*Am. J. Trop. Med. Hyg.*, 90(1), 2014, pp. 43–47
doi:10.4269/ajtmh.13-0271


# Molecular Characterization of *Giardia duodenalis* and *Cryptosporidium parvum* in Fecal Samples of Individuals in Mongolia

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*Abstract.* The *Giardia* and *Cryptosporidium* species are widespread and frequent diarrhea-related parasites affecting humans and other mammalian species. The prevalence of these parasites in Mongolia is currently unknown. Therefore, we performed molecular analyses of *G. duodenalis* and *C. parvum* in stool samples from 138 patients hospitalized with diarrhea in Mongolia using nested polymerase chain reaction (PCR). A total of 5 (3.62%) and 7 (5.07%) fecal samples were positive for *G. duodenalis* and *C. parvum*, respectively. *Giardia duodenalis* and *C. parvum* infections were prevalent in children < 9 years of age. The assemblage-specific fragment patterns for the β-giardin gene of *G. duodenalis* revealed that all five samples testing positive belonged to Assemblage A by the PCR-restriction fragment polymorphism method. For sequencing and phylogenetic analysis of the 18S rDNA and HSP70 genes of all seven patients testing positive the genes were further identified to be of the *C. parvum* bovine genotype. This study is the first to report the prevalence of *G. duodenalis* and *C. parvum* and its molecular characterization of fecal samples from individuals with diarrhea in Mongolia.

## INTRODUCTION

*Giardia* and *Cryptosporidium*, genera of common protozoan parasites that infect domestic and wild animals and humans, generally cause diarrhea.[1–3] The *Giardia* genus is composed of intestinal flagellates that infect a wide range of vertebrate hosts. The *Giardia* genus currently comprises six species that are distinguished on the basis of the morphology and ultrastructure of their trophozoites.[4,5] *Giardia duodenalis*, *Giardia intestinalis*, *and Giardia lamblia* should be considered as a species complex, with little variation in morphology among them. Recently, genetic analyses using polymerase chain reaction (PCR) characterized isolates of *Giardia* directly from feces, allowing the identification of a comprehensive range of genotypes from humans and animals.[6–8] The species *G. duodenalis* has assigned even assemblages from A to H. Assemblage A and B have been identified to infect humans and other mammalian hosts.[9,10] Although "Assemblage C" infects only dogs, Assemblage F infects only cats, and Assemblage D infects both dogs and cats.[11] Assemblage E infects cattle, sheep, and goats, and Assemblage G infects rats. Recently, Assemblage H infecting marine vertebrates has been reported.[12] Regarding the *Cryptosporidium* species, 22 valid species have been identified on the basis of differences in oocyst morphology, the site of infection, vertebrate class specificity, and genetic differences.[1] Among the *Cryptosporidium* species, *Cryptosporidium parvum* and *Cryptosporidium hominis* are known to infect cattle, humans, and other mammals.

The *Giardia* and *Cryptosporidium* are shed in feces as oocysts and cysts and can be directly transmitted by the fecal–oral route by contaminated water or food, especially raw vegetables.[13] Clinical giardiasis and cryptosporidiosis accompanied by diarrhea are major public health concerns in developing nations.[14,15] Approximately 200 million people currently have symptomatic giardiasis in Asia, Africa, and Latin America, and ~500,000 new cases are reported each year[16]; alternatively, 300,000 persons in the United States are expected to be infected with *Cryptosporidium* species annually.[17] In addition, the occurrence of *Giardia* and *Cryptosporidium* species has been reported in Russia and China.[18,19]

In Mongolia, which is located in central Asia and borders Russia to the north and China to the south; many people work in the livestock industry, such as pasturage of cattle, sheep, goats, and horses in steppes, and the agriculture industry. Therefore, individuals in Mongolia may be considered to have a naturally high risk of contact with zoonotic parasites. However, no studies to date have examined specific *G. duodenalis* and *C. parvum* infections among individuals who have diarrhea in Mongolia. The aim of this study was to perform molecular detection and phylogenetic characterization of *G. duodenalis* and *C. parvum* from diarrheal fecal samples of individuals in Mongolia.

