Acta Scientiae Veterinariae, 2023. 51: 1916.

RESEARCH ARTICLE
Pub. 1916

ISSN 1679-9216

Cryptosporidium spp. in Dogs - Prevalence and Genotype Distribution

Özgür Yaşar Çelik[1], Akın Koçhan[2], Burçak Aslan Çelik[3], Adnan Ayan[4], Gürkan Akyildiz[5], Özlem Orunç Kilinç[6], Kerem Ercan[1], Vedat Baldaz[1] & Özge Oktay Ayan[7]

ABSTRACT

***Background*:** *Cryptosporidium* spp. is a zoonotic protozoan parasite that affects the gastrointestinal tract of humans and animals. The disease can cause acute and chronic diarrhoea and even death in both humans and animals. In this study, it was aimed to determine the prevalence and genotype distribution of Cryptosporidiosis in shelter dogs in Diyarbakır province located in the Southeastern Anatolia Region of Turkey.

***Materials, Methods & Results*:** The animal material of the study consisted of 100 dogs of different breeds and sexes. Faecal samples were collected from the rectum with disposable latex gloves and placed in individual sample containers. All of the samples were examined for *Cryptosporidium* spp. by Kinyoun Acid Fast and Nested PCR methods. In the Kinyoun Acid Fast staining method, firstly, smear preparations were prepared from fresh faecal samples, fixed in pure methanol for 1 min and allowed to dry. The slides were kept in Kinyoun Carbol-Fuxin for 5 min, dipped in 50% ethyl alcohol, shaken, washed in tap water, kept in 1% sulphuric acid for 2 min and washed in tap water. The slides were kept in methylene blue for 1 min, washed in tap water and allowed to dry. After drying, immersion oil was dripped and examined under a microscope at 100 magnification. DNA extraction was performed from all samples using GeneMATRIX Stool DNA Purification Kit according to the manufacturer's protocol. After Nested PCR analysis was performed. In the PCR step, primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'- CCCATTTCCTTCCTTCGAAACAGGA-3' were used to amplify the 1325 bp gene region. In the nested PCR step, primers 5'- GGAAGGGTTGTATTTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' were used to amplify the 826-864 bp gene region. As a result of both methods, a prevalence of 3% was determined. The infection rate was higher in males (3.57%) than females (2.27%) and in younger than 1 year (5.56%) than in older than 1 year (1.56%). The DNA sequences obtained from the sequence analysis of 3 positive PCR samples were analysed in BioEdit software. A phylogenetic tree was constructed with the data set created by using the 18s rRNA gene sequences obtained from the NCBI genbank database and the DNA sequences obtained as a result of the study, and it was shown which *Cryptosporidium* species the study samples were related to. Today, many *Cryptosporidium* species have been identified and most of these species have host adaptation. Although *C. canis* is the most common species in dogs, *C. muris*, *C. meleagridis*, and *C. parvum* have also been detected. Among these species, *C. parvum* is recognized as a zoonotic species infecting a wide range of mammals. In this study, DNA sequencing of nested PCR positive samples revealed that 3 samples were zoonotic *C. parvum*.

***Discussion*:** This suggests that dogs may be a reservoir for zoonotic transmission of *Cryptosporidium*. Consequently, it is recommended that people should be informed about the potential for transmission of this protozoan to humans and animals and that control programmes should be implemented, including the prevention of free entry of stray dogs into public places and homes.

Keywords: *Cryptosporidium parvum*, molecular analysis, canine, Diyarbakır, Turkey.

DOI: 10.22456/1679-9216.132334

Received: 28 April 2023 Accepted: 5 July 2023 Published: 21 July 2023

[1]Department of Internal Medicine & [3]Department of Parasitology, Faculty of Veterinary Medicine, Siirt University, Siirt, Turkey. [2]Department of Internal Medicine, Faculty of Veterinary Medicine, Dicle University, Diyarbakır, Turkey. [4]Department of Genetics, Faculty of Veterinary Medicine; [6]Özalp Vocational School & [7]Department of Parasitology, Van Yüzüncü Yıl University, Faculty of Medicine, Van, Turkey. [5]Department of Basic Health Sciences, Faculty of Health Sciences, Marmara University, İstanbul, Turkey. CORRESPONDENCE: Ö.Y. Çelik [oyc@siirt.edu.tr]. Department of Internal Medicine, Faculty of Veterinary Medicine, Siirt University. 56000 Siirt, Turkey.

