수의학석사학위논문

국내 야생 설치류 및 식충류의 크립토스포리디움 감염 진단 및 분자생물학적 연구

Detection and Molecular Characterization of *Cryptosporidium* spp. Among Wild Rodents and Insectivores in South Korea

2015년 2월

서울대학교 대학원
수의학과 실험동물의학 전공
송 주 하

Detection and Molecular Characterization of *Cryptosporidium* spp. Among Wild Rodents and Insectivores in South Korea

A dissertation Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master of Laboratory Animal Medicine

To the Faculty of College of Veterinary Medicine
Department of Laboratory Animal Medicine
The Graduate School
Seoul National University

By
Juha Song

February 2015

국내 야생 설치류 및 식충류의 크렙토스포리디움 감염 진단 및 분자생물학적 연구

지도교수: 박 재 학

이 논문을 수의학석사 학위논문으로 제출함
2014 년 10 월

서울대학교 대학원 수의학과
실험동물의학 전공
송 주 하

송주하의 수의학 석사 학위논문을 인준함
2015 년 2 월

위 원 장: 윤 희 정 (인)

부위원장: 박 재 학 (인)

위 원: 신 남 식 (인)

ABSTRACT

Detection and Molecular Characterization of *Cryptosporidium* spp. Among Wild, Rodents and Insectivores in South Korea

Juha Song, D.V.M.

Department of Laboratory Animal Medicine
College of Veterinary Medicine
The Graduate School of Seoul National University

In order to examine the prevalence of *Cryptosporidium* in wild rodents and insectivores of South Korea and to assess their potential role as a source of human cryptosporidiosis, a total of 199 wild rodents and insectivore specimens were collected and screened for *Cryptosporidium* infection, from 10 regions of South Korea over the period of 2 years (2012-2013). A nested-PCR amplification of *Cryptosporidium* oocyst wall protein (COWP) gene fragment revealed an overall prevalence of 34.2% (68/199). The sequence analysis of 18S rRNA gene locus of

Cryptosporidium were performed in those samples that tested positive by COWP amplification PCR and identified 4 species/genotypes; chipmunk genotype 1, cervine genotype 1, *C. muris* and a new genotype which is closely related to bear genotype. The new genotype isolated from 12 *Apodemus agrarius* and 2 *Apodemus chejuensis* was not previously identified as known species or genotype, and therefore, it is supposed to be a novel genotype. In addition, the host spectrum of *Cryptosporidium* was expended to *A. agrarius* and *C. lasiura,* which had not been reported before. In this study, we found that the Korean wild rodents and insectivores were infected with various *Cryptosporidium* spp. with large intra-genotypic variation, indicating that they may function as potential reservoirs transmitting zoonotic *Cryptosporidium* to livestock and humans.

Keywords: *Cryptosporidium*, Rodents, Insectivores, COWP, 18S rRNA, Korea

***Student Number*: 2012-21542**

CONTENTS

ABSTRACT……..…….... i

CONTENTS..……... iii

LIST OF TABLES……...…….…….. iv

LIST OF FIGURES...……..…… v

INTRODUCTION……..……. 1

MATERIALS AND METHODS……...……. 4

1. Animals and sample collection……..…… 4
2. DNA extraction ……..…… 5
3. PCR amplification for molecular detection of *Cryptosporidium* spp..…… 5
4. Sequence and phylogenetic analysis……..…… 6
5. Histological and morphological analysis……...…… 8
6. Nucleotide sequence accession number……...……. 9

RESULTS ……..……... 10

1. Prevalence of Cryptosporidium in rodents and insectivores ……. 10
2. Sequence and phylogenetic analysis of 18S rRNA gene locus…..… 10
3. Histological and morphological analysis ……..…… 11

DISCUSSION……...…… 13

REFERENCES..……...…….…….. 24

국문초록……... 34

LIST OF TABLES

Table 1. Prevalence of *Cryptosporidium* in wild rodents and insectivores captured in South Korea. ..... ……....**18**

Table 2. *Cryptosporidium* species and genotypes identified in wild rodents and insectivores in this present study. ……..…....**19**

Table 3. Comparison of the nucleotide sequences of the partial 18S rRNA gene of new Cryptosporidium genotype obtained in this study and related reference genotypes in the GenBank..……. **20**

LIST OF FIGURES

Figure 1. Ten geographic locations in South Korea where trapping of wild rodents and insectivores was conducted in 2012-2013……. **21**

Figure 2. Evolutionary relationships of *Cryptosporidium* isolates inferred by a neighbor-joining analysis of Tamura-Nei distances calculated from pairwise comparison of 18S rRNA sequences……. **22**

Figure 3. Histological examination of *Cryptosporidium*……....**23**

INTRODUCTION

Cryptosporidium is an apicomplexan protozoan parasite that infects a wide range of vertebrate species, including humans (1). Invading the epithelium of the gastrointestinal tract, infection causes mild-to-severe diarrheal disease depending on the host's immune status (2). When ingested through contaminated water or food in immunocompetent individuals, it leads to acute and self-limiting illness. However, in immunocompromised individuals, cryptosporidiosis can become a chronic and life-threatening disease (3, 4). For this reason, cryptosporidiosis has been a major public health concern in water utilities (5). Currently, through phylogenetic analysis, at least 26 species and 50 different genotypes of *Cryptosporidium* have been recognized worldwide (6). Among them, *C. parvum* and *C. hominis* are widely known as the causative agents of diarrheal illness in human (7), though recent studies have suggested that all *Cryptosporidium* parasites should be considered hazardous to humans (8, 9).

Wild mammals, particularly rodents and insectivores, have received attention as important reservoirs of *Cryptosporidium*, especially of *C. parvum* and *C. muris* (10, 11). Many other species have also been found to infect rodents as well as humans, including *C. hominis, C. meleagridis, C. felis, C. canis, C. ubiquitum, C. cuniculus* and *C. viatorum* (6, 8, 12-14). Several studies conducted in different

countries including Spain, Japan, China and the Philippines revealed that wild rodents are naturally infected with *Cryptosporidium*, and the prevalence rate ranged from 1% to 63% (1, 6, 10, 15). These results imply the potential threat of wild rodents to human health in urban and rural environments. In fact, increased participation in activities such as camping, hiking or engaging in outdoor sports in natural areas can result in higher exposure to *Cryptosporidium* parasites, transmitted through water or wildlife (16).

