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Single-strand conformation polymorphism-based genetic characterization of the *Cyclospora cayetanensis* strains collected from different provinces in Turkey

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Abstract

Introduction and objective. *Cyclospora cayetanensis*, a coccidian protozoan species, has been recently found to cause diarrhea in all age groups in immunocompetent and immunocompromised individuals in most regions of the world. This study aimed to conduct the molecular detection of *C. cayetanensis* and to determine the genetic diversity of the 18S ribosomal RNA (rRNA) gene sequence of *C. cayetanensis* isolated from individuals living in different provinces in Turkey by using PCR–single-strand conformation polymorphism (SSCP).

Materials and method. A total of 22 subjects were included in the study. Fourteen of the subjects were female and eight were male, with ages ranging between 7–65 years. Stool specimens were examined using wet mount and modified acid-fast staining methods, which revealed the presence of oocysts in the samples. The 18S rRNA ITS-1 Ccits37f-GCTTGCTATGTTTTAGCATGTGG and Ccits501r-GCACAATGAATGCACACACA gene regions were used as primers. The PCR products were analyzed by agarose gel electrophoresis and visualized on a UV transilluminator. For the SSCP, the PCR products were denatured with formamide, run for 16 h in 6% (49:1) polyacrylamide gel, and then imaged with silver staining.

Results. SSCP assay was performed given that the DNA strands demonstrated different folds; the DNA strands contain different nucleotides based on the PCR-SSCP results for the Cyclospora strains collected in 4 provinces. Moreover, 3 different band profiles were observed in the investigated samples. A slight mutation difference was observed among the strains collected.

Conclusions. Further comprehensive studies involving more *C. cayetanensis*-positive specimens and utilizing different mutation screening methods are warranted to demonstrate mutation differences in Cyclopora strains in Turkey.

Key words

Cyclospora cayetanensis, Molecular characterization, Turkiye

INTRODUCTION

Cyclospora cayetanensis is a coccidian protozoan that causes severe gastroenteritis in both immunocompetent and immunocompromised individuals [1, 2]. The oocysts of *C. cayetanensis* are round-oval in shape and 8–10 µm in diameter, and they are not sporulated when excreted in faeces. As a result, infection does not spread from person-to-person; rather, infection occurs through ingestion of infected oocysts and of contaminated water and food [2, 3]. It has been reported that in recent years *C. cayetanensis* has caused foodborne outbreaks in the United States, Canada and Korea, and these outbreaks were associated with tourists and imported products [3, 4, 5, 6].

Up to 19 *Cyclospora* species have been found to cause diseases. It has been reported that *C. cercopitheci*, *C. colobia*, *C. papionis*, and *C. macacae*, which are found in monkeys, are morphologically and molecularly similar to *C. cayetanensis* [7, 8]. Given that the genetic relationship of *C. cayetanensis* with other coccidian parasites and its possible zoonotic reservoirs have not yet been determined, information on the biology and epidemiology of *C. cayetanensis* remains insufficient [9]. Data obtained by Cinar et al. indicated that the *C. cayetanensis* mitochondrial genome is closely related to the mitochondrial genomes of the Eimeria species [10]. Another investigation by Cinar et al. suggested that genomic analyses of mitochondrial genome sequences facilitate finding the source of *C.cayetanensis* outbreaks by providing a genomics tool linking *C.cayetanensis* in clinical and food samples during outbreak investigations [11].

Gene analyses through polymerize chain reaction (PCR) – single strand conformation polymorphism (SSCP) are among the mutation screening methods used to detect the source of an infection, and to demonstrate differences of mutation between strains [12, 13]. It is important to determine the genetic diversity of *C. cayetanensis*, to identify its genetic relationship with other coccidian protozoans, to pinpoint the source of infection in outbreak situations, and to reveal its zoonotic aspect.

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OBJECTIVE

The aim of the study was to conduct the molecular detection of *C. cayetanensis,* and to determine the genetic diversity of the 18S ribosomal RNA (rRNA) gene region in *C. cayetanensis* isolates obtained from individuals living in different provinces in Turkey.

MATERIALS AND METHOD

A total of 22 samples were included in the study: 13 from Diyarbakır, 5 from Van, 1 from Malatya, and 3 from Istanbul. Fourteen of the subjects were female and 8 were male, ages rangingtween 7–65 years. All specimens were microscopically examined using the wet mount and Kinyoun acid-fast staining methods.

