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Identification of Spore-Forming Intestinal Parasites with Pentaplex Real-Time PCR

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Abstract

Objective: Spore-forming intestinal parasites; Cryptosporidium spp., Cyclospora spp., Cytoisospora spp., Encephalitozoon spp., and Sarcoystis spp. are very common in immunocompromised patients, but these parasites are overlooked by healthcare proffesionals. It was aimed to develop a new pentaplex real-time PCR panel for the identification of spore-forming intestinal parasites in this study.

Methods: Primer-probes for pentaplex real-time PCR were designed using the "PrimerQuest Tool (Integrated DNA technologies, Coralville, USA) software program" and "Multiple sequence alignment use a computer software Primer Express™Software v3.0.1 Lience (ThermoFisher Scientific, Waltham, USA)". The primer-probes designed in the study were spore-forming Cryptosporidium spp. (ATCC®87715™), Cyclospora spp. (ATCC®PRA-3000SD™), Cystoispora spp. (KF648871), Encephalitozoon spp. (FJ026010) and Sarcocystis spp. (ATCC®CCL-70) parasites were amplified with DNA isolates from the American Type Culture Collection (ATCC) and then these primer-probes were validated with 232 DNA samples obtained from the stools of the patient samples.

Results: It was found that Cycle Threshold (Ct) ±25.7, Standard curve (R2): ±0,993, and Efficiency (E): %96,1 according to the results of multiplex real-time PCR analysis. Similar results were found in pentaplex real-time PCR analysis of DNA isolates of stool samples. When the pentaplex real-time PCR results of DNA samples isolates from stool samples were compared with the positivie predictive value results of traditional methods, it was found that the pentaplex results were higher.

Conclusion: The new designed pentaplex real-time PCR panel can be used in the diagnosis of spore-forming intestinal parasites, which are very common in immunocompromised patients. Thus, the diagnosis of five different parasites can be made faster, more economically and faster with a single reaction.

Keywords: Spore-forming intestinal parasites, pentaplex real-time PCR, rapid diagnostic

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Spor Oluşturan Bağırsak Parazitlerinin Pentaplex Real-Time PCR ile Tanımlanması

Öz

Amaç: Spor oluşturan bağırsak parazitleri; Cryptosporidium spp., Cyclospora spp., Cytoisospora spp., Encephalitozoon spp., ve Sarcoystis spp. bağışıklığı baskılanmış hastalarda çok yaygındır, ancak bu parazitler sağlık personeli tarafından göz ardı edilir. Bu çalışmada spor oluşturan bağırsak parazitlerinin tanımlanması için yeni bir pentaplex real-time PCR panelinin geliştirilmesi amaçlanmıştır.

Yöntemler: Pentaplex real-time PCR için primer-problar "Primer Quest Tool (Integrated DNA technologies, Coralville, USA) software programı" ve "Multiple sequence alignment use a computer software Primer Express™Software v3.0.1 Lience (ThermoFisher Scientific, Waltham, USA)" programı kullanılarak tasarlandı. Çalışmada tasarlanan primer-problar, Amerikan Tipi Kültür Koleksiyonu (ATCC)'nunda temin edilen, spor oluşturan Cryptosporidium spp. (ATCC®87715™), Cyclospora spp. (ATCC®PRA-3000SD™), Cystoispora spp. (KF648871), Encephalitozoon spp. (FJ026010), and Sarcocystis spp. (ATCC®CCL-70) parazitlerinin DNA izolatları ile amplifiye edildikten sonra hasta örneklerinden elde edilen 232 DNA örneği ile doğrulandı.

Bulgular: Pentaplex real-time PCR analizi sonuçlarına göre analizin döngü Eşiği (Ct) ±25,7, standart eğrisi (R2): ±0,993 ve verimliliği (E): %96,1 olarak bulundu. Dışkı örneklerinin DNA izolatlarının pentaplex real-time PCR analizinde de benzer sonuçlar bulundu. Dışkı örneklerinden izole edilen DNA örneklerinin pentaplex sonuçları ile geleneksel yöntemlerin pozitif öngörme değerinin yüksek olduğu bulundu.

