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 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 lambia* 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 year16; 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
the forward primer Gia7 (5'–AAGCCCAAGCAGCTTACC
GCGCACGCTC3') and the reverse primer Gia759 (5'–GA
GGCCGCTCTGGATTCTGAGAC3'), as previously described.20 In the nested PCR reaction, a 511 bp fragment was
amplified using the forward primer (5'–GAACGAAAGAGA
TGAGTTCCG3') and the reverse primer (5'–CTCGA
CGAGCTTCCGTGTT3'). 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
amplified using the forward primer (5'–GCTGTGATACACT
TGAGATGC3') and reverse primer (5'–CTCTTTGACTACAC
CAATCGATAC3') 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 pro-
ger (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 pro-
gram, Histone, Cambridgeshire, UK). Phylogenetic trees were
constructed using the neighbor-joining method25 with max-
imum composite likelihood distance correction in the Molecu-
lar Evolutionary Genetics Analysis (MEGA) program,26 with robustness of groupings assessed using 1,000 bootstrap repli-
cates 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.](image-url) 

**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.
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).

**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. In a study in Malaysia, the prevalence rates were 0.7% for *Giardia* species and 0.3% for *Cryptosporidium* species. 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. Furthermore, it has been reported that cryptosporidiosis generally affects children <4 years of age. 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. 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. 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. 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. For example, the occurrence of Assemblage A and B of *G. duodenalis* have been reported in Thailand, China, and the Philippines. In this study population, only Assemblage A was identified, and this result is similar to the previous studies from Korea, Japan, Egypt, and Brazil.

*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). Particularly, Morgan and others 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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