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RESEARCH ARTICLE

Detection of *Cryptosporidium* spp. in Humans and Calves Through Nested PCR and Carbol Fuchsin Staining Methods in Ankara, Turkey

Yasemin SAKARYA * Sirri KAR ** Mehmet TANYUKSEL ***
Zafer KARAER * Cahit BABUR **** Zati VATANSEVER *****

* Ankara University, Faculty of Veterinary Medicine, Department of Parasitology, 06110 Dışkapı, Ankara - TÜRKİYE
** Namik Kemal University, Department of Biology, 59030 Tekirdag - TÜRKİYE
*** Gulhane Military Medical Academy, Department of Medical Parasitology, 06018 Ankara - TÜRKİYE
**** The Ministry of Health Refik Saydam Hifzissihha Centre, Department of Parasitology, 06100 Ankara - TÜRKİYE
***** Kafkas University, Faculty of Veterinary Medicine, Department of Parasitology, 36300 Kars - TÜRKİYE

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Summary

In this study, a total of 130 fecal samples, obtained from 98 humans and 32 calves from different hospitals and dairy farms in Ankara, respectively were analyzed with carbol fuchsin staining and nested PCR to determine the presence of Cryptosporidium spp. Nested PCR revealed a total of 13 (10.0%) positive amplification results; 12 (37.5%) were from calves and 1 (1.02%) from humans. Oocysts were observed in 8 (6.15%) fecal samples belonging to 7 (21.88%) calves and 1 (1.02%) human by carbol fuchsin staining. Concerning the detection technics, it was concluded that nested PCR can be successfully used in diagnosis of cryptosporidiosis, and although carbol fuchsin staining method has sufficient specificity it is less sensitive, especially in cases characterized with excretion of fewer oocysts.

Keywords: *Cryptosporidium, Nested PCR, Carbol fuchsin, Ankara*

Ankara'da İnsan ve Buzağılarda *Cryptosporidium* spp. Varlığının Nested PCR ve Carbol Fuchsin Boyama Yöntemleri ile Belirlenmesi

Özet

Bu çalışmada, Ankara'nın değişik hastanelerine ishal şikayeti ile başvuran 98 hastadan ve yine Ankara'da bulunan değişik sığırcılık işletmelerindeki 32 buzağıdan alınan toplam 130 dışkı, carbol fuchsin boyama yöntemi ve nested PCR yardımı ile *Cryptosporidium* spp. yönünden taranmıştır. Sonuç olarak nested PCR yöntemiyle taranan örneklerden toplam 13 (%10.0)'ünde pozitif amplifikasyon ürününe rastlanmış, ilgili pozitiflerin 12 (%37.5)'si buzağılardan, 1 (%1.02)'i ise insan dışkılarından elde edilmiştir. Karbol fuksin boyama yöntemi ile yapılan taramalarda ise insan dışkılarının birinden (%1.02) ve buzağı dışkılarının 7 (%21.88)'sinde olmak üzere, toplam 8 (%6.15) dışkı örneğinde oocystlere rastlanmıştır. Kullanılan tanı teknikleri ile ilgili olarak, nested PCR'ın cryptosporidiosisin tanısında etkili bir şekilde kullanılabileceği, carbol fuchsin boyama yönteminin ise yeterli derecede özgül olmasına rağmen, özellikle az oocyst atılımı ile karakterize olgularda duyarlılığının zayıf kaldığı sonucuna varılmıştır.

Anahtar sözcükler: *Cryptosporidium, Nested PCR, Karbol fuksin, Ankara*

INTRODUCTION

Cryptosporidiosis is a widespread zoonotic protozoan disease throughout the world. With importance especially for immunosuppressive adults and children, it can be seen in many vertebrate species and can be fatal for especially calves, lambs and goat kids [1,2]. Although more than 21 different species have been reported, definitely confirmed species are less in number [3,4].

İletişim (Correspondence)
+90 282 2933866/203
sirrikar@yahoo.com

The disease was firstly noted in humans in 1976 and today it is stated that the disease has an important dimension in 90 countries from 6 continents [5]. It is also mentioned that cattle cryptosporidiosis is widespread throughout the world, and similar to human cryptosporidiosis the incidence has rather differences from regions to regions [6].

Cryptosporidium has biological monoxenous features. The infection occurs mainly by taking oocysts through water at foremost and food orally. These oocysts are excreted through feces and they become infectious from excretion onwards. Infectious oocysts are 4-6 µm in dimension, round-elliptical in structure, containing 4 bare sporozoites. Even taking fewer highly infectious oocysts can cause the disease. Although the pathogen is placed in the digestive system it leads to different types of symptoms affecting the immune system, age and likely infected tissues other than the intestine. The widespread diagnostic evidence is diarrhoea characterized with the ejection of plenty of oocysts [1,7]. That is why diagnosis is generally based on direct fecal examination. For this purpose especially fecal staining methods such as acid fast, carbol fuchsin and fluorescent staining can be utilized [7]. In epidemiological screenings and genetical examinations PCR with its high sensitivity and specificity is highly beneficial [8].