Irrespective of the potential risk, however, few epidemiologic studies on *Cryptosporidium* in small wild animals have been conducted in Korea. Previous studies conducted on humans in South Korea showed the percentage of *Cryptosporidium parvum* infection ranging from 0.6 to 25.9% on healthy subjects and 1 to 5.7% on gastroenteritis patients (17-20). In livestock including cattle and pig, infection rates were between 9.3% and 94% (19, 21). However, these studies have some limitations that they did not perform species/genotype identification which can speculate the epidemiological characteristics of *Cryptosporidium* and ignored the small animals including wild rodents that can serve as reservoir hosts. Therefore, the present study was designed to investigate the prevalence of *Cryptosporidium* in commonly found wild rodents and insectivores in South Korea, to determine their genotypes and evolutionary relationships, and to understand their potential role in the transmission of *Cryptosporidium*. Here we

present the results of a 2-year (2012-2013) investigation of *Cryptosporidium* in wild rodents and insectivores from 10 different regions of South Korea.

MATERIALS AND METHODS

Animals and sample collection.

From 2012 to 2013, the 199 rodents and insectivores living near houses and farms were captured with Sherman traps from 10 regions in South Korea. Each region was designated by the letters from A to J in order of their documentation (Fig. 1). Every capture sites was recorded using global positioning system (GPS) (Montana 650, Garmin International Inc., Olathe, KS) and all animals were trapped in the same GPS sites in each region for 2 years. They were included in two rodent species (striped field mouse [*Apodemus agrarius*], jeju striped field mouse [*Apodemus chejuensis*]) and two insectivore species (Ussuri white-toothed shrew [*Crocidura lasiura*], lesser white-toothed Shrew [*Crocidura shantungensis*]) respectively, which are widespread and abundant species in South Korea. All rodents and insectivores were euthanized by CO_2 inhalation. The fecal samples from a rectum, cecum samples and the large intestinal sections closest to the rectum were collected aseptically, placed in individual 1.7 ml Eppendorf tubes (Axygen Scientific, Inc., Union City, CA) and stored at -70℃ until the examination. The other tissue remnants including brain, heart, trachea, lung, thymus, kidney, esophagus, intestines and reproductive organ were fixed in phosphate-buffered formalin for histological analysis. All animal procedures were

performed according to the guidelines for animal experiments at Seoul National University.

DNA extraction.

Total DNA was extracted from the fecal or cecum samples using i-genomic Stool DNA Extraction Mini Kit (iNtRON Biotechnology, Seoul, Korea) according to manufacturer's instructions with minor modifications. Briefly, approximately 180 mg of fecal or cecum sample was transferred into 1.5 ml tube and added 200 µl Buffer SPL. Then, they were incubated at 70℃ for 10 min to ensure complete lysis of the thick-walled *Cryptosporidium* oocysts and release of DNA. The final elution volume was adjusted to 30 µl of Buffer SE from the kit manufacturer's recommended volume of 50 µl in order to increase DNA concentration. The extracted DNA was kept at -20℃ before being used in PCRs.

PCR amplification for molecular detection of *Cryptosporidium* spp.

The presence of the parasite DNA in fecal and cecum samples was confirmed by nested-PCR method using T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) allowing amplification of *Cryptosporidium* oocyst wall protein (COWP) gene, based on published methods (22). For the primary PCR, a PCR product of 401 bp was amplified using the forward primer COWPF1 (5′-

ACATTTTCAGGAAAGCAGTGTG-3′), and the reverse primer COWPR1 (5′-CCTTGCAGTGTGAAATTTGG-3′). These primer sequences were based on the published COWP sequence (GenBank accession no. AF266264.1). Positive controls (*C. muris* DNA) and negative controls (water instead of DNA template) were included in each analysis. The first PCR was carried out using AccuPower® PCR PreMix (Bioneer, Seoul, Korea) with 2 μl of template DNA and external primer set. PCR amplification was performed under the following conditions: initial denaturation at 94℃ for 5 min to activate DNA polymerase, followed by 35 cycles of amplification with denaturation at 94℃ for 50 sec, primer annealing at 54℃ for 30 sec, extension reaction at 72℃ for 50 sec, and then a final extension at 72℃ for 10 min. For the secondary PCR, a fragment of 205 bp was amplified using 2 μl of primary PCR product and nested forward COWPF2 (5'-CTGATACTGCACCTCCCAAC-3') and nested reverse COWPF2 (5'-GCTGATTCAGGTGCCATACA-3') primers. The conditions for the secondary PCR were identical to those for the primary PCR. The PCR products were separated by electrophoresis in a 2% agarose gel and visualized under a UV lamp after ethidium bromide staining.

Sequence and phylogenetic analysis.

Samples that were confirmed as positive by COWP PCR were further analyzed to verify the specificity of the PCR products. A fragment of the 18S rRNA gene locus was amplified by nested PCR, as described previously (23). Briefly, the primary PCR, performed with primers 18SiCF2 (5'-GACATATCATTCAAGTTTCTGACC-3') and 18SiCR2 (5'-CTGAAGGAGTAAGGAACAACC-3'), amplified a 763-bp fragment using AccuPower® PCR PreMix with 2 μl of template DNA. Primary PCR cycling conditions consisted of an initial denaturation of 94℃ for 5 min, followed by 35 cycles of 94℃ for 30 s, 54℃ for 30 s, and 72℃ 45 s, with a final extension of 72℃ for 10 min. An internal 587-bp fragment was amplified from the primary PCR product in a secondary reaction with primers 18SiCF1 (5'-CCTATCAGCTTTAGACGGTAGG-3') and 18SiCR1 (5'-TCTAAGAATTTCACCTCTGACTG-3'). The reaction conditions were similar to those described above for the primary PCR step, with the exception that the extension time was 30 s. All secondary PCR products positive for *cryptosporidium* were purified using the QIAquick PCR Purification kit (QIAGEN Korea Ltd., Seoul, Korea) and sequenced in both directions by commercial company (CosmoGenetech, Seoul, Korea).

The nucleic acid sequences obtained were aligned with reference sequences of *Cryptosporidium* species and genotypes from GenBank using ClustalW algorithm

of the BioEdit Sequence Alignment Editor software (available at http://www.mbio.ncsu.edu/bioedit/bioedit.html). Neighbor joining analysis with evolutionary distances calculated using Tamura-Nei parameter model was conducted using MEGA version 5.2 (24). A sequence of *Monocystis agilis* (GenBank accession no.AF457127) was used as an outgroup for the 18S rRNA analysis. Bootstrapping using 1000 replicates was carried out to assess the reliability of inferred tree topologies. The percentage of sequence similarity was calculated using MegAlign, version 5.08 (DNASTAR Inc., Madison, WI).