INTRODUCTION

Cryptosporidium is a protozoan belonging to the phylum Apicomplexa, which is widespread worldwide, infects domestic and wild animals as well as humans, and is associated with foetal diarrhoea in animals and humans [1,2,6,12,14,17,20].

Currently, at least 26 *Cryptosporidium* species have been described. Most of these species are host adapted and 20 have been reported in humans [2,6,12,18]. Among these species, *Cryptosporidium parvum* is recognised as a zoonotic species that infects a wide range of mammals. [6,16]. Although *C. canis* is the most common species in dogs, *C. muris*, *C. meleagridis* and zoonotic *C. parvum* have also been detected [12,16,18]. This raises concerns that dogs may be potential reservoirs for the transmission of infection to humans [18,19].

Contact with animals has been identified as an important route of transmission in the epidemiology of human Cryptosporidiosis [13]. Transmission can be by direct faecal-oral route or by ingestion of water or food contaminated with oocysts [1,7,10,14,20].

Cryptosporidium spp. infect the epithelial cells of the gastrointestinal tract of the hosts [19]. Infection is self-limiting in the immunocompetent host but can result in chronic diarrhoea, malabsorption and death in the immunocompromised host [6]. In addition, this disease is recognised as a public health problem for children and immunocompromised individuals [14,16,17].

Because of the possible role of dogs in the spread of the parasite, it is necessary to know the prevalence of infection. Epidemiological studies on *Cryptosporidium* spp. have been carried out in many parts of the world, but the studies carried out in Turkey are quite limited. In this study, it was aimed to determine the prevalence of *Cryptosporidium* spp. in shelter dogs in Diyarbakır province by microscopic and molecular methods.

MATERIAL AND METHODS

Study area and animal material

This study was conducted in the province of Diyarbakır, located in the Southeastern Anatolia Region of Turkey. The animal material of the study consisted of 100 dogs of different breeds and sexes in Diyarbakır Metropolitan Municipality Animal Care and Rehabilitation Centre. Faecal samples were collected from the rectum of the dogs with disposable latex gloves and placed in individual sample containers. The sex and age of the dog were recorded for each sample. The samples were then brought to the laboratory for examination.

Microscopic examination

All of the samples were examined for *Cryptosporidium* spp. by Kinyoun Acid Fast staining method. Firstly, smear preparations were prepared from fresh faecal samples, fixed in pure methanol for 1 min and allowed to dry. The slides were kept in Kinyoun Carbol-Fuxin for 5 min, dipped in 50% ethyl alcohol, shaken, washed in tap water, kept in 1% sulphuric acid for 2 min and washed in tap water. The slides were kept in methylene blue for 1 min, washed in tap water and allowed to dry. After drying, immersion oil was dripped and examined under a microscope (Leica DM500)[1] at 100 magnification.

DNA extraction

DNA extraction was performed from all samples using GeneMATRIX Stool DNA Purification Kit[2] according to the manufacturer's protocol. The obtained DNAs were stored at -20°C until the next steps.

Nested PCR reaction

Nested PCR analysis was performed using primers described by Xiao *et al*. [21]. In the PCR step, primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'- CCCATTTCCTTCCTTCGAAACAGGA-3' were used to amplify the 1325 bp gene region. In the nested PCR step, primers 5'- GGAAGGGTTG-TATTTATTTATTAGATAAAG-3' and 5'-AAGGAG-TAAGGAACAACCTCCA-3' were used to amplify the 826-864 bp gene region. In both reactions; 4 pmol forward and reverse primers, 4 µL 5x FIREPol® Master Mix[3] (12.5 mM MgCl2), 1.6 µL DNA and Nuclease Free Water were used in 20 µL mastermix. In both reactions, following pre-denaturation at 95°C for 5 min, each cycle consisted of 35 cycles of denaturation (95°C for 1 min), binding (55°C for 1 min) and elongation (72°C for 1 min) with a final elongation of 7 min at 72°C. The PCR products obtained were stained with RedSafe™ Nucleic Acid Staining Solution[4] and images were obtained on 1.5% agarose gel.

