Quantification and genotyping of Cryptosporidium spp. in river water by quenching probe PCR and denaturing gradient gel electrophoresis

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Abstract A new detection method was developed for the simultaneous quantification and genotyping of Cryptosporidium spp. in river water. Several modifications made to the US EPA Method 1623 enabled high and stable recovery of Cryptosporidium from 40 L of river water (geometric mean = 35%, standard deviation = 8.7%). Quenching probe PCR (QProbe PCR) was used to quantify the 18S rRNA gene of Cryptosporidium spp. This method could successfully detect single oocysts in a sample, and the lower quantitation limit was as low as 2.5 oocysts/sample. In addition, denaturing gradient gel electrophoresis (DGGE) followed by DNA sequencing was used to identify the genotypes. These methods were applied to detect Cryptosporidium spp. in the Koyama River, Japan. The positive ratio was 69% (11/16) with the maximum concentration of 59 oocysts/100 L. Seven genotypes including two novel ones were identified. These results showed that this detection method could provide valuable information on Cryptosporidium in river water, both in the concentration and in the genotypes, which is essential for the precise assessment of waterborne risk to human health.

Keywords Cryptosporidium; denaturing gradient gel electrophoresis (DGGE); genotyping; quenching probe PCR (QProbe PCR); real time PCR

Introduction
Genus Cryptosporidium is a pathogenic parasite that causes gastroenteritis in many mammals, including humans. Many Cryptosporidium-associated outbreaks have been reported in the last two decades (Solo-Gabriele and Neumeister, 1996; Craun et al., 1998; Fayer et al., 2000; Rose et al., 2002). In Japan, a large outbreak associated with this microorganism in drinking water occurred in the town of Ogose in 1996, which forced Japanese water treatment facilities to take an urgent action against this parasite (Japanese Ministry of Health, Labour and Welfare, 2001). It is therefore crucial to evaluate the level of contamination by Cryptosporidium in drinking water.

Thirteen species have been reported in the genus Cryptosporidium with a variety of host species (Xiao et al., 2004). Human infection occurs mostly with C. hominis and C. parvum, and their infectivity differs among each species or sub-species (Okhuysen et al., 1999, 2002; Engelhardt and Swartout, 2004). Therefore, it is necessary to identify the species or the genotypes of Cryptosporidium in each sample in order to evaluate the threat of waterborne Cryptosporidium to human health.

Many attempts have been made to detect Cryptosporidium in water. The US EPA Method 1623 (1999) and the Standard Operating Protocols of Drinking Water Inspectorate (1999) are widely used for the detection. These methods are based on the immuno-fluorescent assays (IFA) and microscopy-based counting, but these techniques have difficulty distinguishing between species of Cryptosporidium. Meanwhile,
PCR-based genotyping approaches, such as restricted fragment length polymorphism (RFLP) and DNA sequencing, have been developed for discriminating Cryptosporidium species in water samples. However, these genotyping methods lack in quantifying the concentration of Cryptosporidium in water samples. There have been no reported methods for simultaneous quantification and genotyping of Cryptosporidium in a sample.

In this study, a new detection method was established to determine Cryptosporidium spp. in river water both in concentration and in the genotypes. Several modifications of the US EPA Method 1623 (1999) were made to improve the recovery from large volumes of river water. MF hollow fiber membrane was used for filtration of river water. Discontinuous density gradient centrifugation in a high density (1.20 g/cm³) Percoll-sucrose solution was added prior to IMS. A new real-time PCR method, QProbe PCR, was developed to quantify the 18S rRNA gene of Cryptosporidium spp. in water samples. QProbe PCR is a real-time PCR method that utilises a quenching probe (QProbe) (Kurata et al., 2001). This method is based on the characteristics of a fluorescent dye, BODIPY FL, in that its fluorescence is quenched when it interacts with guanine (Torimura et al., 2001). To identify the species of Cryptosporidium present in the samples, denaturing gradient gel electrophoresis (DGGE) was used to isolate the DNA fragments prior to sequencing. The sequential combination of the QProbe PCR method, the DGGE method and DNA sequencing enabled the simultaneous quantification and genotyping of Cryptosporidium spp. in the same water sample.

This method was used in a field survey conducted in the Koyama River, a tributary of the Tone River, one of the main water sources for drinking water in Tokyo Metropolitan area. Cryptosporidium in the river was analysed both for the total concentration and for the genotype.

Materials and methods

Sample collection

Water samples (23.8–66.1 L) were collected at the Koyama River, a tributary of the Tone River that is a major water source for waterworks in the Tokyo metropolitan area. Sixteen samples were collected from August to November 2004. Each water sample was filtered on site through an MF hollow fiber membrane filter (nominal pore size: 0.1 μm, effective filtration area: 4,000 cm², Mitsubishi Rayon Co., Ltd., Tokyo, Japan).

