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Prevalence of *Cryptosporidium* spp. in Diarrheic Dogs in Van Province

Van Bölgesindeki İshalli Köpeklerde Bulunan *Cryptosporidium* spp.'nin Yaygınlığı

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ABSTRACT Objective: Cryptosporidiosis is a zoonotic apicomplexan protozoon that causes significant gastrointestinal problems in humans and animals alike. In the present study, we investigated the prevalence of *Cryptosporidium* spp. in diarrheic dogs in Van province by using two different methods. **Material and Methods:** A total of 153 fecal samples were collected from diarrheic stray dogs aging up to 4 months between 2018-2019 at Animal Care and Rehabilitation Center of Van Metropolitan Municipality in Van province. Samples were stained with with Kinyoun's acid-fast method and examined at x100 magnification under the microscope. DNA were extracted from all the 153 samples using RTA stool DNA isolation kit and Nested PCR was carried out using suitable primers. **Results:** Microscopic examination revealed the presence of *Cryptosporidium* spp. oocysts in 81 out of 153 (52.94%) samples whereas *Cryptosporidium* spp. specific 826-864 bp size bands were obtained in 99 (64.7%) of 153 samples using Nested PCR. **Conclusion:** The prevalence of *Cryptosporidiosis* in dogs up to 4 months of age is quite high that suggests further research on the species of *Cryptosporidium* in dogs.

Keywords: *Cryptosporidium* spp., dog, nested PCR, Turkey, Van province

ÖZET Amaç: Cryptosporidiosis, insan ve hayvanlarda önemli gastrointestinal sorunlara sebep olabilen apikompleksan bir protozoon tarafından oluşturulan zoonoz bir hastalıktır. Bu çalışmada, Van bölgesindeki ishalli köpeklerde *Cryptosporidium* spp.'nin prevalansının iki farklı yöntem kullanılarak araştırılması amaçlanmıştır. **Gereç ve Yöntemler:** Çalışmanın materyalini 2018-2019 yılları arasında Van İlindeki Van Büyükşehir Belediyesi Hayvan Bakımevi ve Rehabilitasyon Merkezindeki 4 aylığa kadar olan 153 adet ishalli sokak köpek dışkısı oluşturmaktadır. Dışkı örnekleri Kinyoun'un acid-fast metodu ile boyanarak mikroskop altında x100'lük büyütmede incelendi. 153 örneğin tamamından RTA dışkı DNA izolasyon kiti kullanılarak DNA ekstraksiyonu yapıldı. Ardından ilgili primerlerle Nested PCR yapıldı. **Bulgular:** Mikroskobik incelemede 153 örneğin 81'inde %52,94 *Cryptosporidium* spp. ookistlerine rastlanıldı. Nested PCR sonucuna göre örneklerin 99'unda (%64,7) *Cryptosporidium* spp. için spesifik 826-864 bp büyüklüğünde bantlar elde edildi. **Sonuç:** Van ilindeki 4 aylığa kadar olan ishalli köpeklerdeki *Cryptosporidiosis* prevalansının oldukça yüksek olduğu ve köpeklerdeki *Cryptosporidium* türleri hakkında daha fazla araştırma yapılması gerektiği kanısına varılmıştır.

Anahtar Kelimeler: *Cryptosporidium* spp, köpek, nested PCR, Türkiye, Van ili

Cryptosporidiosis is a zoonotic disease caused by an apicomplexan protozoon affecting the gastrointestinal tract in humans and animals.[1-3] Transmission of *Cryptosporidium spp*. occurs through fecal-oral route between animals and humans and can cause water and foodborne outbreaks.[3] Previous studies reported the host specificity of *Cryptosporidium spp*. that has been contradicted by the recent molecular studies. *C. parvum* has been isolated from many pets including dogs despite the fact that it is dominantly seen in humans.[4,5] Similarly, *C. canis*, assumed to infect the dogs only, has been recently isolated from humans.[6] Dogs are given the status of potential reservoir host for

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Cryptosporidiosis due to contact between dogs and humans. The risk of cryptosporidiosis is higher especially in immunocompromised individuals. Several studies have reported the isolation of *C. canis* from humans.[7-9] *C. canis* is the dominant etiologic agent in dogs, however, *C. parvum*, *C. meleagridis*, and *C. muris* have also been reported in dogs.[1]

Cryptosporidium infection is generally asymptomatic in dogs; however, it can cause severe diarrhea, malabsorption, and weight loss especially in younger puppies.[10] Particularly, infection can be severe in 2-14 days old puppies and less than 6 months of age dogs compared to adults.[10,11.]