In this study, it is aimed to reveal to a certain extent if *Cryptosporidium* spp. is widespread among people and cattles in Ankara, and also to determine the efficiency of nested PCR and carbol fuchsin staining methods in such a screening.

MATERIAL and METHODS

Material

The study was carried with the fecal samples obtained from 98 people who came to different hospitals with the complaints of diarrhoea and from 32 calves with the disease of diarrhoea in different dairy farms in Ankara. Of these people, 38 patients were 1 year old and above; the others were younger. 22 of the calves were 1-4 weeks old, and 10 of them were 1-6 months old. The fecal samples from sick people and the calves were brought to the laboratory within sterile containers and kept at +4°C during the experimets.

Method

In order to reveal the presence of *Cryptosporidium* in the feces, methods of carbol fuchsin staining and nested PCR were employed. Before diagnosis, these feces brought to the laboratory were washed with distilled water by centrifugation in order to concentrate the oocysts and to remove fecal-derived PCR inhibitors to the utmost. For this purpose, 1 ml of each mixed sample was taken and washed in centrifuge tubes of 10 ml with the help of distilled water for 10 min at 2500 rpm, and this was repeated until supernatant became clear. At the last stage, 1 ml fecal suspension was attained by adding distilled water onto the pellet, and the following processes were carried out with this suspension.

Carbol Fuchsin Staining

The carbol fuchsin staining method used to display oocyst existence in feces was performed according to the methods described by Heine [9]. Briefly, 50 µl of thoroughly homogenized feces sample from the stock fecal suspension of 1 ml was taken over a slide, and then carbol fuchsin (Merck) of equal amount was added. After mixing, thin smear was obtained by spreading. Shortly after air dried, this ready smear mounted with a little drop of immersion oil was examined for *Cryptosporidium* oocysts under a light microscope at x40 magnification. Oocysts were counted at 20 fields, and average number of each field was calculated; more than 20 oocysts were regarded as (++++), 6-20 oocysts as (+++), 1-5 oocysts as (++) and less than 1 oocyst as (+).

Nested PCR

At this stage, 200 µl was taken from each homogenized fecal samples and DNA extraction was performed by using QIAamp DNA Stool mini kit. This DNA was preserved at -20°C for PCR. Nested PCR technic was carried out by applying protocol and primers described by Xiao et al.[10]. The samples which were proved to be *Cryptosporidium* positive in previous studies carried out in the Protozoology Laboratory of the Faculty of Veterinary Medicine in Ankara University were used as positive control.

At the first stage of PCR, the primers CryptoF 5'-TTCTAGAGCTAATACATGCG-3' and CryptoR 5'-CCCATTTCCTTCGAAACAGGA-3' were used, which amplify DNA fragment encoding SSU rRNA as 1.325 bp in length. For each reaction, master mix was prepared as 25 µl including 200 nM from each primer, 0.2 mM from each dNTP, 0.025 U Taq DNA polymerase, 6 mM $MgCl_2$, 1x PCR buffer and the sample DNA of 1.5 µl, 1 µl of the reaction products were later subjected to nested PCR method.

During Nested PCR, a base set of CryptoNF 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and CryptoNR 5'-AAGGAGTAAGGAACAACCTCCA-3' were used, which amplify a region at about 826-864 bp in length in the former product. The reaction was carried out at the same way as the the previous process; however, the quantity of the sample DNA was taken as 1 µl. Reaction products of 15 µl were used for gel electrophoresis.

The reactions were performed in a Biometra TGradient (Whatmann Biometra) PCR thermocycler with a heater lid. Both steps of PCR consisted of a total of 35 cycles (45 sec at 94°C, 45 sec at 55°C and 1 min at 72°C). 5 min of denaturation and 10 min extension period at 72°C were performed.

RESULTS

In the research, 1 (2.5 years old) of 98 human feces (1.02%) and 7 (1-4 weeks) of 32 calves feces (21.88%) examined by carbol fuchsin staining method were found positive in regards to *Cryptosporidium* spp. oocysts. In the slides prepared from the positive samples the number of oocysts at x40 magnification varied between 0.9-6.9 at average for each field *(Table 1)*.

Results of nested PCR of 130 fecal samples showed a total of 13 (10.0%) positive amplifications; 12 (37.5%) were from calves and 1 (1.02%) from humans. Of the positive samples, 8 were also found as positive through carbol fuchsin staining method. Eleven of the positive results were obtained from the calves at the age group of 1-4 weeks and one of them was from a 4.5 month calf. The results of both nested-PCR and carbol fuchsin staining method are given in *Table 1*.

