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▣ BRIEF COMMUNICATION

Molecular Prevalence and Genotypes of *Cryptosporidium parvum* and *Giardia duodenalis* in Patients with Acute Diarrhea in Korea, 2013-2016

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Abstract: *Cryptosporidium parvum* and *Giardia duodenalis* are the main diarrhea-causing parasitic pathogens; however, their prevalence in Korea is unknown. Here, we conducted a survey to determine the prevalence and genotype distribution of these 2 pathogens causing acute diarrhea in 8,571 patients hospitalized in 17 Regional Institute of Health Environment sites in Korea, during 2013-2016. *C. parvum* and *G. duodenalis* were detected and genotyped by nested PCR, and the isolate were molecularly characterized by sequencing the glycoprotein 60 (Gp60) and β-giardin genes, respectively. The overall prevalence of *C. parvum* and *G. duodenalis* was 0.37% (n=32) and 0.55% (n=47), respectively, and both pathogens were more prevalent in children under 9 years old. Molecular epidemiological analysis showed that the *C. parvum* isolates belonged to the IIa family and were subtyped as IIaA13G2R1, IIaA14G2R1, IIaA15G2R1, and IIaA18G3R1. Analysis of the β-giardin gene fragment from *G. duodenalis* showed that all positive strains belong to assemblage A. This is the first report on the molecular epidemiology and subtyping of *C. parvum* and *G. duodenalis* in such a large number of diarrheal patients in Korea. These results highlight the need for continuous monitoring of these zoonotic pathogens and provide a basis for implementing control and prevention strategies. Further, the results might be useful for epidemiological investigation of the source of outbreak.

Key words: *Cryptosporidium parvum*, *Giardia duodenalis*, glycoprotein 60, β-giardin

The protozoan genera *Cryptosporidium* and *Giardia* are important causes of diarrhea worldwide [1,2]. The main clinical symptoms of infections with *Cryptosporidium* spp. include diarrhea, along with vomiting and abdominal cramps, loss of appetite, and low-grade fever. Similarly, *Giardia* spp. cause diarrhea, bloating and fatigue, stomach cramps, nausea, and weight loss during chronic infections [3,4]. Previous reports have shown that *Giardia duodenalis* is the etiological agent in 35.2%, while *Cryptosporidium* spp. was the causal agent in 60.3%, of cases among the 199 documented worldwide outbreaks due to waterborne transmission from 2004 to 2010 [5]. More recently, at least 381 outbreaks of parasitic protozoa were documented globally from January 2011 to December 2016 and the most common etiology was *Cryptosporidium* spp., which was responsible for 63% of cases, and *Giardia duodenalis* was detected in 37% of cases [6].

In Korea, a national program to analyze stool samples for the control and prevention of diarrheal diseases revealed a positive rate of *C. parvum* and *G. duodenalis* of 0.28% and 0.61%, respectively, based on enzyme immunoassays [7]. In another report, *G. lamblia* and *C. parvum* were detected by enzyme-linked immunosorbent assay (ELISA) in 2.5% and 0.4% of 6,071 stool samples collected from a general hospital in Gyeonggi province [8]. Moreover, *Cryptosporidium* oocysts and *Giardia* cysts have been detected in raw vegetables, environmental soil, and intake water samples [9,10]. Two other recent accidental outbreaks of these protozoa in Korea have been reported to date [11,12]. The first was a waterborne outbreak in April 2010 including 9 cases of *giardia*sis that occurred among individuals drinking valley water stored in a water tank without sterilization as a temporary water supply [11]. The second was also a waterborne outbreak from May to June 2012 including 124 cases of cryptosporidiosis that originated from an apartment water tank with worn plumbing systems that became contaminated by *Cryptosporidium* oocysts [12]. In Korea, the outbreaks caused by *C. parvum* and *G. duodenalis* infection

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are typically accompanied by acute diarrhea; however, surveys of the prevalence of such cases and the causal strains, such as *C. parvum* and *G. duodenalis*, are rare because the tap water supply system is generally well established throughout the country. Therefore, there has been no molecular epidemiological surveillance to date among the Korean population with respect to acute diarrhea. Accordingly, the aim of the present study was to determine the molecular infection rate and subtypes of *C. parvum* and *G. duodenalis* responsible for acute diarrhea in Korean patients from 2013 to 2016.

