INTRODUCTION

In humans, cyclosporiasis and cystoisosporiasis are frequently associated with immunocompromised patients worldwide [1]. Recently, canine cyclosporiasis and cystoisosporiasis have been reported. *Cyclospora cayetanensis* is considered an important pathogen in humans and vertebrates since human cyclosporiasis was first identified in diarrheal patients from Papua New Guinea [2]. The genus *Cyclospora* comprises obligate intracellular coccidian protozoa that reside in the intestinal epithelium and bile duct of hosts [2]. *C. cayetanensis* is transmitted through water-borne, soil-borne, and food-borne routes in developing and developed countries [2]. To date, *Cyclospora*-like organisms or *C. cayetanensis* have been detected in dairy cattle, *Macaca mulatta* thess monkey, wild chicken, and dogs [2].

*Cystoisospora canis*, *C. ohioensis*, and *C. burrowsi* cause diarrhea in puppies < 6 months of age and immunocompromised dogs [3]. Although *C. canis* is the prevalent *Cystoisospora* species in dogs, *C. ohioensis* is frequently detected in Chinese dogs [4].

Recently, Japanese raccoon dogs were identified as the reservoir host of *C. ohioensis* [5]. However, no clinical cases of canine cystoisosporiasis have been reported to date in Korea although experimental infection and sporulation of *C. ohioensis* isolates have been performed in Korean puppies [6]. It is difficult for veterinary clinicians to diagnose cyclosporiasis and cystoisosporiasis based on clinical signs alone. The morphological similarity of *Cyclospora* and *Cystoisospora* oocysts hinders microscopic diagnosis using stools. Thus, both microscopic examination and molecular techniques for differential diagnosis are used [4,5]. The present study aimed to describe a clinical case in which the protozoan pathogen *C. ohioensis* was identified in a Korean dog, using microscopic examination and phylogenetic analysis.

CASE RECORD

A 3-month-old female Maltese puppy was hospitalized with persistent diarrhea in a local veterinary clinic. Blood chemistry and hematology profile were analyzed and fecal smear was examined. Diarrheal stools were examined in a diagnostic laboratory, using multiplex real-time polymerase chain reaction (PCR) against 23 diarrheal pathogens. Sequence analysis was performed using nested PCR amplicon of 18S ribosomal RNA. Coccidian oocysts were identified in the fecal smear. Although multiplex real-time PCR was positive for *Cyclospora cayetanensis*, the final diagnosis was *Cystoisospora ohioensis* infection, confirmed by phylogenetic analysis of 18S rRNA. To our knowledge, this is the first case report of *C. ohioensis* in Korea, using microscopic examination and phylogenetic analysis.
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Blood chemistry analysis showed that total protein and sodium concentrations were slightly lower than the normal range (Table 1). Because vomiting and diarrhea lead to decreased total protein and sodium concentrations, these results were consistent with the observed symptoms. In contrast, the concentration of alkaline phosphatase (ALP) (484 U/L) was higher than the normal concentration. Although high ALP concentration is associated with hepatic damage, it is frequently observed in normal puppies. Total white blood cells and red blood cells were in the normal range. Remarkably, the number of lymphocytes and neutrophils were 6.7 × 10³/μl and 6.6 × 10³/μl, respectively. The percentage of lymphocytes and neutrophils were 47.6% and 47.5%, respectively.

Fecal smears fixed with 3% acid alcohol were stained with boiling safranin and counter-stained with hematoxylin. The microscopic image was acquired and analyzed by LAS V4.1 software (Leica Microsystems, Frankfurt, German). Phase-contrast microscopy showed that the fecal smears were negative for spirochetes. Coccidian oocysts were clearly observed in the fecal smears (Fig. 1). All oocysts were 20 to 22 μm in diameter, with an oval structure, and appeared refractile. The oocysts were stained red, with more intense staining at the edges than at the center. However, because the internal structure of the oocysts was not clearly determined, the genus of the pathogen could not be confirmed based on microscopic morphology.

Diarrheal stools were collected and transported to POBANILAB, an animal hospital specialized in infection and allergy diagnostics. Immunochromatographic assay, multiplex real-time polymerase chain reaction (PCR), and reverse transcription PCR (RT-PCR) were performed at POBANILAB using POBGEN canine enteric pathogen detection kits (POSTBIO, Hanam, Korea) for 23 enteric pathogens, including canine distemper, parvovirus, coronavirus, norovirus, circovirus, astrovirus, sapovirus, group A rotavirus, Clostridium perfringens, Campylobacter jejuni, Campylobacter coli, Salmonella species, enterotoxigenic Escherichia coli, enteropathogenic E. coli, enterohemorrhagic E. coli, enteroinvasive E. coli, Lawsonia intracellularis, Cryptosporidium parvum, Giardia lamblia, Entamoeba histolytica, C. cayetanensis, Toxoplasma gondii, and Toxocara canis. Although all laboratory tests were negative for 8 canine viruses, 9 bacteria, and 5 other parasites, multiplex real-time PCR showed a positive reaction only for C. cayetanensis. Considering that the threshold cycle value of C. cayetanensis was as high as 28.8, C. cayetanensis was suspected to be responsible for the diarrhea.

