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Genotype analysis of *Cryptosporidium* spp. prevalent in a rural village in Hwasun-gun, Republic of Korea

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Abstract: Two species of *Cryptosporidium* are known to infect man; *C. hominis* which shows anthroponotic transmission between humans, and *C. parvum* which shows zoonotic transmission between animals or between animals and man. In this study, we focused on identifying genotypes of *Cryptosporidium* prevalent among inhabitants and domestic animals (cattle and goats), to elucidate transmittal routes in a known endemic area in Hwasun-gun, Jeollanam-do, Republic of Korea. The existence of *Cryptosporidium* oocysts was confirmed using a modified Ziehl-Neelsen stain. Human infections were found in 7 (25.9%) of 27 people examined. Cattle cryptosporidiosis cases constituted 7 (41.2%) of 17 examined, and goat cases 3 (42.9%) of 7 examined. Species characterizations were performed on the small subunit of the rRNA gene using both PCR-RFLP and sequence analysis. Most of the human isolates were mixtures of *C. hominis* and *C. parvum* genotypes and similar PCR-RFLP patterns were observed in cattle and goat isolates. However, sequence analyses identified only *C. hominis* in all isolates examined. The natural infection of cattle and goats with *C. hominis* is a new and unique finding in the present study. It is suggested that human cryptosporidiosis in the studied area is caused by mixtures of *C. hominis* and *C. parvum* oocysts originating from both inhabitants and domestic animals.

Key words: *Cryptosporidium hominis*, *Cryptosporidium parvum*, human, cattle, goat, PCR-RFLP, small subunit rRNA, sequence, Republic of Korea

INTRODUCTION

Cryptosporidium is a small protozoan parasite found in the brush-border of mucosal epithelia of various species of mammals, birds, reptiles, and fishes (Morgan et al., 1999). After Tyzzer (1907) found *Cryptosporidium muris* in the gastric gland of mice, more than 20 *Cryptosporidium* species have been proposed based on their host specificities, oocyst morphologies, and genetic characteristics (Morgan et al., 1999; Xiao et al., 2004). Human infection cases have been reported in *Cryptosporidium parvum* (including *C. hominis*), *C. meleagridis*, *C. muris* (renamed as *C. andersoni*), and *C. felis*, but most cases were caused by *C. parvum* (Morgan et al., 2000a; Xiao et al., 2001) or *C. hominis* (Morgan-Ryan, 2002). Cryptosporidiosis is known to be an opportunistic parasitosis of humans

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(Widmer, 1998) with a worldwide distribution (Griffiths, 1998). This disease occurs in 5-50% of AIDS patients (Spano and Crisanti, 2000), and an important cause of AIDS-associated deaths due to severe diarrhea (Widmer et al., 1998).

Several studies have reported the characteristics of *C. parvum* with respect to its transmission, and 2 genotypes of *C. parvum* were suggested to exist in humans (Peng et al., 1997; McLauchlin et al., 2000). One was named as the human genotype, i.e., *Cryptosporidium hominis*, which is transmissible between humans, and the other was a bovine genotype, redesignated as *C. parvum*, which is transmissible between man and vertebrates, especially cattle (Morgan-Ryan et al., 2002). Several studies had revealed the differential characteristics between *C. hominis* and *C. parvum* particularly in terms of genetic differences, whereas others have focused on its pathogenic and epidemiologic properties (Okhuysen et al., 1999; Morgan et al., 2000a; Eisenberg et al., 2005). The different transmission cycles of *C. parvum* and *C. hominis* between man and animals could allow us to trace sources of oocyst contaminations.

In the Republic of Korea, the first documentation of the presence of *Cryptosporidium* was done by a case report of chicken cryptosporidiosis (Mo et al., 1988) and subsequently *C. parvum* was reported in mice (Chai et al., 1990). Thereafter, human *C. parvum* infections were reported in epidemiological studies (Chai et al., 1996, 2001; Seo et al., 2001). In particular, a study in a small rural village of Hwasun-gun verified the existence of *C. parvum* in both humans and cattle, suggesting that the responsible parasite might be the bovine genotype (Chai et al., 2001). However, genetic approaches of *Cryptosporidium* in this village have not been progressed.