Concentration, purification and DNA extraction

The particles remaining in the filter modules were recovered by twice manually shaking the filter modules for 1 min in 200 mL of elution buffer as specified in the US EPA Method 1623 (1999), the particles were then concentrated by centrifugation for 15 min at 1,050 g. The components of the concentrate were first separated by discontinuous density gradient centrifugation in a high density (1.20 g/cm³) Percoll-sucrose solution for 10 min at 1,050 g, followed by immunomagnetic separation (IMS) with an IMS kit (Dynabeads anti-Cryptosporidium kit, Dynal Biotech, Tokyo, Japan). The genomic DNA was extracted by five cycles of freezing at −80°C for 5 min followed by thawing at 95°C for 5 min in the presence of 25% w/v of Chelex 100 resin (Bio-Rad, Tokyo, Japan). The resin was removed by filtration through an MF centrifugal filtering devices (GHP Nanosep MF centrifugal device, Pall, Tokyo, Japan). The extracted DNA was purified using a commercial kit (QIAamp DNA Mini Kit, QIAGEN, Tokyo, Japan) and further concentrated by a UF centrifugal filter unit (Microcon YM-100, Millipore, Tokyo, Japan). The final volume of the concentrate was approximately 10 μL.
Quantification of Cryptosporidium spp. by QProbe PCR

QProbe PCR, targeting approximately 1,280 bp of the 18S rRNA gene of Cryptosporidium spp. was used to quantify the extracted DNA. Primers were selected from those developed by Xiao et al. (1999a, b). Since their primers were originally for the nested PCR, the forward primer for the second PCR (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3') and the reverse primer for the first PCR (5'-CCT GCT TTA AGC ACT CTA ATT TTC-3') were selected based on the amplification efficiency of preliminary experiments (data not shown).

The sequence of the QProbe was developed in this study. Sequences of Cryptosporidium spp. that have the identical region to both the forward and reverse primer were listed from the DDBJ database (Miyazaki et al., 2003) using NCBI-BLAST 2.0 (Altschul et al., 1997). The regions that were common to all selected sequences were obtained using ClustalW 1.7 (Thompson et al., 1994). Since QProbe PCR method utilises the characteristic of BODIPY FL dye, where its fluorescence is quenched by interacting with a guanine, QProbe must have a BODIPY FL-labeled cytosine at either of its 5'- or 3'-end. The sequence of the QProbe (5'-CGA ACC CTA ATT CCC CGT TAC CC-BODIPY FL-3') was then developed concerning the melting temperature of the probe and the generative capacity of the primer-dimer. The QProbe was obtained from a commercial laboratory (J-Bio 21, Tsukuba, Japan).

The LightCycler System (Roche Diagnostics, Tokyo, Japan) was used for the real-time PCR. The PCR mixture (20 µL) contained 0.4 U of DNA polymerase (KOD-Plus-, TOYOBO, Osaka, Japan), 2 µL of 10 × PCR buffer, 200 nM each dNTPs, 1 mM MgSO4 and 0.25 mg/mL BSA. The concentrations of the forward and reverse primer and the QProbe were 600, 200 nM and 100 nM, respectively. PCR conditions consisted of an initial hot-start for 5 min at 94°C; 60 cycles of 30 s at 94°C, 30 s at 57°C and 75 s at 68°C; and a final extension of 5 min at 68°C. After PCR, the data were analysed using a program provided by the QProbe manufacturer (J-Bio 21, Tsukuba, Japan).

Genotyping Cryptosporidium spp. by PCR-DGGE and DNA sequencing

Nested PCR was conducted for the products of the QProbe PCR, with primers targeting the 18S rRNA gene of Cryptosporidium spp. (Morgan et al., 1997). A GC clamp (5'-CGC CCG CGC GCG GGC GGG GCG GCA CGT TAC CC-3', Muyzer et al., 1993) was added at the 5'-end of the forward primer (5'-AGT GAC AAG AAA TAA CAA TAC AGG-3'). The reverse primer (5'-CCT GCT TTA AGC ACT CTA ATT TTC-3') was used without any modification. The PCR mixture (50 µL) contained 1 U of DNA polymerase (AmpliTaq Gold, Applied Biosystems, Tokyo, Japan), 5 µL of 10 × PCR buffer, 200 nM each dNTPs and 2 mM MgCl2. The concentration of both the forward and reverse primer was 500 nM. PCR conditions consisted of an initial hot-start for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C; and a final extension of 5 min at 72°C.

