

Investigation of *Cryptosporidium* spp. positivity in Formol-Fixed Stool samples by Polymerase Chain Reaction Method

Formolle tespit edilmiş dışkı örneklerinde *Cryptosporidium* spp.'nin Polimeraz Zincir Reaksiyonu Tekniği kullanılarak pozitifliğinin araştırılması

Ülkü Karaman¹, Zeynep Kolören², Emine Ayaz²

¹Department of Medical Parasitology, Faculty of Medicine, Ordu University, Ordu, Turkey

²Faculty of Arts and Sciences, Department of Biology, Ordu University, Ordu, Turkey

Corresponding author: Ülkü Karaman, MD, Department of Medical Parasitology, Faculty of Medicine, Ordu University, Ordu, Turkey

E-mail: ulkukaraman44@hotmail.com

Received/Accepted: October 31, 2018 / May 16, 2019

Conflict of interest: There is not a conflict of interest.

SUMMARY


Objective: It was aimed to determine *Cryptosporidium* spp., which is thought to cause digestive system complaints in primary school children, by Modified Acid-Fast (MAF) staining method and Polymerase Chain Reaction (PCR).


Method: One hundred sixty-four stool samples taken from children were examined under light microscopy with MAF. Positive samples were fixed in 10% formalin and stored at + 4 °C until analysis. These samples were then purified using the Sucrose Gradient method and, DNA isolation was performed using the modified QIAamp DNA Mini Kit (Qiagen) protocol. In order to increase the sensitivity of the study, the SSU rRNA gene region was amplified by PCR method.


Results: Microscopic examination showed that 89 of the 164 stool samples (54,26%) were found to be positive for *Cryptosporidium* spp. oocysts. Eighty-nine samples which were found to be positive by microscopic examination and fixed in %10 formalin were studied by PCR, and 74 (45,12%) of them were positive.

Conclusions: Cryptosporidiosis, which is known to cause severe infections, especially in immunocompromised individuals and children, is transmitted by the fecal-oral route from infected humans and animals, and contaminated water and nutrients. Cryptosporidiosis, which is the result of fecal pollution, causes a great risk for human health even with a low number of oocysts due to the fact that oocysts are resistant to chlorine and they can survive for a long time in the outer environment. It was observed that primary school children living in the area of the study were under risk for cryptosporidiosis.

Keywords: Modified Acid-Fast (MAF), Polymerase Chain Reaction (PCR), *Cryptosporidium* spp.

 Ülkü Karaman

 Zeynep Kolören

 Emine Ayaz

ORCID IDs of the authors:

Ü.K. 0000-0001-7027-1613

Z.K. 0000-0001-9708-2716

E.A. 0000-0003-3982-5459

ÖZET

Amaç: Çalışmada ilköğretim okulu çocuklarında sindirim sistemi rahatsızlığına neden olabileceği düşünülen *Cryptosporidium* spp.'nin Modifiye Asit Fast (MAF) ve Polimeraz Zincir Reaksiyonu (PZR) ile tespit edilmesi amaçlanmıştır.

Yöntem: Çocuklardan alınan 164 dışkı örneği Modifiye Asit-Fast (MAF) boyama yöntemiyle ışık mikroskobu altında incelenmiştir. Pozitif bulunan örnekler %10'luk formolde tespit edilerek inceleme yapılana kadar +4°C'de muhafaza edilmiştir. Daha sonra Sükroz Gradient yöntemi kullanılarak saflaştırılan örnekler, modifiye edilmiş QIAamp DNA Mini Kit (Qiagen) protokolü uygulanarak DNA izolasyonu yapılmıştır. Çalışmanın hassasiyetini arttırmak amacıyla SSU rRNA gen bölgesi, PZR yöntemi ile çoğaltılarak örnekler incelenmiştir.

Bulgular: Mikroskopik bakı sonucunda 164 dışkı örneğinin 89'unda (%54,26) *Cryptosporidium* spp.'nin ookistlerine rastlanılmıştır. Mikroskopik inceleme ile pozitif bulunan ve %10'luk formolde tespit edilen 89 örnek PZR yöntemi ile çalışılmış ve 74'ü (%45,12) pozitif olarak belirlenmiştir.

