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Original Article

Subtyping of *Cryptosporidium parvum* Obtained from Humans and Calves in Van, Turkey

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Abstract

Background: We aimed to investigate the prevalence of *Cryptosporidium* species detected in humans and calves in the Van region of Turkey.

Methods: A total of 150 patients, comprising 60 who were immunosuppressed, 50 who were immunosuppressed and had diarrhea, and 40 who had only diarrhea, were enrolled in this study in the Department of Medical Parasitology, Van Yuzuncu Yil University Faculty of Medicine, Turkey. Stool samples were taken from the rectums of a total of 50 calves that had 30 diarrhea and 20 that did not have diarrhea, from the stables and farms of 10 central villages of Van, Turkey. All samples were analyzed using modified acid-fast staining, immunochromatographic test, and PCR. Cryptosporidium positive samples were also subtyped.

Results: Only *C. parvum* subtypes were detected in all positive samples. *C. parvum* was detected in 30 (20%) of the 150 human stool samples, while it was detected in 5 (10%) of the 50 samples from the calves. The GP60 gene region was amplified and sent for sequence analysis to identify the *C. parvum* subtypes.

Conclusion: As a result, *C. parvum* is found to be an active species that caused cryptosporidiosis is in the Van region. IIdA24G1 subtype of *C. parvum* were found in both human and calf. Therefore, due to the zoonotic feature of the *C. parvum* IIdA24G1 subtype, it has been shown that the calves in the region are a significant risk for humans.

Introduction

*C*ryptosporidium species are one of the most important agents of diarrhea in both immunosuppressed and non-immunosuppressed individuals. Human beings can be infected by animals (zoonotic transmission), infected persons (anthroponotic transmission), or via the fecal oral intake of contaminated food and water. The most important species that cause cryptosporidiosis in humans are *C. hominis* and *C. parvum*. However, species such as *C. meleagridis*, *C. felis*, and *C. canis* cause very low incidence of disease in humans (1).

At present, at least 38 *Cryptosporidium* species and more than 40 genotypes are known. The most common revealed causes of human cryptosporidiosis worldwide are related to *C. parvum* and *C. hominis*. *C. hominis* is primarily a human pathogen and hence the main cause of anthroponotic transmission. The reservoir of *C. parvum* comprises several mammal species, with cattle as a main host, which means that zoonotic transmission is thought about a common mode of spread. Other species have also been described as a public health threat, including *C. meleagridis* and *C. cuniculus* (2).

The Van region has a surface area of 2.092.700,00 km² and a population of over 1.000.000 people. Again, this region has nearly 197.000 cattle assets (3). The economy of the region is largely based on animal husbandry, industry processing animal products and agriculture. The importance of zoonotic diseases is also increasing in the region where animal husbandry is intensely carried out.

The distribution of *Cryptosporidium* species may vary according to the geographical features and socioeconomic status of the region. For example, while *C. parvum* species are mostly seen in humans in central Asian countries, *C. hominis* species are seen in most developing or developed countries (4). Cryptosporidiosis is a very common disease that causes diarrhoea in humans and domestic animals around the World. Especially in underdeveloped or developing countries, it is more common in immunosuppressed and malnourished people. The agent is transmitted via fecal oral route. While the prevalence of cryptosporidiosis is 1-2% in developed countries, it varies between 3-20% in developing countries (2). In studies conducted in immunosuppressed patients, the incidence was determined to be between 15-49% (5, 6).

Although cryptosporidiosis is not taken into consideration in clinical examinations, it can cause fatal diarrhea, especially in immunosuppressed patients. Since this disease is not considered in clinical examinations, ineffective and long-term treatments are tried. For this reason, unnecessary medication is applied to the patient, and the patient's condition becomes more serious because the appropriate medication is not administered in the treatment. In addition, due to unnecessary and ineffective drug use, both the patient and the national economy are harmed (5).

There has been no previous study on subtyping of *Cryptosporidium* species in our region. With this study, essential data were provided regarding the parasite genotype, which made it possible to conduct a comparison based on the general or special conditions of the host. In this way, the direct or indirect effects of the parasite subtypes on the host were revealed using statistical methods. Subtyping of *Cryptosporidium* species was carried out for the first time in our region with the current study. In addition, identifying the subtypes of the parasite enabled a possibility of comparison with other studies.