DGGE of the PCR products was performed using the DCode mutation detection system (Bio-Rad, Tokyo, Japan). Denaturant gradient gels (5–25%) containing 8% (w/v) acrylamide were formed (100% denaturant is defined as 7 M urea and 40% (v/v) formamide). The gels were electrophoresed at 130 V and 60°C for 10 h. The gels were stained with Vistra Green (GE Healthcare, Tokyo, Japan) and the stained gels were analysed using Fluorimager 595 (GE Healthcare, Tokyo, Japan). After DGGE, each band was cut out of the DGGE gel and the DNA fragments were sequenced using ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Tokyo, Japan).
Recovery efficiency of the filtration, concentration and purification methods
Three river water samples (40 L each) were collected at the Tone Division Weir. The turbidity of the water was 6.1 NTU. Oocysts of Cryptosporidium parvum (ColorSeed, BTF, New South Wales, Australia) were seeded before the filtration. The filtration, concentration and purification methods were described above. As the seeded oocysts were gamma-inactivated, the number of recovered Cryptosporidium was counted using a fluorescent microscope (BX60, OLYMPUS, Tokyo, Japan) after staining with EasyStain (BTF, North Ryde, Australia).

Detection limit and quantitation limit of the QProbe PCR method
The detection and quantitation limits of the QProbe PCR method were determined using known concentrations (0.83–8300 oocysts/PCR tube) of the genomic DNA of Cryptosporidium parvum IOWA isolate (Waterborne, New Orleans, Los Angeles). The concentration of oocysts in the stock solution was determined by microscopy (BX60, OLYMPUS, Tokyo, Japan). The genomic DNA was extracted by the method described above, and the extracted DNA was diluted with de-ionised water to the required concentration.

The Ct values are usually unstable when the initial concentration is very low, resulting in low quantifiability. Thus, the quantitation limit of the QProbe PCR method was evaluated separately from the detection limit. The detection limit was defined as the lowest initial concentration at which all samples were successfully detected. The quantitation limit was defined based on the ratio of the detected concentration to the seeded concentration of each sample. The quantifiability at a certain concentration was approved when the confidence interval of the ratio was within 50–200%, corresponding to the difference of approximately one PCR cycle.

Discriminating capability of the DGGE method
DGGE was conducted using the genomic DNA of nine species of Cryptosporidium, C. parvum (AF161856), C. hominis (AF093491), C. canis (AF112576), C. meleagridis (AF112574), C. felis (AF112575), C. sp. strain 938 (AY120913), C. andersoni (AB089285), C. serpentis (AF093502) and C. saurophilum (AF112573), to evaluate the ability to discriminate each species/genotype. The amplification of the DNA and the DGGE method was conducted following the procedure described above.

Results and discussion
Recovery efficiency of the filtration, concentration and purification methods
The recovery efficiency of the filtration, concentration and purification method was 35% (n = 3, S.D. = 8.8%). Considering the large volume (40 L) and turbidity (6.1 NTU) of the tested river water, the method developed in this study showed comparable recovery efficiency. Quintero-Betancourt et al. (2003) reported a high recovery (57%) of Cryptosporidium from 100 L of tertially treated wastewater with low turbidity of less than 2.5 NTU. Lee et al. (2004) reported that the recovery from reference sediment samples corresponding to 65–71 L of surface water was 38.0%. In these reports, Envirochek HV capsule filter (Pall) was used for filtration, and the US EPA Method 1623 (1999) was basically followed.

Detection limit and quantitation limit of the QProbe PCR method
The seeded DNA was successfully detected from all samples regardless of the initial concentration (8,300–0.83 oocysts/sample). Therefore, the detection limit of this method was
found to be less than the lowest concentration tested, 0.83 oocysts/sample. This result shows that single oocysts can be successfully detected by using this method. The confidence interval of the ratio of the detected concentration to the seeded concentration at each initial concentration (Figure 1) demonstrated that the lower quantitation limit of this method was 2.5 oocysts/sample. These detection and quantitation limits were comparable to the real time PCR methods using TaqMan probe and the LightCycler hybridisation probes (Higgins et al., 2001; Fontaine and Guillot, 2002; Limor et al., 2002; Tanrıverdi et al., 2002; Guy et al., 2003).

Figure 1 The confidence interval of the ratio of quantified concentration to the seeded concentration. The grey bar shows the 95% confidence interval of the quantified/seeded ratio. If this bar was within the dotted lines, the quantifiability at the concentration was approved.

