INTRODUCTION

Emerging waterborne protozoa, such as microsporidia, *Cyclospora*, and *Cryptosporidium*, have become a challenge to human health worldwide. These protozoans have several common characteristics biologically. Their major habitat is intestinal epithelial cells, and they are all intracellular parasites [1]. In addition, they produce infectious spores that are excreted from the hosts in their stools [2]. Although these protozoa are a concern for AIDS-infected individuals, they are gaining recognition as important infective organisms in immunocompetent individuals as well [1,3,4].

Of AIDS patients with chronic diarrhea, about 50% are diagnosed as infected with microsporidia, and 10-20% are infected with *Cryptosporidium parvum* [1]. A large waterborne outbreak of *C. parvum* occurred in 1993 [5], and outbreaks of *Cyclospora* that was due to consumption of imported raspberries have been reported [6,7]. Microsporidia has been confirmed as a waterborne protozoon based on its detection in tertiary sewage effluent, surface water, and ground water [8]. Among the various genera in microsporidia, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, or both *Cryptosporidium hominis* and *Cryptosporidium parvum*, respectively. Restriction enzyme digestion of PCR products with BsaBI or BsiEI makes it possible to distinguish the 2 species of microsporidia or *Cryptosporidium*, respectively. This simple, rapid, and cost-effective multiplex PCR method will be useful for detecting outbreaks or sporadic cases of waterborne protozoa infections.

Key words: *Cyclospora*, *Cryptosporidium*, microsporidia, multiplex PCR, waterborne protozoa

MATERIALS AND METHODS

E. intestinalis spores were purchased from ATCC (Cat. No. 50506; Manassas, Virginia, USA) and maintained through in...
combined in a 70-μl volume containing 5 μl of template DNA, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP, and dTTP, 0.7-1 μM of each primer (0.7 μM for microsporidia and 1 μM for Cryptosporidium and Cyclosporidium), and 5 U Taq DNA polymerase (Promega). The second-round multiplex PCR amplification was performed under the same conditions, except 2 μl of template DNA (product of the first-round PCR) was used in a 30-μl final volume. Both amplifications were performed using a C-1000 DNA thermal cycler (Bio-Rad, Hercules, California, USA) with initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94°C for 30 sec, 53°C for 30 sec, and 72°C for 90 sec and a final extension at 72°C for 10 min. The second-round PCR cycling conditions were identical to the first-round PCR except for the annealing temperature (55°C). Amplified DNA was analyzed by electrophoresis in a 2% (w/v) agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under ImageQuant300 (GE Healthcare, Giles, UK).

An aliquot (5 μl) of the second-round PCR product of *E. intestinalis* or *C. parvum* was used for enzyme digestion with BsaBI or BsiEI (New England BioLabs, Massachusetts, USA) at 60°C for 2 hr. DNA fragments were analyzed by electrophoresis in a 2% (w/v) agarose gel as described above.

**RESULTS**

From the first-round PCR amplification, we obtained the following products with the predicted sizes: 644-657 bp, 636 bp, and 415-427 bp for microsporidia, *C. cayetanensis*, and *Cryptosporidium*, respectively (data not shown). From the second-round PCR, the following predicted PCR products were amplified: 410-420 bp, 294 bp, and 171-183 bp for microsporidia,

**Table 1.** Primer sets used for multiplex PCR

| Parasite                  | Primer name | Sequence                      | PCR product size |
|---------------------------|-------------|--------------------------------|------------------|
| Microsporidia             | 1st         | Mic A 5'-GGAGCCCTAGAGATGCT-3'  | 644-657 bp       |
|                           |             | Mic E 5'-AACGGCCATGACAC-3'     |                  |
|                           |             | Mic C 5'-GGTGCCACGCAGCCGG-3'   | 410-420 bp       |
|                           |             | Mic D 5'-GCACAATCCACTCTC-3'    |                  |
|                           | 2nd         | CYCF1E 5'-TACCAAATGGAAAAAGGTT-3' | 636 bp          |
|                           |             | CYCR2B 5'-CAGGGAAGCCAGCCGATG-3' |               |
|                           |             | CYCF3E 5'-CCCTCCGCGCTCCGCTC-3' | 294 bp          |
|                           |             | CYCR4B 5'-CAGTCTCCAACCCCCCTACTG-3' |             |
| *Cyclospora cayetanensis* | 1st         | cp2415F 5'-CCCACCCGAAAGTTAAG-3' | 415-427 bp       |
|                           |             | cp2415R 5'-CTAGTATGCTCGACCC-3'  |                  |
|                           | 2nd         | cp2171F 5'-CAACCCGAAAGTTAAGT-3' | 171-183 bp       |
|                           |             | cp2171R 5'-CTAGTATGCTCGACCC-3'  |                  |
C. cayetanensis, and Cryptosporidium, respectively (Fig. 1). The lower detection limit of nested PCR amplification that was performed with template DNA from each individual protozoa was $10^2$ spores for microsporidia, $10^0$ oocysts for Cyclospora, and $10^1$ oocysts for Cryptosporidium (Fig. 1). The lower detection limit of multiplex PCR amplification that was performed with mixed DNA from each protozoan was $10^2$ for microsporidia and Cyclospora and $10^1$ for Cryptosporidium (Fig. 2).

