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Detection and genotyping of *Cryptosporidium* spp. in diarrheic stools by PCR/RFLP analyses

Selma USLUCA, Ümit AKSOY

Aim: To compare microscopy and polymerase chain reaction (PCR) as methods for diagnosing *Cryptosporidium* spp. and identifying the genotype of the parasite in diarrheic stool.

Materials and methods: Stool samples from diarrheic patients admitted to Dokuz Eylül University Medical Faculty were collected on a specific day of the week. Stool samples sent to our laboratory from patients affected by a diarrhea epidemic in İzmir were also included in the study. A total of 162 stool samples were examined by microscopic and molecular methods, and the validity of the molecular method was investigated.

Results: Using Kinyoun acid-fast dye, *Cryptosporidium* spp. was found in 18 stool samples. Using PCR, *Cryptosporidium* spp.-specific bands were found in 15 of these cases. In stool samples that showed no *Cryptosporidium* spp. by Kinyoun acid-fast dye, a parasite-specific band was obtained in 6 of 144 cases. The restriction fragment length polymorphism (RFLP) method was applied in the cases in which the parasite was found with PCR. *Cryptosporidium* meleagridis was found in the stool sample of 1 case, and *Cryptosporidium* parvum-specific bands were seen in the stool samples of 20 cases. The mean age of those who were diagnosed with *Cryptosporidium* spp. was significantly lower than the mean age of study participants who were not diagnosed with the parasite (P = 0.006). It was found that the families of 44.4% of those diagnosed with *Cryptosporidium* spp. had similar complaints. This rate was statistically significant (P = 0.01). It was found that 88.9% of those diagnosed with *Cryptosporidium* spp. drank artesian and well water, and 11.1% drank bottled water. The rate of *Cryptosporidium* spp. in those who drank artesian and well water was significantly higher than the rate of those who drank city or bottled water (P < 0.001). While the Kinyoun acid-fast method is considered the gold standard, the sensitivity of the PCR method is 83.3% and the selectivity is 95.8%.

Conclusion: It can be concluded that Kinyoun acid-fast dye, which can be used as a referential diagnostic method, has some limitations and that PCR is more sensitive and allows for identification of the parasite genotype.

Key words: *Cryptosporidium* spp., Kinyoun acid-fast, PCR-RFLP

İshalli dışkılarda PCR/RFLP yöntemi ile *Cryptosporidium* spp.'nin saptanması ve tür ayrımının yapılması

Amaç: İshalli dışkılarda *Cryptosporidium* spp.'nin tanısında mikroskobi ve polimeraz zincir reaksiyonu (PCR) yöntemlerinin karşılaştırılması ve parazitin tür ayrımının yapılması amaçlanmıştır.

Yöntem ve gereç: Dokuz Eylül Üniversitesi Tıp Fakültesi'nin çeşitli kliniklerine başvuran ishalli hastalardan, haftanın belli bir gününde dışkı örnekleri toplanmıştır. Ayrıca İzmir'deki bir ishal salgınında laboratuarımıza yönlendirilen olguların dışkı örnekleri de çalışma kapsamına alınmıştır. Toplam 162 dışkı örneğinin mikroskobi ve moleküler yöntemle değerlendirilmesi ile tanıda moleküler yöntemin geçerlilik çalışması planlanmıştır.

Bulgular: Kinyoun acid-fast boya ile 18 olgunun dışkı örneğinde *Cryptosporidium* spp. saptanmıştır. PCR ile bu olgulardan 15'inin dışkı örneğinde *Cryptosporidium* spp.'ye özgü bant elde edilmiştir. Kinyoun acid-fast boya ile

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Department of Parasitology, Faculty of Medicine, Dokuz Eylül University, 35304, İnciraltı, İzmir - TURKEY

Correspondence: *Selma USLUCA, Department of Parasitology, Faculty of Medicine, Dokuz Eylül University, 35304, İnciraltı, İzmir - TURKEY*
E-mail: selmausluca@yahoo.com