Sonuç: Özellikle bağışıklık sistemi baskılanmış kişilerde ve çocuklarda ağır enfeksiyonlara neden olduğu bilinen *Cryptosporidiosis*, enfekte insan ve hayvanlarla yakın temasla, kontamine su ve besin yoluyla fekal-oral yolla bulaştığı bilinmektedir. Fekal kirlilik sonucunda meydana gelen bu enfeksiyonun, düşük sayıda ookistle bile meydana gelebilmesi, ookistlerin kloro karşı dirençli oluşu ve dış ortamda uzun süre canlı kalabilmesi nedeniyle insan sağlığı için büyük risk oluşturmaktadır. Elde edilen veriler değerlendirildiğinde araştırma alanında yaşayan ilköğretim çocuklarının *Cryptosporidiosis* açısından risk altında olduğu gözlenmiştir.

Anahtar sözcükler: Modifiye Asit Fast (MAF); Polimeraz Zincir Reaksiyonu (PZR); *Cryptosporidium* spp.

INTRODUCTION

Cryptosporidium spp. which is an obligate intracellular parasite belongs to the coccidia subgroup of Apicomplexa class. This parasitosis was identified for the first time in gastric crypts of laboratory mice by Ernest Edward Tyzzer in 1907.¹ Oocysts of the parasite which causes gastroenteritis in many vertebrates and humans are 4-6 µm in size and transmit between organisms with consumption of stool-contaminated water and food.²⁻⁴

The incidence of *Cryptosporidium* spp. is high among some occupational groups (persons dealing with animal husbandry, veterinarians, laboratory personnel, day care center personnel), children and elderly people, persons traveling to the endemic areas, those living in environments with poor hygienic conditions, and persons who contact with infected individuals. There have been *Cryptosporidium* epidemics known to be originated from public swimming pools, food eaten from the same plates, jointly eaten meals, and non-hygienic water resources.⁶ Transitions from animals to humans in rural areas have also been reported.⁵

Cryptosporidiosis symptoms vary depending on the type, immunity, and age of the infected host.^{7,8} The disease causes asymptomatic infection or spontaneously resolved diarrhea in persons with healthy immune system, it may lead to chronic diarrhea with severe progression; pancreas, gallbladder and respiratory tract infections; and mortality.⁹ Lack of clean water and sanitation facilities, crowded home environment, and close

contact with reservoir animals are known to increase the prevalence of the parasite.⁹ Several studies from Turkey have reported the prevalence between 0%-35.5%.^{8,10,11}

In this study, we aimed to determine *Cryptosporidium* spp., which is thought to cause digestive system complaints by Modified Acid-Fast (MAF) staining method and Polymerase Chain Reaction (PCR) in children attending to Ordu Primary School.

MATERIAL AND METHODS

Before the beginning of the study, 2018 dated and 174 numbered approval was received from the Ordu University Clinic Research Ethics Committee.

Sample Collection and Purification of the Oocysts with Sucrose Gradient Centrifugation

Stool samples were collected from the school children, and centrifuged; smears from the sediments were prepared on the slides, dried and examined under a light microscope with X100 magnification using MAF staining method. The positive samples were fixed in 10% formalin and kept at +4°C until the analysis. Each sample found positive with the microscopic examination was subjected to serial centrifugation in order to remove PCR inhibitors and formol. Stool samples were purified with Sheather's sugar flotation technique described by Karanis and Kimura¹²; and the supernatant portion was removed. The samples were then put into 1 mL pellet Eppendorf tubes and stored at -80°C until DNA isolation.

DNA Isolation and Imaging of *Cryptosporidium* oocysts with PCR Method

DNAs were extracted from all stool samples that were purified with sucrose gradient method, with the QIAamp DNA Mini Kit using the protocol modified by Plutzer et al.¹³

Hot Start Taq DNA Polymerase Kit (Qiagen), 25 mM dNTP mix, 10 pmol CPBD AGF, and CPBDIAGR primers;

(CPBDIAGF: 5'-AAGCTCGTAGTTGGATTTCTG-3',

CPBDIAGR: 5'-TAAGGTGCTGAAGGAGTAAGG-3') and 1 μ l DNA was used for PCR. Nuclease-free water (Hyclone, catalog no: SH30538.02) was added so as to provide a total volume of 25 μ L. The reaction mixture prepared was vortexed and left to incubation in PEQlab PCR device at 95°C for 15 minutes, at 95°C for 30 seconds, at 48.7°C for 1

minute, at 72°C for 30 seconds (45 cycles), and at 72°C for 10 minutes. The PRC products obtained were kept at +4°C until the analysis. Positive and negative controls were used for each test. The products were then stained with ethidium bromide and loaded to 1.5% agar. After running in the gel electrophoresis at 100 V for 6a minutes, the bands were displayed under UV (Prizma/Quantum ST4).