The primers for microsporidia and Cryptosporidium used in the present study are capable of detecting both E. bieneusi and E. intestinalis, and C. parvum and C. hominis, respectively. Restriction enzyme digestion of the resulting nested PCR products can distinguish the 2 kinds of microsporidia and Cryptosporidium spp. Restriction enzyme digestion with BsaBl showed 2 fragmented bands of 167 and 253 bp in E. intestinalis (Fig. 3A), and with BsiEl showed no fragmented bands in C. parvum (Fig. 3B). Whereas it was confirmed with Clone Manager 6 (Sci-Ed, North Carolina, USA) that there will be no fragmented bands in E. bieneusi with BsaBl digestion and 2 fragmented bands of 48 and 137 bp in C. hominis with BsiEl digestion. Each primer set for microsporidia, C. cayetanensis, and Cryptosporidium used in the present study showed no cross-reactivity with each of the other parasite DNA (Fig. 4).

### DISCUSSION

There are various detection methods currently available for microsporidia, Cyclospora, and Cryptosporidium. These include
Their detection sensitivity for as few as 10 oocysts from singleplex PCRs with each parasite described a detection sensitivity of 0.8 log lower than our results. In addition, Orlandi and Lampel [9] reported a sensitivity level for Cryptosporidium that was as low as 3 oocysts using nested PCR with the same primer set, which is similar to the level of sensitivity in our study. Although it is inexpensive and fast, the classical staining method depends on highly trained experts for accurate diagnosis. Immunofluorescence-based antibody-labeling methods depend on the sensitivity and specificity of antibodies against parasites for the accurate diagnosis, and cross-reactivity or nonspecific labeling can be a challenge. Multiplex PCR would eliminate the need for a highly trained expert and would reduce the diagnostic time by being able to catch 3 kinds of protozoa at the same time. If well-designed multiplex PCR having a high sensitivity and specificity is available, it would be an excellent diagnostic method for these waterborne protozoa.

The primers used for Cyclospora in this study were designed by Relman et al. [14]. Orlandi and Lampel [9] reported a sensitivity level for Cyclospora that was as low as 3 oocysts using nested PCR with the same primer set, which is similar to the level of sensitivity in our study. In addition, Orlandi and Lampel [9] described a detection sensitivity of C. parvum and E. intestinalis of as few as 10 oocysts from singleplex PCRs with each parasite DNA [9]. Their detection sensitivity for E. intestinalis is 10-fold higher than our results; however, they did not report the sensitivity of multiplex PCR using mixed DNA of each protozoan. Furthermore, agarose gel electrophoresis analysis of PCR products from both the first and second rounds of PCR is necessary to confirm the detection results with the method described by Olandi and Lampel [9], because that method amplified the C. parvum target only during the first round of PCR.

Generally the sensitivity of multiplex PCR is not exactly the same with individual PCR done with each parasite DNA. Multiplex PCR developed in the present study showed the same detection sensitivity with that of individual PCR in case of microsporidia and Cryptosporidium. Agarose gel electrophoresis of the second-round PCR products could provide all of the detection results, when the multiplex PCR tests reported here were applied.

Another advantage of the multiplex PCR test developed in the present study is that the 2 species of microsporidia, such as E. bieneusi and E. intestinalis, and the 2 species of Cryptosporidium, such as C. parvum and C. hominis, can be differentiated after PCR by restriction enzyme digestion. We did not show restriction enzyme-digested PCR products of E. bieneusi and C. hominis in the present study as we did not secure DNAs of these 2 organisms. Instead, we confirmed the results of restriction enzyme digestion of these 2 organisms after PCR using a restriction enzyme cutting software program (Clone Manager 6).

Although there is a well-established genomic database for Cryptosporidium (http://cryptodb.org) that was established through the efforts of many devoted and excellent scientists, there are still only limited genomic data available for Cyclospora and microsporidia. Therefore it is quite difficult to develop specific primers for these 2 protozoa. The difficulty is especially challenging for Cyclospora because PCR detection of the 18S rRNA gene could be confused with that of Eimeria spp., which show 98% similarity with the 18S rRNA gene sequence of Cyclospora. Thus sequence analysis after multiplex PCR should be performed for the accurate differential diagnosis of Cyclospora and Eimeria spp.

In conclusion, the multiplex PCR test developed in the present study can detect microsporidia, Cyclospora, and Cryptosporidium, all of which cause severe waterborne diarrheal disease. The test is very simple and rapid and offers high sensitivity and specificity. This test could help improve detection of diarrheal outbreaks or sporadic diarrheal disease that is due to infection with these major waterborne protozoa which have been considered as unknown etiology for the difficulty in detection previously.

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