Table 1. *Positive results of carbol fuchsin staining and nested PCR methods*

Tablo 1. *Carbol fuchsin ve nested PCR yöntemlerine ait pozitif sonuçlar*

Source	Carbol Fuchsin Staining		Nested-PCR
	The Average Number of Oocysts in Each Field (x40)	Evaluation	
Calf 1	0.9	+	+
Calf 2	1.9	++	+
Calf 3	2.7	++	+
Calf 4	3.2	++	+
Calf 5	4.9	++	+
Calf 6	5	++	+
Calf 7	6.9	+++	+
Human 1	4.9	++	+
Calf 8	-	-	+
Calf 9	-	-	+
Calf 10	-	-	+
Calf 11	-	-	+
Calf 12	-	-	+

DISCUSSION

It is notified that the prevalence of cryptosporidiosis in Turkey, which is regarded as widespread throughout the world [1,7], varies between 0-35.5% in people [11-15] and 7-63.3% on calves [16,17]. However, our molecular research reveals that the disease shows a prevalence of 37.5% (12/32) in calves with diarrhoea and 1.02% (1/98) in people with a complaint of diarrhoea in Ankara province.

Cryptosporidiosis is reported to be the scourge of especially young people and the adults who are immune deficient [1,7]. In this study, 38 of 98 human feces were from one year old and above; 60 were from younger people and the only positive result belonged to a 2.5 year old boy. As for the calves, 22 of them were 1-4 weeks and 10 were 1-6 months. While 11 of 12 positive results, examined by PCR, were from 1-4 week calves, only one positive result was obtained from 4.5 month calf. Less number of positive results among people renders it difficult to interpret. However, our results reveal that the disease is more widespread in the calves examined, especially a few months old. In fact, studies on the cattle cryptosporidiosis indicate that the disease is especially more widespread among the young cattles, being noteworthy in calves 4 days and 4 weeks old [18].

The symptom of the disease is characterized by homogeneous, yellowish and aqueous diarhoea, but these signs are not sufficent alone for a definite clinical diagnosis [7]. In this study, although every patient examined had complaints of diarrhoea, only 13 (10.0%) of them were found positive by nested PCR. But, this number was less with carbol fuchsin staining method (8 positive, 6.15%). Nonetheless, the signs of diarrhoea in the patients who were carbol fuchsin positive were closer to characteristics of diarrhoea abovementioned. But as for the patients who were negative by staining method, colour, density and appearance of diarrhoea was more different. On the other hand, the feces of 4.5 month calf detected as positive with the staining method was also aqueous, but had an appearance closer to normal colour and contained normal feces particles.

In order to diagnose *cryptosporidiosis,* many different

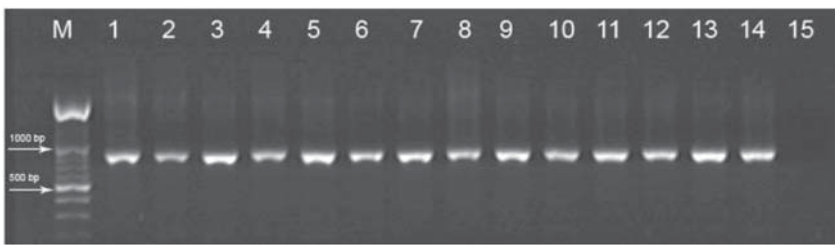

Fig 1. Nested PCR scanning results of positive fecal samples. **M**: 100 bp DNA size marker (Invitrogen). **1**: Positive control, **2-14**: Positive samples, **15**: Negative control

Şekil 1. Pozitif dışkı örneklerinde nested PCR sonuçları. **M**: 100 bp DNA marker (Invitrogen). **1**: Pozitif kontrol, **2-14**: Pozitif örnekler, **15**: Negatif kontrol

methods can be applied. Of those, staining, PCR, IFAT ve ELISA applied to feces are the foremost diagnostic technics. While staining methods such as acid fast, carbol fuchsin and fluorescent staining are accepted to be effective in diagnosis [1,7], and even if they are frequently applied, their sensivities and specifities are known to be low [19].

Carbol fuchsin staining technic is evaluated by laboratories to be advantageous due to its simplicity and practical importance. With this technic, the oocysts can be defined by the specialists easily. However, it has some restrictions in that as the other staining methods, the number of oocysts in the slides prepared from the same feces can vary. In addition, the structure of oocysts can be degenerated when the slides are kept longer, and the number of oocysts should be 10.000-500.000 per gram of feces in order to have positive results from microscopic examination [5,7,20,21]. Our results confirmed that carbol fuchsin staining method can be used for diagnosis of clinical cases. In diagnosis of subclinical cases, PCR was reported to be more sensitive than conventional staining methods and other diagnostic technics [10]. Furthermore, nested PCR is 4-5 times more sensitive than classical PCR [22], and can detecteasily the carriers showing no clinical symptoms. On the other hand, gene regions chosen as a target for PCR are also one of the important effects on the sensitivity and specifity of these molecular technics. As pointed out by some researchers previously [23,24], the primers targeted SSU rDNA coding region yielded desired amount of DNA in this study.

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