RESULTS

Microscopic examination revealed *Cryptosporidium* spp. positivity in 89 (54.26%) of 164 stool samples. Eighteen-nine positive stool samples were fixed in 10% formalin until PCR analysis. In order to increase the sensitivity of the study, the SSU rRNA gene region was amplified with the PCR method, and 74 (45.12%) of 89 stool samples (Figures 1 and 2). We observed that the data obtained with staining and PCR methods were closely similar.

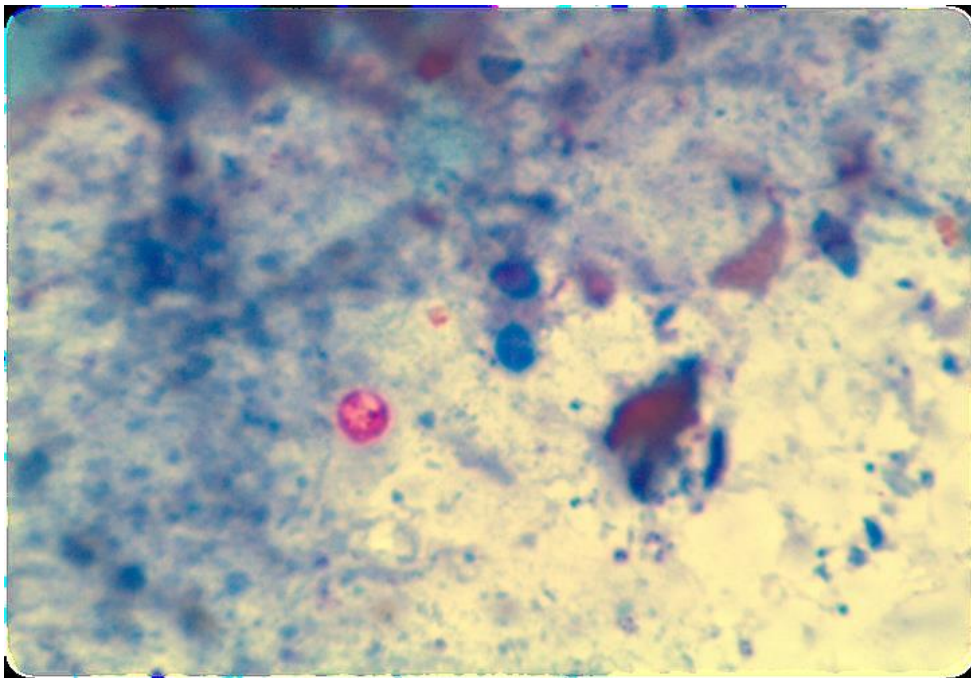


Figure 1: *Cryptosporidium* spp. detected with the modified acid-fast method.

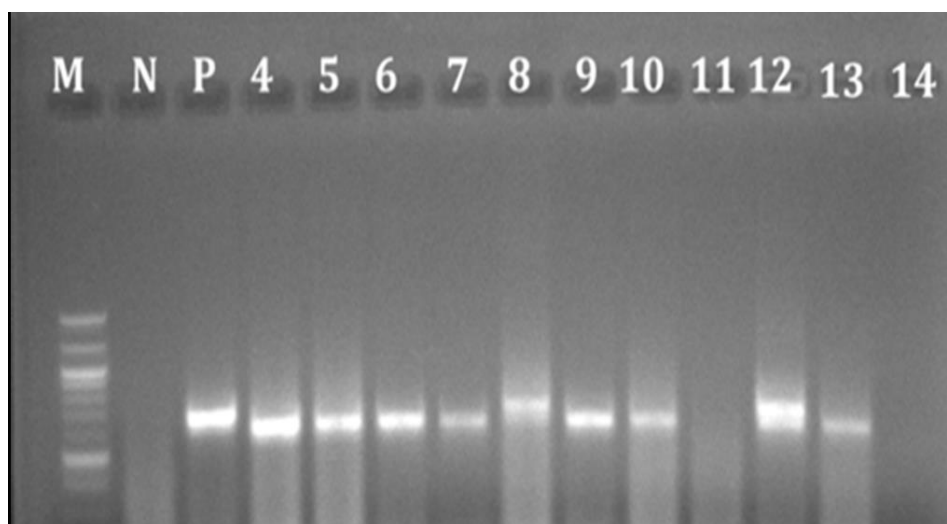


Figure 2: Image of Polymerase Chain Reaction products of stool samples collected from children attending to Ordu Province Kokenli Village primary school, on an agarose gel. M: 100-bp DNA marker; N: distilled water (negative), P: *Cryptosporidium* IOWA DNA (positive), 4-14 stool samples studied.