DNA sequence analysis and phylogeny

The DNA sequences obtained from the sequence analysis of 3 positive PCR samples were analysed in BioEdit software. A phylogenetic tree was constructed with the data set created by using the 18s rRNA gene sequences obtained from the NCBI genbank database and the DNA sequences obtained as a result of the study, and it was shown which *Cryptosporidium* species the study samples were related to.

Statistical analysis

The data obtained in the study were analyzed using the SPSS V16.0[5] program. The relationship between grouped variables was calculated using chi-square test. The difference was considered statistically significant when $P < 0.05$.

RESULTS

Both microscopic examination and Nested-PCR analysis of all samples revealed 3% (3/100) positivity (Figure 1). As a result of the study, a higher prevalence was found in males (3.57%) compared to females (2.27%) and in age groups, a higher prevalence was found in those younger than 1 year (5.56%) compared to those older than 1 year (1.56%) [$P > 0.05$] (Table 1). When the DNA sequences of the SSU rRNA gene obtained in the study were compared with the NCBI Basic Local Alignment Search Tool database, it was observed that 3 samples overlapped with *C. parvum* (Table 2). As can be seen in the phylogenetic tree, all samples were related to *C. parvum* (Figure 2).

Table 1. Infection rates of *Cryptosporidium* spp. in dogs by sex and age.

Factor	Examined (n)	Positive		*P*
		(n)	(%)	
Sex				
Female	44	1	2.27	0.706
Male	56	2	3.57	
Age (Year)				
≤ 1	36	2	5.56	0.261
>1	64	1	1.56	
Total	100	3	3.00	

Table 2. Comparison of study samples with NCBI Basic Local Alignment Search Tool.

Sample	Access codes of the most similar sample	Species	Similarity ratio
32	OL689400, EU553550, DQ656354	*C. parvum*	% 100
33	MT648442, MT648441, MT002720	*C. parvum*	% 100
44	OP861564	*C. parvum*	% 99.76

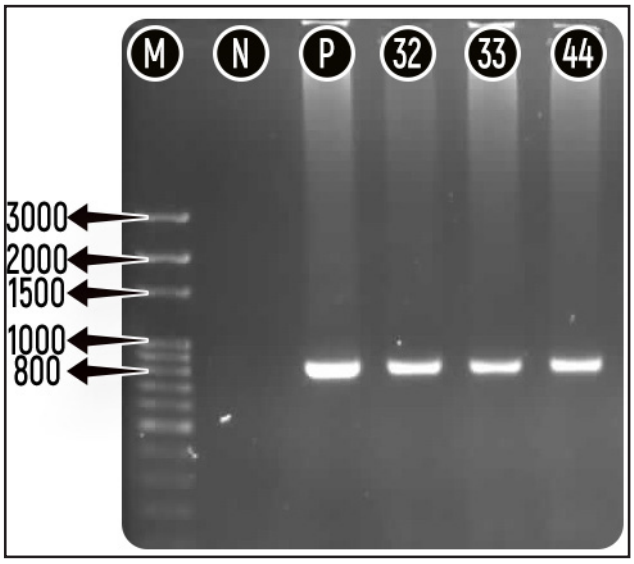

Figure 1. Nested PCR agarose gel image. M: Marker, N: Negative control, P: Positive control, 32, 33 and 44 positive samples (826-864 bp).

DISCUSSION

Parasitic diseases caused by enteric protozoans are widespread in humans and animals worldwide [2]. Dogs, which are considered friends and close companions of humans [12], are hosts of *Cryptosporidium* species and have therefore long been thought to be reservoirs of human *Cryptosporidium* infections [13]. It is therefore important to appropriately identify infectious agents with zoonotic potential to reduce the risks of transmission to humans and other animals [2].

In studies conducted worldwide; 9.3% in Japan [1], 3.3% in Italy [7], 52.7% in Romania [20], 3.8% in China [12], 18.5% in Nigeria [15], 2.14 - 13% in Iran [14,16,19], 4.1% in Spain [8], 31.2% in Thailand [18], 2.41 - 5.6% in Brazil [2,11] and 34% in Egypt [6] prevalence have been reported. Studies on dogs in Turkey are quite limited and prevalence rates of 15.5 - 64.7% have been reported [3,5,9].

