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▣ BRIEF COMMUNICATION

Cryptosporidium hominis Infection Diagnosed by Real-Time PCR-RFLP

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Abstract: There are approximately 20 known species of the genus *Cryptosporidium*, and among these, 8 infect immunocompetent or immunocompromised humans. *C. hominis* and *C. parvum* most commonly infect humans. Differentiating between them is important for evaluating potential sources of infection. We report here the development of a simple and accurate real-time PCR-based restriction fragment length polymorphism (RFLP) method to distinguish between *C. parvum* and *C. hominis*. Using the *CP2* gene as the target, we found that both *Cryptosporidium* species yielded 224 bp products. In the subsequent RFLP method using *Taq*I, 2 bands (99 and 125 bp) specific to *C. hominis* were detected. Using this method, we detected *C. hominis* infection in 1 of 21 patients with diarrhea, suggesting that this method could facilitate the detection of *C. hominis* infections.

Key words: *Cryptosporidium parvum*, *Cryptosporidium hominis*, real-time PCR, RFLP, *Taq*I

Cryptosporidium parvum is a parasitic protozoan that infects gastrointestinal epithelial cells of many vertebrates, including humans [1]. It causes watery diarrhea and can be fatal to immunocompromised individuals [1]. There are 20 known *Cryptosporidium* species and at least 44 genotypes, which differ significantly in their molecular signatures but have not been assigned species status [2]. Eight fully characterized *Cryptosporidium* species (*C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. suis, C. muris,* and *C. andersoni*) and 5 partially characterized species (from the deer, monkey, skunk, rabbit, and chipmunk) infect humans [2-5], among which *C. hominis* and *C. parvum* are the most commonly detected [3]. A real-time PCR (qPCR) method using primers derived from the *CP2* gene is highly sensitive, specific, and accurate for the detection of cryptosporidiosis but cannot distinguish among species [6]. Therefore, we developed a qPCR-based restriction fragment length polymorphism (RFLP) method for the 2 *Cryptosporidium* spp. differentiation and we detected *C. hominis* in 1 of 21 patients with diarrhea.

DNA was prepared from *C. parvum* oocysts of (KKU isolates) collected from laboratory mice (C57BL6/J) that were infected with the parasite and maintained as described [6]. DNA was extracted from the oocysts and fecal materials by using a QIAquick Stool Mini Kit (QIAGEN Inc., Hamburg, Germany USA). The qPCR reactions were performed according to the method of Lee et al. [6] using a LightCycler® (Roche, Basel, Switzerland, USA). The results were analyzed using the LightCycler® software (version 4.05, Roche). DNase/RNase-free water was used in place of template DNA as a negative control. *CP2* sequences of *C. parvum* (AY471868) and *C. hominis* (XM_661199) were aligned using Clone Manager Suite 7 (Sci-Ed Software, Cary, North Carolina, USA), and restriction enzyme cleavage sites were identified using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/). An aliquot (15 µl) of the qPCR product was digested with *Taq*I (Takara Bio Inc., Shiga, Japan) at 65℃ for 2 hr, and DNA fragments were analyzed using 2.5% agarose gels. Stool samples from 21 patients with diarrhea in the Busan area of Korea were collected from June 1 to 30, 2011, by the Korea Centers for Disease Prevention and Control. Modified acid-fast staining was performed on stool samples that were positive for *C. hominis* by using qPCR-based RFLP.

The sequences of the *CP2* genes of *C. parvum* (GenBank AY-

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471868) and *C. hominis* (GenBank XM_661199) are 94% identical (data not shown). Because analysis using NEBcutter V2.0 identified a *Taq*I site only in the sequence of *C. hominis CP2* (Table 1), *Taq*I was chosen for restriction fragment length polymorphism (RFLP) analysis.

Using qPCR, we found that 1 of 21 patients with diarrhea tested was positive for *CP2* (data not shown). The patient with positive results was a 6-year-old girl with watery diarrhea. No other information about the patient was available. To further identify the species of *Cryptosporidium* responsible for the infection, the sample was digested using *Taq*I, which generated 99 bp and 125 bp bands, indicating that the patient was infected by *C. hominis* (Fig. 1). *C. hominis* oocysts were also detected in the diarrheal stool sample by using the modified acid-fast staining (Fig. 2).