DISCUSSION

According to the literature that we could access, the first study about Cryptosporidiosis infection in Turkey was conducted by Ozcan et al. in Adana province and *Cryptosporidium* spp. oocysts were detected in 8.2% of children with diarrhea, and 4.10% of children without diarrhea. In the later years, studies for this infection have been speeded up, because of the increase in the number of immunocompromised cases and the introduction of new laboratory diagnostic methods.¹⁴ In a study by Karaman et al. conducted in Malatya, 2281 stool samples were examined with MAF in order to determine the epidemiology of *Cryptosporidium* spp., and 161 (7.1%) was found positive.¹⁵ Again, in a study performed in Van to determine the presence of Cryptosporidiosis, stool samples of 2000 children with 870 being girls and 1130 boys aged between 0 and 15 years who had complaints of diarrhea were studied. In addition, stool samples of randomly selected 100 children were used as the controls. Sample stool collected from the children were studied with MAF and Enzyme-Linked Immunosorbent Assay (ELISA). In conclusion, the presence of *Cryptosporidium* spp. oocysts were determined in 97 (4.9%) of 200 children, while staining method found positivity only in 39 (1.95%) children. Both methods could not find parasites in the control group.¹⁶ In a study by Elgun and Koltas in Adana province between October 2008 and July 2009, *Cryptosporidium* spp. oocysts were found in 8 (5.19%) of 154 stool samples with MAF staining method. The authors detected *Cryptosporidium* spp. the antigen in 37 (24%) of the samples with

ELISA (Microwell ELISA) method.¹⁷ Similarly, Şahin et al. examined stool samples of 191 villagers living in a village of Buca County who had diarrhea in İzmir Province with Kinyoun acid-fast stain method, and detected *Cryptosporidium* spp. in 15 (7.9%) and *Cyclospora cayetanensis* in 9 (4.71%) of the patients. The authors stated that the possible suspected origin of this outbreak was the contaminated mains water reservoir which provided water to the village.¹⁴ In the present study, *Cryptosporidium* spp. was detected in 89 (54.26%) of 164 stool samples with microscopic examination. Differences among the results of various studies might result from study populations, a region of the study, and the methods used.

Looking at the studies conducted using PCR method; Sungur et al. examined stool samples of 18 children with diarrhea and 27 calves with the diarrheal disease with Nested PCR. The authors identified *Cryptosporidium* spp. in a total of 9 (20%) stool samples with 8 (29.70%) being from stool samples of the calves and one (5.60%) from a child.¹⁸ Again, Sakarya et al. studied a total of 130 stool samples collected from 98 patients who presented to various hospitals in Ankara Province and 32 calves in various cattle breeding farms.¹⁹ The authors found positive amplification products in 13 (10%) of the samples with 12 (37.5%) being from the calves and one (1.02%) from the human stools, using Nested PCR method. In a study from Iran, stool samples collected from children < 12 yo with diarrhea were studied acid-fast staining method in order to determine the presence of *Cryptosporidium* infection in children, and