Microscopic, immunological and molecular methods are used in the diagnosis of cryptosporidiosis.[12,13] In conventional microscopic methods, staining techniques, being inexpensive and fast, are frequently used. Nonetheless, specificity and sensitivity can vary due to technique used and skills of the observer. Molecular techniques like polymerase chain reaction (PCR) accurately diagnose the *Cryptosporidium* spp., however, it is not preferred in routine screening of patients being labor-intense and expensive. Such techniques are usually used in epidemiological studies.[12]

In this study, we aimed to investigate the prevalence of Cryptosporidiosis in Van province using two different techniques, it will highlight the importance of this infection from public health perspective.

MATERIAL AND METHODS

COLLECTION OF SAMPLES

Fecal samples were manually collected from the rectum of 153 diarrheic stray dogs aging up to 4 months between 2018-2019 at Animal Care and Rehabilitation Center of Van Metropolitan Municipality in Van province. All dogs had diarrhea. Single-use latex gloves were used for this purpose. The samples were stored at +4ºC until further processing.

MICROSCOPIC EXAMINATION

All samples were stained with Kinyoun's acid-fast method and examined under the microscope at x100 magnification.

DNA EXTRACTION

DNA were extracted from all the samples using RTA stool DNA isolation kit (Cat 09028050, Turkey) according to manufacturer-recommended protocol. Extracted DNA were stored at -20ºC until further analysis.

NESTED PCR REACTION

Primers identified by Xiao et al.[14] were used in order to amplify the SSU rRNA gene region. The first step involved the use of 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3'primers to amplify the 1325 bp gene region in Nested PCR. In the second stage of Nested PCR, 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' primers were used to amplify the 826-864 bp gene region. Following reagents were used in both reactions: 25 µL mastermix, 200 µM dNTPs, 3 mM $MgCl_2$, 5 pmol forward and reverse primers, 1U Taq Polymerase, and 10X PCR buffer (500 mM Tris-HCl, pH 8.8, 160 mM $(NH_4)SO_4$ and 0.1% Tween®20), nuclease free water, and 2 µL DNA. The procedures involved in both stages of nested PCR were: 15 minutes pre-denaturation at 95ºC followed by 35 cycles of processes each consisting of denaturation (at 95ºC for 1 minute), binding (at 60ºC for 1 minute), and elongation (at 72ºC for 1 minute). Following these cycles, final elongation was performed at 72ºC for 7 minutes in each stage of the nested PCR.[15] These reactions were carried out using Eppendorf Mastercycler® pro brand automatic Thermal cycler. The PCR products were run on 1.5% agarose gel stained with Safe-T-Stain and images were obtained using gel imaging device (Syngene bio imaging system).

ETHICAL CONSIDERATIONS

All procedures involved in the study were approved by the local ethics committee of Van Yuzuncu Yil

University, Van, Turkey vide letter no. VAN YUHADYEK/2018/07 dated 26 July 2018.

RESULTS

PREVALENCE USING MICROSCOPIC EXAMINATION

Oocysts of *Cryptosporidium spp.* were observed in 81 out of 153 (52.94%) fecal samples at x100 magnification (Figure 1).

PREVALENCE USING NESTED PCR

A total of 99 samples of 153 (64.7%) yielded the *Cryptosporidium spp.* specific 826-864 bp sized bands (Figure 2). It confirms that Nested PCR is more reliable in the diagnosis of *Cryptosporidium* spp. than microscopic technique.

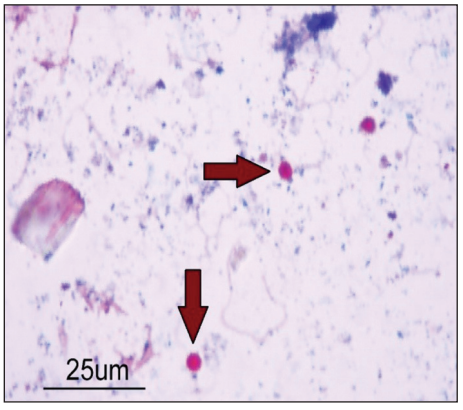

FIGURE 1: *Cryptosporidium* spp. oocyst in Kinyoun's acid-fast staining method.