Acid-washed glass beads (Sigma Glass beads, 425–600 µm), freeze-thaw, and a commercial DNA isolation kit (QIAamp DNA Stool Mini Kit, Qiagen) were used to facilitate the easy disassembly of the oocyst wall for DNA isolation from faeces. The presence of the investigated agent was demonstrated using the *Ccits37f-GCTTGCTATGTTTTAGCATGTGG* and *Ccits501r-GCACAATGAATGCACACACA* primers, which are complementary to the rDNA region of *C. cayetanensis* and which are *C. cayetanensis*-specific primers [14]. The PCR products amplified by the nested PCR method, which were used in a previous study, were examined with the SSCP method to determine whether a mutation had occurred in the rDNA sequence of *C. cayetanensis*. A region consisting of approximately 1,000 bases in the 18S ribosomal DNA region of the agent was amplified using the first *ExCyc F 5'-AAT GTA AAA CCC TTC CAG AGT AAC-3' and ExCycR 5'-GCA ATA ATC TAT CCC CAT CAC G-3* primers for a nested PCR. To eliminate other coccidian protozoa from the PCR products produced with the ExCyc primers, a PCR was performed using the *C. cayetanensis*-specific NesCycF *5'-AAT TCC AGC TCC AAT AGT GTA T-3* and NesCycR (*5'-CAG GAG AAG CCA AGG TAG GCR TTT-3* primers under the conditions described by Lalonde et al. [14].

The PCR products were run in 1.5% agarose gel containing 0.2 g/mL ethidium bromide and visualized in a UV transilluminator. Subsequently, the PCR products were added to the 6% gel containing 10% glycerol placed in a 33 cm × 39 cm × 0.4 cm metal block for DNA SSCP. The electrophoresis was performed for 16–20 h at room temperature. After the gel was washed with a solution containing 100% ethanol and 100% acetic acid, it was stained with 0.1% $AgNO_3$. The PCR products were denatured with formamide for the SSCP method, run for 16 h in a 6% (49:1) polyacrylamide gel, and then imaged with silver staining.

RESULTS

After the structures with a diameter of 8–10 µm were identified as being specific for *C. cayetanensis* by using the wet mount method (Fig. 1), they were examined with modified acid-fast staining. Dark red-purple stained oocysts were identified as *C. cayetanensis* oocysts (Fig. 2). Only *C. cayetanensis* oocysts were identified in 13 patients. In the other 9 patients, other intestinal parasites apart from *C. cayetanensis* oocysts were detected.

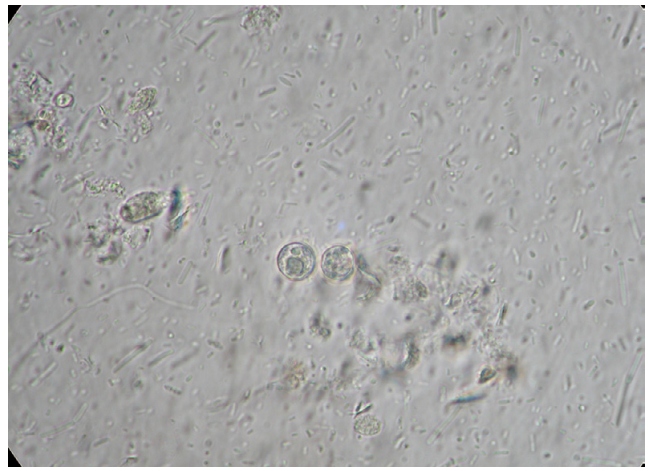

Figure 1. *Cyclospora* oocysts X100 diagnosed with wet slide

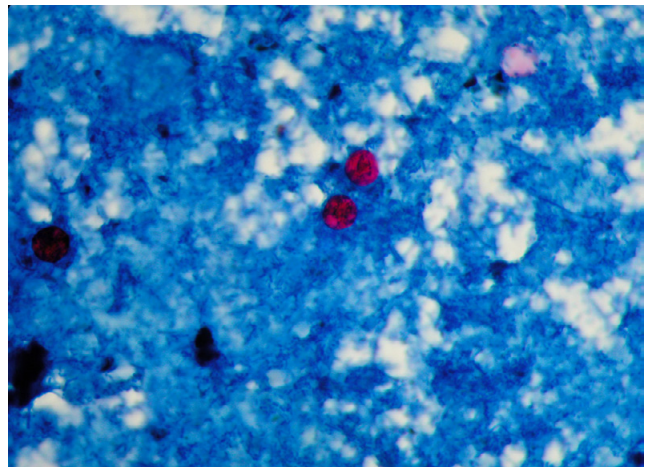

Figure 2. *Cyclospora* oocysts X100 diagnosed with Kinyoun acid fast staining method