positive samples were subjected to DNA isolation. Next, Thrombospondin-Related Adhesive Protein of *Cryptosporidium*-2 (TRAP-C2) gene was amplified with Nested PCR using *Cryptosporidium* spp. specific primers. In conclusion, 12 samples were found to be positive for *Cryptosporidium* spp. The products obtained were subjected to Restriction Fragment Length Polymorphism (RFLP) technique using (266-366 bp) *Bst*EII and *Hae*III enzymes. *C. Parvum* infection was detected in 10 (83.3%), *C. hominis* infection in 1 (8.3%) and mixed infection in 1 (8.3%) of 12 samples that were found positive with Nested PCR amplification.²⁰ Similarly, Ulcay et al. tried to determine which laboratory method should be used to determine intestinal protozoa that may cause gastroenteritis in immunodeficient patients with complaints of diarrhea.²¹ The authors studied 36 immunodeficient patients who had diarrhea for more than 10 days and 44 immunodeficient patients who had no diarrhea: Nativ-Lugol, trichrome, MAF, ELISA, DFA, and PCR techniques for investigation of the stool samples. The authors reported the most common agents responsible for chronic diarrhea in the immunodeficient patients as intestinal protozoa such as *Giardia intestinalis* and *C. Parvum*. In addition, DNA was isolated from 162 diarrheal stool samples collected from the patients who presented to the Medical Faculty in Izmir, in order to determine and classify *Cryptosporidium* spp. Fifteen of 18 samples found positive with Kinyoun acid-fast stain method was found positive also with PCR technique, whereas 6 of 144 samples that could not found positive with staining method were positive with PCR. PCR products of *Cryptosporidium* spp. oocyst wall protein (COWP) gene region was then cut with *Rsa*I enzyme, and *Cryptosporidium meleagridis* was found in 1 and *C. parvum* in 20 samples.²² In our study also 89 stool samples found positive with microscopic examination and fixed in 10% formalin and studied with the PCR method, and 74 (45.12%) samples were found positive.

Another study was performed in order to investigate the efficacy of PCR method in fresh stool samples and samples stored in formaldehyde in the diagnosis of Cryptosporidiosis. In that study, a total of 33 stool samples collected from 22 patients with 23 of the samples being fresh and 10 being fixed in 10% formaldehyde in the Ege University Medical Faculty Hospital, Department of Parasitology with Nested PCR method. At the end of the study, sensitivity and specificity of PCR were found as 100% in the fresh stool samples, while sensitivity was found as 50% in

the formaldehyde-fixed samples.²³ In our study also 89 stool samples found positive with microscopic examination and fixed in 10% formaline and studied with PCR method, and 74 (45.12%) samples were found positive. We found the sensitivity of PCR as 83%. This may be interpreted as that formol-fixed method may increase negativity in PCR outcomes of the parasite.

CONCLUSION

Cryptosporidiosis, which is known to cause severe infections, especially in immunocompromised individuals and children, is transmitted by fecal-oral route from infected humans and animals, and contaminated water and nutrients. Ranching is widespread in the area of this study. Previous studies have reported the incidence of *Cryptosporidium* spp. as 65.5% in water found in Ordu and Sinop provinces. In our study, the incidence was found as 54.26% with MAF method, and 45.12% with PCR technique. This results suggest that this parasite might be transmitted to the patients with contaminated water and animals. We could not find any previous study investigating epidemiology in this parasite in pediatric patients. This study is the first conducted in this area and provided clues about routes of the parasite to infect humans. Accordingly, this study will guide programs about protection against and control of the parasite. In addition, we found that primary school children in the study region might be at risk for Cryptosporidiosis. In the study, children were informed about the transition and protection ways of the parasite, and they were checked up and treated.

REFERENCES

1. Hashwey R, Smith NH, Cron S, Graviss EA, Chappell, CL, White AC. Cryptosporidiosis in Houston. Texas a report of 95 cases *Medicine* 1997; 76(2): 118-139.
2. Özçelik S, Poyraz Ö, Kalkan K, Malatyali E, Değerli S. Hayvancılıkla uğraşanlarda ve sığırlarda *Cryptosporidium* spp. Yaygınlığının ELISA ile araştırılması. *Kafkas Univ Vet Fak Derg* 2012; 18:A61-A64, DOI:10.9775/kvfd.2011.5981.
3. Clark DP. New insights into human cryptosporidiosis. *Clin Microbiol Rev* 1999; 12:554-563.
4. Griffiths JK. Human cryptosporidiosis: epidemiology, transmission, clinical disease,