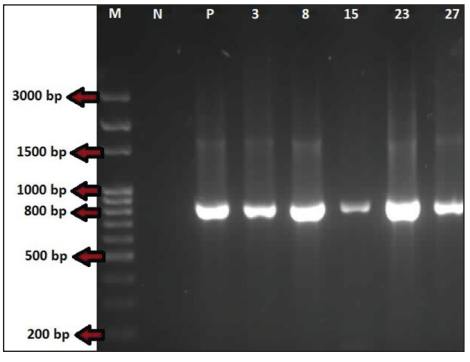

FIGURE 2: *Cryptosporidium* spp. Nested PCR agarose gel images (M: Marker. N: Negative control. P: Positive control. 3,8,15,23,27: Positive samples).

DISCUSSION

Cryptosporidiosis is a worldwide zoonotic disease caused by a parasitic protozoon *Cryptosporidium* spp. and *C. canis* is the most common type in dogs.[16,17] *Cryptosporidium spp.* are monoxenous protozoa that cause food- and water-borne infections in humans and animals. Routine diagnosis of *Cryptosporidium spp.* is made using acid-fast staining technique, however, PCR based techniques are specific and sensitive methods to detect the infection and *Cryptosporidium* types.[18-21] The most commonly used molecular test in the diagnosis of *Cryptosporidium spp.* is nested PCR that uses specific primers to amplify the 18S rRNA gene region. In this study, the prevalence of *Cryptosporidium spp.* was investigated in diarrheic dogs in Van, Turkey using two different methods as the parasite possesses the potential for zoonotic transmission through environmental contamination. The prevalence of *Cryptosporidium spp.* in Van, Turkey was determined s 52.94% by using Kinyoun's acid-fast method and 64.7% by nested-PCR.

In Turkey, there are increasing number of studies on Cryptosporidiosis in farm animals and its importance.[15,22-25] However, no molecular study is available regarding Cryptosporidiosis in dogs. Earlier reports are available reporting the use of PCR in investigating the *Cryptosporidium spp.* to amplify the 18S rRNA gene region. Various researchers have reported the presence of *Cryptosporidium spp.* in dogs; Ayinmode et al.[1] in 2.5% of 203 dogs in Ibadan region of Nigeria, Itoh et al. in 21% of 314 kennel dogs in Japan, Julien et al. in 6.12% dogs in Iqaluit region of Canada, Homayouni et al. 0.6% of 315 dogs in Shiraz region of Iran, Tangtrongsup et al. 7.6% of 301 dogs in Chiang Mai region of Thailand, and Li et al. found *Cryptosporidium spp.* in 6.9% of 641 dogs in Guangdong region of China.[6,26-29] Zaglool et al. and Quílez et al. in their studies on *Cryptosporidium* noted the low sensitivity of mZN test as 73.3% and 79.3%, respectively.[30,31] Miambo et al. reported the presence of *Cryptosporidium* spp. in 0.6% of 156 puppies in Mozambique using modified Ziehl-Neelsen technique whereas Eze et al. determined the oocysts of *Cryptosporidium* spp. in 74 out of 203 dogs (36.5%).[3,32] In contrast, we detected the oocysts in 52.94% of 153 dogs with Kinyoun's acid-fast method. Our findings are consistent with those of Ramirez et al. Hamnes et al. and Jian et al. who

reported that puppies are more prone to *Cryptosporidium* infections than adults.[11,33,34] Direct smear and molecular techniques showed the presence of *Cryptosporidium* in 8% and 12.3%, respectively.[35] Similarly, the prevalence of *Cryptosporidium spp.* in HIV patients was 7.6% and 18.3% using modified Ziehl-Neelsen staining and PCR, respectively.[12] Likewise, the prevalence of *Cryptosporidium spp.* in calves was 5% using modified acid-fast staining, 7% with IFAT, and 7.6% with PCR.[36] It explains that PCR is the most favorable test in epidemiological studies involving *Cryptosporidium*. In the present study, *Cryptosporidium* spp. was found to be prevalent in 99 of 153 samples (64.7) using PCR whereas 52.94% using Kinyoun's acid-fast method. A higher prevalence in our study might be attributable to younger age of puppies with the signs of diarrhea. The findings also reveal that *Cryptosporidium spp.* is an important diarrhea causing agent in puppies.

CONCLUSION

In conclusion, the results of our study reiterate that PCR is a very sensitive test in the diagnosis of *Cryptosporidium*. The results suggest the necessity of further research on infection causing *Cryptosporidium* species in dogs. The reported prevalence in the literature varies greatly depending on the geographical structure, hygienic condition, animal population, area of study, skills of the people conducting the study, and laboratory conditions.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

All authors contributed equally while this study preparing.

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