Sonuç: Bağışıklık sistemi baskılanmış hastalarda çok sık görülen sporlu bağırsak parazitlerinin tanısında yeni tasarlanan pentaplex real-time PCR paneli kullanılabilir. Böylece beş farklı parazitin tanısı tek bir reaksiyon ile daha hızlı, daha ekonomik ve daha hızlı konulabilir.

Anahtar kelimeler: Sporlu bağırsak parazitleri, pentapleks gerçek zamanlı PCR, hızlı teşhis.

INTRODUCTION

Intestinal parasitic infections are characterized by clinical symptoms such as abdominal pain, vomiting, fever, chills and diarrhea[1,2]. Cryptosporidium spp., Cyclospora spp., Cytoisospora spp., Encephalitozoon spp., and Sarcoystis spp. are spore-forming intestinal parasites and these are known to be the most common cause of intestinal parasite infection. All of them are obligate intracellular parasites of intestinal epithelial cells. These intestinal parasites are opportunistic pathogens, mainly seen in immunocompromised patients such as HIV patients, organ transplant recipients, and cancer patients[3,4]. The clinical symptoms of intestinal infections are overlooked by health professionals because that resemble the clinical symptoms of other diseases or are suppressed by the symptoms of other infections[3]. Therefore, clinical symptoms are not sufficient in the diagnosis of intestinal parasite infection and laboratory investigations are needed for diagnosis of these infection.

The diseaes caused by these parasites are cosmopolitan diseases and the prevalence of the diseases varies according to the geographical region, the population studied, the sample selection technique and the diagnostic methods used[5-7]. The diagnosis of these diseases can be based directly on the presence of parasites spores in stool samples or indirectly by serological methods. However, these methods can show low sensitivity depending on the clinical course of the infection, the intensity of the infection, the type of parasite, the life cycle of the parasite, and the experience of the microscopist[8-10].

The real-time PCR, which is one of the molecular methods in the diagnosis of intestinal parasite infections, has been used in recent years[11]. The multiplex real-time PCR is used in the diagnosis of other diseases because it is a high-throughput test with faster return[12]. The multiplex real-

time PCR can identify many pathogens in a single reaction with more than one primer-probe and takes names like pentaplex, hexaplex depending on the number of probe used[13,14].

It is difficult to distinguish Cryptosporidium spp., Cyclospora spp., and Encephalitozoon spp., which are morphologically similar, with traditional methods in laboratories where specialists are not available. Detection of these parasites with faster and cheaper methods such as pentaplex rather than conventional methods will be beneficial for public health and national economy. Therefore, in this study, it was aimed to develop a new pentaplex real-time pcr panel to identify spore-forming intestinal parasites (Cryptosporidium spp., Cyclospora spp., Cytoisospora spp., Encephalitozoon spp., Sarcoystis spp.) more economically and faster.

METHODS

Parasite Strains and DNA extraction

Strains of the spore-forming intestinal parasites Cryptosporidium spp. (ATCC®87715™), Cyclospora spp. (ATCC®PRA-3000SD™), Cystoispora spp. (KF648871), Encephalitozoon spp. (FJ026010), and Sarcocystis spp. (ATCC®CCL-70) were purchased from the American Type Culture Collection (ATCC). Positive controls of intestinal parasites obtained from ATCC and these controls were prepared according to product sheet. In addition, sterile water was used as negative control in this study.

Each spore-forming intestinal parasite strains obtained from ATCC was prepared using different media according to the product sheet. Parasites grown in culture were collected with a sterile pipette and washed with 100 µL of 1 × TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.5) for DNA isolation. Total DNA extraction was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's intrustions. The concentrations of parasite DNA were evaluated with nanodrop spectrophotometers (Thermo Scientific, Wilmington, USA).

Design of primers and probes

The target gene sequences of the parasites to be identified in this study were obtained from the GenBank website (http:// blast.ncbi.nlm.nih.gov/Blast.cgi). The primer-probes were designed from the nucleotide sequences in the GenBank and these primer-probes cover 98-99% nucleotides of each parasite. The each real-time PCR primers and probes were designed using the online "PrimerQuest Tool (Integrated DNA technologies, Coralville, USA) software programme". The melting temperature (Tm) of primers was designed to be 60°C and the probes were specially designed with a Tm of 65°C. Therefore, the predicted amplicon size was limited to 100-150 bp for each primer pair to potentially increase the reaction sensitivity.