Cryptosporidium spp. saptanmayan 144 olgudan 6'sının dışkı örneğinde parazite özgü bant elde edilmiştir. PCR ile parazit saptanan olgulara RFLP yöntemi de uygulanmıştır. Olgulardan birinde *Cryptosporidium* meleagridis'e, 20 olgunun dışkı örneğinde ise *Cryptosporidium* parvum'a özgü bantlar saptanmıştır. *Cryptosporidium* spp. saptanan olguların yaş ortalamaları, saptanmayanlara göre anlamlı olarak daha düşük olarak belirlenmiştir (P = 0,006). *Cryptosporidium* spp. saptanan olguların % 44,4'ünün aile bireylerinde de benzer yakınmalar saptanmıştır. Bu oran istatistiksel olarak anlamlı bulunmuştur (P = 0,01). *Cryptosporidium* spp. saptanan olguların % 88,9'unun artezyen veya kuyu suyu içtikleri, % 11,1'inin damacana suyu içtikleri belirlenmiştir. Artezyen veya kuyu suyu içenlerde, damacana suyu içenlere göre *Cryptosporidium* spp. saptanma oranı anlamlı olarak yüksek bulunmuştur (P < 0,001).

Sonuç: Referans tanısal yöntem olarak kullanılan Kinyoun acid-fast boya yönteminin bir takım kısıtlılıkları olduğu ve PCR yönteminin parazitin tür ayrımının yapılmasında daha duyarlı bir yöntem olduğu kanısına varılmıştır.

Anahtar sözcükler: *Cryptosporidium* spp., Kinyoun acid-fast, PCR-RFLP.

Introduction

Cryptosporidium spp. is a coccidian parasite infecting mammals, birds, reptiles, and fish (1). It is known to spread through fecal-oral routes. While *Cryptosporidium* spp. causes short-term diarrhea in immunocompetent persons, it may cause life-threatening diarrhea in immunocompromised patients (2).

In diagnosing cryptosporidiosis, the Kinyoun acid-fast dye method is the accepted gold standard (3). However, microscopy is a time-consuming diagnostic method that requires expertise and may prove inadequate in diagnosing a small number of parasites (4). The use of molecular methods has increased recently (5). Polymerase chain reaction (PCR) is a sensitive diagnostic method capable of determining *Cryptosporidium* spp. with high selectivity in environmental and clinical samples, and it allows for genotyping (4). Of the 2 major *Cryptosporidium* spp. genotypes that cause infection in humans, *C. hominis* causes infections in humans only (anthroponotic infection), while *C. parvum* causes infections in humans and animals (zoonotic infection) (5). When evaluated pathogenetically and clinically, there appear to be other differences between *C. hominis* and *C. parvum*. *C. hominis* causes long-term diarrhea and more oocyst excretion, while *C. parvum* causes short-term diarrhea and less oocyst excretion (6). However, other *Cryptosporidium* species have been associated with human infections: *Cryptosporidium felis*, *Cryptosporidium canis*, and *Cryptosporidium meleagridis* in human immunodeficiency virus-positive individuals and *C. meleagridis* in immunocompetent individuals (7). In addition, *Cryptosporidium* species such as *C. felis*, *C. canis*, and *C. meleagridis* have been able to infect healthy children and adults. Transmission from pets is more likely in children; however, the exact mechanism for transmission in elderly and immunocompromised individuals has not yet been clearly identified (5).

The aim of this study was to evaluate microscopy and PCR as methods for detecting *Cryptosporidium* spp. in stool samples of diarrheic cases whose demographic features were tracked. Identification of the genotype of the parasite was also carried out.

Materials and methods

Data collection

Between October 2005 and January 2009, stool samples of diarrheic patients admitted to the various units of Dokuz Eylül University Medical Faculty were collected once, on a specific day of the week. The stool samples of patients affected by a diarrhea epidemic in the village of Karaağaç in Buca, İzmir, and sent by the İzmir Health Directorate to Dokuz Eylül University Medical Faculty's Parasitology Laboratory were also included in the study.

The degree of diarrhea affecting subjects who participated in the study was evaluated using the Bristol Stool Scale (8). Rural participants made up 7.4% of the cases and were affected by the epidemic, whereas urban participants made up 92.6% and came to our hospital with a number of gastrointestinal complaints. Of the studied cases, 48.8% of patients were female (between ages 1-84, std: 25.44) and 51.2% were male (between ages 1-76, std: 22.38). The

mean age was 44.50 (min: 1, max: 84).

The stool samples from a total of 162 patients were investigated using microscopic and molecular methods, and the validity of the molecular method in diagnosis was evaluated. All patients completed a questionnaire designed to evaluate symptoms and create a demographic profile of the participants (ethical committee approval date: 03.05.2008).