During the 4 years of the study, from January 2013 to December 2016, a total of 8,571 stool samples were collected from acute diarrhea patients in 17 Regional Institute of Health Environment sites located throughout the country. Each stool sample (1 g) was suspended in 5 ml of phosphate-buffered saline (PBS) and filtered through gauze to remove large particles. The filtered liquid was centrifuged at 800 g for 10 min. The supernatant was carefully removed, and the remaining sediment was mixed with 1 ml PBS. The pellet was subjected to 10 boiling (100°C) and freezing (-70°C) cycles to break the hard surface wall of *Giardia* and *Cryptosporidium* cysts.

Genomic DNA was extracted from each stool sample using DNAzol (MRC, Cincinnati, Ohio, USA) and stored at -20°C until use. The target DNA for nested PCR was the partial *Cryptosporidium* oocyst wall protein (COWP) gene. In the first PCR, a 550-bp fragment was amplified using the Cry-15 (5′-GTAGATAATGGAAGA GATTGT G-3′) and Cry-9 (5′-GGACTGAAATACAGGCATTATCTT G-3′) primers. In the second PCR, a 310-bp fragment was amplified using the cowpnest-F1 (5′-TGTGTTCAATCAGACACAGC-3′) and cowpnest-R2 (5′-TCTGTATATCCTGGTGGGC-3′) primers [13]. *G. duodenalis* was detected by nested PCR of the β-giardin gene. The first primer set was G376A forward (5′-CCATCCATAACGACGCCATCGCGGCTCTC-3′) and GGR789-809B reverse (5′-GGC GCT TAG TGC TTT GTG ACC-3′), and the second primer set was G376B (5′-CGA CGC CAT CGC GGC TCT CAG GAA GGA GG-3′) and G759A (5′-CGC CCT GGA TCT TCG AGA CGA CGT CCT-3′), amplifying fragments of 415 bp and 374 bp [11]. The PCR cycle conditions for the PCR were identical to those used for the primary PCR. PCR products and restriction fragments were subjected to electrophoretic separation on 2% agarose gels, which were stained with Safe-Pinky (GenDEPOT Barker, Texas, USA) and visualized on a gel documentation system (Sangene; G-Box; Cambridge, UK).

Table 1. Detection of *Cryptosporidium parvum* and *Giardia duodenalis* infections using PCR in human fecal samples in 2013-2016

Year	No. of sample	Positive no. of *C. parvum* (%)	Positive no. of *G. duodenalis* (%)
2013	3,093	23 (0.74)	27 (0.87)
2014	2,046	2 (0.1)	9 (0.44)
2015	1,722	2 (0.12)	1 (0.06)
2016	1,710	5 (0.29)	10 (0.68)
Total	8,571	32 (0.37)	47 (0.55)

The total positive rate of all samples was 0.89%, with a *C. parvum* infection rate of 0.37% and a *G. duodenalis* infection rate of 0.55%. There were only 2 cases of mixed infection (Table 1). The positive rates and pattern obtained here using PCR are similar to those reported previously by Cheun et al. [7] in Korea, using methods other than PCR; however, the rates were lower than those reported in China (1.4%) and in Libya (1%) [14,15].