Histological and morphological analysis.

Formalin-fixed stomach and ileum specimens were embedded in paraffin. Then, serial sections (3 μM) were cut and stained with hematoxylin and eosin (H&E) for overall morphology.

Cryptosporidium-positive fecal samples were also prepared for Ziehl-Neelsen stain. Feces collected directly from rectum were suspended in sterilized distilled water. Then, oocysts were concentrated by sucrose floatation method as described previously (25). Ten microliters of well-mixed specimen were spread over an area approximately 1 cm in diameter and incubated at 70℃ for 5 min, followed by air-dry at room temperature (RT) for attachment. Well-dried smear samples were then stained using the modified Ziehl-Neelsen technique as previously described (26, 27).

Nucleotide sequence accession number.

The unique partial 18S rRNA sequence obtained from wild rodents in this study was deposited in the GenBank database under accession number KP317127.

RESULTS

Prevalence of *Cryptosporidium* in rodents and insectivores.

In total, 199 wild rodents and insectivores were captured from the 10 regions of South Korea for 2 years. They included 169 *A. agrarius*, 19 *A. chejuensis*, 10 *C. lasiura* and 1 *C. shantungensis*. The nested-PCR screening at the COWP gene locus detected 68 *Cryptosporidium* positives from the 199 fecal or cecum DNA samples obtained from 2012 to 2013; an overall prevalence of 34.2% (Table 1). Most of the *Cryptosporidium*-positive animals had no apparent clinical signs such as diarrhea or lethargy at the time of sample collection. On the area A through J, the average infection rate of each region was 43.5%, 40.0%, 15.4%, 62.5%, 55.0%, 47.1%, 15.0%, 17.6%, 18.8% and 31.6% respectively. In wild rodents, the prevalence of *Cryptosporidium* in *A. agrarius* and *A. chejuensis* were 33.1% (56/169) and 31.6% (6/19), respectively. In wild insectivores, 6 of the 10 *C. lasiura* showed *Cryptosporidium* positive (Table 2).

Sequence and phylogenetic analysis of 18S rRNA gene locus.

The effective length for sequencing was approximately 500 bp because the primer pair (18SiCF1 and 18SiCR1) amplified 587 bp fragments. Of the 68 positive samples identified at the 18S rRNA gene locus, sequence analysis was

successful for 44 of these. Fig. 2 shows the phylogenetic relationships between various *Cryptosporidium* species/genotypes and the 44 isolates from Korean wild rodents and insectivores. The Korean isolates were identified to cluster with 4 different *Cryptosporidium* species and genotypes; Chipmunk genotype I (n = 8), cervine genotype I (n = 9), bear genotype (n = 14) and *C. muris* (n =13). The percent identity values of isolates clustering chipmunk genotype I exhibited ranging between 98.8% and 99.0% (mean nt divergence of 0.95 ± 0.03%). Isolates clustered into cervine genotype I featured genotypic similarities ranging from 99.2% to 99.5% (mean nt divergence of 0.64 ± 0.06%). In the case of 14 isolates clustering with bear genotype, the similarities were low; they were between 92.9% and 98.6% (mean nt divergence of 1.16 ± 0.31%). Of 13 Korean isolates grouped with *C. muris* clade, 9 (KWRA5/13, KWRB6/13, KWRD3/13, KWRE4/13, KWRE7/13, KWRE10/13, KWRH3/13, KWRH10/13 and KWRJ6/13) shared 100% similarities with JFM 3 isolate, previously reported in *A. speciosus* in Japan (15). The remaining 4 isolates displayed sequence similarities ranging between 98.8% and 99.8% (mean nt divergence of 0.52 ± 0.26%) to JFM 3 isolate. The differences between reference sequences and new sequence obtained in this study are shown in Table 3.

Histological and morphological analysis.

Microscopic examination of H&E stained stomach biopsies of *Cryptosporidium*-positive samples revealed the presence of numerous *Cryptosporidia* with eosinophilic small oval structures lining the mucosal epithelium of gastric glands and gastric pits (Fig. 3A). The gastric epithelial cells at heavily affected area had scanty cytoplasm and there were infiltrations of eosinophils in the base of lamina propria and occasionally in the tips of the glands. In affected intestine, however, marked epithelial exfoliation and inflammatory cell infiltration were observed. These findings are probably due to the multiple infestations of other parasites, such as nematodes, trematodes, cestodes and other protozoa (data not shown).

Oocyst in fecal samples stained by modified Ziehl-Neelsen technique showed typical characteristics of *Cryptosporidium* (Fig. 3B). The oocyst appeared deep red colored spherical morphology with approximately 4 to 6 μm in diameter. It contained central black granule and the crescent shaped sporozoites.

DISCUSSION

Wild rodents and insectivores have been reported to serve as reservoir hosts that may transmit various zoonotic diseases by contacting humans and livestock (28-31). For this reason, the health surveillance of these wild animals has been conducted steadily so far. As one of the zoonotic parasitic agent, *Cryptosporidium* has been reported to trigger the acute or chronic digestive system disorders in humans and animals when infected (2). Using molecular and histological techniques, we investigated the current status of infection with cryptosporidiosis in the pathogenic mediators such as *A. agrarius*, *A. chejuensis*, *C. lasiura* and *C. shantungensis*. According to the results, the overall prevalence of the infection among rodents and insectivores was 34.2% (68/199) in South Korea, which was within the reported range of 1% to 63% (1, 6, 11, 12, 32-34). The prevalence of the infection among *A. agrarius* and *A. chejuensis* in South Korea was found to be 33.1% (56/169) and 31.6% (6/19), respectively. This result is reasonable when compared with the prevalence of the infection in other wild rodents belonging to the genus *Apodemu*s, which was 35.2% in Spain and 27% in Japan (10, 15). The prevalence of the infection with *C. russula* belonging to the genus *Crocidura* was 14.8% in Spain (10). In this study, however, the prevalence of the infection among *C. lasiura* and *C. shantungensis* was rather different, which were at 60%

(6/10) and 0% (0/1), respectively. This is probably due to lack of sufficient sample size that can obtain reliable average value.