## MATERIALS AND METHODS

**Fecal sample collection and DNA isolation.** A total of 138 stool samples from 138 patients admitted to the intestinal ward of the National Center for Communicable Diseases located in Mongolia who had diarrhea were collected and transported to the Laboratory of Parasitology for diagnosis of parasitic diseases. Each fresh stool sample (5 g) was suspended in 15 mL of phosphate buffered saline and filtered using four layers of gauze to remove coarse material. The filtrate was then centrifuged at 3,000 rpm for 10 min. The supernatant was eliminated, and the sediment was mixed with 5 mL of phosphate buffered saline. The pellet underwent repeated boiling (100°C) and deep freezing (–70°C) 10 times to break the thick wall of the *Cryptosporidium and Giardia* cyst. Total genomic DNA was isolated from the pellet using DNAzol (MRC, Cincinnati, OH) and stored at –20°C until use.

**PCR and characterization of *G. duodenalis* by PCR-restriction fragment length polymorphism (RFLP) assay.** The amplification of the β-giardin gene was performed using a nested PCR protocol. In the primary PCR reaction, a 753 basepair (bp) fragment was amplified using Accure PCR Master Mix (Bioneer, Daejeon, Korea) containing 1 μM of

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TABLE 1

Detection of *Giardia duodenalis and Cryptosporidium parvum* infections using polymerase chain reaction analysis in human fecal samples from Mongolia, by age and sex

| Variables | No. of sample | Positive no. of *G. duodenalis* (%) | Positive no. of *C. parvum* (%) |
|---|---|---|---|
| Age (year) | | | |
| < 4 | 65 | 4 (6.15) | 3 (4.62) |
| 5–9 | 17 | – | 3 (17.65) |
| 10–14 | 3 | | – |
| 15–19 | 7 | – | – |
| 20–29 | 19 | 1 (5.26) | – |
| 30–39 | 15 | – | 1 (6.67) |
| 40–49 | 4 | – | – |
| 50–59 | 7 | – | – |
| 60+ | 1 | – | – |
| Sex | | | |
| Female | 64 | 1 (1.56) | 2 (3.13) |
| Male | 74 | 4 (5.41) | 5 (6.76) |
| Total | 138 | 5 (3.62) | 7 (5.07) |

the forward primer Gia7 (5′-AAGCCCGACGACCTCACC CGCAGTGC-3′) and the reverse primer Gia759 (5′-GA GGCCGCCCTGGATCTTCGAGACGAC-3′), as previously described.[20] In the nested PCR reaction, a 511 bp fragment was amplified using the forward primer (5′-GAACGAACGAGA TCGAGGTCCG-3′) and the reverse primer (5′-CTCGA CGAGCTT CGTGTT-3′).Thermal cycle reactions were set to an initial denaturing step (95°C for 5 min), 35 cycles of a denaturing step (95°C for 30 s), an annealing step (55°C for 30 s), an extension step (72°C for 60 s), and finally an extension step (72°C for 7 min). Amplification products were electrophoresed by an auto electrophoresis machine (QIAxcel, Hilden, Germany), as previously described.[21] The PCR products were purified using the agarose gel extraction kit (Qiagen, Hilden, Germany) and digested using 10 U/μL of *Hae* III (Enzynomics, Daejeon, Korea) in a final volume of 20 μL for 4 h at 37°C for assemblage analysis, according to previous reports.[22]

**Amplification of the 18S rDNA and heat-shock protein (HSP70) genes for *C. parvum*.** The primers used to amplify a 695 bp fragment from the 18S rDNA gene were 18SSF, forward primer (5′-AGTCATAGTCTTGTCTCAAAGATT-3′) and 18SR3B, reverse primer (5′-TTAACAAATCTAAGAA TTTCACC-3′).[23] Thermal cycle reactions were set to an initial denaturing step (96°C for 2 min), 35 cycles of a denaturing step (94°C for 30 s), an annealing step (55°C for 30 s), an extension step (72°C for 45 s), and finally an extension step (72°C for 10 min). A nested PCR protocol was used to amplify the HSP70 gene from genomic DNA of selected *Cryptosporidium* isolates for nucleotide sequencing.[24] For the primary PCR reaction, a 448 bp fragment was amplified using the forward primer HSPF4 (5′-GGTGGTGGTACTTTTGATGTATC-3′) and reverse primer HSPR4 (5′-GCCTGAACCTTTGGAATACG-3′). Thermal cycle reactions were set to an initial denaturing step (94°C for 5 min), 40 cycles of a denaturing step (94°C for 30 s), an annealing step (56°C for 30 s), an extension step (72°C for 30 s), and finally an extension step (72°C for 10 min). For the secondary PCR, a 325 bp fragment was amplified using the primary PCR product and HSPF3 (5′-GCTGSTGATACTCACT TGGGTGG-3′) and HSPR3 (5′ CTCTTGTCCATACCAGCATCC-3′) primers. The condition for the secondary PCR was identical to the primary PCR. Secondary PCR products were sequenced directly in both directions.