Materials and Methods

This study was conducted at the laboratories of the Department of Medical Parasitology,

Faculty of Medicine, Van Yuzuncu Yıl University, Turkey. Samples from humans and calves were collected between Jan 21st, 2019 and Jul 1st, 2020. The required material was obtained from samples collected from the Parasitology Laboratory of Dursun Odabas Medical Center, Van, Turkey. Additionally, calf feces samples were taken from animal breeding facilities in 10 central villages of Van, Turkey after the necessary permissions were obtained.

Samples were taken from one-yr-old calves from cattle ranches. Overall of 150 patients were included in the study, comprising 60 who were immunosuppressed, 50 who were immunosuppressed and had diarrhea, and 40 who had only diarrhea. Immunosuppressed patient group consists hemodialysis, diabetes, chronic heart failure, chronic lung disease, and patients who use immunosuppressed drugs. In addition, a total of 50 calves, comprising 30 calves that had diarrhea and 20 calves that did not have diarrhea, were included in the study.

The samples brought to the laboratory were stained with modified acid-fast staining for preliminary examination. In addition, the immunochromatographic card test (Certest crypto Biotec, Spain) was used for the detection of the *Cryptosporidium* antigen in the stool. DNA isolation was performed using a stool DNA purification kit (GeneMATRIX, Poland) for all of the stool samples for the molecular methods. The obtained DNA was kept in a refrigerator at 4 °C for a short time until the PCR assays were performed, with the caps of the tubes tightly closed and parafilmed.

The *Cryptosporidium* oocyst wall protein (*COWP*) gene region of *Cryptosporidium* spp. was amplified for determination of the *Cryptosporidium* species (7).

The PCR products that were found to be positive by nested PCR were cut with enzymes and the *Cryptosporidium* species were typed. Thermo Scientific Fast Digest restriction *RsaI* enzyme was used for this procedure. The study procedure was modified by mixing 16.8 μL of H_2O, 2.5 μL of buffer, 0.7 μL of *RsaI*

enzyme, and 10 μL of DNA, and the total volume was adjusted to 30 μL. The prepared mixture was incubated at 37 °C for 30 min. After applying PCR-RFLP, the samples were run on 2% agarose gel. To determine the *C. parvum* subtypes, the 850 bp region of the 60-kDa glycoprotein (*GP60*) gene region was amplified using nested PCR. The sanger sequence analysis results of these samples were evaluated using SnapGene 4.1 software.

Cryptosporidium spp. bidirectional sequence analysis of the *COWP* gene region was conducted by sending it to a commercial firm. Sequence analysis results were analyzed using SnapGene 4.1 software (GSL Biotech LLC, Chicago, IL, USA).

The sequencing part of the hyper-variable 60 kDa glycoprotein *GP60* gene region of *Cryptosporidium* spp. was used for the subtyping. This region of *Cryptosporidium* spp. was amplified (8).

The *GP60* gene regions of *Cryptosporidium* spp., obtained via PCR, were sent for sequence analysis. Phylogenetic analysis was performed using SnapGene 4.1 software to support the results.

Van Yuzuncu Yil University Non-Interventional Clinical Research Ethics Committee approved this study (03.08.2018 / Decision no: 2018/03). Informed consents were obtained from the patients.

Statistical analysis

MINITAB (ver: 14) statistics package program was used for data statistical analysis. The frequency of parasite prevalence was expressed as number and percentage according to the relevant categorical variables. Significance was calculated using the Chi-square (χ^2) test to compare between quantitative data, and Z-ratio test to compare the rates of noise. Odds values were calculated for the risk of occurrence of parasites. The sensitivity and specificity values of the methods used (e.g., PCR) were calculated to determine its diagnostic efficacy. Statistical significance level was

taken as 5% (*P*<0.05) in calculations and SPSS Ver. 21 (IBM Corp., Armonk, NY, USA) was used for calculations.

Results

C. parvum was detected in the stool samples of 10 (16.7%) of the 60 immunosuppressed patients, 16 (32%) of the 50 immunosuppressed patients who had diarrhea, and 4 (10%) of the 40 who had only diarrhea. Thus, *C. parvum* was detected in the stool samples of 30 (20%) of the 150 patients in total. While parasites were observed in the stool samples of 5 (16.7%) of the 30 calves that had diarrhea, no parasites were found in the stool samples of the 20 calves that did not have diarrhea.

Hence, *C. parvum* was found in the stool samples of 5 (10%) of the 50 calves in total.

In this study, 27 positive stool samples, which were detected using modified acid-fast staining, were also found to be positive using the immunochromatographic test and nested-PCR. However, 8 of the stool samples that were found to be negative using modified acid-fast staining were found to be positive with the other 2 methods. It was observed that the immunochromatographic test, which gives much more practical and rapid results, and works with the antigen screening principle in stool, was more sensitive than the modified acid-fast staining. The sensitivity and specificity were 100%. Moreover, the same results were obtained with nested-PCR (Table 1).

