Genotypic Characterization of Cryptosporidium Oocysts Isolated from Healthy People in Three Different Counties of Korea

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(Received 2 August 2006/Accepted 4 July 2007)

ABSTRACT. To investigate Cryptosporidium infection among healthy people, we collected stool samples from 150 healthy individuals in Gokseong, Muan, and Imshil Counties, southwest Korea, where neighbors on both an animal farm and a river respectively. In 12 of 150 samples, Cryptosporidium oocysts were detected by means of modified acid-fast staining. The bovine genotype, Cryptosporidium parvum, was identified by PCR/RFLP and 18S rRNA sequencing. C. parvum existed endemically in these areas, and the residents showed a relatively higher infection rate for C. parvum than that for C. hominis. Our results indicate that countermeasures against Cryptosporidium infection must be taken in these areas to ensure human health.

KEY WORDS: Cryptosporidium parvum, PCR/RFLP, 18S rRNA.

The protozoan parasite Cryptosporidium causes acute diarrhea in animals and humans. Immunodeficient humans, such as AIDS patients, suffer particularly severe complications [8]. Recently, an increase of Cryptosporidium hominis was noted in the United Kingdom due to the use of mixed lake water for drinking [2], and Cryptosporidium sp. oocysts have been detected in lakes, rivers, and livestock farms [9].

The genus Cryptosporidium comprises 13 species, namely C. hominis, C. parvum, C. wrairi, C. felis, C. canis, C. andersoni, C. muris, C. baileyi, C. meleagris, C. galli, C. serpentis, C. saurophilum, and C. molnari, and host specificity for human, bovine, bird, reptile, and fish has been determined by 18S ribosomal RNA (rRNA) analysis [4, 9, 12, 14]. The human type, C. hominis, has been detected only in humans, but the cattle type, C. parvum, is parasitic in cattle, humans, and other mammals [9].

In 1992 and 1993, several epidemiological surveys were carried out in north and south area of Korea, in which the detection rate in the cattle for C. parvum oocysts ranged from 2 to 40%, indicating high rate of contamination [3, 13], and the positive rate in the diarrhea patients without immunosuppression problems for C. parvum was 1% of 942 stools [6].

In the present study, in order to basic investigation of Cryptosporidium sp. infection status of healthy people who reside near both an animal husbandry area and a river, we selected three counties, namely Imshil (Jeollabuk-do), Gokseong (Jeollanam-do), and Muan (Jeollanam-do), where have been developed for animal husbandry and are close to the Seomjin or Yongsan Rivers (Fig. 1).

We detected Cryptosporidium oocysts in 12 (eight in Gokseong, three in Muan, and one in Imshil) of 150 fecal specimens collected from February to October 2004 from

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Fig. 1. Three areas, Imshil (Jeollabuk-do), Muan (Jeollanam-do), and Gokseong (Jeollanam-do) Counties in Korea, where the stool specimens were collected from a total of 150 people.
adults without diarrhea or watery stool. The stools were collected by visit to house. The stool specimens were stained with a modified acid-fast staining method and examined with a light microscope at 400×. Suspended specimens containing Cryptosporidium oocysts were selected and preserved in a 2.5% phosphate dichromate solution at 4°C for further analysis several weeks later. Each sample was washed three times with phosphate buffered saline (PBS, pH 7.0) and filtered through gauze. The harvested oocysts were added to a sucrose solution with a specific gravity of 1.2, then mixed thoroughly and centrifuged at 750 × g for 5 min. Floated oocysts on the surface of sucrose solution were collected [1] and subjected to ten freeze/thaw cycles at 4°C water bath. The samples were then sonicated on ice with a Virsonic 50 Cell Disrupter (VirTis Company, Gardiner, NY, U.S.A.) at 50% power output, ten times for 10 sec at 1-min intervals, then centrifuged at 14,000 rpm at 4°C for 5 min. Total DNA was extracted with the QIAamp DNA kit according to the manufacturer’s protocol (Qiagen, Germany). C. parvum HNJ-1 was used as positive control. Oligonucleotide primers for the variable region of the 18S rRNA of Cryptosporidium [1] were used to confirm the sequence as shown in Table 1. PCR was performed in a 25-µl reaction solution containing 5 µl of DNA template, 20 pmol of each primer, 1.25 U of Ex Taq DNA polymerase (Takara Co., Japan), 2 mM MgCl₂, and 250 µM of each dNTP. The following PCR cycle was performed using an iCycler thermal cycler (BioRad Ltd., U.S.A.): 1 × 94°C for 2 min, 40 × (94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec), 1 × 72°C for 7 min. Nested PCR products were purified by a QIAquick Gel Extraction kit (Qiagen, Germany), and the PCR products were sequenced using an automated sequencer with an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer Ltd., U.S.A.). The nucleotide sequences of isolates were aligned by Clustal-X (DNAstar, ver. 5.0).