Microscopic examination of the stool

Following routine parasitological examination, stool samples were dyed with Kinyoun acid-fast after being concentrated with modified formalin-ethyl acetate (9). Preparations were evaluated by light microscope, magnified at ×100. The density of parasites in the dyed preparation was determined by a method developed by McLauchlin et al. (10). The PCR method was applied to all stool samples, which were stored at −80 °C without preservatives. The restriction fragment length polymorphism (RFLP) method was applied to samples found to be PCR-positive.

DNA extraction

QIAamp DNA Stool Mini Kit (QIAGEN Ltd., Crawley, West Sussex, UK) was used on the stool samples, and DNA was obtained in accordance with the procedure suggested by the manufacturer.

PCR

Cry9 (5-GACTGAAATACAGGCATTATCTTG-3') and Cry15 (5'-GTAGATAATGGAAGAGATTGTG-3') primers were used on the *Cryptosporidium* spp. oocyst wall proteins (7). The PCR mixtures contained 10 µL of DNA sample, 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 10 pmol/µL of each primer, and 5 U/µL of Taq DNA polymerase (Roche). PCR was performed under the following conditions: 35 cycles at 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min, followed by 10 min at 72 °C (7). Positive and negative controls were included in each batch of tests. A 10-µL aliquot of PCR product was analyzed for 18S rRNA and COWP gene (550 bp) fragments by electrophoresis in 1% agarose/ethidium bromide gels.

Restriction fragment length polymorphism

RsaI digestion fragments (GT-AC) were resolved by electrophoresis in 2% agarose/ethidium bromide gels. All gels were recorded using UV transillumination (7).

Statistical analysis

SPSS 11.00 was used for statistical analyses. A chi-square test was performed for the correlation between the demographic features of the cases and parasite determination. A t-test was performed for the mean ages. In comparing the PCR methods with Kinyoun acid-fast dye, sensitivity and selectivity were taken into consideration.

Results

Evaluation of the degree of diarrhea in the participants revealed that 50% of them were compatible with a Bristol Scale score of 5, 30.2% with Bristol Scale 6, and 19.8% with Bristol Scale 7. The rate of *Cryptosporidium* spp. in those from rural areas was significantly higher than those from urban areas ($P < 0.001$).

It was decided that the number of symptomatic and asymptomatic patients, as well as gastrointestinal symptoms, should be determined. In addition, the distribution of *Cryptosporidium* spp. and whether it correlated with symptomatic or asymptomatic cases was investigated.

All patients complained of diarrhea. The complaints accompanying the diarrhea were evaluated and it was determined that 52.4% had colic, 33.3% meteorism, 23.4% nausea, 12.3% vomiting, 6.8% fever, and 1.8% itching and bleeding in the anal area. It was seen that those who were diagnosed with *Cryptosporidium* spp. had complaints such as colic (55.6%), meteorism (38.9%), vomiting (16.7%), fever (11.1%), and itching and bleeding in the anal area (5.5%). The relationship between each clinical complaint and *Cryptosporidium* spp. diagnosis is given in Table 1.

A comparison between *Cryptosporidium* spp. diagnosis and diarrhea duration revealed that the presence of the parasite had no influence on the duration of diarrhea ($P = 0.185$).

In our study, *Cryptosporidium* spp. was found in 18 cases by using microscopy and 21 cases by the molecular method. It was observed that 53% of the positive cases were traced to a waterborne epidemic

Table 1. The relationship between other clinical complaints and *Cryptosporidium* spp.; some patients had more than one complaint.

Complaints*		*Cryptosporidium* spp.			
		Positive	**Negative**	**Total**	P-value
Abdominal pain	(+)	10	75	85	P = 0.978
	(-)	8	69	77	
Meteorism	(+)	4	50	54	P = 0.426
	(-)	14	94	108	
Nausea	(+)	7	31	38	P = 0.137
	(-)	11	113	124	
Vomiting	(+)	3	17	20	P = 0.469
	(-)	15	127	142	
Fever	(+)	2	9	11	P = 0.351
	(-)	16	135	151	
Other	(+)	1	2	3	P = 0.299
	(-)	**17**	**142**	**159**	

and 47% were sporadic cases. It was seen that 61.1% of the positive patients resided in rural areas and 38% resided in urban areas. It was found that 88.9% of those diagnosed with *Cryptosporidium* spp. drank artesian and well water, and 11.1% drank city and bottled water. Of those who drank artesian and well water, 91.7% resided in Karaağaç, Buca, and 8.3% in Kırıklar, Buca.