Figure 2 Discrimination of Cryptosporidium spp. by DGGE
Discriminating capability of the DGGE method
The bands from all tested species appeared at the denaturant gradient between 8 and 20% (Figure 2). Since the band positions of nine species were different from each other, it was approved that this DGGE method could effectively isolate the DNA fragments of multiple Cryptosporidium spp. in a sample. This result also suggests that by using the DNA fragments from typical species as a marker, the genotypes in a sample could be determined without the sequencing analysis. Previously, cloning was widely used for isolation of the DNA fragments of Cryptosporidium, but it is not cost-effective because it often requires many samples to be sequenced. The DGGE method could reduce the number of samples for sequencing to identify all genotypes in a sample because of the preceding isolation of DNA fragments by the electrophoresis.

Concentration and genotypes of Cryptosporidium in the Koyama River
Cryptosporidium was detected from 11 samples (69%) out of 16 samples collected from the Koyama River (Table 1). Two samples contained Cryptosporidium more than the lower quantitation limit (2.5 oocysts/sample), and the maximum concentration was 59 oocysts/100 L. Seven genotypes (C. hominis, C. parvum, C. andersoni, C. sp. 938, C. sp. PG1-26, C. sp. t03, C. sp. t04), including two novel ones (C. sp. t03 and C. sp. t04), were identified. The most frequently detected species was C. andersoni (seven samples), followed by C. sp. 938 (four samples) and C. parvum (two samples). The human-specific species, C. hominis, was detected from only one sample, showing that risk assessment based on quantitative data without information on the genotypes may lead to an overestimation of the risk to human health. Five samples contained multiple genotypes, suggesting that the direct sequencing of the PCR product may overlook the existence of other genotypes.

Table 1 The concentration and the detected species in the Koyama River

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Sample volume [L]</th>
<th>Detection</th>
<th>Detected genotypes</th>
</tr>
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<tbody>
<tr>
<td>3rd Aug., 11:00</td>
<td>45.2</td>
<td></td>
<td>C. hominis, C. sp. 938</td>
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<td>3rd Aug., 12:30</td>
<td>32.0</td>
<td></td>
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<tr>
<td>3rd Aug., 14:00</td>
<td>43.3</td>
<td>C. hominis</td>
<td></td>
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<tr>
<td>3rd Aug., 15:30</td>
<td>33.5</td>
<td>C. sp. 938</td>
<td></td>
</tr>
<tr>
<td>26th Aug., 11:00</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>26th Aug., 12:30</td>
<td>43.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26th Aug., 14:00</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>16th Sep., 10:30</td>
<td>41.6</td>
<td>C. andersoni</td>
<td></td>
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<tr>
<td>16th Sep., 12:00</td>
<td>47.4</td>
<td>C. parvum, C. sp. 938</td>
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<tr>
<td>16th Sep., 13:30</td>
<td>47.4</td>
<td>C. andersoni</td>
<td></td>
</tr>
<tr>
<td>16th Sep., 15:00</td>
<td>66.1</td>
<td>C. sp. PG1-26</td>
<td></td>
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<tr>
<td>27th Oct., 10:45</td>
<td>30.9</td>
<td>59</td>
<td>C. andersoni, C. sp. 938, C. sp. t03, C. sp. t04</td>
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<tr>
<td>27th Oct., 12:15</td>
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<td>C. andersoni</td>
<td></td>
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<td>27th Oct., 13:45</td>
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<td>C. andersoni, C. parvum</td>
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<tr>
<td>27th Oct., 15:15</td>
<td>44.9</td>
<td>14</td>
<td>C. andersoni</td>
</tr>
</tbody>
</table>

a – is for samples negative for Cryptosporidium, + is for samples positive for Cryptosporidium, but the concentration was less than the quantitation limit (<2.5 oocysts/sample) and values represents the quantified concentration in oocysts/100 L
b The accession number of each species is; C. parvum (AF164102), C. hominis (AY204231), C. andersoni (AB089285), C. sp. 938 (AY120913), C. sp. PG1-26 (AY271721), C. sp. t03 (AB231612) and C. sp. t04 (AB231613)

A novel genotype with the sequence similar to C. sp. 1665 (AY120915, identity = 97%)
A novel genotype with the sequence similar to C. sp. 1665 (AY120915, identity = 97%)
Conclusion
This sequential combination of the QProbe PCR method, the DGGE method and the DNA sequencing enabled the simultaneous quantification and genotyping of Cryptosporidium spp. in the same water sample. It is known that not all species are infective to humans, and the infectivity varies according to the species, and sometimes the subspecies. Therefore, from the viewpoint of assessing waterborne risk to human health, it is essential to determine not only the concentration of Cryptosporidium in water samples but also the species and genotypes. The detection method developed in this study is promising for obtaining the valuable information for the precise assessment of the waterborne microbial risk to human health.

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References