Morgan-Ryan et al. [7] proposed a new species of *Cryptosporidium*, *C. hominis*, to indicate its isolation from human feces. However, *C. hominis* and *C. parvum* oocysts are morphologically indistinguishable [7]. Species discrimination is important for molecular epidemiological purposes to evaluate potential sources of infections [8]. Real-time PCR increases the speed of sample analysis and decreases the risks of contamination with DNA present in the laboratory [8]. The present study showed that both major *Cryptosporidium* species can be detected simultaneously and distinguished from each other by using *Taq*I to digest the *CP2* gene of *C. hominis*. Because the *CP2* gene is highly specific, no genetic information is available for other *Cryptosporidium* species, except for *C. parvum* and *C. hominis* in GenBank (www.ncbi.nlm.nih.gov/genbank/).

The genotypes of *Cryptosporidium* in Korea have been reported [9-11]. Cheun et al. [11] studied *Cryptosporidium* sp. in 3 rural areas by using a PCR-RFLP method to detect 18S rDNA sequences and identified *C. parvum* in 12 patients with *Cryptosporidium* infection. Therefore, the case confirmed by the present study is very important, because it indicates the presence of *C. hominis* infection in Korea.

In the present study, we developed a simple and accurate qPCR-based RFLP method for differentiating *C. parvum* from *C. hominis*. This method could be helpful in facilitating the detection of *C. hominis* infection in Korea.

Table 1. DNA fragments produced using a qPCR-based *Taq*I-RFLP assay for the *CP2* genes of *C. parvum* and *C. hominis*

	C. hominis	*C. parvum*
Size of PCR product	224 bp	224 bp
Enzyme cutting (*Taq*I)	99 bp, 125 bp	224 bp

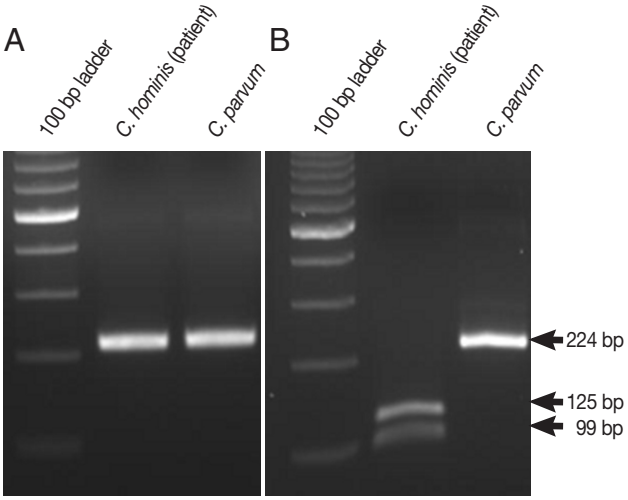

Fig. 1. DNA fragments generated by *Taq*I digestion of real-time PCR-amplified *CP2*. The results are shown for fecal samples taken from 21 patients with diarrhea. *C. hominis* was detected in the stool samples. *C. parvum* isolated from laboratory mice was used as the control. (A) The qPCR product was confirmed using agarose gel electrophoresis. (B) *Taq*I digestion profile of the qPCR product.

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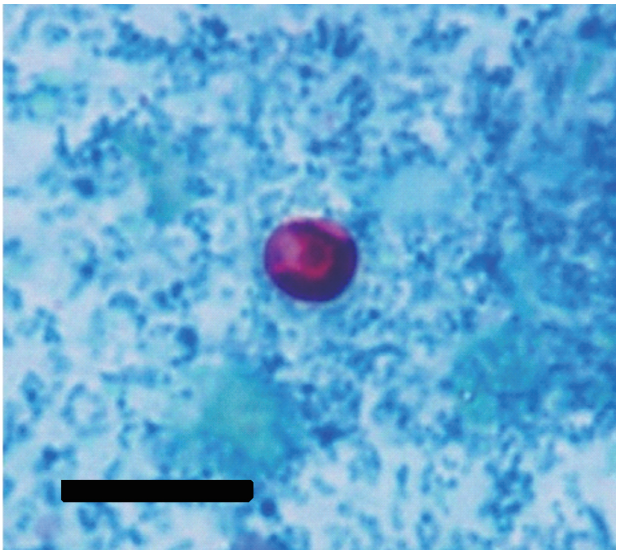

Fig. 2. Modified acid-fast staining of a stool sample of a patient infected with *C. hominis*. Bar = 10 μm.

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