACT
A quantitative survey was performed to understand the annual and diurnal profiles of Cryptosporidium and Giardia, representative waterborne infectious protozoans, in river water which is used for drinking water sources in Japan. To investigate the annual profiles, 84 river water samples were collected at 7 sites in the tributary rivers of the Tone River basin in Japan from June 2008 to February 2010. Cryptosporidium and Giardia were detected in 59 (70%) and 64 (76%) out of the 84 samples (10 liters each), showing the highest concentration of 344 oocysts/10 L and 144 cysts/10 L, respectively. Annual variation of the concentrations of Cryptosporidium and Giardia was high. The ratio of the maximum concentration to the mean value at each sampling site ranged from 2–8 except for one sampling site in which the frequency of detection was extremely low. To investigate diurnal profiles, 15 river water samples were collected at 3 sites in the tributary rivers of the Tone River on October 9th, 2008. The maximum concentrations of Cryptosporidium and Giardia in some sampling sites were approximately 10-fold higher than the lowest value. The correlation between the anaerobic spore-forming bacteria and these infectious protozoans was stronger than other microbial indicators (total coliforms, Escherichia coli and heterotrophic bacteria).

Keywords: cryptosporidiosis, Cryptosporidium, drinking water, Giardia, giardiasis.

INTRODUCTION
Cryptosporidium and Giardia are enteric protozoans that cause diarrheal disease and significant adverse health effects in humans (Fayer, 2004; Craun et al., 2005; Sunderland et al., 2007). These parasites are widely distributed throughout the world and transmitted through contaminated water and food. Waterborne cryptosporidiosis and giardiasis are particularly important because their transmissive stages, i.e., oocysts and cysts, respectively, are resistant to disinfectants (such as chlorine) commonly used for water treatment (Peeters et al., 1989; Carpenter et al., 1999). The most notorious waterborne outbreak occurred in Milwaukee, Wisconsin, in 1993 where more than 400,000 suspected and 5,000 confirmed cases of clinical cryptosporidiosis were reported (MacKenzie et al., 1995; Cicirello et al., 1997; Corso et al., 2003). In Japan, an outbreak occurred in Ogose Town, Saitama Prefecture, in 1996 where more than 8,800 suspected cases were reported, accounting for approximately 70% of population of the town.

Cryptosporidium oocysts and Giardia cysts are shed in large numbers in the feces of infected humans and animals (Castro-Hermida et al., 2008). Due to their high resistance...
to environmental stressors, they are ubiquitous in the environment. Many investigators have reported that these protozoans exist in surface waters all over the world (Rose et al., 1991; Castro-Hermida et al., 2009; Mons et al., 2009). In Japan, several surveys on these protozoans in surface waters for drinking water sources have been conducted since 1996 (Ono et al., 2001; Masago et al., 2006). However, there is little information available on annual and diurnal variations in these protozoan concentrations, which makes it difficult to estimate the health risk of protozoan diseases via water supply systems.

The specific objective of this study is to investigate the annual and diurnal profiles of Cryptosporidium and Giardia in river water which is used for drinking water sources. For this purpose, frequent monitoring of protozoans was performed in tributary rivers of the Tone River basin in Japan where the surface water is utilized for the main production of drinking water for people living in the Tokyo metropolitan area. In addition, the relationship between microbial indicators and protozoans was investigated.

MATERIALS AND METHODS
Collection of River Water Samples
To investigate the annual profiles of protozoans, a total of 84 river water samples were collected at 7 sites (sites A–G) in tributary rivers of the Tone River basin in Japan from June 2008 to February 2010 (Fig. 1). Although the water sampling was performed under a normal weather condition, heavy rainfall was observed at the upper area of Tone River basin on the day before the sampling date in June 2009, which increased river flow rates at sites A and B. To investigate the diurnal profiles, a total of 15 river water samples were collected at 3 sites (sites H–J) in tributary rivers of the Tone River on October 9th, 2008. The Tone River has a total length of 322 km and a catchment area of 16,840 km², with approximately 800 tributary rivers. There are many livestock production facilities (cow and pig farms) in the catchment area, especially in the Koyama River and River X basins. Sites A and B are located along the main Tone River. Site B is located near the Tone Diversion Weir, where the surface water is utilized for the production of drinking water for people living in the Tokyo metropolitan area. Sites C and D are located along the Koyama River, which meets the main river between sites A and B, whereas sites E–J are located along the River X, which meets the main river between sites C and D. Sites H and I are located in the area where many livestock production facilities exist, while site J is located closely on the discharge point of treated sewage wastewater.

Fig. 1 - Sampling sites in tributary rivers of Tone River basin
All samples were stored in plastic bottles on ice and delivered to the laboratory within two days of collection. As soon as the samples arrived, microbial tests were conducted.