- treatment, and diagnosis. *AdvParasitol* 1998; 40:37–85.
5. Miron D, Kenes J, Dagan R. Calves as a source of an outbreak of cryptosporidiosis among young children in an agricultural closed community. *Pediatr Infect Dis J* 1991; 10:438–441.
 6. Çeliksöz A, Çelik S. Cumhuriyet Üniversitesi Hastanesi'nde gastroenteritli ve malnütrisyonlu hastalarda *Cryptosporidium* spp. Araştırması. *Türkiye Parazitoloj Derg* 2003; 27: 85-88.
 7. Egyed Z, Sreter T, Szell Z, Varga L. Characterization of *Cryptosporidium* spp.-recent developments and future needs. *Vet. Parasitol* 2003; 111:103-114.
 8. Usluca S. İshalli Dışkılarda *Microsporidium* spp. ve *Cryptosporidium* spp.'nin Saptanması PCR Yöntemi İle Tür Tayininin Yapılması. Doktora tezi. 2009.
 9. Börekçi G, Otağ F, Emekdaş G. Mersin'de bir gecekondu mahallesinde yaşayan ailelerde *Cryptosporidium* prevalansı. *İnfek Derg* 2005; 19(1):39-46.
 10. Tanyüksel M, Haznedaroğlu T, Gün H. Neoplastik hastalarda *Cryptosporidium* spp. Araştırılması. *Türkiye Parazitoloj Derg* 1995; 19 (1): 56-63.
 11. Hazer Y. Afyonkarahisar bölgesindeki risk gruplarında *Cryptosporidium parvum* araştırılması. Afyonkarahisar: Afyonkarahisar Kocatepe Üniversitesi Sağlık Bilimleri Enstitüsü. 2007.
 12. Karanis P, Kimura A. Evaluation of three flocculation methods for the purification of *Cryptosporidium parvum* oocysts from water samples. *Lett Appl Microbiol* 2002; 34:444–449.
 13. Plutzer J. *Cryptosporidium* and *Giardia* as water contaminant pathogens in Hungary: 2008; 22-25.
 14. Dağ A. Şanlıurfa yöresinde immunsuprese hastalarda *Cryptosporidium* spp. sıklığının kinin youn asit-fast boyama ve elisa yöntemleri ile araştırılması. Uzmanlık Tezi, Harran Üniversitesi, Tıp Fakültesi, Tıbbi Mikrobiyoloji Anabilim Dalı, Şanlıurfa. 2010.
 15. Karaman U, Dakdal N, Özer A, Enginyurt Ö, Ertürk Ö. Incidence of *Cryptosporidium* spp. in the human population of malatya in Turkey, *Acta Med Mediterr* 2015, 31: 263
 16. Yılmaz, H., Taş, C.Z., Çiçek, M. Investigation of cryptosporidiosis by enzyme-linked immunosorbent assay and microscopy in children with diarrhea. *Saudi Med J* 2008; 29(4): 526-529.
 17. Elgün, G., Koltaş, I.S. Investigation of *Cryptosporidium* spp. antigen by ELISA method in stool specimens obtained from patients with diarrhea. *Parasitol Res* 2011; 108(2): 395-7.
 18. Sungur T, Kar S, Güven E, Aktaş M, Karaer Z, Vatansever Z. *Cryptosporidium* spp.'nin dışkıdan Nested PCR ve carbolfuchsin boyama yöntemi ile teşhis edilmesi. *Türkiye Parazitoloj Derg* 2008; 32(4): 305-308.
 19. Sakarya Y, Kar S, Tanyüksel M. ve ark. Detection of *Cryptosporidium* spp. in humans and calves through Nested PCR and carbolfuchsin staining methods in Ankara, Turkey. *Kafkas Univ Vet Fak Derg* 2010; 977-980.
 20. Mojarad, N.E., Keshavarz, A., Taghipour, N., Haghghi, A., Kazemi, B., Athari, A. Genotyping of *Cryptosporidium* spp. in clinical samples: PCR-RFLP analysis of the TRAP-C2 gene. *Gastroenterol Hepatol Bed Bench* 2011; 4(1): 29-33.
 21. Ülçay A., Görenek L., Coşkun Ö., Araz E., Acar A., Eyigün, C.P. İmmünyetmezlikli hastalarda intestinal protozoonların tanısı. *Türkiye Parazitoloj Derg* 2008; 32(4): 328–333.
 22. Usluca, A., Aksoy, Ü. Detection and genotyping of *Cryptosporidium* spp. in diarrheic stools by PCR/RFLP analyses. *Turk J Med Sci* 2011; 41(6): 1029-1036.
 23. Dirim, E.D., Dağcı, H., Turgay, N., Akarca, U.S., Alkan, M.Z. The molecular diagnosis of cryptosporidiosis in fresh and formalin preserved fecal samples. *Türkiye Parazitoloj Derg* 2009; 33(2): 120-4.