Using native-lugol and trichrome dye methods, 4.9% of the patients that participated in the study were diagnosed with *Blastocystis hominis*, 1.8% with *Giardia intestinalis*, 1.2% with *Trichomonas hominis*, 0.6% with *Endolimax nana*, 0.6% with *Entamoeba histolytica*, and 0.6% with *Entamoeba coli*. With the Kinyoun acid-fast method 11.1% were diagnosed with *Cryptosporidium* spp. and 1.2% with *Cyclospora cayetanensis*.

Table 2 shows the evaluation of *Cryptosporidium* spp. density by Kinyoun dye.

Table 2. Evaluation of the density of *Cryptosporidium* spp. using Kinyoun dye.

Parasite density	Number (%)
No oocysts	144 (88.9)
<1 oocyst per field	15 (9.3)
1-10 oocysts per field	2 (1.2)
11-100 oocysts per field	1 (0.6)
Total	162 (100)

In Kinyoun acid-fast staining, *Cryptosporidium* spp. oocysts appear as bright pink spheres approximately 5 µm in diameter on a blue background (Figure 1).

PCR images from patients with *Cryptosporidium* spp. are shown in Figure 2.

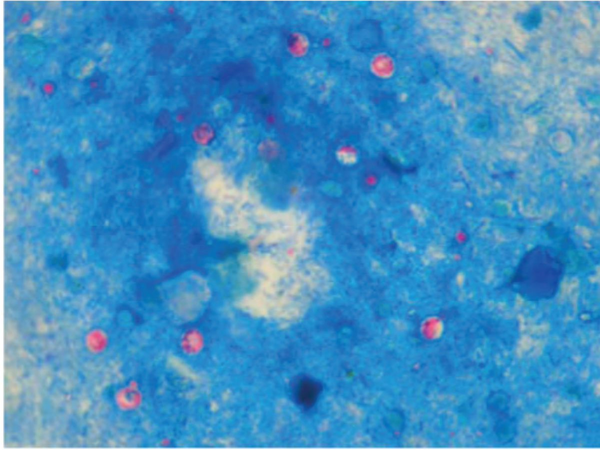

Figure 1. *Cryptosporidium* spp. oocysts with Kinyoun acid-fast staining.

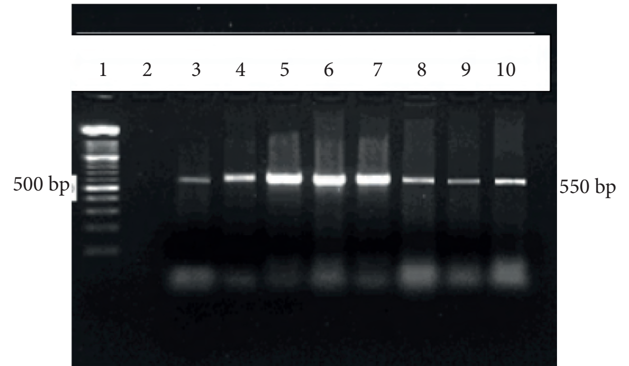

Figure 2. Gels (1% agarose/ethidium bromide electrophoresis) from PCR amplification of *Cryptosporidium* COWP gene; **lane 1:** marker (100 bp ladder), **lane 2:** negative control, **lane 3:** *Cryptosporidium* spp. positive control, **lanes 4-10:** *Cryptosporidium* spp.-positive patients.

In order to genotype the parasite, the RFLP method was applied. In 1 case (0.6%), *C. meleagridis*-specific bands of 34, 147, and 372 bp were found, while 20 (12.3%) had *C. parvum*-specific bands of 34, 106, and 413 bp (Figures 3 and 4).

The use of the Kinyoun acid-fast method and PCR for diagnosing *Cryptosporidium* spp. is shown in Table 3.

Using the Kinyoun acid-fast method, *Cryptosporidium* spp. was found in 55.6% of the female and 44.4% of the male study participants. There was no significant difference between females and males in terms of the prevalence of *Cryptosporidium* spp. (P = 0.718). The mean age of those who were diagnosed with *Cryptosporidium* spp. was 28.5, and the mean age of negative cases was 40.2. The mean age of patients with *Cryptosporidium* spp. was significantly lower than the mean age of patients without *Cryptosporidium* spp. (P = 0.006).