Multiple sequence alignment use a computer software "Primer Express™Software v3.0.1 Lience (ThermoFisher Scientific, Waltham, USA) multiple primer analyzer". The primers and probes used for pentaplex real-time PCR in this study were designed to simultaneously detect the following five parasites: Cryptosporidium spp. (ATCC®87715™), Cyclospora spp. (ATCC®PRA-3000SD™), Cystoispora spp. (KF648871), Encephalitozoon spp. (FJ026010) and Sarcocystis spp. (ATCC®CCL-70).

The primers and probes in each panel were checked for potential secondary structures cross primer dimers and self dimers formations prior to synthesis. The annealing temperature of pentaplex real-time PCR was optimized as 60°C in this study. Since more than one primer-probe is placed in the same tube in pentaplex real-time PCR, each probe were marked with different fluoroscent dyes. The recognition wavelengths of fluorescent dyes were checked using the Spectral Overlay Tool for multiplexed

QPCR (Biosearch Technologies, Middleton, USA). All of intestinal parasite nucleotides were synthesized by Integrated DNA Technologies. The information of the primer and probe sequences, amplicon sizes, targeted genes, and dye of oligonucleotide used for diagnosis of spore forming intestinal parasites in Table 1.

Table I: The combination of primers-probes using the pentaplex real-time PCR assay for the spore-forming intestinalis parasites in this study

Parasites	Target gene-Dye	Primer-Pobe Sequence-Dye	Amplicon Length
Cryptosporidium spp.	ssuRNA	F: GCTTTAGACGGTAGGGTATTGG P: TTAGGGTTCGATTCCGGAGAGGGA-hex R: CTTAGATGTGGTAGCCGTTTCT	131 bp
Cyclospora spp.	ssuRNA	F: TGGTTTCTAGGACCAAGGTAATG P: TCACCTCTGACAGTTAAATACGAATGCCC-fam R: GGCAAATGCTTTCGCAGTAG	115 bp
Cystoispora spp	18srRNA	F: AGCTTTCGACGGTAGTGTATTG P: TTAGGGTTCGATTCCGGAGAGGGA-rox R: CCTTAGATGTGGTAGCCGTTTC	105 bp
Encephalitozoon spp.	Alpha-tubulin gene	F: CCACCCTGGACAGCTTATTT P: TGCAGCAAATAACTATGCCCGAGGG-cy5 R: TCTCCTTCCCAACAGTGTAATG	91 bp
Sarcocystis spp.	18SrRNA	F: ATGGTCGCAAGGCTGAAA P: TAAAGGAATTGACGGAAGGGCACCA-vıc R: CCTGTCATCCTTCCATGTCTG	121 bp

Pentaplex real-time PCR assay and using stool samples

The pentaplex real-time PCR was performed in a total volume of 20 µL containing each 0.5 µM of forward and reverse primers, 0.25 µM of each probe, 1xMultiplex RT-PCR mixes (Qiagen, Hilden, Germany) and 5 µL DNA samples. The pentaplex real-time PCR assay was performed on a Rotor-Gene Q5plex Platform real-time PCR detection system (Qiagen, Hilden, Gemrnay) according to the following thermocycler programme; an inital denaturation step of 5 min at 95°C; followed by 40 cycles of 10 s at 95°C; and 15 s 60°C. The results of amplification and cycle threshold (Ct) values were calculated using Rotor-Gene Q5Plex Platform real-time PCR detection system software. In this study, a total of 232 DNA samples isolated from stool samples in the archive of Aksaray University Faculty of Medicine Parasitology Laboratory were used to investigate whether the new designed pentaplex real-time PCR method analyzed in patient samples.

