TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2016) 46: 1050-1058

doi:10.3906/sag-1504-69

The high prevalence of *Encephalitozoon intestinalis* in patients receiving chemotherapy and children with growth retardation and the validity of real-time PCR in its diagnosis

Ülfet ÇETİNKAYA[1], Süleyman YAZAR[1,*], Salih KUK[1], Eda SİVCAN[1], Leylagül KAYNAR[2], Duran ARSLAN[3], İzzet ŞAHİN[1]

[1]Department of Parasitology, Faculty of Medicine, Erciyes University, Kayseri, Turkey
[2]Department of Internal Medicine, Faculty of Medicine, Erciyes University, Kayseri, Turkey
[3]Department of Pediatrics, Faculty of Medicine, Erciyes University, Kayseri, Turkey

Received: 15.04.2015 • **Accepted/Published Online:** 04.10.2015 • **Final Version:** 23.06.2016

Background/aim: The aim of this study was to investigate the presence of *Encephalitozoon intestinalis* in different patient groups consisting of immunocompromised and immunocompetent individuals.

Materials and methods: The stool samples of 100 patients consisting of 25 patients receiving chemotherapy and with acute gastrointestinal complaints, 25 with bone marrow transplant and acute gastrointestinal complaints, 25 with urticaria, and 25 with growth retardation were included in the study. As control groups, 25 subjects without any chronic disease but with acute gastrointestinal complaints and 25 healthy volunteers, making a total of 50 subjects, were included in the study. *E. intestinalis* was investigated by IFA-MAbs and molecular methods.

Results: Forty percent of patients receiving chemotherapy and with acute gastrointestinal complaints, 24% of patients with bone marrow transplant and acute gastrointestinal complaints, 20% of patients with urticaria, 40% of children with growth retardation, and 28% of patients without any chronic disease but with acute gastrointestinal complaints were determined as positive.

Conclusion: To the best of our knowledge, this is the first report to assess the relationship between *E. intestinalis* and growth retardation. We think that the reliability of the use of molecular methods, especially real-time PCR, should be improved for the diagnosis of *E. intestinalis*.

Key words: *Encephalitozoon intestinalis*, prevalence, IFA-MAbs, nested PCR, probe-based real-time PCR

1. Introduction

Microsporidian pathogens are obligate intracellular parasites that infect all ages in humans and animals and have highly resistant and small spores. Of the more than 1200 species of microsporidia belonging to 144 genera, 14 species are known to cause human infections. *Encephalitozoon intestinalis*, *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, and *Enterocytozoon bieneusi* are responsible for over 90% of microsporidia infections in humans (1,2).

Encephalitozoon intestinalis causes opportunistic infections in immunocompromised patients. *E. intestinalis* may cause enteritis, cholangitis, cholecystitis, nephritis, bronchitis, sinusitis, rhinitis, and keratoconjunctivitis and disseminated infection in addition to persistent life-threatening diarrhea (1,2). It is the second most prevalent microsporidia species in humans (1,3).

The diagnosis of microsporidiosis is difficult. Light and fluorescent microscopy and trichrome staining are often used as a routine method for the detection of microsporidia. The use of molecular methods for the diagnosis of microsporidian pathogens in recent years has been accelerated (2,4).

The aim of this study was to investigate the prevalence of *E. intestinalis* in different patient groups consisting of immunocompromised and immunocompetent individuals with different diagnostic methods and to determine the sensitivity and specificity of the diagnostic methods used in this study.

2. Materials and methods

2.1. Patient and control groups

The study was conducted between 2012 and 2014 in the Erciyes University Medical Faculty Department of Parasitology. It was performed after being approved (application deadline: 05.04.2011, decision no: 2011 / 196) by the Ethics Committee of the Erciyes University Medical

* Correspondence: syazar@erciyes.edu.tr

School. Fecal samples collected from patients admitted to the Parasitology Laboratory and those of healthy volunteers were included in this study. Patient groups were formed according to the clinical diagnosis. The patient and control groups are given in Table 1.

E. intestinalis was investigated by IFA-MAbs and molecular methods (nested PCR and probe-based real-time PCR).

2.2. IFA-MAbs

All samples were evaluated by the use of a commercial kit (Bordier Affinity Products, Switzerland) following the manufacturer's instructions.

2.3. Molecular methods

2.3.1. DNA extraction

Stool samples were stored at –20 °C until DNA isolation. These samples were concentrated with the water-ether sedimentation method. The precipitate was diluted in 100 µL of distillated water. DNA was prepared using the QIAmp stool kit (QIAGEN, Germany) with minor modifications to the manufacturer's protocol. Briefly, the pellet was resuspended in 1.4 mL of ASL buffer and 0.250 g of 425–600 µm acid-washed glass beads (Sigma, USA) was added. The samples were homogenized in a mini bead-beater (BioSpec, Taiwan) for 2 min at high speed. The homogenized samples were transferred into 15 mL Falcon tubes and frozen in liquid nitrogen. The samples were then put into boiling water and boiled for 5 min. At the end of the boiling process, the samples were homogenized in a mini bead-beater for 20 s at high speed. The tubes were then centrifuged at 20000 × *g* for 2 min, and the upper phase of each sample was transferred to a different tube. For the steps of downstream processing, DNA was prepared following the manufacturer's instructions. DNA was eluted in 100 µL of AE buffer and stored at –20 °C until applied for PCR.

