Original Article

Evaluation of nested PCR for diagnosis of *Cyclospora cayetanensis* in a sample of immunosuppressed and diarrheic patients in Turkey

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ABSTRACT

Background: *Cyclospora cayetanensis* is a food-borne coccidian parasite that causes cyclosporiasis in humans and possibly in other animals. It presents with watery diarrhea and other related symptoms. Since detection of oocysts may be difficult with histological stains, a negative result should not exclude the possibility of *C. cayetanensis*. PCR methods can achieve more sensitive detection of the parasite.
Objective: The presence of *C. cayetanensis* was investigated in an immunosuppressed patient group, diarrhea patient group, and in both immunosuppressed and diarrhea patient group using the modified acid-fast staining and nested polymerase chain reaction (nPCR) methods.
Subjects and Methods: Included in the study were 80 patients with immune suppression, 50 patients with diarrhea, and 70 patients with both immune suppression and diarrhea. The clinical findings of these patients were recorded, stool samples were collected and examined using both the modified acid-fast (AF) staining and nPCR methods.
Results: The overall detection rate of *C. cayetanensis* was 8% and 12% using the modified AF and nPCR, respectively. *C. cayetanensis* was detected in 5% of immunosuppressed patients, 12%, in patients with diarrhea and 20% in patients with both immune suppression and diarrhea. Statistically significant relationships were identified between the frequency of *C. cayetanensis* and abdominal pain (P<0.01), nausea (P<0.01), fatigue (P<0.01), diarrhea (P<0.05), and weight loss (P<0.01).
Conclusion: nPCR gave a higher rate of cyclosporiasis, and it is more appropriate especially in cases with recurrent prolonged symptoms.

Keywords: *Cyclospora cayetanensis*, diarrhea, immune suppressed, modified acid-fast staining, nested PCR.

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INTRODUCTION

Cyclospora cayetanensis taxonomically belongs to the subgroup Coccidia of the family Eimeriidae, of Apicomplexa. Although cyclosporiasis is common all over the world, it is especially common in tropical and subtropical countries. In Turkey, cyclosporiasis cases have been detected sporadically since 1998[1].

Although cyclosporiasis was originally not considered important, later it was defined as tourist diarrhea. It has become an important pathogen in all age groups, causing food- and water-borne diseases in healthy and immunosuppressed subjects of all ages. In recent years, the importance of the disease has increased even more after sporadic cases were observed in individuals with healthy immune systems besides the increased rate in immunosuppressed patients[2,3].

The onset of clinical symptoms is sudden in 68% of adult patients and slow in the remaining 32%, initially associated with clinical symptoms similar to influenza infection. In symptomatic cases, the incubation period lasts an average of one week. The most common clinical manifestation of cyclosporiasis was recognized as recurrent diarrhea with excessive fluid output, that may occur approximately six times a day, and is often accompanied by weight loss[4].

Cyclosporiasis is diagnosed by the presence of oocysts using different staining methods for stool, duodenum aspiration fluid, or biopsy samples. The most widely preferred classical method of diagnosis is by microscopic detection of modified AF staining of the oocysts. However, it requires experienced personnel and extended time to examine the samples. Another disadvantage is that the parasite may be overlooked when the number of oocysts is low. The fact that no comprehensive study has been performed on either immunocompromised or immunocompetent patients in Turkey suggests the underrated importance of the

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disease and that the rates of cyclosporiasis determined in Turkey do not reflect reality.

The aim of our study was to investigate the presence of *C. cayetanensis* in an immunosuppressed non-diarrheic patient group, in diarrheic patient group, and in both immunosuppressed and diarrheic patient group using nPCR and modified AF methods.

SUBJECTS AND METHODS

This cross-sectional study was conducted in the Parasitology Laboratory of the Dursun Odabas Medical Center of the Van Yuzuncu Yil University, between January 2018 and May 2019.

Sample and patient groups: Included in the study were 80 patients with immune suppression, 50 patients with diarrhea, and 70 patients with both immune suppression and diarrhea. Stool samples were collected from these patient groups. Sex, age, patient status, and clinical findings were recorded for each patient.

Microscopic stool examination: For identification of *C. cayetanensis* oocysts fecal suspensions of the formol-ether concentration technique[5] were stained with modified AF staining[6]. The slides were examined under a Leica DM500 microscope (Leica Microsystems, Wetzlar, Germany) at a magnification of X1000.