Other parasitological diagnostic methods

To validate the new pentaplex real-time PCR method designed in the study, 232 stool samples were analyzed with other parasitological diagnostic methods. Spore-forming intestinal parasites were investigated in stool samples taken from patients using ziehl neelsen staining and examined under a light microscope.

Cryptosporidium spp., Cyclospora spp., Cystoispora spp., Encephalitozoon spp., and Sarcocystis spp. (Handpicked Antibodies, Germany) were investigated using Enzyme Linked İmmuno Sorbent Assay (ELISA) stool human kit according to the manufacturer's instructions. The optical density (OD) values of ELISA methods were evaluated at 450 nm using an automatic ELISA reader.

The gene regions specific of Cryptosporidium spp., Cyclospora spp., Cystoispora spp., Encephalitozoon spp., and Sarcocystis spp. were targeted and primers were designed for PCR method[15-17]. After the PCR method is studied according to reference, PCR amplification products were evaluated by electrophoresis method with 1.5% agarose gel stained using 0.5 µg/mL ethidium bromide strain.

Statistical Analysis

All data were entered to computer and analyzed with Statistical Package fort he Social Science v.22.0 software (SPSS Inc., Chicago, USA) and p-value were used for comparisons diagnostic methods and pentaplex real-time PCR. The results of ziehl-neelsen, ELISA, PCR and pentaplex real-time PCR assay was analyzed using the chi-square ($\chi 2$) test. The statistical significance limit will be taken as $p<0.05$ for this study. According to recent Clinical and Laboratory Standards Instıtue (CLSI) approved guidelines for molecular diagnostic assays, parasite detection specificity of pentaplex real-time PCR assay was compared with other diagnostic methods and expressed as negative predictive value and positive predictive value[18].

RESULTS

The primers and probes designed in the study were optimized with pentaplex real-time PCR methods and optimized sequences of primer-probes were determined by having Ct values of less than ≤ 26. All of strains of reference parasites showed correlation for DNA concentration and Ct values (R2: 0.9921-0.1) along with high real-time PCR efficiencies ranging from 93.3% to 96.1% (Table 2). The pentaplex real-time PCR results of parasite strains used as control group and DNA samples isolated from patients were found to be similar (p=0.01). According to the pentaplex real-time PCR results of DNA isolates of stool samples, the Ct value was found to be between 19-32, while the R2 value was found to be ±0.999 and E: %95. The results of DNA isolates from stool samples by pentaplex real-time PCR has been shown in figure 1.

Table II: The pentaplex real-time PCR efficiency (%) obtained from dependence of DNA concentrations on threshold cycle (Ct) values

Parasites	Average Ct of DNA concentrations					R^2	Slope	Efficiency (%)
	100 fg	10 fg	1 fg	100 ag	10 ag			
Cryptosporidium spp.	25.0	28.2	31.1	35.3	39.1	0.997	-3.6	93.3
Cyclospora spp.	26.2	28.1	30.2	34.5	38.1	0.995	-3.5	96.1
Cystoispora spp.	24.3	27.1	29.3	32.1	37.3	0.991	-3.6	95.2
Encephalitozoon spp.	25.4	28.2	30.1	32.3	35.4	0.990	-3.3	96.1
Sarcocystis spp.	24.5	27.3	31.1	33.5	38.2	0.993	-3.6	94.2

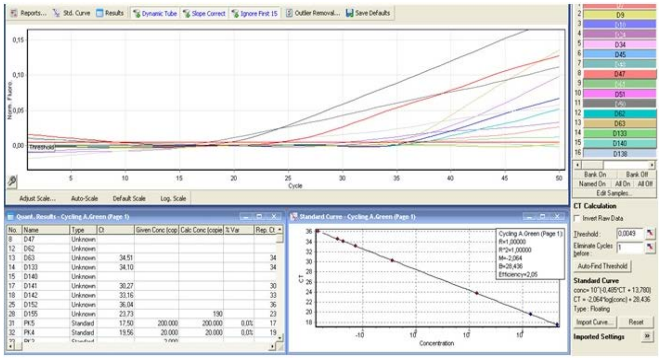

Figure 1: The results of DNA isolates from stool samples by pentaplex real-time PCR.