None of the *C. cayetanensis*-positive patients had a travel history. In all patients, the common complaints were diarrhea, abdominal pain, anorexia, and fatigue. Nausea and fever were present in 14 patients, and vomiting was observed in 5 patients. None of the cases had any immunodeficiency or chronic illness. All cases were diagnosed in the months of June, July and August in 2017. Moreover, the cases from Diyarbakır and Van belonged to the same family. No systemic pathology other than gastrointestinal system disease was found during the systemic examination of the *C. cayetanensis*-positive patients. Their complete blood count and urinalysis and liver and renal function tests results were normal. Moreover, no reproduction was observed in stool cultures, and the faecal occult blood test produced negative results.

ITS primers and the agent were present in all studied samples as indicated by the results of the nested PCR conducted with ITS primers (Fig. 3). Moreover, the PCR products obtained by the nested PCR had 3 different band profile, as revealed by the SSCP performed on a 6% polyacrylamide gel (49:1). The presence of the investigated agent in the studied samples was demonstrated by PCR that used *C. cayetanensis*-specific ITS primers. The approximately 500-nucleotide regions of the 18S rDNA region, consisting of approximately 1,800 bases, were reproduced using nested PCR and then examined. SSCP analysis was conducted given that the DNA strands

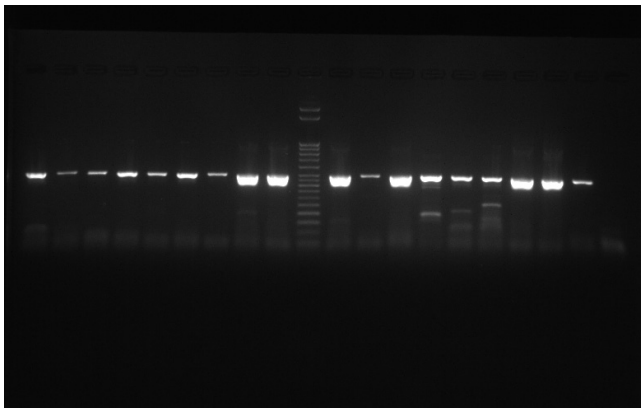

Figure 3. *Cyclospora* PCR results in agarose gel electrophoresis

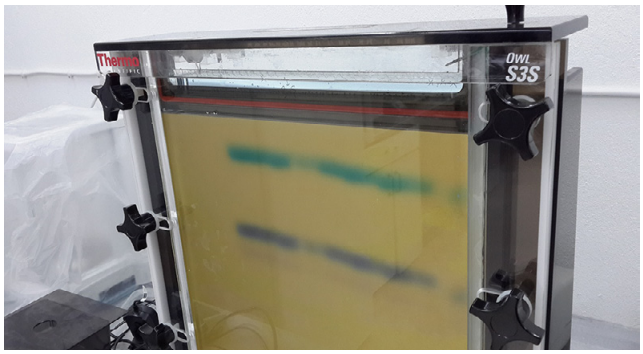

Figure 4. Execution of DNA in vertical electrophoresis

containing different nucleotides exhibited different folds, and 3 different band profiles were observed in the studied samples (Fig. 4).

DISCUSSION

The sporadic prevalence of *C. cayetanensis* in Turkey has been reported for 20 years [15, 16, 17, 18]. However, it is believed that this phenomenon was not well recognized and that in some laboratories *C. cayetanensis* was confused with other organisms. It may therefore be consideredhat the prevalence was higher than expected. More comprehensive epidemiological studies on this agent are needed in Turkey.