In general, diarrhea caused by infection by waterborne protozoa mainly occurs in the periods between spring and summer, and between summer and fall during the season transition [7]. Consistently, in the present study, the highest positive rates of the 2 pathogens were observed in May and October

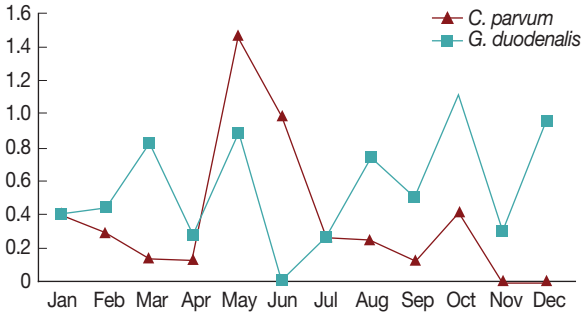

Fig. 1. Monthly positive rates of *Cryptosporidium parvum* and *Giardia duodenalis* in stool samples of Korean patients with acute diarrhea sampled during 2013-2016.

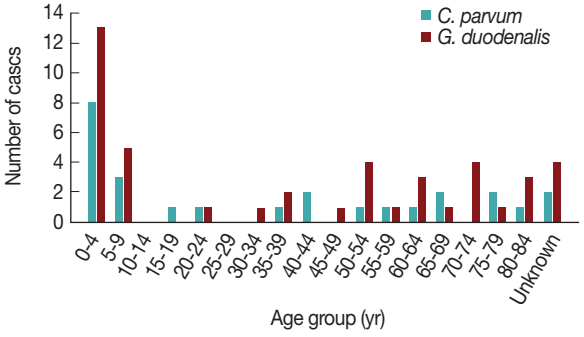

Fig. 2. Number of *Cryptosporidium parvum* and *Giardia duodenalis* cases by age group in Korea, 2013-2016.

(Fig. 1). This might be due to the change in seasons from spring to summer in May, and from fall to winter in October; however, more research is needed to explain the seasonal nature of protozoan infection [7,16]. Furthermore, the pathogens were more prevalent in patients under 10 years of age compared to other ages (Fig. 2). Previously, the infection rate of *G. duodenalis* was found to be the highest in the less than 10-year-old group [17] and to occur most frequently in the summer [16]. One of the reasons for the high infection rate in children under 10 years of age may be due to their sensitivity to exposure of these pathogens via food and environmental risk factors such as food contamination and accidental ingestion of these organisms [18]. This general pattern indicates that more attention should be paid to warning young children about the possibility of waterborne pathogen infection.

Interestingly, the positive rates of *C. parvum* and *G. duodenalis* were very low and did not correlate with increase in age (Table 1). Although acute diarrhea cases caused by *C. parvum* and *G. duodenalis* in Korea are rare, these results demonstrate that regular epidemiological surveillance among patients with acute diarrhea could offer guidance for the control of waterborne diseases. Subtyping has been extensively performed in studies of *C. parvum* and *G. duodenalis* transmission in humans and animals, and nearly 20 *C. parvum* subtype families and 8 *G. duodenalis* genetic assemblages have been described at the respective loci [19,20]. We conducted phylogenetic analysis of the GP60 gene of *C. parvum* using PCR amplification of ~400 base pairs (bp). Nested PCR was performed using the AL3531 forward primer (5′-ATAGTCTCCGCTGTATTC-3′) and AL3533 reverse primer (5′-GAGATATATCTTGGT GCG-3′) and the second set of primers were AL3532 forward primer (5′-TCCGCTGTATTCTCAGCC-3′) and LX0029 reverse primer (5′-CGAACCACATTACAAATGAAGT-3′) [21]. Nested PCR amplification of β -giardin gene was used to analyze the molecular characteristics of *G. duodenalis*. In the primary PCR reaction, a 753 bp fragment was amplified using the G7 forward primer (5′ -AAGCCCGACGACCTCACCCGCAGTGC-3′) and G-759 reverse primer (5′-GAGGCCGCCCTGGATCTTCGAGACGAC-3′) and the second set of primers were G511F forward primer (5′ -GAACGAACGAGATCGAGGTCC-3′) and G-511R reverse primer (5′-CTCGACGCG CTTCGTGTT -3′) [22]. All PCR-positive products were purified using a DNA purification kit (Qiagen, Hilden, Germany) after electrophoresis, and then purified again on an agarose gel and sequenced using the ABIPRISM 3730xl Analyzer system (Applied Biosystems, Foster City, California, USA). DNA sequences were compared using the BLAST tool, and phylogenetic trees were constructed using the CLUSTAL W multiple sequence alignment computer program