Table 1: Sensitivity and specificity values of the modified acid-fast staining, cassette test, and nested PCR methods

Variabel	Modified acid-fast staining %	Casette test %	Nested PCR %
Sensitivity	77.1	100.0	100.0
Specificity	100.0	100.0	100.0
False positivity	0.0	0.0	0.0
Undetectable positivity	22.9	0.0	0.0
Negative predictive	95.4	100.0	100.0
Positive predictive	100.0	100.0	100.0
Accuracy rate	96.0	100.0	

A statistically significant correlation was found between the incidence of *Cryptosporidium* spp. and clinical symptoms such as vomiting, nausea, weakness, anorexia, abdominal pain, mucus and diarrhea (Table 2).

The genomic DNA obtained from the stool samples was amplified using nested RCR and amplification of the region of 553 bp was successfully performed.

When PCR-RFLP was applied using the *RsaI* enzyme after nested PCR, it was observed that bands were formed in the regions of 34, 106, and 410 bp in *C. parvum*, and 34,

106, 125, and 285 bp in *C. hominis*. These samples were determined to be *C. parvum* using agarose gel imaging. In addition, these results were confirmed by sanger sequence analysis. To determine the *C. parvum* subtypes, the 850 bp region of the *GP60* gene region was amplified using nested PCR. The sanger sequence analysis results of these samples were evaluated using SnapGene 4.1 software. There were no statistical relationships between the *Cryptosporidium* subtypes and the clinical findings of diarrhea, mucus, vomiting, abdominal pain, nausea, weakness, or anorexia in humans.

The *Cryptosporidium* subtypes of the 30 positive human stool samples were IIdA15G1, IIdA24G1, IIaA18G3R1, and IIdA18G2. The *Cryptosporidium* subtype of 5 positive calf stool samples were IIdA24G1.

Table 2: Relationship of the prevalence of *Cryptosporidium* species with some clinical symptoms

Clinical signs and symptoms		Cryptosporidium sp.		Total	P-value
		Positive (number and %)	Negative (number and %)		
Vomiting	Positive (Number and %)	23 (76.7)	7 (23.3)	30	0.001
	Negative (Number and %)	7 (5.8)	113 (94.2)	120	
Nausea	Positive (Number and %)	15 (55.6)	12 (44.4)	27	0.0001
	Negative (Number and %)	15 (12.2)	108 (87.8)	123	
Weakness	Positive (Number and %)	20 (69)	9 (31)	29	0.001
	Negative (Number and %)	10 (8.2)	111 (91.8)	121	
Anorexia	Positive (Number and %)	14 (58.3)	10 (41.7)	24	0.001
	Negative (Number and %)	16 (12.7)	110 (87.3)	126	
Abdominal pain	Positive (Number and %)	20 (71.4)	8 (28.6)	28	0.001
	Negative (Number and %)	10 (8.2)	112 (91.8)	122	
Mucus	Positive (Number and %)	21 (77.8)	6 (22.2)	27	0.001
	Negative (Number and %)	9 (7.3)	114 (92.7)	123	
Diarrhea	Positive (Number and %)	19 (70.4)	8 (29.6)	27	0.001
	Negative (Number and %)	11 (8.9)	112 (91.1)	123	

Discussion

In the current study, *Cryptosporidium* spp. was found in the stool samples of 30 (20%) of the 150 patients included in the study. *Cryptosporidium* infections were generally higher in Turkey (9, 10). The low socioeconomic status of the Van region, insufficient infrastructure, lack of attention to personal hygiene, and widespread animal husbandry are among the reasons of high infection rates in Van province. In addition, animal husbandry is very common in the region, the continuation of traditional methods in livestock farms, the adjacent house and animal shelters, and the late diagnosis and treatment in case of the disease in animals are the reasons for the higher rate of the cryptosporidiosis in the province of Van. The fact that *C. parvum* was the parasite species that

caused infection in all of the human patients in the study supported this opinion. In addition, we believe that similar high results would be found if the methods used in this study were used in the many regions of Turkey where cattle breeding is widespread.

The rate of the spread of cryptosporidiosis varies depending on the patient group, geographical location, climatic conditions, socio-economic status, and hygiene practices (5). *Cryptosporidium* oocysts were found at a rate of 1.8%–46.8% in some studies (9, 11-15). There are many species of *Cryptosporidium*, and it is not possible to separate these species from each other without the use of molecular methods (4).