**Phylogenetic analysis of the 18S rDNA and HSP70 genes of *C. parvum*.** The PCR products were analyzed by electrophoresis, purified using an agarose gel DNA purification kit (Qiagen), and sequenced with an ABI PRISM 3730xl Analyzer (Applied Biosystems, Foster City, CA). A search of highly similar 18S rDNA gene fragment sequences was performed using nucleotide BLAST (National Center for Biotechnology Information, Bethesda, MD) to confirm the genotype. *Cryptosporidium* 18S rDNA sequences were obtained from GenBank. Sequence alignment was performed using CLUSTAL W (Multiple sequence alignment computer program, Histon, Cambridgeshire, UK). Phylogenetic trees were constructed using the neighbor-joining method[25] with maximum composite likelihood distance correction in the Molecular Evolutionary Genetics Analysis (MEGA) program,[26] with robustness of groupings assessed using 1,000 bootstrap replicates of the data.[27]

## RESULTS

**Prevalence of *G. duodenalis* and *C. parvum* in human fecal samples in Mongolia.** The 138 patients comprised 85 children

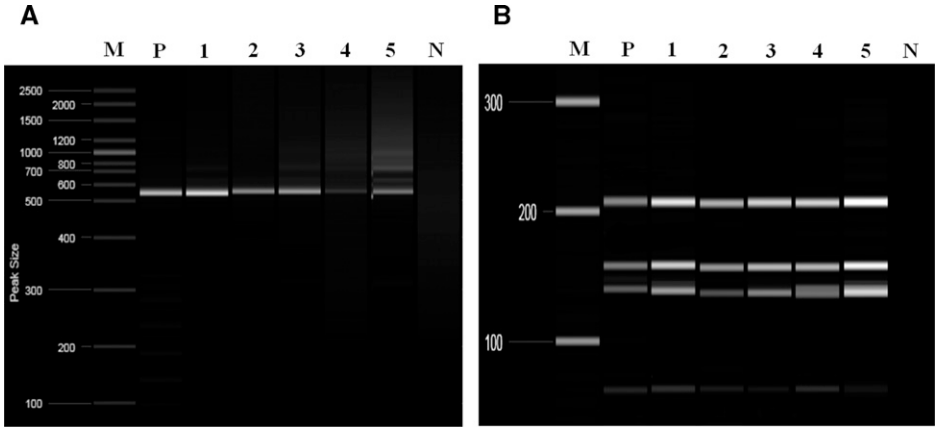


FIGURE 1. (**A**) Electrophoretic identification of *Giardia* β-giardin gene products (511 bp) by second polymerase chain reaction (PCR) using an auto electrophoresis machine. (**B**) Electrophoretic separation of *Giardia* β-giardin gene products after restriction with the *Hae* III enzyme using PCR-restriction fragment length polymorphism. Lane M, 2.5 kb molecular marker; lane P, positive for *Giardia duodenalis*; lanes 1–5, human fecal samples; lane N, negative.

TABLE 2

Genotyping of the 18S rDNA and HSP70 genes for each human fecal sample of patients from Mongolia testing positive for *Cryptosporidium parvum* using nested polymerase chain reaction

| Specimen ID | Genotype (18S rDNA) | Genotype (HSP70) |
|---|---|---|
| Mongol-H05 | *C. parvum* | *C. parvum* |
| Mongol-H07 | *C. parvum* | *C. parvum* |
| Mongol-H08 | *C. parvum* | *C. parvum* |
| Mongol-H16 | *C. parvum* | *C. parvum* |
| Mongol-H28 | *C. parvum* | *C. parvum* |
| Mongol-H32 | ND | *C. parvum* |
| Mongol-H39 | *C. parvum* | ND |

ND = no detection.

1–15 years of age (mean age, 3.6 years) and 53 adults 16–74 years of age (mean age, 32.5 years). Of the 138 patients included, 5 (3.62%) and 7 (5.07%) tested positive for *G. duodenalis* and *C. parvum*, respectively. Four of the 5 patients with a *G. duodenalis* infection were < 4 years of age, and 3 of the 7 patients with a *C. parvum* infection were < 4 years of age and 3 patients were 5–9 years of age except 1 patient. Our results showed that the positive rate of *G. duodenalis* and *C. parvum* in children was higher than that in adults (Table 1).