Table 2. Subtyping of the GP60 genes and genotyping of the β-giardin for human stool sample of diarrheal patients from Korea testing positive for *Cryptosporidium parvum* and *Giardia duodenalis* using nested PCR

	Specimen ID	Subtypes (GP60)		Specimen ID	Genotypes (β-giardin)
C. parvum	13C1	IIaA14G2R1	*G. duodenalis*	13G1	Assemblage A
	13C2	IIaA14G2R1		13G2	Assemblage A
	13C3	IIaA14G2R1		13G3	Assemblage A
	13C4	IIaA14G2R1		13G4	Assemblage A
	13C5	IIaA14G2R1		13G5	Assemblage A
	13C6	IIaA14G2R1		13G6	Assemblage A
	13C7	IIaA14G2R1		13G7	Assemblage A
	14C1	IIaA13G2R1		13G8	Assemblage A
	15C1	IIaA18G3R1		13G9	Assemblage A
	16C1	IIaA15G2R1		14G1	Assemblage A
	16C2	IIaA15G2R1		15G1	Assemblage A
				16G1	Assemblage A
				16G2	Assemblage A
				16G3	Assemblage A
				16G4	Assemblage A
				16G5	Assemblage A
				16G6	Assemblage A
				16G7	Assemblage A
				16G8	Assemblage A
				16G9	Assemblage A

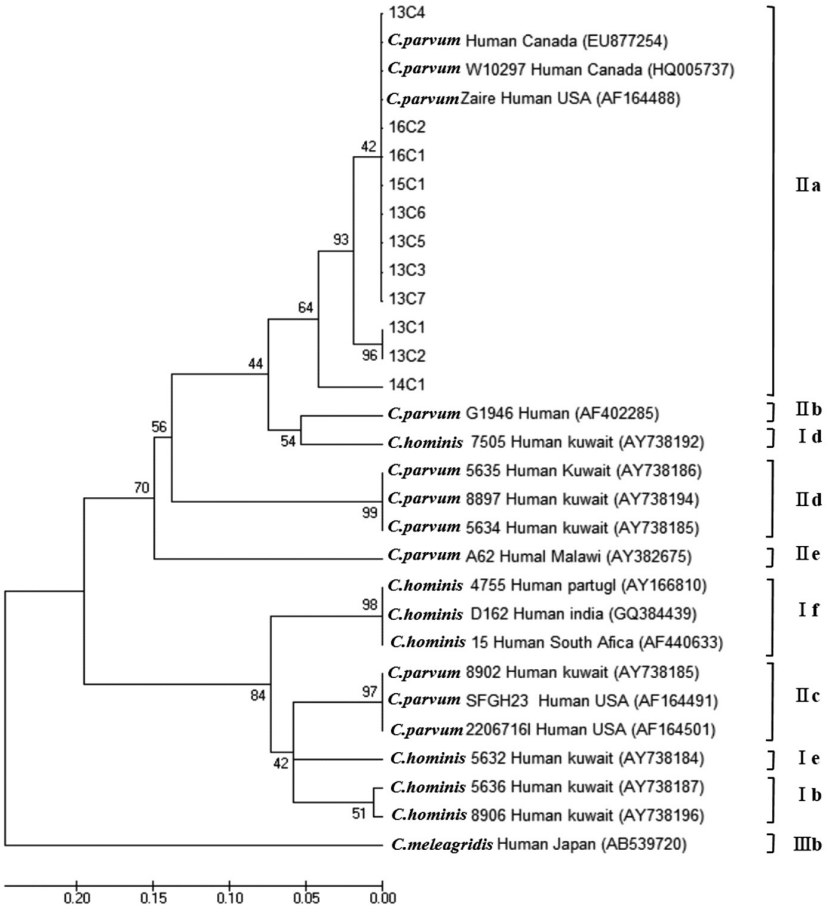

Fig. 3. Phylogenetic relationships of the GP60 locus of *Cryptosporidium* species strains isolated from Korean patients with diarrhea. The phylogenetic tree was constructed using the maximum-likelihood method with 1,000 replicates (implemented using Molecular Evolutionary Genetics Analysis [MEGA]). Other *Cryptosporidium* species sequences were obtained from GenBank.