DNA extraction: DNA extraction was performed as described in the GeneMATRIX Stool DNA Purification Kit (Gdańsk, Poland) manual from whole stool samples. The Lyticase enzyme from Arthrobacter Luteus (L2524; Sigma-Aldrich, St. Louis, MO, USA) was used to weaken or break down the oocyst wall before extraction. Enzymes were added to the samples and incubated at 25°C for 15 min. The samples were then incubated at 95°C for 30 min in a dry block heater and vortexed at five-min intervals during the incubation period. All other procedures were carried out according to the kit's procedure instructions.

PCR and electrophoresis: nPCR was performed using the methods and primers specified by Orlandi *et al*.[7]. In the first stage of the PCR, F1E 5′-TACCCAATGAAAACAGTTT-3′ and R2B 5′-CAGGAGAAGCCAAGGTAGG-3′ were the primers used to amplify the ~636 bp region of the 18S rRNA gene region of *Cyclospora* and *Eimeria* species. The reaction was adjusted to a total volume of 50 µL, containing 25 µL of Tag 2x Master Mix (12.5 mM $MgCl_2$), 0.5 mM $MgCl_2$ and 0.2µM of each primer, and 1 µL of sample DNA. Next, 1 µL of the amplicon obtained for the second stage of the nPCR was used. In the second stage of the nPCR, the primers were used to amplify the region of ~298 bp from the 18S rRNA gene region of *Cycylospora* species. The second nPCR reaction was carried out under the conditions specified in the previous step.

Reactions were performed on the Applied Biosystems SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA). The first PCR was programmed for a total of 35 cycles, each at 94°C for 30 s, then 53°C for 30 s, and 72°C for 90 s. nPCR was adjusted for a total of 25 cycles, at 94°C for 15 s and 66°C for 15 s. Since the primary binding temperature in the nPCR phase was close to the activity temperature of the Tag polymerase, no extension temperature (72°C) was required. In both PCR procedures, an additional administration was done at 95°C for 15 min before the first cycle for the denaturation step. Following the last cycle, an extension step at 72°C (66°C for nPCR) for 10 min was performed.

To display the results of the nPCR procedure, 15 µL of PCR reaction products was run on agarose gel (1%) electrophoresis and visualized in a UVP Gel documentation system (Ultra-Violet Products Ltd., Upland, CA, USA).

Statistical analysis: MINITAB (ver: 14) statistics package program was used for data statistical analysis. The frequency of parasite prevalence was expressed as number and percentage according to the relevant categorical variables. Significance was calculated using the Chi-square (X^2) test to compare between quantitative data, and Z-ratio test to compare the rates of noise. Odds values were calculated for the risk of occurrence of parasites. The sensitivity and specificity values of the methods used (e.g., PCR) were calculated to determine its diagnostic efficacy. Statistical significance level was taken as 5% (P<0.05) in calculations and SPSS (IBM SPSS for Windows, Ver. 21) statistics package program was used for calculations.

Ethical considerations: Ethical approval was obtained from The Van Yuzuncu Yil University Ethics Committee approved the study (No: 18). Informed consent form was obtained from the patients included in the study.

RESULTS

In this study, *C. cayetanensis* oocysts were detected using both modified AF staining (Figure 1) in 16/200 (8%) samples and nPCR (Figure 2) in 24/200 (12%) samples. Oocysts were detected with modified AF staining in 2/80 (2.5%) patients with immune suppression, in 3/50 (6%) patients with diarrhea, and in 11/70 (15.7%) patients with both immunosuppression and diarrhea. Presence of oocysts was detected using nPCR in 4/80 (5%) patients with immune suppression in 6/50 (12%) patients with diarrhea, and in 14/70 (20%) patients with both immunosuppression and diarrhea.

The results obtained using modified acid-fast staining were compared with those obtained using nPCR and a statistically significant difference was

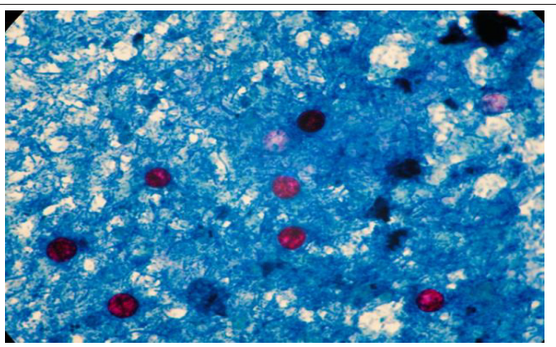

Fig. 1. *C. cayetanensis* oocysts stained by modified acid-fast technique.