The present study was thus focused on investigating *Cryptosporidium* species by analyzing genotypes in the known endemic village of Hwasun-gun, by applying PCR-RFLP and sequence analysis of the small subunit (SSU) rRNA gene of *Cryptosporidium* oocysts collected from man and domestic animals.

MATERIALS AND METHODS

The area studied was a small rural village; Ssangbong-ri, Iyang-myon, Hwasun-gun, Jeollanam-do, a well-known endemic area of cryptosporidiosis (Chai et al., 2001). The previous *Cryptosporidium* oocyst positive rate was 57% (Chai et al., 2001). In December 2000, fecal samples were collected from 27 people, 17 domestic cattle, and 7 domestic goats living in the village. The samples were mixed well with 2 volumes of 2.5% $K_2Cr_2O_7$ and stored at 4°C to maintain oocyst vitality. The specimens were examined for the presence of oocysts, which was confirmed by modified Ziehl-Neelsen staining. Oocysts were then purified using Arrowood and Sterling (1987)'s discontinuous sucrose and CsCl gradient methods. Collected oocysts were re-suspended in 1 ml of 2.5% $K_2Cr_2O_7$ and stored at -70°C until required. Genomic DNA was extracted as previously described (Kim et al., 1992).

For species-specific identifications, primer sets were designed based on the SSU rRNA gene locus. Primer sequences unique to *Cryptosporidium* species, as identified by Xiao et al. (2001), were used in a nested PCR protocol. The primer sets used for the primary PCR were 5`-TTCTAGAGCTAATACATGCG-3` and 5`-CCCTAATCCTTCGAAACAGGA-3` and those used for the secondary PCR were 5`-TTCTAGAGCTAATACATGCG-3` and 5`-CCCATTTCCTTCGAAACAGGA-3`. For restriction fragment analyses, 5 μl of the second PCR products were digested for 4 hr in 20 μl reaction mixtures containing 10 U of *Ssp* I (Roche, Mannheim, Germany) or 10 U of *Vsp* I (Gibco BRL, Grand island, New York, U.S.A.), as recommended by the manufacturers. Digested mixtures were loaded on 2 % agarose gels and visualized by ethidium bromide staining after electrophoresis. Amplified PCR products in agarose gels were extracted using the QIAGEN Gel Elution Kit (QIAGEN K.K., Tokyo, Japan).

PCR products eluted from gels were directly sequenced using a model ABI Prime 377 Automatic Sequencer (Perkin Elmer, Foster City, California, U.S.A.), when they were suitable for sequence analyses. However, if eluted products were too small or unsuitable for direct sequencing, the pGEM®-T Easy