**Identification of *G. duodenalis* assemblages by PCR-RFLP.** The five samples with *G. duodenalis* were confirmed by β-giardin gene amplification by nested PCR (Figure 1A). After digestion by *Hae* III, the assemblage-specific patterns were obtained, showing patterns of 201, 150, 110, and 50 bp (Figure 1B). All five samples with *G. duodenalis* belonged to Assemblage A.

**Identification and phylogenetic analysis of *C. parvum*.** A total of seven fecal samples (sample numbers Mongol-H05, H07, H08, H16, H28, H32, and H39) tested positive for the 18S rDNA and HSP70 genes of *C. parvum* in the nested PCR. A sequence analysis of these seven samples suggested the presence of *C. parvum* in all patients, with homologies from 97% to 99% (Table 2). Phylogenetic analysis showed that the 18S rDNA gene fragments were of the *C. parvum* bovine genotype in all patients except Mongol-H32 (Figure 2A). An analysis of the HSP70 gene showed similar results (all patients except Mongol-H39) (Figure 2B).

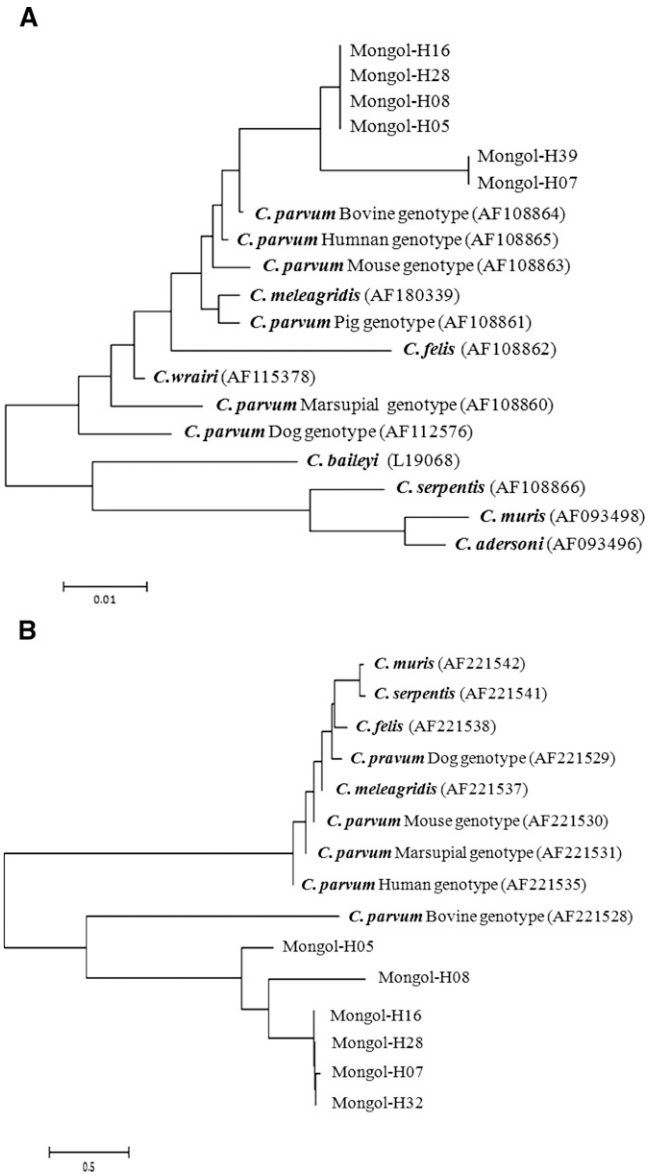


FIGURE 2. The phylogenetic relationships among *Cryptosporidium* species and genotypes according to the neighbor-joining analysis and the maximum composite likelihood distance correction (implemented using Molecular Evolutionary Genetics Analysis [MEGA]) of (**A**) a fragment from the partial 18S rDNA sequence and (**B**) HSP70 sequence. Sequences of other *Cryptosporidium* species and genotypes were obtained from GenBank.