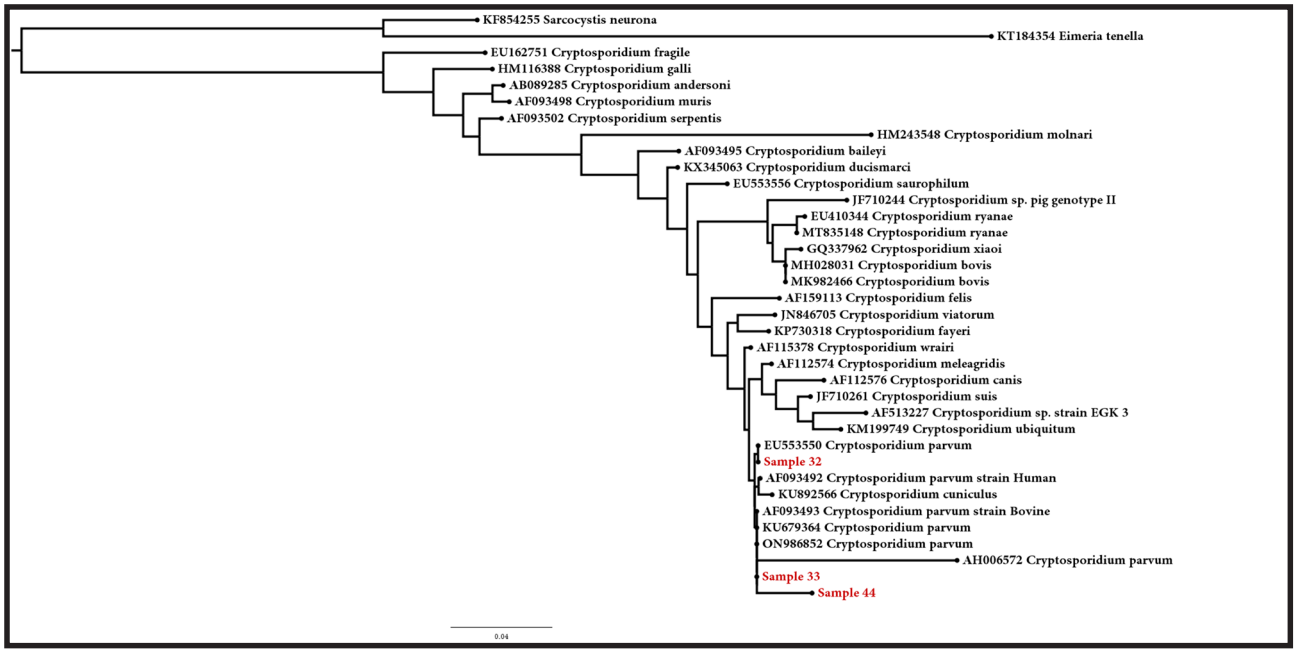

Figure 2. Phylogenetic relationships of *Cryptosporidium* spp. isolates using Maximum Likelihood Method analysis based on SSU rRNA gene region. Numbers in nodes represent Bootstrap values (1000 replicates). *Eimeria tenella* and *Sarcocystis neurona* were used as outgroups.

Methods such as Ziehl-Neelsen staining [2,14,19], Kinyoun staining [16], ELISA [15,20] and PCR [1,7,8,18] are used to diagnose the disease. Each of these methods differs in terms of sensitivity and specificity and there is no universally accepted 'gold standard' [14].

In this study, Kinyoun Acid Fast staining and Nested PCR methods were used and 3% prevalence was determined as a result of both methods. This result is similar to some previous studies [7,11,12,14,16]. The reason for the low prevalence in this study may be due to the fact that only one faecal examination was performed from each dog.

Although *C. canis* is the most common species in dogs, *C. muris*, *C. meleagridis*, *C. hominis* and zoonotic *C. parvum* are also known [8,12,16,18]. The studies carried out on dogs in Turkey have been carried out to determine the prevalence, and no study was found in which species identification was made. In this study, 3 samples were identified as zoonotic *C. parvum* as a result of sequence analyses. This result is similar to the findings of researchers [1,2,6,7,16,18].

While some studies reported higher prevalence in females [12,14,15,18,19], other studies reported higher prevalence in males [4,6,8,20]. In this study, a slightly higher prevalence was found in males (3.57%) than females (2.27%) [$P > 0.05$]. This result is similar to previous studies [6,8,20]. This indicates that sex is not an important factor affecting the prevalence of *Cryptosporidium* in dogs [6].