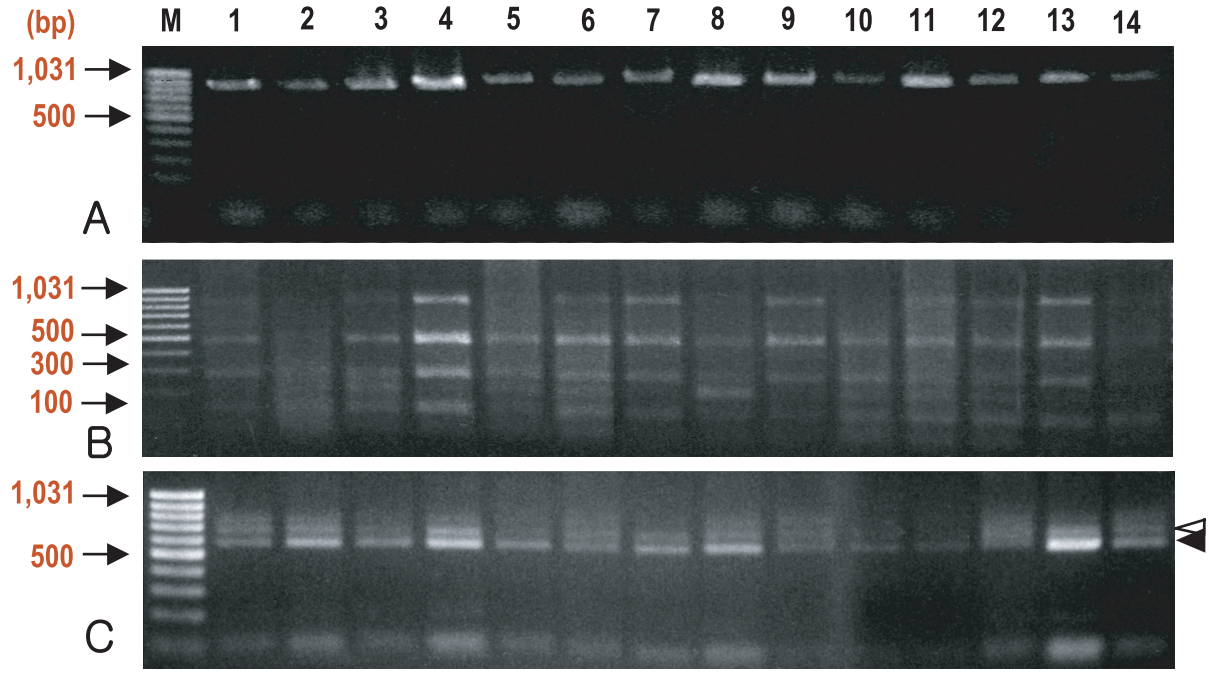

Fig. 1. Gel electrophoresis of nested PCR-RFLP based on the small subunit (SSU) rRNA gene of *Cryptosporidium*. Stained with ethidium bromide on 2% agarose gel. The species of *Cryptosporidium* was determined by digestion with endonuclease, *Ssp* I **(B)** or *Vsp* I **(C)**, of nested PCR products **(A)**. Most of isolates had both *C. hominis* and *C. parvum* patterns **(B, C)**. The white arrowhead (ca. 628 bp) is *C. parvum*-specific and the black arrowhead (ca. 556 bp) indicates *C. hominis*-specific bands obtained by *Vsp* I **(C)** digestion. M, DNA size marker; lanes 1-5, isolates from villagers; lanes 6-11, isolates from domestic cattle; lanes 12-14, isolates from domestic goats.

Vector Systems I (Promega, Madison, Wisconsin, U.S.A.) was used. All sequences were aligned and analyzed using Megalign (DNA Star, Madison, Wisconsin, U.S.A.) and Sequence Navigator (Applied BioSystems, California, U.S.A.) programs.

RESULTS

Cryptosporidium oocysts were found in 7 human fecal specimens (25.9% in positive rate), 7 cattle (41.2%), and 3 goats (42.9%) (Table 1), and these were encoded serially (Fig. 1). In most cases of smeared samples, the number of oocysts did not exceed 20 per smear, and oocyst sizes ranged 4-6 μm for both *C. parvum* and *C. hominis*. No other protozoan cysts were detected, but unidentified helminth eggs were observed in 2 goats. A sucrose gradient separation method and a CsCl gradient method were found to be useful for purifying oocysts from fecal samples; however, oocyst losses were incurred at each step.

Nested PCR using genomic DNA, extracted from purified oocysts, was sufficient for endonuclease

Table 1. Prevalence of *Cryptosporidium* spp. among people and domestic animals in the study area, Ssangbong-ri, Iyang-myon, Hwasun-gun, Jeollanam-do

Villagers[1)]		Cattle		Goats	
No. examined	No. (%) positive	No. examined	No. (%) positive	No. examined	No. (%) positive
27	7 (25.9)	17	7 (41.2)	7	3 (42.9)

[1)] Human fecal samples were collected from villagers who were oocyst positive more than twice in a long-term survey conducted from November 1996 to October 1997 (Chai et al., 2001).

Table 2. Sequence alignments of *Cryptosporidium* spp. small subunit (SSU) rRNA partial gene deposited at GenBank