## DISCUSSION

*Giardia* and *Cryptosporidium* are significant worldwide causes of diarrhea and nutritional disorders in humans. In Asia, among patients with diarrhea in a study from the Philippines, the prevalence rates for *Giardia* and *Cryptosporidium* species were 2.0% and 1.9%, respectively[28]; furthermore, the prevalence rates from a study in Malaysia were 0.7% for *Giardia* species and 0.3% for *Cryptosporidium* species.[29] In our study, the percentage of patients with diarrhea infected with *G. duodenalis* and *C. parvum* in Mongolia was higher than the above rates from the Philippines and Malaysia. Most outbreaks of human giardiasis in developing countries have mainly been detected in children < 2 years of age.[30] Furthermore, it has been reported that cryptosporidiosis generally affects children < 4 years of age.[31] In our data, children were more frequently infected than adults, and it is a finding similar to the findings from studies in other countries. In previous reports, the reasons for high prevalence of giardiasis and cryptosporidiosis in young children may be caused by the lack of immunity, and because they are easily exposed to contaminated water through playing water games.[31] In addition, Faubert reported that numerous factors contributed to infection with *Giardia* species, including the number of cysts ingested, the age of the host, the virulence of the *Giardia* strain, and the situation of the immune system at the time of infection.[32] Interestingly, in the current study, there was only one case of an adult infected with *G. duodenalis*, whereas the rest were all children. The reason for the high prevalence in children was unclear because we did not acquire any information on the patients except that they had diarrhea. Almost all of the infected children were living in Gers or houses equipped with indoor latrines and without tap water located in a steppe. The poor hygiene conditions of a

steppe, such as the low quality of water, poor cleanliness of containers for transporting water, and poor hand washing facilities, should be considered as contributing factors to infection with various pathogens, and these may be critical causes of infections. Further surveys for the detection of pathogens and the transmission through contamination of waters in poor environmental conditions should be performed. Additionally, in 3 of the 5 cases of *C. parvum*, *Shigella flexneri* ($N = 2$) and *Salmonella enteritidis* ($N = 1$), and in 2 of the 7 cases of *G. duodenalis, S. flexneri* were also detected (data not shown). These findings indicate that we detected the existence of mixed infections, both bacterial and parasitic, in patients with diarrhea in Mongolia. To understand the epidemiologic characteristics of the infections and to implement control measures, it is important to determine whether *G. duodenalis* and *C. parvum* can infect humans through a zoonotic route. Therefore, further epidemiologic studies examining the risk factors of infection among these protozoa in individuals with diarrhea should be carried out in the near future for improving public health.

Recently, molecular epidemiologic studies with *Giardia* DNA directly extracted from feces have been performed to amplify techniques, and several PCR assays have been developed.[20,33] In this study, we successfully performed a molecular analysis of the β-giardin gene and pattern analysis using a PCR-RFLP assay with *Hae* III on *Giardia* DNA from fecal samples. An investigation of human isolates from stool samples in diverse geographic areas established that only *G. duodenalis* Assemblages A and B are related to almost all human infections.[34] For example, the occurrence of Assemblage A and B of *G. duodenalis* have been reported in Thailand, China, and the Philippines.[19,35,36] In this study population, only Assemblage A was identified, and this result is similar to the previous studies from Korea, Japan, Egypt, and Brazil.[37–40]

*Cryptosporidium* species are classified on the basis of different oocyst morphology, sites of infection, vertebrate class, and genetic differences; such classifications of *Cryptosporidium* species include *C. parvum*, (a parasite of humans, cattle, and other mammals), *C. hominis* (a parasite of humans), and *Cryptosporidium felis* (a parasite of cats).[15,41] Particularly, Morgan and others[42] reported that the *C. parvum* bovine genotype and *C. hominis* are responsible for the majority of human infections. Our results showed that the *Cryptosporidium* DNA isolated from diarrheal fecal samples belonged to the bovine genotype using phylogenetic analysis. Our results are meaningful because they reveal that zoonotic parasitic infection cycles from cattle to humans may be possible in Mongolia.

In this study, *G. duodenalis* and *C. parvum* genes from human diarrheal fecal samples in patients from Mongolia were identified by molecular analysis. In particular, *G. duodenalis* was classified as a zoonotic pathogen belonging to Assemblage A, and the *C. parvum* bovine genotype was discovered through phylogenetic analysis. From our results, we assume that *C. parvum* can possibly emerge important human pathogens with contact between humans and animals in Mongolia. Further epidemiological studies subject to humans and animals in different areas and/or a big population in Mongolia are needed to better characterize the transmission of *Giardiasis* and *Cryptosporidiosis* in humans. To our knowledge, this is the first study to report the prevalence and genetic identification of *G. duodenalis* and *C. parvum,* and it may contribute to the understanding of the epidemiologic characteristics and improve the preventive control of both parasites in Mongolia.

Received May 20, 2013. Accepted for publication July 5, 2013.

Published online November 18, 2013.

Financial support: This work is performed to collaborative research both Korea CDC and Mongolia NCCD and this is supported by funding (4847-302-210-13, 2012) from the Korea National Institute of Health, Korea Centers for Disease Control and Prevention.

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