When regional differences of the infection rates were compared, the prevalence of *Cryptosporidium* in Korea's northern and central areas (indicated as A, B, D, E and F) ranged from 43.5% to 62.5%, which is higher than the average. However, its prevalence in the Southern part of the country and the northeast area 'C' ranged from 15% to 31.6%, which was lower than the average. The epidemiological study that can precisely analyze the reason why the infection rates in these four areas are low is regarded to be necessary (35).

The COWP gene used in the present study is a widely used target in identifying *Cryptosporidium* species/genotypes (22, 36). In order to screen the infection status with *Cryptosporidium*, we designed a specific primer pairs amplifying the fragment of COWP gene. We confirmed that this primer pairs for nested-PCR amplified the DNA with high sensitivity and without cross-reactivity with other parasite DNA such as *Eimeria* or *Cyclospora* (37). However, the predicted PCR product size was about 300 bp, which does not meet the recommended minimum size of 400-500bp (38). For this reason, instead of COWP gene, 18S rRNA gene which is the most commonly used target in sequence and phylogenetic analysis was used for the *Cryptosporidium* species/genotypes identification (23, 39-41). In phylogenetic analysis of 18S rRNA gene locus, a total of four *Cryptosporidium* species/genotypes were identified from wild rodents and insectivores in South

Korea. Chipmunk genotype I, identified from a total of 8 isolates in this study, was first reported in water after a storm in New York (42). This genotype has been identified from wild animals, such as eastern chipmunks, eastern squirrels and deer mice of New York State and red squirrels in Italy (43, 44). There have also been reported cases where humans were infected with this species as well (7). In the present study, 3 of the 35 properly sequenced *A. agrarius* samples (8.6%) were infected with this genotype. Interestingly, among the 11 captured insectivores, 5 positive subjects infected with *Cryptosporidium* species/genotypes were all found to belong to Chipmunk genotype I. The sequences obtained from these isolates only had 98.8 to 99.0% similarity with reference sequence, which were previously identified in Sweden from human infection (45). This discrepancy can be considered to be from sequence heterogeneity of 18S rRNA gene locus (46). This is the first report of chipmunk genotype I identified in small wild animals of genus *Apodemus* and genus *Crocidura*, thus expanding its host range.

Cervine genotype I is known to infect people as well as various mammals so as to be called *C. ubiquitum* (47, 48). After this species was first detected from a pediatric patient in Canada, it was identified in human feces in New Zealand, England, Slovenia, UK and the US (7, 49-53). The infections of this genotype in many rodent species such as squirrels, chipmunks, and woochucks were also described (44). According to our survey, 22.9% (8/35) of *A. agrarius* and 25%

(1/4) of *A. chejuensis* were infected with cervine genotype I which was previously reported from surface water in Japan, exhibiting 99.2% to 99.5% sequence similarity. These findings that *Cryptosporidium* from Korean isolates is more similar to that of Japan than of other countries imply the existence of geographical difference in distribution of *Cryptosporidium* subgenotypes.

Bear genotype which was first isolated from black bears in the U.S. has not been detected in other hosts thus far (54). In the present study, *Cryptosporidium* isolates identified from 12 *A. agrarius* and 2 *A. chejuensis* were found to be clustered with this bear genotype with rather low similarity of 92.9% to 98.6%. They are considered to be distinct new genotypes that have not been reported in other hosts, and further sequence analysis is required to elucidate their genetic characteristics.

C. muris was first recognized in laboratory mice and was also identified from diverse mammals such as mice, rats, Japanese field mice, cats and cynomolgus monkeys (44, 55, 56). In addition, detection of *C. muris* in the feces of the immunosuppressed humans with HIV has been reported (57, 58). In this study, among the 13 isolates identified as *C. muris*, *Cryptosporidium* isolated from 8 *A. agrarius* and 1 *A. chejuensis* exhibited 100% similarity with *C. muris* genotype that was previously reported from *A. speciosus* in Japan, and the rest of the 4 *A. agrarius* showed 98.8% to 99.8% similarity with intra-genotypic variation (15).

Our results are the first reported data that verified the infection status of *Cryptosporidium* in wild rodents and insectivores in South Korea. The current investigation revealed that high genetic diversity of *Cryptosporidium* spp. existed in Korea's wild small animals. Especially, it is meaningful in that it widened the scope of hosts of *Cryptosporidium*. Furthermore, Chipmunk genotype I, cervine genotype I, and *C. muris* identified in this study are known to be able to trigger diseases in livestock and humans when infected. These findings suggest that the rodents and insectivores in South Korea may play a role as a potential reservoir hosts that can mediate transmission of *Cryptosporidium* among them. A continuous examination of the surveillance and epidemiological study of *Cryptosporidium* in biological vector, particularly rodents and insectivores, is required to prevent the unexpected outbreak from animals including humans.

Table 1. Prevalence of *Cryptosporidium* in wild rodents and insectivores captured in South Korea.

Location	Host species	Total no. of samples	No. of positive samples	Prevalence (%)	Overall prevalence per region (%)
A (Paju)	*Apodemus agrarius*	21	8	38.1	43.5
	Crocidura lasiura	2	2	100.0	
B (Chuncheon)	*Apodemus agrarius*	21	8	38.1	40.0
	Crocidura lasiura	4	2	50.0	
C (Yangyang)	*Apodemus agrarius*	26	4	15.4	15.4
D (Gongju)	*Apodemus agrarius*	16	10	62.5	62.5
E (Mungyeong)	*Apodemus agrarius*	18	9	50.0	55.0
	Crocidura lasiura	2	2	100.0	
F (Yeongyang)	*Apodemus agrarius*	16	8	50.0	47.1
	Crocidura lasiura	1	0	0.0	
G (Gwangju)	*Apodemus agrarius*	20	3	15.0	15.0
H (Sancheong)	*Apodemus agrarius*	15	3	20.0	
	Crocidura lasiura	1	0	0.0	17.6
	Crocidura shantungensis	1	0	0.0	
I (Ulsan)	*Apodemus agrarius*	16	3	18.8	18.8
J (Jeju)	*Apodemus chejuensis*	19	6	31.6	31.6
Total		**199**	**68**		**34.2**

Table 2. *Cryptosporidium* species and genotypes identified in wild rodents and insectivores in this present study.