Sequence origin at GenBank (isolate name)	Host	Location of mutations in the SSU rRNA gene[a)]		
		260-267	37-653	680-699
C. hominis (sample no. 1; Fig. 1)[b)]	Human	AAT--T--AAT--G	AAAATATTTTTGAT-GAAT	ATTACTATTTTTT---TTTTTAG
C. hominis (sample no. 11; Fig. 1)[c)]	Cattle	AAT--T--AAT--G	AAAATATTTTTGAT-GAAT	ATTACTATTTTTT---TTTTTAG
C. hominis (sample no. 16; Fig. 1)[d)]	Goat	AAT--T--AAT--G	AAAATATTTTTGAT-GAAT	ATTACTATTTTTT---TTTTTAG
C. hominis (HCNV4)	Human	AAT--T--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATTTTTT---TTTTTAG
C. hominis (H7)	Human	AAT--T--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATTTTTT---TTTTTAG
C. hominis (L16997)	Human	AAT--T--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATTTTTT---TTTTTAG
C. parvum (C1)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum (BOH6)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum (MT)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum (TAMU)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum (UCP)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum (GCH1)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum (IOWA)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum (L16996)	Bovine	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum	Deer	ATT--A--AAT--G	AAAATATTTT-GAT-GAAT	ATTACTATATATT---TTAGTAT
C. parvum	Dog	ATT--TT--AT--G	ATAATATTTAA-----CAT	ATTACTAT--------TTAT-AG
C. parvum	Ferret	ATA--A--ATT--G	AAAATATTTT-GAT-TAAT	ATTACTAAATTTT---TGTTTGG
C. parvum	Mouse	ATT--A--AAT--G	AAAATATTTT-AAT-TAAT	ATTACTATA-ATT---ATTTT-T
C. parvum	Pig	ATT--TTTAAT--G	ATAATATTTTT-----AAT	ATTACTATAATTT---TTATTAG
C. parvum	Monkey	AAT--T--AAT--G	AATATATTTT-GAT-GAAT	ATTACTATTTTTT---TTTA-GT
C. baileyi	Chicken	A----TTTA---TG	ACAATACCAC------GGT	ATTACT--T-----A---TTTAA
C. felis (C8)	Feline	AATAATTTATTTTG	ATAATATTTTTTTTTAAAT	ATTTTTTAAGACTGAATTTTTAG
C. meleagridis	Turkey	AAT--T--TAT--G	ATAATATTTG--AT-TAAT	ATTACTAAATTTA---TTAGTAT
C. muris	Rat	ATC--TCTGA--TG	ATAATATTACCAA---GGT	ACTATTATATTCTAAAT----AT
C. serpentis	Snake	ATC--TCTGA--TG	ATAATATTATTAA---GGT	ACTATTATATTTTTAAT----AT
C. wrairi	Guinea pig	ATA--A--ATT--G	ATAATATTTT--GA-AAAT	ATTACTATATATT---TTTA-GT

[a)] The nucleotide positions of aligned sequences are numbered using sequences of the *C. hominis* HCNV4 isolate in the SSU rRNA gene.
[b)] GenBank accession No.: DQ054817.
[c)] Genbank accession No.: DQ054818.
[d)] Genbank accession No.: DQ054819.

digestion (Fig. 1A). However, the amplification failed for 1 isolate from a man (data not shown) even though a small number of oocysts were detected by modified Ziehl-Neelsen staining. Digestion with *Ssp* I fragmented the second PCR products (ca. 830 bp) into 3 bands (448, 247, and 106 bps) (Fig. 1B), which is characteristic for both *C. parvum* and *C. hominis* (Xiao et al., 1999), except in 2 samples, one each from a man and a cattle (data not shown). Digestion of the PCR products with *Vsp* I produced 3 bands, 2 of which (ca. 628 and 104 bps) are specific for *C. parvum*, and 2 (ca. 556 and 104 bps) for *C. hominis* (Xiao et al., 1999) (Fig. 1C).

The sequences of the PCR products were analyzed in 5 human isolates (sample Nos. 1, 2, 3, 4, and 5; Fig. 1), 6 cattle isolates (6, 7, 8, 9, 10, and 11), and 3 goat isolates (12, 13, and 14). The PCR products from sample 1 were sequenced directly and others were sequenced using the cloned PCR products. A few variations were observed at a repeated thymine locus. However, sequences in the polymorphic regions

(positions 260-267, 637-653, and 680-699) were all *C. hominis* type (Table 2), and no sequences for *C. parvum* type were obtained. Sequences of *C. hominis* from humans, cattle, and goats were accessed in GenBank under accession numbers DQ054817 (human isolate), DQ054818 (cattle isolate), and DQ054819 (goat isolate).