It was found that the families of 44.4% of those diagnosed with *Cryptosporidium* spp. had similar complaints. This rate was statistically significant (P

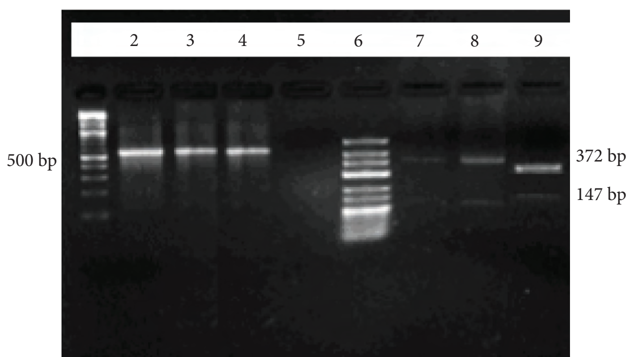

Figure 3. Gels (2% agarose/ethidium bromide electrophoresis) from PCR amplification of *Cryptosporidium* COWP gene and *RsaI* digestion of PCR products; **lane 1:** marker (100 bp ladder), **lane 2:** *Cryptosporidium* spp.-positive control, **lanes 3 and 4:** *Cryptosporidium* spp. from whole feces of the patients, **lane 5:** negative control, **lane 6:** marker (25 bp ladder), **lane 7:** *C. parvum*-positive control, **lane 8:** *C. parvum*-positive patient, **lane 9:** *C. meleagridis*-positive patient.

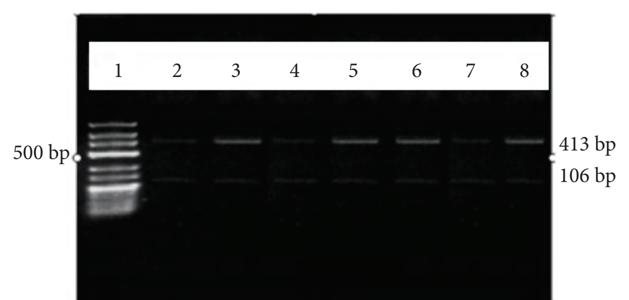

Figure 4. Gels (2% agarose/ethidium bromide electrophoresis) from PCR amplification of *Cryptosporidium parvum*-positive patients; **lane 1:** marker (25 bp ladder), **lanes 2-8:** *C. parvum*-positive patients.

Table 3. Comparison of Kinyoun acid-fast staining method with PCR for diagnosis of *Cryptosporidium* spp.

Positive		Kinyoun acid-fast		
		Negative		Total
PCR	**Positive**	15 (83.3%)	6 (4.2%)	21 (13%)
	Negative	3 (16.7%)	138 (95.8%)	141 (87%)
Total		18 (100%)	144 (100%)	162 (100%)

= 0.01). It was found that 88.9% of those diagnosed with *Cryptosporidium* spp. drank artesian and well water and 11.1% drank city and bottled water. The rate of *Cryptosporidium* spp. in those who drank artesian and well water was significantly higher than the rate in those who drank city and bottled water ($P < 0.001$).

While the Kinyoun acid-fast method is considered the gold standard, the sensitivity of the PCR method is 83.3%, the selectivity is 95.8%, the positive predictive value is 71.4%, and the negative predictive value is 97.9%.

Discussion

Cryptosporidium spp., frequently accompanied by diarrhea, is more often seen in developing countries in malnutrition cases, among the old, and in immunocompromised patients (11). Waterborne *Cryptosporidium* epidemics, seen frequently in recent years, are considered a serious public health problem (12).

The identification of *Cryptosporidium* types is important in that it reveals the epidemiology of *Cryptosporidium* infections in humans and animals (13). Studies that investigated the relationship between the presence of the infection and the complaints of family members found a correlation rate between 0.5% and 37.5% (14-16). Our study revealed that the families of 44.4% of those diagnosed with *Cryptosporidium* spp. reported similar complaints, and a significant correlation was found between *Cryptosporidium* spp. diagnosis and familial complaints. In our study group, family contact, derived from factors such as contact with contaminated hands and use of a communal bathroom, was considered to have a significant influence on the spread of infection. This type of infection is seen among urban people.