Host species	Total no. of samples	No. of positive samples	Prevalence (%)	No. and name of *Cryptosporidium* species/genotype(s)
Apodemus agrarius	169	56	33.1	chipmunk genotype Ⅰ (3), cervine genotype Ⅰ (8), *C. muris* (12), new genotype (11)
Apodemus chejuensis	19	6	31.6	cervine genotype Ⅰ (1), *C. muris* (1), new genotype (2)
Crocidura lasiura	10	6	60.0	chipmunk genotype Ⅰ (5)
Crocidura shantungensis	1	0	0.0	
Total	**199**	**68**	**34.2**	**4 genotypes**

Table 3· Comparison of the nucleotide sequences of the partial 18S rRNA gene of new *Cryptosporidium* genotype obtained in this study and related reference genotypes in the GenBank.

Organism or genotype (accession no.)	Nucleotide sequence at the indicated position(s)[a]					
	114-130	275-324	352-377	419-438	443-482	493-510
Chipmunk genotype Ⅰ (JX978272)	GGACTTTTT----GGTT	TA-ATAATTTATATAT--AATATT- -TTGATGAATATTT--A-TATAA-T	ACTATATTT---- -TTTAGTA--TAT	ATT--AGCCT TGAATACTCC	-ATGGAATAATATTAAA-GA TTTTTATCTTT----CTT--	AG-ATAAGA ATA-ATGAT
Cervine genotype Ⅰ (AB694733)	........--AAATA...	..-.........-.....---.C.A.A T....ATG......A---.....-.	....A.-..TTAA -...G...--...	....	-.....A...-.. .....----T..--	..-....A. ...-.....
C. muris (AB697054)	..G.C.AA----C...C	.GT.....C.----------...A.A T.ACCAAGG...A.A--T....T-.	-....T.C.A--- -...A.A..TA.GG	.AA-CT....	-.....AGT..G.. C....G.....----CTT--	G.-.C..A. G..-...G.
Bear genotype (AF247535)	........--AA-TA...	..-.........-.....---...ATA -..TTAAT......A---.....-.	.....A------ -C.....--...	..A--T.... .....AG	-.....A....-.. .....TTTTT----	..-...G.. ...-.....
Cryptosporidium sp. KSFM (KP317127)	.........AAA-TA...	..-.........-.....---...A.A -..TTAAT......A---.....-.	.....A-T- T.....--...	..AATT.... .....A.	-.....-.. .....CTTTT..--	..-....A. ...-.....

[a]Dots indicate nucleotide identity to the Chipmunk genotype Ⅰ sequence (JX978272) and dashes represent nucleotide deletions.

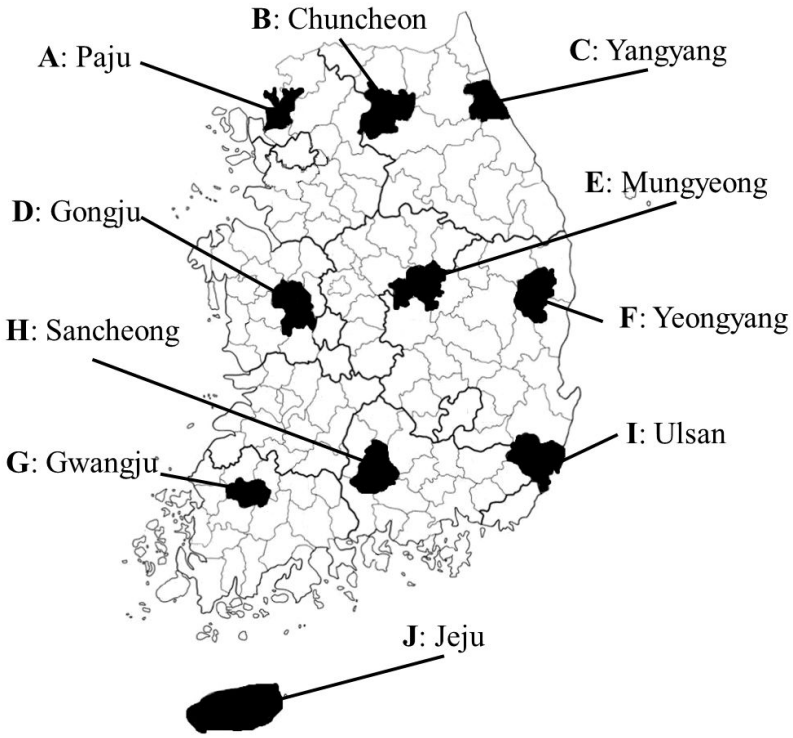

Figure 1. Ten geographic locations in South Korea where trapping of wild rodents and insectivores was conducted in 2012-2013. Each region was designated by the capital letters (A-J).