In addition, we confirmed the species on the basis of 18S rRNA gene from the isolated Cryptosporidium oocysts by nested PCR and restriction fragment length polymorphism analysis (RFLP). The following PCR cycle was used: 1 × 94°C for 2 min, 40 × (94°C for 30 sec, 60°C for 45 sec, 72°C for 90 sec), 1 × 72°C for 7 min. Nested PCR using the primers listed in Table 1 was performed in a 25 µl reaction solution formerly [4]. The conditions for nested PCR were: 1 × 94°C for 2 min, 40 × (94°C for 30 sec, 58°C for 30 sec, 72°C for 60 sec), and 1 × 72°C for 7 min. The appropriate digestion enzymes were used to analyze the genotype of 18S rRNA products.

As a result, total DNA extracted was amplified to about 214 bp by nested PCR. In PCR/RFLP with AseI and Taq I enzymes, all the bands detected from respective samples were identical with that of Cryptosporidium parvum (Fig. 2). Also the oocysts were genotyped using 18S RNA sequence analysis. Sequence alignments were 100% identical with that of C. parvum (GenBank accession No. AF108864) in the 444 to 739 bp region of 18S RNA (data not shown). This region has been used to distinguish C. hominis, C. parvum, C. canis, C. baileyi, C. galli, C. serpentis in humans, mammals, birds, and fish [5, 7, 10, 11, 15, 16].

The selected area for the survey, Gokseong (30,000 population, 11,000 cattle), Muam (11,000 population, 3,700 cattle), and Imshil (32,000 population, 6,000 cattle) Counties has animal farms, and is located near a river, respectively. In Gokseong County, it has been reported that 98.2% of cattle were infected with C. parvum and its infection rate was particularly high in June due to heavy rainfall [17], and that the percentage of C. parvum-infected humans in normal inhabitants was 1.3–8.2% [17].

Thus, we speculated that groundwater and/or rivers in these environments might be contaminated with wastewater containing animal feces. Actually water supplied to domestic animals in these regions was often groundwater. We were unable to examine whether people were exposed to Cryptosporidium-contaminated groundwater or not, because our basic investigation for healthy individuals was performed on a small scale.

Future wide-scale genetic and epidemiological studies for Cryptosporidium sp. infection of healthy people, as well as seasonal testing of rivers, would improve our understanding of cryptosporidiosis and make it possible to undertake appropriate countermeasures to ensure human and animal health in Korea.

REFERENCES


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<th>PCR</th>
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Table 1. Primers used in this study.
Fig. 2. Restriction digests of *Cryptosporidium* oocyst genes amplified using SCL2 and SCR2 primers. Lane M, 100-bp ladder; lanes 1–8, stool samples from Goseong county (Jeollanam-do); lanes 9–11, stool samples from Muan county (Jeollabuk-do); lane 12, stool sample from Imshil county (Jeollanam-do); lane C, *C. parvum* strain HNJ-1 (positive control).