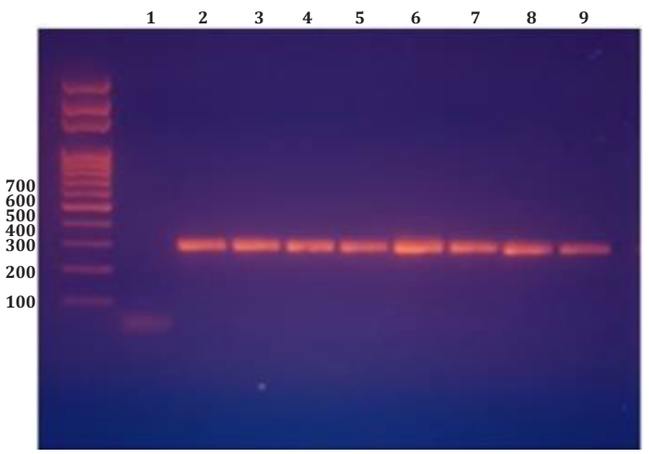

Fig. 2. nPCR results of *C. cayetanensis* on agarose gel. **Lane 1:** negative control; **lane 2:** positive control; **lanes 3-9:** positive samples.

found between both (P<0.05). Stool samples found to be positive using modified AF staining were also found to be positive by nPCR. However, eight samples were found to be negative using modified AF staining. We determined a sensitivity and specificity of 100% and 66.7%, respectively, for the modified AF staining (Table 1).

Significant correlations were determined between the incidence of *C. cayetanensis* and symptoms such as abdominal pain, nausea, fatigue, and weight loss with P valeus <0.001, and diarrhea (P<0.05). However, there was no statistically significant relationship with sex and age (P>0.05) (Table 2).

Table 1. Sensitivity and specificity values of modified acid-fast staining compared to the nPCR results.

Modified acid-fast staining	Values
Sensitivity	**100.0**
Specificity	**66.7**
False positivity	**33.3**
Undetectable positivity	**27.6**
Negative predictive value	**100.0**
Positive predictive value	**95.6**
Diagnostic accuracy	**96.0**

Table 2. Relationship between the frequency of *C. cayetanensis* and some clinical symptoms.

Clinical symptoms		*C. cayetanensis*			Statistical analysis
		Positive N. (%)	Negative N. (%)	Total N.	*P* values
Abdominal pain	**Positive [N. (%)]**	20 (57.1)	15 (42.9)	35	**<0.001**
	Negative [N. (%)]	4 (2.5)	161 (97.5)	165	
Nausea	**Positive [N. (%)]**	13 (35.1)	24 (64.9)	37	**<0.001**
	Negative [N. (%)]	11 (6.7)	152 (93.3)	163	
Fatigue	**Positive [N. (%)]**	23 (45.0)	28 (55.0)	51	**<0.001**
	Negative [N. (%)]	1 (0.7)	148 (99.3)	149	
Diarrhea	**Positive [N. (%)]**	20 (15.4)	110 (84.6)	130	**<0.05**
	Negative [N. (%)]	4 (5.7)	66 (94.3)	70	

DISCUSSION

C. cayetanensis is transmitted to humans by fecal-oral ingestion of contaminated food and water, causing a gastrointestinal disease called cyclosporiasis. Other potential risk factors for the disease are contact with domestic animals and/or contaminated soil, and poorly washed fruit and vegetables[8]. Although it is known that more than 2 million children died due to diarrheal diseases around the world, the role of cyclosporiasis has not yet been determined. Again, due to the lack of epidemiological studies on the disease worldwide, limited data exists on the effects of the disease on human health[9].