(Histon, Cambridgeshire, UK) and the Molecular Evolutionary Genetics Analysis (MEGA) program with the maximum-likelihood method; the robustness of groupings was assessed using 1,000 bootstrap replicates of the data [23]. Unfortunately, phylogenetic analysis could only be performed in 47 of the 77 positive samples. A total of 11 samples for *C. parvum* and 20 samples for *G. duodenalis* were successfully amplified by nested PCR. *C. parvum* was classified into 4 subtype families: 1 in IIaA13G2R1, 7 in IIaA14G2R1, 2 in IIaA15G2R1, and 1 in IIaA18G3R1, representing the most common zoonotic subtype family (Table 2; Fig. 3). According to the recent review of genetic diversity in *Cryptosporidium*, the IIaA15G2R1 subtype is particularly dominant in calves and lambs as well as in humans; IIaA13G2R1 was reported mostly in calves and AIDS patients; IIaA18G3R1 in cows and in people of Northern Ireland; and IIaA14G2R1 was reported for the first time in cattle from Germany, in AIDS patients from Malaysia and Italy, as well as in fish (Nile tilapia) in Papua New Guinea [19].

Main source of outbreaks with *Cryptosporidium* spp. infection were identified as recreational waters and animal contact, and other sources such as environmental contact, person-to-person spread, food and drinking water supplies comprised a very low proportion [24]. Especially among the subtypes of *C. parvum*, IIaA15G2R1 was the predominant in England and Wales, IIdA24G1 was the most common type in the outbreak in Sweden [24,25], and IIaA14G2R1 was identified as dominant in Korea through the present surveillance. With respect to the molecular subtyping analysis, based on these previous reports, we cautiously speculate that the *C. parvum* detected from Korean diarrhea stool samples may have originated from contact with animals such as calves, cattle, and lambs raised in Korea. Unfortunately, there was no molecular subtyping con-

Fig. 4. Phylogenetic relationships of the β-giardin locus of *Giardia* species strains isolated from Korean patients with diarrhea. The phylogenetic tree was constructed using the maximum-likelihood method with 1,000 replicates (implemented using Molecular Evolutionary Genetics Analysis [MEGA]). Other *Giardia* species sequences were obtained from GenBank.

ducted in the first outbreak of cryptosporidosis in Korea for source of origin [12].

Among the 8 genetic assemblages of *Giardia*, assemblages A and B are known to be the main causes of human infections [26]. Although the majority of studies on the inter-species transmission of *G. duodenalis* have focused on animal-to-human transmission, there is increasing evidence that *Giardia* cysts of human origin can also contaminate the environment and infect wild mammals [27]. In the present investigation, all positive cases with *G. duodenalis* infection were identified to belong to assemblage A (Table 2; Fig. 4). This is in line with previous results from the feces DNA of Japanese, Mongolian, and Korean patients [28,29]. Because *Giardia* cysts have also been detected in natural water resources, it is possible that these patients were naturally infected or exposed to environmental contamination of the pathogen; however, there was no information available on the site of infection or route and timing of exposure. Unfortunately, molecular subtyping was not conducted in the first outbreak of giardiasis in Korea for comparison [11].

In summary, this is the first molecular epidemiological investigation and subtyping of *C. parvum* using the Gp60 gene and of *G. duodenalis* using the β-giardin gene in stool samples from diarrheal patients in Korea. Both pathogens were found to belong to a zoonotic subtype family. These findings emphasize the importance of continuous molecular epidemiological surveillance and subtyping for waterborne parasitic pathogens among acute diarrheal patients to identify the origin of waterborne outbreaks of *C. parvum* and *G. duodenalis* infections.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

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