In order to confirm coccidian organisms in fecal samples, sequence analysis was performed as described previously [7]. For primary PCR, ExCycF (5’-AAT GTA CCC TTC TTC CAG AGT
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AAC-3') and ExCyR (5'-GCA AIA ATG TAC CCC CAT CAC G-3') were used to amplify 18S ribosomal RNA locus. Nested PCR was performed with NesCycF (5'-AAT TCC AGC TCC AAT AGT GTA T-3') and NesCycR (5'-CAG GAG AAG CCA AGG TAG GCR TTT-3'). A 498-bp nested PCR product was confirmed on a 1% agarose gel and purified. The purified PCR product was cloned in competent E. coli using an All in One™ PCR Cloning Kit (Biofact, Deajeon, Korea). Sequencing was performed with ABI 3730XL DNA Analyzer (Applied Biosystems, California, USA) using the Sanger dideoxy method. C. cayetanensis, C. ohiensis, C. canis, C. timonii, and T. gondii were used as outgroup species. Sequence alignment of 18S rRNA was performed using ClustalX2.1 (http://softadvice.informer.com/Clustalx_2.1.html). The phylogenetic tree was analyzed using the neighbor joining method and generated using MEGA7 (http://www.megasoftware.net/).

Sequence data of 18S rRNA obtained in this study were deposited in Genbank at the National Center for Biotechnology Information (Accession no. MH497583.1). Phylogenetic analysis of 18S rRNA sequence showed a 98.79% homology with the C. ohiensis sequence (GenBank Accession No. AY618555.1) (Fig. 2). However, sequence analysis did not match with any of C. cayetanensis sequences. On the basis of all data, the final diagnosis of this case was confirmed as C. ohiensis infection.

**DISCUSSION**

This study focused on the detection of C. cayetanensis by multiplex PCR; however, the final diagnosis of this case was C. ohiensis infection, confirmed by sequence analysis. Recently, C. cayetanensis was identified in the diarrheal stools of dogs in Nepal and Brazil [1]. It has also been reported that some human cyclosporiasis cases in Guatemala, Peru, Nepal, Jordan, and Egypt may be associated with contact with C. cayetanensis-infected animals [2]. However, C. cayetanensis experimental infection in mouse, rat, sand rat, chicken, duck, rabbit, hamster, ferret, pig, dog, owl monkey, rhesus monkey, and cynomolgus monkey have failed [9]. Although zoonotic potential of C. cayetanensis seems to be low, public health guidelines emphasize on preventing its transmission from companion animals to humans. Canine cyclosporiasis has not been documented in Korea to date; however, it is necessary to monitor it in future research.

To our knowledge, this is the first phylogenetic analysis of C. ohiensis isolates confirmed in a veterinary clinic in Korea. Previous studies have reported high prevalence of canine cyclosporiasis: 8.7% in Austria, 5.7% in north-eastern Italy, and 5.1% in the United Kingdom [3]. C. canis was the prevalent species in the United Kingdom, whereas C. ohiensis was prev-
lent in Australia, Japan, and China [3-5]. Although other coccidian parasites are not associated with peripheral eosinophilia, it is known that cytosiosisporiasis causes eosinophilic enteritis [1]. However, veterinary clinicians could not observe peripheral eosinophilia in the hematology profile (Table 1). The veterinary hematology analyzer commonly used in Korea has only 3 channels for lymphocytes, monocytes, and neutrophils; this limitation might hinder the diagnosis of C. ohiensis in dogs.

Notably, nested PCR primers described in a previous study [7] detected not only C. cayetanensis but also C. ohiensis because the 18S rRNA gene is frequently used to detect protozoa [4,5,7,8]. In some previous studies, 18S rRNA for C. cayetanensis and 5.8S rRNA and ITS2 region for Cystoisospora belli were used for the development of multiplex PCR, whereas in other studies, 18S rRNA and ITS1 genes of Cystoisospora spp. were used for phylogenetic analysis [4,5,8]. For diagnostic purposes, detection by the 18S rRNA gene has limitations in differentiating C. cayetanensis from C. ohiensis. Therefore, ITS1, which is a high-copy number element of the genome commonly used as a DNA biomarker or DNA barcode, could be a promising target for detecting and differentiating C. ohiensis and C. cayetanensis. After the development of C. ohiensis species-specific PCR, its primer specificity should be tested with C. cayetanensis.

Based on multiplex real-time PCR and fecal smear analyses, trimethoprim-sulfamethoxazole and metronidazole were initially administered to the puppy for treatment of the condition tentatively diagnosed as canine cyclosporiasis. In general, trimethoprim-sulfamethoxazole is recommended for the treatment of cyclosporiasis and cytosiosisporiasis [1]. However, an initial prescription of trimethoprim-sulfamethoxazole did not improve the clinical signs of this case. A combination of clavamox (amoxicillin trihydrate/clavulanate potassium) and metronidazole was effective in the treatment of diarrhea in this case. Ciprofloxacin is an alternative drug for the treatment of diarrhea in HIV-infected patients or patients allergic to sulfax drugs [1]. Recently, nitazoxanide was used in the treatment of a C. cayetanensis-infected patient who was not responsive to trimethoprim-sulfamethoxazole [10]. Although ciprofloxacin or nitazoxanide was not prescribed in this case, notably, trimethoprim-sulfamethoxazole was not effective. Thus, a combination of clavamox and metronidazole can be considered as an alternative treatment for canine cyclosporiasis.

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CONFLICT OF INTEREST

All authors declared that there is no conflict of interest with respect to the research, authorship, and publication of this article.

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