**Detection of Cryptosporidium Oocyst and Giardia Cyst**

Samples were analyzed according to the Japanese standard procedure for the detection of *Cryptosporidium* and *Giardia* in water supply systems (MHLW, 2007). This procedure was adapted from the U.S. EPA method 1623 for the detection of *Giardia* and *Cryptosporidium* in water by filtration, immunomagnetic separation and fluorescent assay (U.S. EPA, 2001). Briefly, 10 L samples were filtered through a hydrophilic polytetrafluoroethylene (PTFE) type membrane filter (1 μm pore size) (Advantec Toyo, Ltd, Japan). Retained oocysts and cysts were eluted by a 50 mL solution containing sodium pyrophosphate (0.02%), Tween 80 (0.01%) and trisodium EDTA (0.03%), and then centrifuged at 1050 × g for 10 min. Oocysts and cysts in the pellet were purified by immunomagnetic separation (IMS) using *Cryptosporidium* and *Giardia* specific antibody-coated magnetic beads according to the manufacturer’s protocol (Dynabeads GC-Combo, Invitrogen, USA).

The IMS-purified oocysts and cysts were stained on a PTFE membrane filter (0.8 μm pore size) (Advantec Toyo Ltd, Japan) on a glass slide by FITC conjugated antibodies (EasyStain™, BTF Pty Ltd, Australia) and the nuclear fluorochrome 40, 60-diamidino-2-phenylindole (DAPI). Slides were examined using differential interference contrast (DIC) and epifluorescence microscopy at 400 × or 1000 × magnification. Oocysts and cysts were identified when the size, shape, fluorescence, and morphology agree with control parasite suspensions. The number of oocysts and cysts in the whole area of the filter was counted. Results were expressed as oocyst and cyst counts per 10 L.

**Bacterial Analysis**

Concentrations of 4 types of microbial (fecal) indicators (total coliforms, *Escherichia coli*, anaerobic spore-forming bacteria, heterotrophic bacteria) were measured in this study. Total coliforms and *E. coli* were enumerated on Chromocult® Coliform agar (Merck, USA) following incubation for 20 h at 37°C. Blue colonies were counted as *E. coli*, while both blue and red colonies were counted as total coliforms. Anaerobic spore-forming bacteria were enumerated on modified Handford Agar (Eiken Chemical Co., LTD, Japan) following incubation for 24 h at 42°C. Heterotrophic bacteria were enumerated on R2A (Difco, BD, USA) following incubation for 7 d at 20°C. Results were expressed as colony-forming units (CFU) per 100 mL of sample.

**Statistical Analysis**

Spearman rank order correlation coefficients (r_s) were calculated to evaluate the correlation among the concentrations of microbial indicators and protozoans. Analysis was performed using Excel Statistics 2010 (SSRI, Tokyo, Japan). Probability value was set at 0.01. This analysis is often used for evaluating the relationship among the concentrations of microorganisms (Mons et al., 2009). In addition, regression analysis was performed. All the microbial analysis data during annual surveys (n = 84) were used for statistical analysis.
RESULTS AND DISCUSSION

Annual Profiles of *Cryptosporidium* Oocyst and *Giardia* Cyst

Concentrations of *Cryptosporidium* oocysts and *Giardia* cysts during the survey period are summarized in Fig. 2. *Cryptosporidium* and *Giardia* were detected in 59 (70%) and 64 (76%) out of the 84 samples (10 liters each), respectively. Although it has been reported that the frequency of two protozoans in some river basins was significantly different (Mons *et al.*, 2009), the frequency in Tone River basin was almost the same. Actually, *Giardia* was detected in 53 out of 59 (90%) river water samples in which *Cryptosporidium* was detected.

![Fig. 2 - Annual profiles of the concentrations of *Cryptosporidium* and *Giardia* in river water](image-url)
The protozoan concentration in main rivers increased after the confluence of tributary rivers, which indicates that the main river water is contaminated due to the confluence of tributary rivers. In River X, the protozoan concentration at site F was much higher than that at site E. This is because a lot of livestock production facilities (cow and pig farms) which seem to be contamination sources are located between sites E and F. Although the prevalence ratio of these protozoans in livestock in the River X basin is unknown, the ratio in Japanese livestock is generally high. Sarashina et al. (2002) have reported that the prevalence ratio of Cryptosporidium in 0 to 2 week-old Japanese calves was 12%. In addition, the average turbidity at site F was 6.4 while that at site E was 1.2. On the other hand, the protozoan concentration at sites F and G was not so different although the discharge point of treated sewage wastewater is located between sites F and G, which indicates that the sewage wastewater treatment plant is not normally a contamination source in the River X basin.

Annual variation of concentrations of Cryptosporidium and Giardia was high. The ratio of the maximum concentration to the mean value at each sampling site ranged from 2 to 8 except for site C at which the frequency of the detection was extremely low, which indicates that the health risk caused by these protozoans via recreation or water supply greatly fluctuates annually. Meanwhile, the concentration of Cryptosporidium and Giardia in a storm event was relatively high as shown in Fig. 2. Reportedly, heavy rainfalls contribute to protozoan runoff from contaminated soils (Curriero et al., 2001; Carmena et al., 2007).

