Characterization of a Giardia-like microorganism causing false positive reactions in the detection of Cryptosporidium/Giardia from water samples

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ABSTRACT

Giardia-like microorganisms were found as a result of a Cryptosporidium/Giardia detection assay on winter reservoir samples. The morphological aspects, DNA sequence of the microorganism, and specificity of immunoassay reagents were investigated. The microorganism was very similar to a Giardia cyst. The cell was oval shaped (11.0 μm in length, 7.7 μm in width), contained organelles and was stained bright apple-green by a fluorescent monoclonal antibody, although no axonemes or curved bristles were observed. Cross-reaction occurred with the anti-Cryptosporidium, but not the anti-Giardia, immunomagnetic antibody. The specificity of the fluorescent monoclonal antibodies differed depending on the product. As a result of the analysis of the partial sequence of 18S rDNA, the microorganism was identified as Lunulospora curvula, mitosporic Ascomycota. These findings will be very helpful for avoiding false positives in future assays.

Key words | cross-reaction, Cryptosporidium, Giardia, immunofluorescence assay, immunomagnetic separation, polymerase chain reaction

INTRODUCTION

Cryptosporidium and Giardia infect the gastrointestinal epithelium and cause diarrhea. Numerous outbreaks of waterborne diseases caused by these microorganisms have been reported (Karanis et al. 2007; Baldursson & Karanis 2011). Immunomagnetic separation and staining with fluorescent monoclonal antibodies is widely used to detect these protozoan parasites (US EPA 2005). Some species of algae (Rodgers et al. 1995) and protozoa (Riggs et al. 1983; Ortega-Mora et al. 1992; Bull et al. 1998; Graczyk et al. 1998) are reported to cross-react with immunofluorescent antibodies, and this is likely to cause a false positive when their morphological characteristics and fluorescence intensity are very similar. However, little is known about these organisms. A profusion of Giardia-like microorganisms were found as a result of the detection assay for Cryptosporidium/Giardia in winter reservoir samples in Kobe City, although there was no contamination source near the reservoir. Therefore, we investigated the characteristics of this microorganism and the specificity of reagents used for separation and staining.

MATERIALS AND METHODS

Sampling

Water samples were collected at the Nunobiki Reservoir, which is used as a water source for Kobe City (34°42′40″ N, 135°11′25″ E). The reservoir is a small oligotrophic lake with a surface area of 0.62 km² and a maximum depth of 29.8 m. Ten to eighty liters of surface water were collected in plastic containers from 23 November to 17 December 2007 and from 19 January to 3 March 2011. Upon arrival at the laboratory, the samples were immediately filtered.
**Filtration and separation**

Water samples were filtered and concentrated by the hydrophilic polytetrafluoroethylene (HPTFE) membrane filtration method (Oda et al. 2002). Then, Giardia-like microorganisms were separated using anti-Cryptosporidium immunomagnetic antibodies (Dynabeads GC-Combo, Invitrogen Dynal AS, Oslo, Norway) following the manufacturer’s instructions.

**Staining and observation**

Giardia-like microorganisms were stained by immunofluorescent reagent (product A) and 4′,6′-diamidino-2-phenylindole (DAPI) on a well slide. Product A is the mixture of immunofluorescent monoclonal antibodies against Cryptosporidium oocysts and Giardia cysts. The microorganisms were observed under differential interference contrast (DIC) using an epi-fluorescence microscope (BX50, Olympus, Tokyo, Japan).

**DNA extraction from environmental water samples**

Microorganisms collected by immunomagnetic antibodies were concentrated by centrifugation at 6,000×g for 10 min in a polymerase chain reaction (PCR) tube, and an aliquot was removed. Ninety microliters of ATL buffer (DNeasy Tissue Kit, Qiagen, Tokyo, Japan) was added to the pellet, followed by five cycles of freezing in liquid nitrogen for 1 min and thawing at 25 °C for 2 min. After that, DNA was extracted with DNeasy Tissue Kit and manufacturer-recommended procedures.

**PCR amplification**

Two sets of primers were used for PCR to examine the presence of Cryptosporidium or Giardia in the water sample. The first primer set was forward primer Cp.E (5′-GGA TGG GTA TCA GGT AAT AAG AA-3′) and reverse primer Cp.Z (5′-CAA CTA GCC CAG TTC TGA CTC TCT CG-3′), which amplifies a 1,200-bp fragment of the TRAP-C1 (thrombospondin-related adhesive protein of Cryptosporidium-1) gene of Cryptosporidium (Spano et al. 1998). The second primer set was forward primer GDH1 (5′-ATC TTC TGC GAG ATG CTT GAG-3′) and reverse primer GDH4 (5′-AGT ACG CGA CGC TGG GAT ACT-3′) which amplifies a 768-bp fragment of the glutamate dehydrogenase gene of Giardia (Homan et al. 1998). Fifty microlitres of PCR mixture contained 5 μl of 10× Ex Taq buffer, 4 μl of dNTP mixture, 25 pmol of each primer, 2.5 μl of extracted DNA, 0.25 μl of Takara Ex Taq polymerase (Takara, Otsu, Japan), and 37.25 μl of distilled water. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Tokyo, Japan). Amplification conditions were 94 °C for 2.5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, with a final extension at 72 °C for 6.5 min. Amplicons were electrophoresed on 1.5% (wt/vol) agarose gel containing ethidium bromide (final conc. 0.5 μg ml⁻¹).