Microscopy methods used to investigate *Cryptosporidium* species in feces have low sensitivity, are time-consuming, and require experienced personnel. In addition, it is necessary to use different staining methods to detect *Cryptosporidium* spp. and some other parasites that not determined cannot be detected by direct examination. In the present study, *Cryptosporidium* spp. was detected in 27 of the 200 stool samples from the humans and calves using modified acid-fast staining. Moreover, 8 cases that could not be determined using modified acid-fast staining were able to be detected using immunochromatographic test and PCR. Thus, it was seen that there might be cases that were overlooked in the examinations that were performed using only modified acid-fast staining. It was concluded that the immunochromatographic test, which searches for specific antigens in stool, should be the more preferred method, since it can be applied more easily than the other diagnostic methods. In addition, it does not require experienced personnel and its reliability is high. The samples that had a low positive result using the immunochromatographic test gave weak bands when they were run in agarose gel after nested-PCR.

Cryptosporidiosis can lead to fatal diarrhea in immunosuppressed patients, as well as diarrhea in children, severe fluid loss, electrolyte imbalance, and even circulatory failure in advanced cases (13). Since cryptosporidiosis cannot be diagnosed with only clinical examinations of the patients, ineffective and long-term treatments are generally attempted. For this reason, unnecessary medication is given to the patient and thus, their condition becomes more serious, because the patient cannot be treated due to incorrect treatment. In addition, the economy of the country is damaged as the result of the cost of this unnecessary and ineffective drug use. Therefore, it is necessary to use rapid diagnostic tests in every hospital.

In this study, *Cryptosporidium* spp. DNA could not be obtained because of the incubation process performed by heating the stool samples, which had been kept at –20 °C for a certain period, at 95 °C for 30 min. However, when fresh fecal samples were brought to the laboratory and subjected to the same incubation process, DNA was successfully obtained from all of the samples. The correct execution of the study was directly related to the quality of the DNA extraction and the purity of the DNA obtained. Since feces has a structure that contains many organisms, it directly affects the purity and quality of the DNA extraction. The intact oocyst wall of some parasites, such as *Cryptosporidium* species, negatively affects the success of DNA extraction (16,17). Therefore, researchers who work with *Cryptosporidium* species may need to perform some procedures to weaken the oocyst wall or make various modifications to the kit procedure before beginning the DNA extraction (18). It is extremely important for researchers who extract DNA from *Cryptosporidium* species to consider these issues in terms of cost, labor, and timesavings.

In a study conducted in Jordan, stool samples were taken from children and *Cryptosporidium* spp. was detected using a microscope and molecular methods (19). A total of 44 positive *Cryptosporidium* isolates were genotyped, and 4 *Cryptosporidium* species were identified. These

species were determined to be *C. parvum* (n=22), *C. hominis* (n=20), *C. meleagridis* (n= 1), and *C. canis* (n=1). In the subtyping conducted, the subspecies of *C. parvum* were identified as IIaA15G1R1 (2), IIaA20G3R1 (1), IIcA5G3a (2), IIdA14G1 (1), IIdA20G1 (5), IIdA24G1 (1), and IIdA29G1 (1). Subtypes of *C. hominis* were identified as IbA6G3 (3), IbA9G3 (3), IbA10G2 (1), IbA20G2 (1), IdA21 (2), and IdA24 (5).

In a study conducted on the stool samples of 4031 individuals in the United Kingdom, 131 were infected with *Cryptosporidium* spp. (20). It was determined that 69 of these samples were infected with *C. parvum*, 60 were infected with *C. hominis*, and 2 were infected with both species. Animal contact, environmental contact, and foodborne outbreaks were only found to occur as the result of *C. parvum* infection. Because of examining the subtypes of *C. parvum* and *C. hominis* by amplifying the *GP60* gene region, it was revealed that *C. parvum* had more subtypes when compared to *C. hominis*. *C. hominis* IbA10G2 was seen in the stool samples of 30 patients and this subtype was more dominant. Among the *C. parvum* subtypes, *C. parvum* IIaA15G2R1 was seen in the stool samples of 17 patients and it was the dominant type. The *C. parvum* IIaA17G1R1 subtype was the cause of 3 or less outbreaks in 12 patients, the IIaA19G1R1 subtype was the cause of outbreaks in 3 patients, and the other subtypes were the cause of outbreaks in 3 patients.