Some previous studies reported [8,14,18,20] a higher prevalence in younger, while another study reported a higher prevalence in adult dogs [19]. In this study, a higher prevalence was found in dogs younger than 1 year of age (5.56%) compared to dogs older than 1 year (1.56%) [$P > 0.05$], this result supports others studies [6,8]. The reason for the higher prevalence in juvenile animals may be due to the immune system of these animals being undeveloped.

CONCLUSION

As a result of this study, both the prevalence of *Cryptosporidium* and the presence of *C.* parvum, which is an important species for public health, were determined in shelter dogs in Diyarbakır province. This situation shows that dogs may be a reservoir for zoonotic transmission of *Cryptosporidium*. Therefore, people need to be educated about Cryptosporidiosis and the potential for transmission of the causative protozoan to humans and animals. It is also recommended that stray dogs should be prevented from freely entering public places and homes, that control programmes should be carried out including the collection and hygienic disposal of dog faeces, as well as repeated faecal examinations.

MANUFACTURERS

[1]Leica Microsystems (Schweiz) AG. Heerbrugg, Switzerland.

[2]Urx. Gdańsk, Poland.

[3]Solis BioDyne OÜ. Tartu, Estonia.

[4]Intron Biotechnology Inc. Gyeonggi-do, Korea.

[5]IBM. Chicago, IL, USA.

***Ethical approval*.** Ethical clearance for the present study was obtained from the Dicle University Health Sciences Application and Research Centre with a document number of 454665.

***Declaration of interest*.** The authors report no conflicts of interest and are alone responsible for the content and writing of the paper.

REFERENCES

1 **Abe N., Sawano Y., Yamada K., Kimata I. & Iseki M. 2002.** *Cryptosporidium* infection in dogs in Osaka, Japan. *Veterinary Parasitology*. 108(3): 185-193. DOI: 10.1016/s0304-4017(02)00204-2.

2 **Alves M.E.M., Martins F.D.C., Bräunig P., Pivoto F.L., Sangioni L.A. & Vogel F.S.F. 2018.** Molecular detection of *Cryptosporidium* spp. and the occurrence of intestinal parasites in fecal samples of naturally infected dogs and cats. *Parasitology Research*. 117: 3033-3038. DOI: 10.1007/s00436-018-5986-4.

3 **Ayan A. & Orunç Kılınç Ö. 2020.** Prevalence of *Cryptosporidium* spp. in Diarrheic Dogs in Van Province. *Turkiye Klinikleri Journal of Veterinary Sciences*. 11(2): 64-68. DOI: 10.5336/vetsci.2020-77151.

4 **Bahrami A., Doosti A., Nahravanian H., Noorian A. & Asbchin S. 2011.** Epidemiological survey of gastro-intestinal parasites in stray dogs and cats. *Australian Journal of Basic and Applied Sciences*. 5(9): 1944-1948.

5 **Denizhan V. & Karakuş A. 2019.** Prevalence of Gastrointestinal Protozoon in Stray Dogs in the Van Province. *Dicle Üniversitesi Veteriner Fakültesi Dergisi*. 12(1): 25-29.

6 **Gharieb R.M., Merwad A.M., Saleh A.A. & Abd El-Ghany A.M. 2018.** Molecular screening and genotyping of *Cryptosporidium* species in household dogs and in-contact children in Egypt: risk factor analysis and zoonotic importance. *Vector-Borne and Zoonotic Diseases*. 18(8): 424-432. DOI: 10.1089/vbz.2017.2254.

7 **Giangaspero A., Iorio R., Paoletti B., Traversa D. & Capelli G. 2006.** Molecular evidence for *Cryptosporidium* infection in dogs in Central Italy. *Parasitology Research*. 99: 297-299. DOI: 10.1007/s00436-006-0169-0.

8 **Gil H., Cano L., de Lucio A., Bailo B., Mingo M.H., Cardona G.A., Fernández-Basterra J.A., Aramburu-Aguirre J., López-Molina N. & Carmena D. 2017.** Detection and molecular diversity of *Giardia duodenalis* and *Cryptosporidium* spp. in sheltered dogs and cats in Northern Spain. *Infection, Genetics and Evolution*. 50: 62-69. DOI: 10.1016/j.meegid.2017.02.013.