**Diurnal Profiles of Cryptosporidium Oocyst and Giardia Cyst**

Fig. 3 shows the concentrations of Cryptosporidium oocysts and Giardia cysts during the diurnal survey day. At sites H and I located in the area where many livestock production facilities exist, the concentration of Cryptosporidium was higher than that of Giardia on the survey day. The apparent fluctuation of the concentration of Cryptosporidium was observed. The maximum concentration was approximately 10-fold higher than the lowest value despite the stability of flow rate of River X. The flow rate during the diurnal survey period was 27.2 ± 1.2 m³/min. In Japan, most livestock wastewater treatment plants which discharge the treated wastewater into rivers are operated at sequencing batch mode, and the effluent discharge is normally performed in the morning and/or evening. Therefore, the concentration might increased in the morning and evening. Actually, in the morning, we confirmed that the effluent was discharged from the livestock production facility which is closely located in the sampling sites.

![Diurnal profiles of Cryptosporidium and Giardia](image.png)

Fig. 3 - Diurnal profiles of the concentrations of Cryptosporidium and Giardia in river water (October 9th, 2008). Sites H and I are located in the area where many livestock production facilities exist, while site J is located closely at the discharge point of treated sewage wastewater.
At site J which is located closely at the discharge point of treated sewage wastewater, *Giardia* was detected in high concentration, while *Cryptosporidium* was rarely detected. Therefore, there would be patients of giardiasis in the watershed. Although epidemiological information available on giardiasis in Japan is limited, reportedly, *Giardia* was frequently detected in the influent at another Japanese sewage treatment plant (Oda et al., 2005). The maximum concentration was approximately 10-fold higher than the lowest value. Oda et al. (2005) have reported that the concentration of *Giardia* in the influent sewage wastewater markedly fluctuated although they did not analyze the prevalence in the effluent wastewater.

**Relationship among the Concentrations of Microbial Indicators, Cryptosporidium Oocyst and Giardia Cyst**

Spearman rank correlation analyses were conducted using all the microbial analysis data during the annual surveys (Table 1). Significant correlations (p < 0.01) were found among protozoans and the microbial indicators with \( r_s \) ranging from 0.354 to 0.616 except for total coliforms. The correlation between these protozoans and anaerobic spore-forming bacteria was the strongest among the microbial indicators. One of the reasons why the correlation was high is the resistibility of anaerobic spore-forming bacteria to environmental stressors. Like *Cryptosporidium* oocysts and *Giardia* cysts, it is well known that anaerobic spore-forming bacteria are resistant to chlorination. On the other hand, the correlation between these protozoans and total coliforms was not significant although total coliforms are currently adopted as environmental quality standard indicating microbiological (fecal) contamination in public water bodies in Japan. As evidenced by the regression analysis data (Figs. 4, 5), it is clear that the correlation of total coliforms with these protozoans is weak compared with other indicators.

<table>
<thead>
<tr>
<th>Types of microorganisms</th>
<th>Cryptosporidium</th>
<th>Giardia</th>
<th>Total coliforms</th>
<th>E. coli</th>
<th>Anaerobic spore-forming bacteria</th>
<th>Heterotrophic bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptosporidium</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giardia</td>
<td>0.653*</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total coliforms</td>
<td>0.277</td>
<td>0.154</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>0.453*</td>
<td>0.354*</td>
<td>0.765*</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anaerobic spore-forming bacteria</td>
<td>0.515*</td>
<td>0.616*</td>
<td>0.466*</td>
<td>0.736*</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Heterotrophic bacteria</td>
<td>0.422*</td>
<td>0.402*</td>
<td>0.594*</td>
<td>0.566*</td>
<td>0.580*</td>
<td>1.000</td>
</tr>
</tbody>
</table>

* Significant correlation was obtained (p < 0.01).
Fig. 4 - Correlation among Cryptosporidium oocysts and microbial indicators

Fig. 5 - Correlation among Giardia cysts and microbial indicators
CONCLUSIONS
This study identified some annual and diurnal differences in the concentrations of Cryptosporidium and Giardia in river water which is used for drinking water sources in Japan. In addition, the relationship between microbial indicators (E. coli, total coliforms, anaerobic spore-forming bacteria and heterotrophic bacteria) and protozoans were revealed. The ratio of the maximum concentration to the mean value at each sampling site during the annual survey period ranged from 2 - 8, which indicates that the health risk caused by these protozoans via recreation or water supply greatly fluctuate annually. In the diurnal survey, the maximum concentrations of Cryptosporidium and Giardia at some sampling sites were approximately 10-fold higher than the lowest value. The correlation between these infectious protozoans and anaerobic spore-forming bacteria was stronger than other microbial indicators, while the correlation between these protozoans and total coliforms was not significant although total coliforms are currently adopted as environmental quality standard indicating microbiological (fecal) contamination in public water bodies in Japan.

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