In a study conducted in northern and central Israel, human-to-human transmission of the *C. hominis* was investigated. The *C. hominis* IdA16 subtype was seen in 6 patients, the *C. hominis* IbA10G2 was seen in one patient, the *C. parvum* IIdA20G and IIaA15G2R1 subtypes were seen in 4 patients, and the *C. hominis* IeA11G3T3 subtype was seen in 24 patients. *C. hominis* IeA11G3T3 subtype was reported to be the most dominant (21).

Studies on the subtyping *Cryptosporidium* species in Turkey are very limited. In a study examining the *gp60* gene region for the determi-

nation of *C. parvum* subtypes in Burdur and Kayseri regions, *C. parvum* IIaA13G2R1 and IIaA14G1R1 subtypes were determined. *C. parvum* IIaA13G2R1 subtype was common in both regions. Additionally, *C. parvum* IIaA13G2R1 subtype was detected in 95.5% of weaned calves with diarrhea (22).

In a study conducted on calf stool samples collected from farms in Balıkesir, Çorum, Ankara, Kırşehir and Kırıkkale, three subtypes of *C. parvum* IIaA13G2R1 (20/23), IIdA18G1 (2/23) and IIdA20G1b (1/23) were found in calves (23).

In Konya, subtypes of *C. parvum* IIaA11G2R1, IIaA11G3R1, IIaA12G3R1, IIaA13G2R1, IIaA13G4R1, IIaA14G1R1, IIaA14G3R1, IIaA15G2R1, IIdA16G1, and IIdA22G were determined (24).

In Kars Province, in Turkey, two subtype families of *C. parvum*, IIa (12/13) and IId (1/13) were determined in the stool samples of cattle (25). Based on these subtype families, 3 subtypes were identified, comprising *C. parvum* IIaA15G2R1 (10/13), IIaA16G3R1 (2/13), and IIdA15G1 (1/13). When considering age, *C. parvum* subtypes IIaA15G2R1 (9/10) and IIaA16G3R1 (1/10) were seen in the stool samples of calves, while *C. parvum* subtypes IIaA16G3R1 (2/3) and IIdA15G1 (1/3) were determined in the stool samples of cows. When the results of the molecular characterization of *C. parvum* were examined based on the clinical conditions of the animals, the *C. parvum* IIa subtype family was found in all of the stool samples of the calves that had diarrhea. The *C. parvum* IIa subtype family (8/8) and *C. parvum* IIaA15G2R1 (7/8) subtypes were more commonly detected in the stool samples of the calves. Because the *C. parvum* IIaA15G2R1 subtype was more common in the stool samples of the calves that had diarrhea, *C. parvum* IIaA15G2R1 may be more pathogenic. In addition, this result also indicated that the calves in the region posed a significant risk for humans due to the zoonotic *Cryptosporidium* spp. potential.

Cryptosporidiosis is a zoonotic infection and has a worldwide distribution. The stools of domestic animals and livestock, especially calves, play a major role in human contamination. Due to the importance of *Cryptosporidium* infections in cattle both in terms of veterinary and human medicine, research on bovine cryptosporidiosis has intensified in the world. Studies are conducted more frequently in calves. Among the species reported in cattle, *C. parvum* is the species with the widest host spectrum and is more common in calves before 2-3 months of age. *Cryptosporidium bovis*, *C. andersoni* and *C. ryanae* species are common in cattle older than three months of age or post-weaning (24).

In this study, 35 positive samples were evaluated using both sequence analysis and PCR-RFLP, and the causative species in all of the cases was *C. parvum*. The *C. parvum* subtypes seen in the Van region were IIdA15G1, IIdA24G1, IIaA18G3R1, IIdA18G2, and IIdA24G1. Furthermore, the transmission of *C. parvum* IIdA24G1 subtypes to humans originated from calves. With this result, the role of calves in the transmission of cryptosporidiosis to humans is once again demonstrated. In the control and protection; isolation and treatment of calves showing cryptosporidiosis clinical symptoms, raising awareness of the society, and screening animals at regular intervals in endemic regions are essential for both public and animal health.

The finding of these species as *C. parvum* was similar to the results of some studies that have been conducted in Turkey (23, 25). Since there have not been many studies on the subtyping of *Cryptosporidium* species in Turkey, it was not possible to provide information about other species that infect humans. Moreover, no significant relationship was found between the subtypes of *C. parvum* and the clinical symptoms observed in the patients, such as diarrhea, mucus, vomiting, abdominal pain, nausea, weakness, and anorexia. It is our belief that this is the first study in the Van region

related with the subtyping of *Cryptosporidium* species. In order to provide more detailed information, it is necessary to conduct other studies on the subtyping of *Cryptosporidium* species.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

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