**Single cell separation**

In total, five cells were separated by the micropipette washing method using Microcaps (Drummond Scientific, PA, USA), the tips of which were made about 20 μm smaller in inside diameter. Samples purified by anti-Cryptosporidium immunomagnetic antibodies were used for separation. The cells were washed one at a time in a drop of distilled water once, then transferred to a PCR tube containing 20 μl of distilled water.

**DNA and RNA extraction**

Five cycles of freezing at −80 °C for 2 min and thawing at 37 °C for 1 min were performed to extract DNA and RNA from the microbial cells. The sample was sonicated for 30 s in an ultrasonic cleaner (IUC-7321, Tocho, Tokyo, Japan), and incubated at 60 °C for 60 min with Proteinase K (final conc. 200 μg ml⁻¹), then heated at 95 °C for 5 min to inactivate the Proteinase K.

**RT-PCR**

Reverse transcription PCR (RT-PCR) was performed to amplify the partial sequence of 18S rDNA from the microorganism. The primer set used was forward primer EukA (5′-AAC CTG GTT GAT CCT GCC AGT-3′) designed by Medlin et al. (1988), and reverse primer Euk516r-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG...
GCA CGG GGGG ACC AGA CTT GCC CTC C-3') designed by Amann et al. (1990), which produces a 590-bp fragment of 18S rDNA. The underlined sequence indicates the GC clamp (Diez et al. 2001). RT-PCR was performed by PrimeScript One-Step RT-PCR Kit Ver. 2 (Takara, Otsu, Japan). Two microlitres of PrimeScript One-Step Enzyme Mix, 25 μl of 2× One-Step Buffer, and 25 pmol of each primer were added to the extracted DNA and RNA (final vol. 50 μl). Amplification conditions were 50 °C for 30 min, 94 °C for 2 min, followed by 50 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using the DCode Universal Mutation Detection System (Bio-Rad, CA, USA). The PCR products were loaded onto 8% (wt/vol) polyacrylamide gels in 1×TAE. The denaturing gradient ranged from 20 to 50%, where 100% denaturant contained 7 M urea and 40% (vol/vol) formamide. Electrophoresis run conditions were 75 V for 15 h at 60 °C. Upon completion of electrophoresis, the gel was soaked for 30 min in SYBR Gold nucleic acid gel stain (1:10,000 dilution) (Molecular Probes, Eugene, USA). The DGGE band was carefully excised under UV illumination, The DNA solution was used as a template in PCR reamplification using the same primer pair (EukA and Euk516r-GC). Fifty microlitres of PCR mixture contained 5 μl of 10× Ex Taq buffer, 4 μl of dNTP mixture, 25 pmol of each primer, 5 μl of template DNA, 0.25 μl of Takara Ex Taq polymerase (Takara), and 34.75 μl of distilled water. Amplification conditions were 94 °C for 2.5 min, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplicons were electrophoresed again on a DGGE gel to verify that the mobility was the same as the original band.

Sequencing and phylogenetic analysis

The amplified DNA fragment was sequenced at Bex Co., Ltd (Tokyo, Japan). The sequence of the DGGE band was aligned to 18S rDNA sequences obtained by a BLAST search of the DNA Data Bank of Japan (DDBJ) database, available at http://www.ddbj.nig.ac.jp/.

Specificity of reagents

Either anti-Cryptosporidium or anti-Giardia immunomagnetic antibodies (Dynabeads GC-Combo, Invitrogen Dynal AS) were added to concentrated samples to examine the specificity of the immunoassay reagents. Then, each sample was stained by product A or product B, both of which are a mixture of immunofluorescent monoclonal antibodies against Cryptosporidium oocysts and Giardia cysts. They are employed in Method 1623 (US EPA 2005). Detection of the microorganisms was confirmed by epifluorescence microscopy.