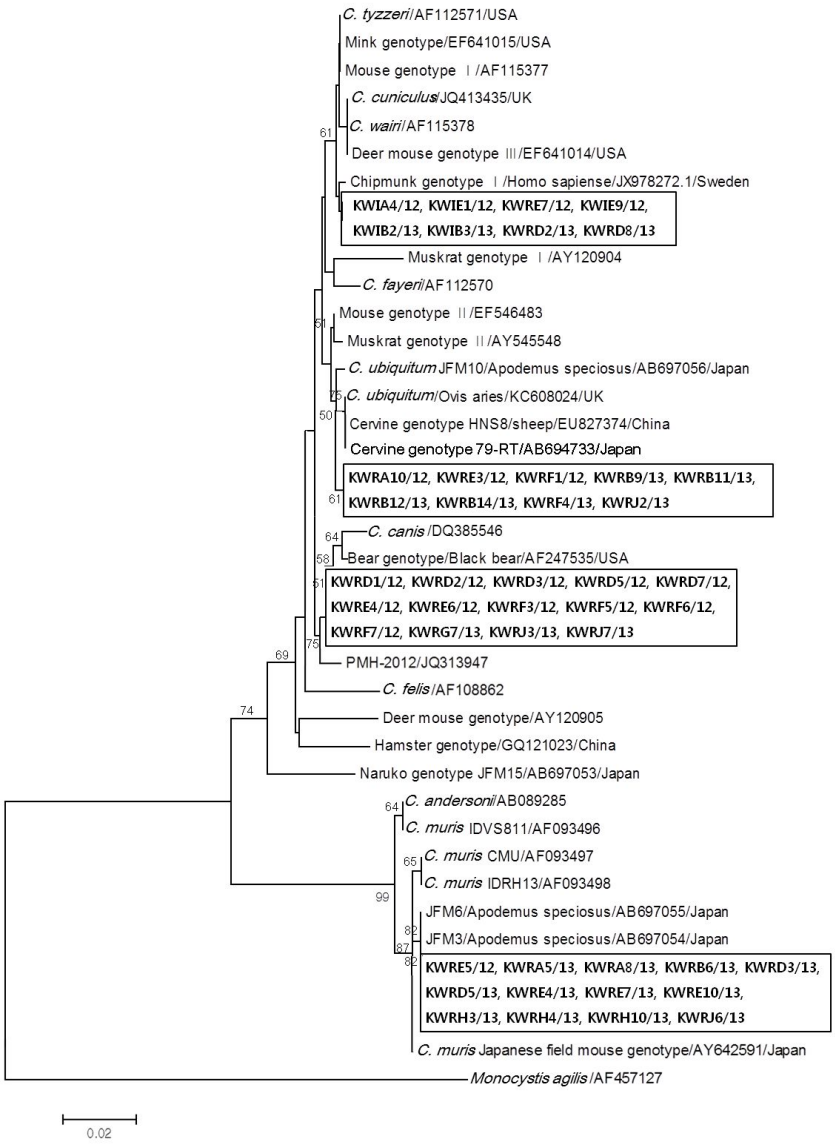

Figure 2. Evolutionary relationships of *Cryptosporidium* isolates inferred by a neighbor-joining analysis of Tamura-Nei distances calculated from pairwise comparison of 18S rRNA sequences. The tree was rooted with the 18S rRNA sequence of *Monocystis agilis* (AF457127). Percentage bootstrap support (>50%) from 1,000 pseudoreplicates is indicated at the left of the supported node.

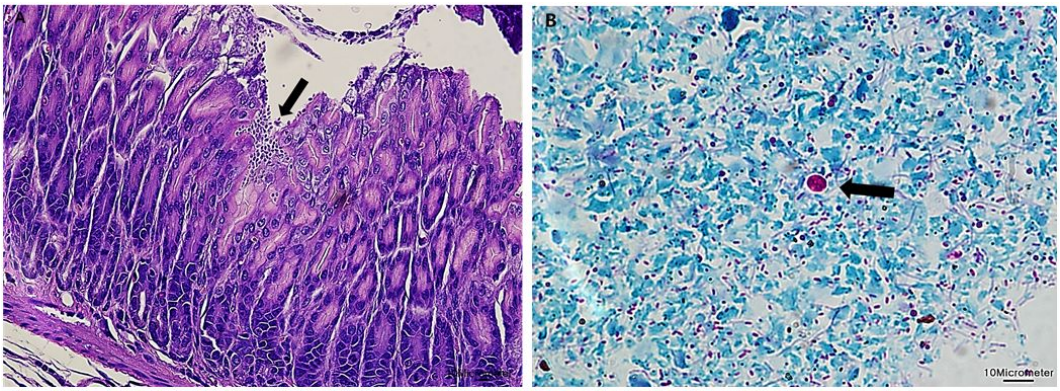

Figure 3. Histological examination of *Cryptosporidium*. (A) Clusters of *Cryptosporidium* (arrow) adherent to the luminal epithelium (hematoxylin-eosin stain; magnification: X40). (B) *Cryptosporidium* oocyst (arrow) showing deep red staining of internal structure (Ziehl-Neelsen stain; magnification: X100). The scale bars represent 10 μm.

REFERENCES

1. **Lv C, Zhang L, Wang R, Jian F, Zhang S, Ning C, Wang H, Feng C, Wang X, Ren X, Qi M, Xiao L.** 2009. *Cryptosporidium* spp. in wild, laboratory, and pet rodents in china: prevalence and molecular characterization. Appl Environ Microbiol **75:**7692-7699.
2. **Leitch GJ, He Q.** 2012. Cryptosporidiosis-an overview. J Biomed Res **25:**1-16.
3. **Kurniawan A, Dwintasari SW, Connelly L, Nichols RA, Yunihastuti E, Karyadi T, Djauzi S.** 2013. *Cryptosporidium* species from human immunodeficiency-infected patients with chronic diarrhea in Jakarta, Indonesia. Ann Epidemiol **23:**720-723.
4. **Elwin K, Hadfield SJ, Robinson G, Chalmers RM.** 2012. The epidemiology of sporadic human infections with unusual *Cryptosporidia* detected during routine typing in England and Wales, 2000-2008. Epidemiol Infect **140:**673-683.
5. **Xiao L, Morgan UM, Fayer R, Thompson RC, Lal AA.** 2000. *Cryptosporidium* systematics and implications for public health. Parasitol Today **16:**287-292.

6. **Ng-Hublin JS, Singleton GR, Ryan U.** 2013. Molecular characterization of *Cryptosporidium* spp. from wild rats and mice from rural communities in the Philippines. Infect Genet Evol **16:**5-12.

7. **Feltus DC, Giddings CW, Schneck BL, Monson T, Warshauer D, McEvoy JM.** 2006. Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin. J Clin Microbiol **44:**4303-4308.

8. **Xiao L, Fayer R, Ryan U, Upton SJ.** 2004. *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbiol Rev **17:**72-97.

9. **Chen XM, Keithly JS, Paya CV, LaRusso NF.** 2002. Cryptosporidiosis. N Engl J Med **346:**1723-1731.

10. **Torres J, Gracenea M, Gomez MS, Arrizabalaga A, Gonzalez-Moreno O.** 2000. The occurrence of *Cryptosporidium parvum* and *C. muris* in wild rodents and insectivores in Spain. Vet Parasitol **92:**253-260.

11. **Kimura A, Edagawa A, Okada K, Takimoto A, Yonesho S, Karanis P.** 2007. Detection and genotyping of *Cryptosporidium* from brown rats (*Rattus norvegicus*) captured in an urban area of Japan. Parasitol Res **100:**1417-1420.

12. **Foo C, Farrell J, Boxell A, Robertson I, Ryan UM.** 2007. Novel *Cryptosporidium* genotype in wild Australian mice (*Mus domesticus*). Appl Environ Microbiol **73:**7693-7696.

13. **Lebbad M, Beser J, Insulander M, Karlsson L, Mattsson JG, Svenungsson B, Axen C.** 2013. Unusual Cryptosporidiosis cases in Swedish patients: extended molecular characterization of *Cryptosporidium viatorum* and *Cryptosporidium* chipmunk genotype I. Parasitology **140:**1735-1740.

14. **Robinson G, Elwin K, Chalmers RM.** 2008. Unusual *Cryptosporidium* genotypes in human cases of diarrhea. Emerg Infect Dis **14:**1800-1802.