Nested PCR, which could reproduce the 294 base pairs of the 18S rRNA gene, was used for the molecular detection of the parasite [14]. Lalonde et al. created a new oligonucleotide design with 116 base pairs of *CCITS2-F (5_-GCAGTCACAGGAGGCATATATCC-3_)* and *CCITS2-R (5_-ATGAGAGACC TCACAGCCAAAC-3_)*, which belong to

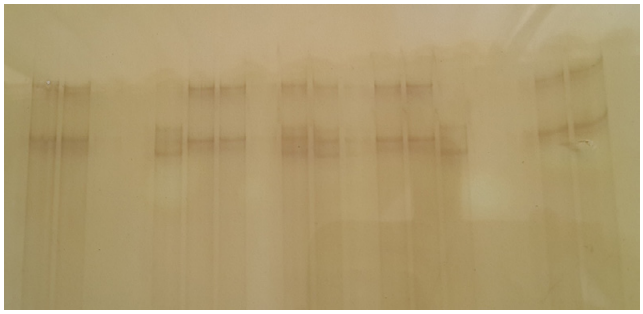

Figure 5. DNA bands obtained with SSCP

the ITS-2 gene region of the 18S rRNA gene. They indicated that this pair of genes distinguishes *Cyclospora* from other *Coccidian* protozoa [14]. The first molecular study on *Cyclospora* in Turkey was conducted by Ozdamar et al. [19], who performed a nested PCR of 17 positively staining samples collected in Istanbul, and obtained the DNA bands by using the same primers described above. However, they did not use any method for determine the genetic variation in the samples. In the present study, a two-step nested PCR was conducted using the same primers to demonstrate the presence of DNA bands in *Cyclospora*.

In their India-based study, Yadav et al, by using the PCR method examined the 18S rRNA region of 19 *Cyclospora* isolates obtained from human diarrhea stool. After sequencing the isolates, they reached 2 conclusions. First, the amino acid sequence of their isolates did not differ from those of the isolates obtained from primates. Second, on the basis of the phylogenetic analysis results, the isolates could be divided into 2 distinct sequence groups. In the first group, the isolate were closely related to *C. cayetanensis* strains, whereas those in the second group were associated with the species found in non-primates [20].

In 2013, Sulaiman et al. reported that the strains obtained from 3 endemic countries, namely, Nepal, Peru, and Mexico, did not display genetic differences as revealed by a DNA sequence analysis that involved a two-step nested PCR based on the characterization of the 70-kDa heat shock protein (HSP70) gene [9]. In 2014, Sulaiman et al. [21] comparatively investigated the strains obtained from the above-mentioned 3 endemic countries by using the 18S rRNA region of the HSP70 gene; they performed a DNA sequence analysis and this time found a minor genetic diversity. Therefore, the HSP70 gene, which is mostly used in bacteria, is not suitable for determining genetic differences; instead, it seems more meaningful to focus on rDNA or on the ITS regions that bind these rDNAs.

In this study, SSCP was performed differently as it involved a two-step nested PCR using the 18S rRNA gene, compared with those performed in other studies, and a minor genetic difference was identified among the strains obtained in 4 provinces in Turkey. In 2016, Hussein et al. [22] reported that *Cyclospora* infections have a clinical picture that can change from being symptomatic to being asymptomatic. They claimed that the possible reason behind this variable clinical picture is the genetic heterogeneity of *C. cayetanensis*. To reveal the genetic variation in *C. cayetanensis*, they amplified the DNA from 70 patients with cyclosporiasis by PCR, and investigated the possibility of genetic variation of *C. cayetanensis* by using a high-resolution melting curve from 18S rRNA genes. They identified 4 genotypic profiles in *C. cayetanensis* as a result. Moreover, they reported that there is a wide variation in *C. cayetanensis* genes and that the clinical variability found in patients with cyclosporiasis can be explained by this genetic variation.

CONCLUSIONS

As mentioned above, the examination of the regions outside the 500-nucleotide region in the 18S rDNA sequence consisting of approximately, nucleotides and the identification of the different band profiles obtained with SSCP, is significant for determination of the polymorphic structure of *Cyclospora*

strains found in Turkey, and their possible subtypes. At the same time, these findings are important for revealing new species in future research, to explain the reason for the difference in clinical findings presented by patient, to reveal new subspecies common to both animals and humans. To determine which species can cause disease in humans and to investigate their genotypic differences, further comprehensive studies involving larger samples, different mutation detection methods, and new protected gene regions that can be used for species identification are required.

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