The majority of the cases diagnosed with *Cryptosporidium* spp. in our study lived and bred animals in rural areas. Lack of hygiene and poor living conditions, direct contact with farm animals where cryptosporidiosis has a high prevalence, and oocyst-contaminated food and water account for the spread of the infection. The rate of diagnosis of *Cryptosporidium* spp. among those who drink artesian and well water is significantly high; thus, the reason for the spread of the parasite, especially in rural areas, is contaminated water. Because the water consumed by *Cryptosporidium* spp.-infected patients from cities was not analyzed, the spread of the parasite remains unexplained and was attributed to possible food contamination. It is also possible that infection by zoonotic types is caused not only by direct contact with animals, but also by consumption of water and food contaminated by animal or human stools. Due to the fact that both *Cryptosporidium* spp. types found in our study have zoonotic potential and the majority of the patients resided in rural areas, it was assumed that the infection had zoonotic spread. However, no definitive conclusions were reached as no samples were collected from animals.

Balabat et al. reported that Kinyoun acid-fast dye could determine 10,000 oocyst/g *Cryptosporidium* spp. in watery stool and 50,000 oocyst/g in formed stool, and recommended that the more sensitive testing method be a requirement in asymptomatic or chronic carriers (17). Thanks to molecular methods, it is possible to diagnose the factors quickly and

reliably, to identify subtypes, and, importantly, to determine the source of and risk factors for an epidemic (5). The sensitivity of the PCR method is about 20 oocysts in 1 mL of stool sample (18). As some studies reported, less positivity is found by PCR as opposed to microscopy, because there are too few oocysts and they are not diffused homogeneously within the stool sample (19). It was seen in our study that there was low oocyst density in cases diagnosed with *Cryptosporidium* spp. The PCR method proved to be more effective in determining the stool samples with a high density of parasites. The explanation for these results is the fact that there are very few oocysts in the stool and they are not diffused homogeneously, as suggested in the literature. Oocysts may have been damaged before DNA extraction, and this could account for differences between PCR results and microscopic evaluation.

COWP and 18S rRNA genes are frequently used for genotyping *Cryptosporidium* spp. (7). The primers used in our study work well with the wall-protein-coding gene and have high sensitivity and selectivity, thus preventing unwanted amplification products from being obtained. A maximum amount of DNA was also obtained by eliminating PCR-inhibiting substances. In a study by Morgan et al., which investigated *Cryptosporidium* in clinical and environmental samples in Australia, PCR was considered as an alternative to microscopic examination. A comparison with PCR revealed that the sensitivity of microscopy was 83.9% and its selectivity was 98.9% (20). With the Kinyoun acid-fast dye used in this study, the sensitivity of PCR was estimated to be 83.3% and its selectivity 95.8%. In light of these results, it was concluded that PCR was a reliable method for identifying *Cryptosporidium* spp. and that it could be used in place of the Kinyoun acid-fast dye method.

When reviewing studies on the diagnosis of *Cryptosporidium* in Turkey, we observed that most dealt with dye methods (21-23). Future molecular studies should focus on the way the parasite spreads and its means of infection. *Cryptosporidium* species differ according to country. In a study in Slovenia, for example, it was reported that diarrheic patients had a *Cryptosporidium* species known to exist in animals (24). In a study carried out in Kenya, Gatei et al. found, as a result of species identification, that 87% of the PCR-positive cases belonged to *C. hominis* and 9% to *C. parvum*; the remaining 4% belonged to *C. canis*, *C. felis*, *C. meleagridis*, and *C. muris*. The results obtained from the study showed that anthroponotic *Cryptosporidium* species in Kenya were frequently encountered and that they were carried from person to person (25). It was reported in our study that 12.3% of the cases were due to *C. parvum* and 0.6% were due to *C. meleagridis.* We concluded that infection spread through zoonotic means, as both species cause zoonotic infections and the majority of our subjects made their living by farming and animal husbandry in rural areas. However, it must be kept in mind that the species that cause zoonotic infections can also cause anthroponotic infections. Our study is the first to report *C. meleagridis* in Turkey.

It can be concluded that Kinyoun acid-fast dye, which can be used as a reference diagnostic method, has some limitations and that PCR is a more sensitive method that allows for the identification of parasite species. We believe that species identification is a prerequisite for discovering infection means and resources. Identification also allows for the development of precautionary measures and the creation of suitable treatment protocols and clinical approaches pertaining to different species.

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