15. **Murakoshi F, Fukuda Y, Matsubara R, Kato Y, Sato R, Sasaki T, Tada C, Nakai Y.** 2013. Detection and genotyping of *Cryptosporidium* spp. in large Japanese field mice, *Apodemus speciosus*. Vet Parasitol **196:**184-188.

16. **Hayes EB.** 2010. Looking the other way: preventing vector-borne disease among travelers to the United States. Travel Med Infect Dis **8:**277-284.

17. **Cheun HI, Cho SH, Lim YY, Lee BC, Kim JY, Ju JW, Na BK, Kimata I, Yu JR, Kim TS.** 2010. *Cryptosporidium parvum* in Korea: prevalence in individuals residing in three major river valleys and genetic characteristics of the isolates. J Vet Med Sci **72:**167-172.

18. **Lee JK, Song HJ, Yu JR.** 2005. Prevalence of diarrhea caused by *Cryptosporidium parvum* in non-HIV patients in Jeollanam-do, Korea. Korean J Parasitol **43:**111-114.

19. **Park JH, Guk SM, Han ET, Shin EH, Kim JL, Chai JY.** 2006. Genotype analysis of *Cryptosporidium* spp. prevalent in a rural village in Hwasun-gun, Republic of Korea. Korean J Parasitol **44:**27-33.

20. **Huh JW, Moon SG, Lim YH.** 2009. A survey of intestinal protozoan infections among gastroenteritis patients during a 3-year period (2004-2006) in Gyeonggi-do (province), South Korea. Korean J Parasitol **47:**303-305.

21. **Yu JR, Lee JK, Seo M, Kim SI, Sohn WM, Huh S, Choi HY, Kim TS.** 2004. Prevalence of Cryptosporidiosis among the villagers and domestic animals in several rural areas of Korea. Korean J Parasitol **42:**1-6.

22. **Xiao L, Limor J, Morgan UM, Sulaiman IM, Thompson RC, Lal AA.** 2000. Sequence differences in the diagnostic target region of the oocyst wall protein gene of *Cryptosporidium* parasites. Appl Environ Microbiol **66:**5499-5502.

23. **Ryan U, Xiao L, Read C, Zhou L, Lal AA, Pavlasek I.** 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl Environ Microbiol **69:**4302-4307.

24. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol **28:**2731-2739.

25. **Fujino T, Matsuo T, Okada M, Matsui T.** 2006. Detection of a small number of *Cryptosporidium parvum* oocysts by sugar flotation and sugar centrifugation methods. J Vet Med Sci **68:**1191-1193.

26. **Kar S, Gawlowska S, Daugschies A, Bangoura B.** 2011. Quantitative comparison of different purification and detection methods for *Cryptosporidium parvum* oocysts. Vet Parasitol **177:**366-370.

27. **Casemore DP, Armstrong M, Sands RL.** 1985. Laboratory diagnosis of Cryptosporidiosis. J Clin Pathol **38:**1337-1341.

28. **Arai S, Gu SH, Baek LJ, Tabara K, Bennett SN, Oh HS, Takada N, Kang HJ, Tanaka-Taya K, Morikawa S, Okabe N, Yanagihara R, Song JW.** 2012. Divergent ancestral lineages of newfound hantaviruses harbored by phylogenetically related crocidurine shrew species in Korea. Virology **424:**99-105.

29. **Klein TA, Kim HC, Chong ST, O'Guinn ML, Lee JS, Turell MJ, Sames WJ, Gu SH, Kang HJ, Moon S, Lee SY, Chun Y, Song JW.** 2012. Hantaan virus surveillance in small mammals at firing points 10 and 60, Yeoncheon, Gyeonggi Province, Republic of Korea. Vector Borne Zoonotic Dis **12:**674-682.

30. **Ko S, Kim HC, Yang YC, Chong ST, Richards AL, Sames WJ, Klein TA, Kang JG, Chae JS.** 2011. Detection of *Rickettsia felis* and *Rickettsia typhi* and seasonal prevalence of fleas collected from small mammals at

Gyeonggi Province in the Republic of Korea. Vector Borne Zoonotic Dis **11:**1243-1251.

31. **O'Guinn ML, Klein TA, Lee JS, Richards AL, Kim HC, Ha SJ, Shim SH, Baek LJ, Song KJ, Chong ST, Turell MJ, Burkett DA, Schuster A, Lee IY, Yi SH, Sames WJ, Song JW.** 2010. Serological surveillance of scrub typhus, murine typhus, and leptospirosis in small mammals captured at firing points 10 and 60, Gyeonggi province, Republic of Korea, 2001-2005. Vector Borne Zoonotic Dis **10:**125-133.

32. **Paparini A, Jackson B, Ward S, Young S, Ryan UM.** 2012. Multiple *Cryptosporidium* genotypes detected in wild black rats (*Rattus rattus*) from northern Australia. Exp Parasitol **131:**404-412.

33. **Sturdee AP, Bodley-Tickell AT, Archer A, Chalmers RM.** 2003. Long-term study of *Cryptosporidium* prevalence on a lowland farm in the United Kingdom. Vet Parasitol **116:**97-113.

34. **Chalmers RM, Sturdee AP, Bull SA, Miller A, Wright SE.** 1997. The prevalence of *Cryptosporidium parvum* and *C. muris* in *Mus domesticus*, *Apodemus sylvaticus* and *Clethrionomys glareolus* in an agricultural system. Parasitol Res **83:**478-482.

35. **Ostfeld RS, Keesing F.** 2012. Effects of Host Diversity on Infectious Disease. Annual Review of Ecology, Evolution, and Systematics, Vol 43 **43:**157-182.

36. **Salyer SJ, Gillespie TR, Rwego IB, Chapman CA, Goldberg TL.** 2012. Epidemiology and molecular relationships of *Cryptosporidium* spp. in people, primates, and livestock from Western Uganda. PLoS Negl Trop Dis **6:**e1597.

37. **Lee SH, Joung M, Yoon S, Choi K, Park WY, Yu JR.** 2010. Multiplex PCR detection of waterborne intestinal protozoa: *microsporidia*, *Cyclospora*, and *Cryptosporidium*. Korean J Parasitol **48:**297-301.

38. **Egyed Z, Sreter T, Szell Z, Varga I.** 2003. Characterization of *Cryptosporidium* spp.--recent developments and future needs. Vet Parasitol **111:**103-114.

39. **Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA.** 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microbiol **65:**1578-1583.