RESULTS AND DISCUSSION

Morphological aspects of Giardia-like microorganism

Figure 1 shows the DIC and epifluorescent micrographs of the Giardia-like microorganism. The cell was oval shaped and stained bright apple-green by the fluorescent monoclonal antibody. The red fluorescence as a result of autofluorescent plastid pigments was not seen under G illumination, indicating that the microorganism was not algae. The cell was 11.0 μm in length, 7.7 μm in width and contained organelles. These characteristics are very similar to those of the Giardia cyst. On the other hand, no axonemes or curved bristles were observed. A slightly protruding structure similar to a handle was found at the end of the cell. Under UV illumination, only a single nucleus was observed and it was mostly ambiguous compared to that of the Giardia cyst (Figure 2).
In a DIC examination, observation at 1,000-fold magnification is necessary. However, a dry cell on a well slide is wrinkled, making it difficult to distinguish the organelles of the microorganism from the axonemes and curved bristles of a *Giardia* cyst. In that case, observation of a fresh cell will make the differentiation easier.

**PCR and sequencing**

No amplicon was found when PCR was performed with the primer pairs targeting the genes of *Cryptosporidium* or *Giardia* (Figure 3), although a profusion of the *Giardia*-like microorganisms were detected in the samples during the experiment period (around 3,000 cells per 10 l). The oocysts of *Cryptosporidium* and cysts of *Giardia* were also not detected in the microscope assay. Therefore, it is concluded that there were no *Cryptosporidium* oocysts or *Giardia* cysts in the water sample. These results are in agreement with the fact that there was no contamination source, such as a stock farm or waste water treatment plant, near the water reservoir.

Only a single band was detected in DGGE, indicating that there was no DNA or RNA contamination in the purification procedure (Figure 4). The excised DGGE band showed 99% sequence similarity to the 18S rDNA of *Lunulospora curvula*, which is mitosporic Ascomycota, and belongs to Sordariomycetes (Figure 5). The microorganism was phylogenetically distant from *Giardia* and *Cryptosporidium*. *L. curvula* inhabits submerged leaves (Patil & Borse 2011), and also the endophytes of riparian plant roots (Satish & Belwal 2005). Aquatic fungi play a major role in the degradation of submerged leaf litter and nutrient release in the aquatic ecosystem (Wurzbacher et al. 2013). The Nunobiki Reservoir is surrounded by forest, which contributes to an increase in leaf litter during autumn and winter. Therefore, *Lunulospora* may have grown on the leaves. Based on the morphological aspects, the microorganism was presumed to be an ascospore.

**Specificity of the reagents for immunoassay**

The microorganism cross-reacted with the anti-*Cryptosporidium*, but not the anti-*Giardia*, immunomagnetic antibody (Table 1). Usually, both anti-*Cryptosporidium* and anti-*Giardia* immunomagnetic antibodies are applied to the sample simultaneously (US EPA 2005). Therefore, if the suspected microorganism is detected in the sample, it is not certain which antibody reacted with the microorganism. In that case, separate application of the immunomagnetic antibodies is the way to avoid a false positive.

The specificity of the fluorescent monoclonal antibodies differed depending on the product. The microorganism was only stained by product A and not by product B. The difference in the specificity of immunofluorescent reagent may be due to the class of antibodies. Product B is a mixture of anti-*Cryptosporidium/Giardia* IgG1 monoclonal antibodies and is reported to be more specific than IgM or IgG3 antibodies (Ferrari et al. 1999).
On the other hand, Product A is a mixture of anti-Cryptosporidium IgM and anti-Giardia IgG1 monoclonal antibodies.

Eimeria (Ortega-Mora et al. 1992), Monocystis sp. (Bull et al. 1998), trophozoite of Hexamita nelsoni (Graczyk et al. 1998) and some algae (Rodgers et al. 1995) are reported.
to cross-react with anti-
Cryptosporidium

Table 1 | Reactivity of the reagents used for immunomagnetic separation and immunofluorescence staining of a Giardia-like microorganism

| Target organism of immunomagnetic antibodies | Fluorescent monoclonal antibody | Detection of Giardia-like microorganism
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<tr>
<td>Cryptosporidium</td>
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<tr>
<td>Giardia</td>
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<tr>
<td>Giardia</td>
<td>Product B</td>
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*Both products are a mixture of direct immunofluorescent antibodies anti-Cryptosporidium oocyst and anti-Giardia cyst.

CONCLUSION

The Giardia-like microorganism detected in the reservoir was identified as L. curvula, mitosporic Ascomycota, from the partial sequence of 18S rDNA. The morphological aspects and immunofluorescence intensity were very similar to those of the Giardia cyst, and care should be taken in observation. The absence of the axonemes and curved bristles, typical organelles in the Giardia cyst, the presence of a slightly protruding structure at the end of the cell, and a single ambiguous nucleus are the criteria for distinguishing the microorganism from the Giardia cyst. The cell cross-reacted with the anti-Cryptosporidium, but not the anti-Giardia, immunomagnetic antibody. The specificity of the fluorescent monoclonal antibodies differed depending on the product. We must possess an accurate knowledge of the characteristics of the reagents used for detection assays, along with careful microscopic observation. These findings will be very helpful for avoiding false positives in future assays.

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