9 **Görkem Ö. & Ulutaş B. 2022.** Prevalence of *Cryptosporidium* spp. in Dogs in The Aegean Region. *Animal Health, Production and Hygiene*. 11(1): 26-31. DOI: 10.53913/aduveterinary.1105182.

10 **Güven E., Avcioğlu H., Balkaya I., Hayirli A., Kar S. & Karaer Z. 2013.** Prevalence of Cryptosporidiosis and molecular characterization of *Cryptosporidium* spp. in calves in Erzurum. *Kafkas Üniveritesi Veteriner Fakültesi Dergisi*. 19(6): 969-974. DOI: 10.9775/kvfd.2013.9187.

11 **Huber F., Bomfim T. & Gomes R. 2005.** Comparison between natural infection by *Cryptosporidium* sp., *Giardia* sp. in dogs in two living situations in the West Zone of the municipality of Rio de Janeiro. *Veterinary Parasitology*. 130(1-2): 69-72. DOI: 10.1016/j.vetpar.2005.03.012.

12 **Jian F., Qi M., He X., Wang R., Zhang S., Dong H. & Zhang L. 2014.** Occurrence and molecular characterization of *Cryptosporidium* in dogs in Henan Province, China. *BMC Veterinary Research*. 10: 1-4. DOI: 10.1186/1746-6148-10-26.

13 **Li J., Ryan U., Guo Y., Feng Y. & Xiao L. 2021.** Advances in molecular epidemiology of cryptosporidiosis in dogs and cats. *International Journal for Parasitology*. 51(10): 787-795. DOI: 10.1016/j.ijpara.2021.03.002.

14 **Mirzaei M. 2012.** Epidemiological survey of *Cryptosporidium* spp. in companion and stray dogs in Kerman, Iran. *Veterinaria Italiana*. 48(3): 291-296.

15 **Olabanji G.M., Maikai B.V. & Otolorin G.R. 2016.** Prevalence and risk factors associated with faecal shedding of *Cryptosporidium* oocysts in dogs in the Federal Capital Territory, Abuja, Nigeria. *Veterinary Medicine International*. 2016: 4591238.DOI: 10.1155/2016/4591238.

16 **Ranjbar R., Mirhendi H., Izadi M., Behrouz B. & Mohammadi Manesh R. 2018.** Molecular identification of *Cryptosporidium* spp. in Iranian dogs using seminested PCR: a first report. *Vector-Borne and Zoonotic Disease*s. 18(2): 96-100. DOI: 10.1089/vbz.2017.2136.

17 Sakarya Y., Kar S., Tanyüksel M., Karaer Z., Babur C. & Vatansever Z. 2010. Detection of *Cryptosporidium* spp. in humans and calves through nested PCR and carbol fuchsin staining methods in Ankara, Turkey. *Kafkas Üniveritesi Veteriner Fakültesi Dergisi*. 16(6): 977-980. DOI: 10.9775/kvfd.2010.2140.

18 Tangtrongsup S., Scorza A.V., Reif J.S., Ballweber L.R., Lappin M.R. & Salman M.D. 2017. Prevalence and multilocus genotyping analysis of *Cryptosporidium* and *Giardia* isolates from dogs in Chiang Mai, Thailand. *Veterinary Sciences*. 4(2): 26. DOI: 10.3390/vetsci4020026.

19 Tavalla M., Kord E., Abdizadeh R. & Asgarian F. 2017. Molecular study of *Cryptosporidium* spp. in dogs from southwest of Iran. *Jundishapur Journal of Microbiology*. 10(4): e43412. DOI: 10.5812/jjm.43412.

20 Titilincu A., Mircean V., Achelaritei D. & Cozma V. 2010. Prevalence of *Cryptosporidium* spp. in asymptomatic dogs by ELISA and risk factors associated with infection. *Lucrari Stiinłifice Medicina Veterinara*. 43(1): 7-12.

21 Xiao L., Singh A., Limor J., Graczyk T.K., Gradus S. & Lal A. 2001. Molecular characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. *Applied and Environmental Microbiology*. 67(3): 1097-1101. DOI: 10.1128/AEM.67.3.1097-1101.2001.