40. **Fayer R, Morgan U, Upton SJ.** 2000. Epidemiology of *Cryptosporidium*: transmission, detection and identification. Int J Parasitol **30:**1305-1322.

41. **Xiao L, Feng Y.** 2008. Zoonotic Cryptosporidiosis. FEMS Immunol Med Microbiol **52:**309-323.

42. **Jiang J, Alderisio KA, Xiao L.** 2005. Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. Appl Environ Microbiol **71:**4446-4454.

43. **Kvac M, Hofmannova L, Bertolino S, Wauters L, Tosi G, Modry D.** 2008. Natural infection with two genotypes of *Cryptosporidium* in red squirrels (*Sciurus vulgaris*) in Italy. Folia Parasitol (Praha) **55:**95-99.

44. **Feng Y, Alderisio KA, Yang W, Blancero LA, Kuhne WG, Nadareski CA, Reid M, Xiao L.** 2007. *Cryptosporidium* genotypes in wildlife from a New York watershed. Appl Environ Microbiol **73:**6475-6483.

45. **Insulander M, Silverlas C, Lebbad M, Karlsson L, Mattsson JG, Svenungsson B.** 2013. Molecular epidemiology and clinical manifestations of human Cryptosporidiosis in Sweden. Epidemiol Infect **141:**1009-1020.

46. **Le Blancq SM, Khramtsov NV, Zamani F, Upton SJ, Wu TW.** 1997. Ribosomal RNA gene organization in *Cryptosporidium parvum*. Mol Biochem Parasitol **90:**463-478.

47. **Slapeta J.** 2013. Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int J Parasitol **43:**957-970.

48. **Fayer R, Santin M, Macarisin D.** 2010. *Cryptosporidium ubiquitum* n. sp. in animals and humans. Vet Parasitol **172:**23-32.

49. **Ong CS, Eisler DL, Alikhani A, Fung VW, Tomblin J, Bowie WR, Isaac-Renton JL.** 2002. Novel *Cryptosporidium* genotypes in sporadic Cryptosporidiosis cases: first report of human infections with a cervine genotype. Emerg Infect Dis **8:**263-268.

50. **Learmonth JJ, Ionas G, Ebbett KA, Kwan ES.** 2004. Genetic characterization and transmission cycles of *Cryptosporidium* species isolated from humans in New Zealand. Appl Environ Microbiol **70:**3973-3978.

51. **Leoni F, Amar C, Nichols G, Pedraza-Diaz S, McLauchlin J.** 2006. Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000. J Med Microbiol **55:**703-707.

52. **Soba B, Petrovec M, Mioc V, Logar J.** 2006. Molecular characterisation of *Cryptosporidium* isolates from humans in Slovenia. Clin Microbiol Infect **12:**918-921.

53. **Davies AP, Campbell B, Evans MR, Bone A, Roche A, Chalmers RM.** 2009. Asymptomatic carriage of protozoan parasites in children in day care centers in the United Kingdom. Pediatr Infect Dis J **28:**838-840.

54. **Xiao L, Limor JR, Sulaiman IM, Duncan RB, Lal AA.** 2000. Molecular characterization of a *Cryptosporidium* isolate from a black bear. J Parasitol **86:**1166-1170.

55. **Iseki M, Maekawa T, Moriya K, Uni S, Takada S.** 1989. Infectivity of *Cryptosporidium muris* (strain RN 66) in various laboratory animals. Parasitol Res **75:**218-222.

56. **Feng Y.** 2010. *Cryptosporidium* in wild placental mammals. Exp Parasitol **124:**128-137.

57. **Palmer CJ, Xiao L, Terashima A, Guerra H, Gotuzzo E, Saldias G, Bonilla JA, Zhou L, Lindquist A, Upton SJ.** 2003. *Cryptosporidium muris*, a rodent pathogen, recovered from a human in Peru. Emerg Infect Dis **9:**1174-1176.

58. **Gatei W, Ashford RW, Beeching NJ, Kamwati SK, Greensill J, Hart CA.** 2002. *Cryptosporidium muris* infection in an HIV-infected adult, Kenya. Emerg Infect Dis **8:**204-206.

국문초록

국내 야생 설치류 및 식충류의 크립토스포리디움 감염 진단 및 분자생물학적 연구

서울대학교 대학원
수의학과 실험동물의학전공

송 주 하
(지도교수: 박 재 학)

국내 야생설치류 및 식충류에서의 크립토스포리디움의 감염 현황을 조사하고 사람으로의 전파를 매개하는 보유숙주로서의 역할을 평가하기 위해, 2 년간(2012-2013) 국내 10 개 지역에서 포획한 총 199 마리의 야생설치류와 식충류를 대상으로 크립토스포리디움의 감염 여부를 조사하였다. *Cryptosporidium* oocyst wall protein (COWP) 유전자의 절편을 증폭시키는 nested-PCR 기법을 통해 확인한 결과, 국내 야생 소형 포유류에서의 크립토스포리디움 유병률은 34.2% (68/199)로 확인되었다. COWP 유전자의 증폭으로 확인된 크립토스포리디움 양성 샘플을 대상으로 18S rRNA 유전자의 염기 서열 분석을 실시한 결과, 총 4 가지의 크립토스포리디움 종/유전자형이 밝혀졌다; chipmunk genotype

Ⅰ, cervine genotype Ⅰ, *C. muris*, 그리고 bear genotype 과 염기서열이 유사한 새로운 유전자형. 이 새로운 유전자형은 총 12 마리의 등줄쥐와 2 마리의 제주등줄쥐에서 확인되었는데, 이전까지 밝혀진 종 또는 유전자형과는 염기서열이 92.9%에서 98.6%정도의 낮은 유사성을 보여 새로운 유전자형임을 확인하였다. 또한 크립토스포리디움이 그동안 밝혀졌던 숙주 외에 *A. agrarius* 와 *C. lasiura* 에도 감염이 되어 있는 것이 확인되어 , 크립토스포리디움의 감염가능 숙주 범위를 넓혔다 . 이번 연구를 통해 국내 야생설치류 및 식충류는 높은 염기서열 내 변이의 특성을 보이는 다양한 크립토스포리디움에 감염되어 있음을 확인하였고, 이러한 결과는 야생설치류 및 식충류가 사람이나 가축에게 크립토스포리디움을 전파시킬 수 있는 잠재적인 보유숙주로서 역할을 할 수 있음을 시사한다.

주요어: 크립토스포리디움, 설치류, 식충류, COWP, 18S rRNA, 한국

학 번 : 2012-21542