DISCUSSION

As shown by the results, oocysts of both *C. hominis* and *C. parvum* genotypes were identified in humans by PCR-RFLP analyses. Isolates from domestic animals also showed the same species genotypes, although sequencing of the PCR products successfully revealed only *C. hominis* genotype. Obtaining only *C. hominis* sequence may suggest predominance of *C. hominis* among the *Cryptosporidium* oocysts in the study area. However, the co-infection of both *C. hominis* and *C. parvum* in man and animals is a unique finding in the present study.

Human cryptosporidiosis, whatever the parasite species, is caused by direct contact with contaminated food or through drinking water. Ingestion of only small numbers of viable *Cryptosporidium* oocysts could cause human cryptosporidiosis (Smith et al., 1993). In the study area, water is supplied from a storage reservoir used by both villagers and domestic animals. Swimming and fishing are prohibited in the reservoir; however, a few domestic animals including cattle and goats are allowed to roam freely around the reservoir. Thus, if the reservoir was contaminated with *Cryptosporidium* oocysts from these animals, villagers and their animals could be easily exposed to oocysts. Sylvatic transmissions of *C. parvum* involving deer and smaller mammals have also been suggested to be important sources of human cryptosporidiosis (Perz and Le Blancq, 2001).

In the waterborne outbreaks in England, human cryptosporidiosis was found to be primarily caused by drinking water contamination and *C. parvum* was identified as the causative parasite species (McLauchlin et al., 2000). The area studied in the present study showed a prevalence and seasonality (Chai et al., 2001) similar to those reported in England (McLauchlin et al., 2000). However, in the present study, it seems that *C. hominis* genotype predominated, as shown by the sequencing data. In this respect, water reservoir contamination by infected animals may not have been the main cause of human cryptosporidiosis. We suggest that cryptosporidiosis in the studied village may have been caused by food handlers directly contacting foods contaminated by oocysts, i.e., during the preparation of family meals and animal foods.

A unique finding in the present study was the presence of *C. hominis* genotype in domestic cattle and goats. Of 7 cattle isolates, 6 contained *C. hominis* genotype as shown by PCR-RFLP (Fig. 1C), and all of the 3 goat isolates contained the same species of *Cryptosporidium*. Observations in other countries have identified species of *Cryptosporidium* in bovines and ovines to be *C. parvum* (McLauchlin et al., 2000). This was an important reason why *C. parvum* and *C. hominis* were proposed to be distinct species in terms of transmittal and genetic characteristics (McLauchlin et al., 2000; Morgan-Ryan et al., 2002). However, several recent studies have demonstrated the possibility of animal infections with *C. hominis*; the examples of animals include interferon-γ knock-out (GKO) mice, immunosuppressed ICR mice, gnotobiotic piglets, a captive primate, a lamb, and calves (Widmer et al., 2000; Giles et al., 2001; Akiyoshi et al., 2002; Guk et al., 2004). *Dugong dugon*, a marine mammal, was also found infected with the human genotype (Morgan et al., 2000b). In the present study, most of the villagers and their domestic animals, including cattle and goats, reside at the same premises, and thus share a limited biological environment, which may have been responsible for their common infections with *C. hominis*.

Another possibility is that the Korean isolate of *Cryptosporidium* might be a third genotype, not discriminated by SSU rRNA gene analyses, i.e., isolates which have genetic characteristics of *C. hominis* type but have an ability to infect domestic animals. In this respect, successful passages of a human isolate of *Cryprosporidium*, a mixture of the human and bovine

genotypes, to calves and then ICR mice were reported in the Republic of Korea (Guk et al., 2004). If these 2 genetically similar *Cryptosporidium* species were admixed for a sufficiently long period, genetic recombination could occur. In fact, an experimental meiotic recombination occurred between human (= *C. hominis*) and bovine genotypes (= *C. parvum*) in the sexual phase, and produced a progeny that infected GKO mice (Feng et al., 2002).

The results of the present study demonstrated that all of the human, cattle, and goat isolates of *Cryptosporidium* studied were mixtures of *C. parvum* and *C. hominis*. Further studies are required to determine further characteristics of the 2 *Cryptosporidium* species existing in the Republic of Korea.

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