Although cyclosporiasis was previously described as tourist diarrhea and was underrated in most cases, it became an important pathogen in all age groups of both the healthy or immunosuppressed individuals. In addition, the fact that infective oocysts can survive for months, depending on the temperature of the environment, renders *C. cayetanensis* an important pathogen[10]. Various clinical signs of cyclosporiasis have been reported in both sporadic cases and in different patient groups. Ortega *et al*.[11] presented 17 patients with cyclosporiasis in their 1997 study. All of the patients had diarrhea, with abdominal distension and flatulence in 16, weight loss in 13, nausea and abdominal pain in 12, incontinence in 11, halitosis in 10, fever in nine, belching in eight, and vomiting and constipation in four. In a study conducted in 1998 by Koumans *et al*. [12] that included 24 patients with cyclosporiasis, 22 had amorphous or watery stools, 20 had loss of appetite and cramps, 17 had diarrhea, 16 had fatigue and gas, 15 had weight loss, 14 had nausea, 10 had headache, 9 had fever, 8 had swelling and tremor, 7 had vomiting, and 5 had joint pain, constipation, and muscle pain. In a cyclosporiasis outbreak among 77 individuals in Peru's capital, Lima, Torres-Slimming *et al*.[13] reported diarrhea in all 77 patients, nausea in 50, restlessness in 46, tremor in 44, fever in 40, abdominal pain in 36, headache in 26, and vomiting in 24. In our study, statistically significant relationship was found between the frequency of *C. cayetanensis* and abdominal pain, nausea, fatigue, weight loss (P<0.001), and diarrhea (P<0.05). It was concluded that *C. cayetanensis* should be taken into consideration in the case of statistically significant clinical symptoms. However, no statistically significant relationship was found between the frequency of *C. cayetanensis* and sex and age (P>0.05).

Sporadic infections in endemic regions, occur as a result of traveling to an endemic area or due to water and food-borne outbreaks in nonendemic regions[2]. Cyclosporiasis is endemic in Bangladesh, Brazil, Chile, Cuba, Egypt, Haiti, Indonesia, Mexico, Nigeria, Nepal, Peru, Thailand, and Venezuela[14]. In recent years, it has also been occasionally encountered in Turkey[10].

Epidemiological studies have been performed on immunocompetent individuals of all age groups around the world, and *C. cayetanensis* was found to be positive in 2% of individuals in Guetamala in 1999 by Bern *et al*.[15], in 5.6% in China in 2002 by Wang *et al*.[16], 1% in Nigeria in 2003 by Alakpa *et al*.[17] and in 10.3% in Peru in 2005 by Alva[18].

In countries where the disease is endemic, both children and adults who are immunocompromised are at risk. In studies on patients with suppressed immune systems, *C. cayetanensis* was found in 3.8% of HIV-positive patients in Guetamala in 2001 by Pratdesaba *et al*.[19], in 3.5% of HIV-positive patients in Cuba in 2003 by Capo de Paz *et al*.[20], in 9.8% of HIV-positive patients in Venezuela in 2006 by Chacin-Bonilla *et al*.[21], and in 4.4% of patients in Indonesia by Kurniawan *et al*.[22]. In our study by nPCR, *C. cayetanensis* was found in 12% of 200 patients. Among these patients, *C. cayetanensis* was found in 5% of 80 patients with immune suppression, in 12% of 50 patients with diarrhea, and in 20% of 70 patients with both immune suppression and diarrhea.

In Turkey, *C. cayetanensis* infections are generally reported as sporadic cases; however, the rates obtained in our study were higher than those previously reported in Turkey[23-25]. Several reasons govern this outcome. For one, the sensitivity of the modified AF staining method varies depending on the expertise and skill of the person screening the slides especially when number of oocysts is low; or in cases with intermittent passage of oocysts. Furthermore, another reason for the limited diagnosis of cyclosporiasis is its insufficient differential diagnosis by clinicians. In Turkey, the lack of adequate studies on cyclosporiasis in both immunocompromised patients and in healthy individuals suggests that the disease is not given enough importance and that the previously determined rates of cyclosporiasis do not reflect reality of spread. Hence, in Turkey, researchers using molecular methods are quite limited and comprehensive research using these methods is needed. Although there is a higher rate of positivity with the PCR method, it is more appropriate to use nPCR together with the modified AF staining method in the diagnosis of this parasite which will increase the positivity rate. Therefore, we recommend that the diagnosis of cyclosporiasis should not be based on routine modified AF staining alone.

In conclusion, cyclosporiasis cases are being encountered from time to time in different countries, as in Turkey. In the presence of suggestive complaints, such as long-term diarrhea, abdominal pain, nausea, loss of appetite, and weight loss in patients with suppressed immune systems, this infection should be considered, and molecular methods in addition to staining methods are recommended for diagnosis.

Author contributions: Yilmaz H, Cengiz ZT and Ekici A initialized the study concept. Yilmaz H, Ekici A, Cengiz ZT, Unlu AH, Beyhan YE contributed in data collection and/or processing. Literature search was done by Beyhan YE, Yilmaz H, Ekici A, Cengiz ZT, and Unlu AH. Ekici A and Unlu AH conducted the practical work, analyzed and interpreted study results, and wrote the manuscript. Cengiz ZT performed the critical review of the manuscript.

Conflict of interest: Authors declare no competing interests.

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