A total of 232 DNA samples isolated from the stool samples of the patients were used for the validation of new designed pentaplex real-time PCR method. Also, stool samples taken from 232 patients were analzed by microscopy (ziehl-neelsen staining), ELISA and PCR methods. It was found that 9.5% (22/232) Cryptosporidium spp., 2.6% (6/232) Cyclospora spp., 5.2% (12/232) Cytoisopora spp., 3.5% (8/232) Encephalitozoon spp., 1.7% (4/232) Sarcoystis spp., according to the ziehl-neelsen staining method. Using ELISA method

10.3% (24/232) Cryptosporidium spp., 2.6% (6/232) Cyclospora spp., 5.6% (13/232) Cytoisopora spp., 3.1% (7/232) Encephalitozoon spp., 2.2% (5/232) Sarcoystis spp., were determined among stool samples of patients. It was found to be 11.6% (27/232) Cryptosporidium spp., 3.5% (8/232) Cyclospora spp., 6.0% (14/232) Cytoisopora spp., 3.9% (9/232) Encephalitozoon spp., 2.2% (5/232) Sarcoystis spp. by PCR method (Table 3).

Table III: The results of conventional diagnostic methods and pentaplex real-time PCR method of DNA isolates from stool samples

Parasite	Ziehl-nelson	ELISA	PCR	Pentaplex RT PCR
	(+) (-) %+ %-	(+) (-) %+ %-	(+) (-) %+ %-	(+) (-) %+ %-
Cryptosporidium spp	22 210 9.5 90.5	24 208 10.3 89.7	27 205 11.6 88.4	27 205 11.6 88.4
Cyclospora spp.	6 226 2.6 97.4	6 226 2.6 97.4	8 224 3.5 96.5	8 224 3.4 96.6
Cytoisospora spp	12 220 5.2 94.8	13 219 5.6 94.4	14 218 6.0 94.0	14 218 6.0 94.0
Encephalitozoon	8 224 3.5 96.5	7 225 3.1 96.9	9 223 3.9 96.1	10 222 4.3 95.7
Sarcocystis spp.	4 228 1.7 98.3	5 227 2.2 97.8	5 227 2.2 97.8	6 226 2.5 97.5

All of the DNA isolates of stool samples were scanned with the new pentaplex real-time PCR panel, and this method showed an accurate identification rate with conventional methods. It was found to be 11.6% (27/232) Cryptosporidium spp., 3.4% (8/232) Cyclospora spp., 6.0% (14/232) Cytoisopora spp., 4.3% (10/232) Encephalitozoon spp., 2.5% (6/232) Sarcoystis spp. according to the result of new pentaplex real-time PCR panel (Table 3). When the results of the pentaplex method are compared with the conventional methods used for the diagnosis of spore-forming intestinal parasites, it has been observed that the positive predictive value of pentaplex diagnostic methods is higher than other diagnostic methods used in the study ($p<0.01$).

DISCUSSION

Spore-forming intestinal parasites infections are one of the main causes of health-service consultations in developing countries and these infections are an important cause of morbidity worldwide. The clinical symptoms of Cryptosporidium spp., Cyclospora spp., Cytoisospora spp., and Sarcoysts spp. parasites include abdominal pain, diarrhea, dehydration, weight loss, fever, vomiting, and these parasites have been reported mostly in immunocompromised patients[19,20]. Immunocompromised patients experience symptoms that are confused with the clinical symptoms of spore-forming intestinal parasites, such as abdominal pain, fever, and diarrhea, due to side effects of treatment or decreased in the CD4+T cell/ CD8+T cell ratio[21]. The mortality rates are increasing in patients due to the mixing of clinical symptoms and ignorance of spore-forming intestinal parasites by heathcare professionals. Therefore, laboratory diagnostic methods are needed in the diagnosis of infections to confirm clinical symptoms.

Laboratory diagnosis of spore-forming intestinal parasites is performed by conventional methods, including culture, antigen test, microscopic examination, and molecular methods[22]. There is a growing interest in alternative methods, such as DNA detection mostly by real-time PCR, to overcome the limitations of conventional methods for diagnosis of intestinal parasite in recently. It has been reported in multiplex real-time PCR method, which can identify many parasites in a single reaction from different countries. Nazeer et al. used multiplex real-time PCR method to

identify intestinal parasites Giardia duodenalis, Cryptosporidium spp., and Entamoeba histolytica in Egypt. Their studies have shown that the triplex real-time PCR method, performed in closed system, which can identify several parasites, is useful for rapid and accurate diagnosis of parasites[23]. Won et al. analyzed eight parasites caused to gastroenteritis using octaplex real-time PCR and they have reported that this method useful to determine to these parasite. They have reported that octaplex real-time PCR rapid method and higher sensitivity than convensiyonel methods[9]. Basuni et al. have been developed a pentaplex real-time PCR method for the identification of soil-transmitted helminths[24]. They reported that this method can be used in epidemiology, diagnosis and treatment programs. The costs of the conventional methods and the pentaplex real-time PCR method were compared and they reported that these methods were close to each other in their study[24].

The real-time PCR method has been used not only for parasite identification but also for the identification of other viruses, bacteria and fungi[14,25,26]. In addition, in many studies on the diagnosis of parasites, it has been reported that the sensitivity of real-time PCR method is higher than PCR and other conventional methods such as microscopy. It is known that the real-time PCR method is more sensitive depending on the use of the probe and the closed system principle of this method[27,28]. According to the results of this study, the positive predictive value of real-time PCR was found to be higher in the identification of parasites and the results of this study support the results of the previous study.

The distribution and prevalence of spore-forming intestinal parasites are affected by personal hygiene practices, dietary habits, education level of the society, socio-economic status and climatic conditions[29,30]. In addition, the severity of spore-forming intestinal parasites infections varies according to the immunological status of the host and can be seen as asymptomatic. In long-term intestinal parasite infections, serious clinical symptoms such as anemia, malabsorption and mental retardation can be seen in children. Intestinal parasite infections are important cause of morbidity and mortality in the world. Morbidity and mortality rates of these infections can be reduced by early and rapid diagnosis. The pentaplex real-time PCR can be used in the diagnosis of intestinal parasite infection, thus preventing health problems caused by these infections.

CONCLUSION

The use of pentaplex real-time PCR method in the definition of spore-forming intestinal parasites can solve problems such as dependency on well-trained microscopists, low sensitivity in diagnosis, and loss of time in diagnosis. Although the microscopy method has limitations in diagnosis, it is still accepted as the reference standard method for routine diagnosis due to its low cost and easy applicability in endemic countries. Over the last decade, pentaplex real-time PCR and microscopy method have been compared in different countries and positive results have been obtained regarding pentaplex real-time PCR method[25,26]. The design and evaluation, optimization and validation of the pentaplex real-time PCR panel requires investment in time and cost. However, after optimization, cost-effectiveness, time saving and high efficiency in diagnosis make the pentaplex real-time PCR method more advantageous than other diagnostic methods. In this study, pentaplex real-time PCR panel was developed a fast, eficient, cost-effective and easy to perform to be used in the diagnosis of spore-forming intestinal parasites, which are very common in immunocompromised patients and overlooked due to the symptoms of other diseases. Moreover, clinical performance of the pentaplex

real-time PCR panel was validated on stool samples from variety patients. The pentaplex real-time PCR panel is suitable for the diagnosis of mixed intestinal parasites infections and its diagnostic potential can be expanded with studies including more clinical samples. The pentaplex real-time PCR can diagnose several types of parasites at the same time. This panel is one of great importance in diagnosis of spore-forming intestinal parasites infections, especially in immunocompromised patients where multiple intestinal parasites infections are common. In addition, this panel can be adapted for epidemiological research and contribute significantly to improving patient management and infection control.

Ethics Committee Approval: All stool samples were taken and processed by our routine diagnostic laboratory for routine diagnostic analysis and DNA extraction. All samples were used anonmously in this pentaplex real-time PCR panel development and permission was obtained from the Dean of Aksaray University Faculty of Medicine and the head of the Department of Medical Parasitology.

Conflict of Interest: The authors declared no conflicts of interest.

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