2.3.2. Nested PCR

First PCR reactions were performed with Micro-F and Micro-R primers (5). These primers were amplified to approximately 1300 bp 16S SSU rRNA fragments. Secondary PCR reactions used SINTF1 and SINTR primers to amplify approximately 530 bp 16S SSU rRNA fragments (6). Both PCR steps were performed in a 25 µL volume containing 20 pmol of each primer, 200 mM concentrations of each deoxynucleoside triphosphate (dNTP), 2 mM $MgCl_2$, and 5 U of Taq DNA polymerase. The primary PCR program consisted of an initial denaturation at 95 °C for 15 min; 45 cycles of denaturation at 94 °C for 60 s, annealing at 54 °C for 90 s, and elongation at 72 °C for 90 s; and a final elongation at 72 °C for 10 min. The secondary PCR program consisted of an activation step at 95 °C for 5 min and 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 60 s with a final elongation at 72 °C for 7 min.

2.3.3. Primer design and real-time PCR

We designed novel primers to amplify 62 bp 16S SSU rRNA fragments and probe as follows: E.int F (5' - cctgactggacggacagaag - 3'), E.int R (5' - ttcgtccttcatcgtcacat - 3'), and Universal ProbeLibrary (UPL) probe (Roche Cat. No. 04692179001; ctgtgctc) (Figure 1).

Amplifications were done in 20 µL reaction mixtures under the following conditions: 20 pmol of each primer, 0.4 µL UPL probe, 1X probe master, and 5 µL of DNA. The real-time PCR program consisted of an initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s with a final cooling at 40 °C for 30 s.

Dilution series of different concentrations that were prepared from plasmids containing the gene region of *E. intestinalis* 16S SSU rRNA and genomic DNA that DNA sequence analysis had confirmed as *E. intestinalis* were used as positive controls. Sterile distilled water; plasmids containing the gene region of *E. cuniculi*, *E. hellem*, and *E. bieneusi* 16S SSU rRNA; and genomic DNA samples that were positive for *Giardia intestinalis*, *Cryptosporidium parvum*, *Blastocystis hominis*, *Toxoplasma gondii*, and *Leishmania* spp. were used as negative controls.

Table 1. Patient and control groups

Groups	Disease condition	GD*	Sex (M/F)	Average age	Diarrhea (+/-)	Total
Oncology	Receiving chemotherapy	+	13/12	62.52 ± 11.36	20/5	25
BMT**	BMT	+	18/7	42.4 ± 13.42	20/5	25
Urticaria	Urticaria	-	11/14	34.66 ± 14.56	0/25	25
Growth retardation	Growth retardation	-	10/15	8.16 ± 3.42	3/22	25
Control 1	Without chronic illness	+	12/13	46.88 ± 16.08	12/13	25
Control 2	Healthy volunteers	-	15/10	29.77 ± 9.9	0/25	25

*GD: Gastrointestinal disorders, **BMT: Bone marrow transplantation.

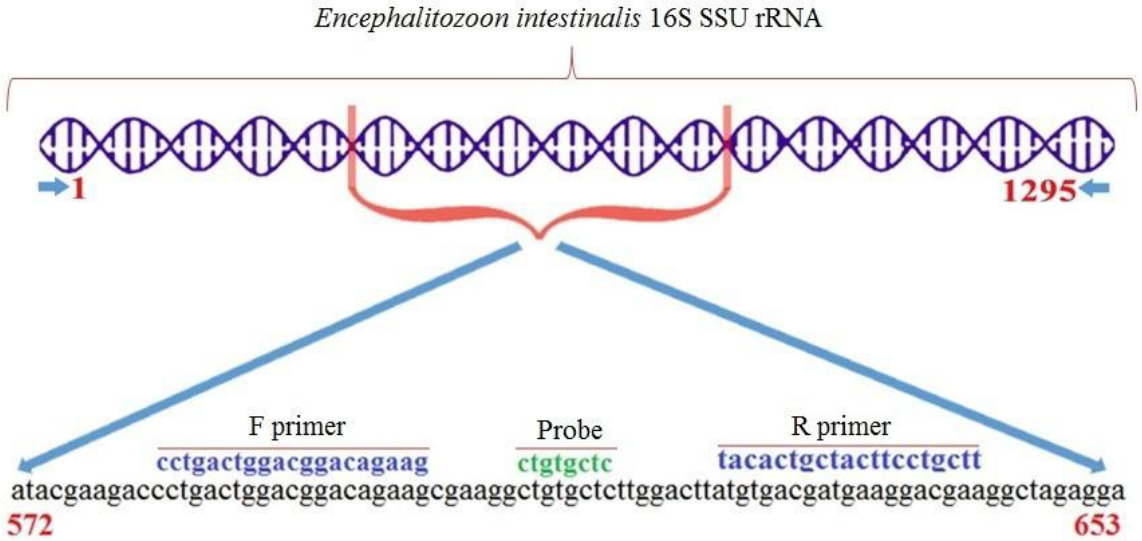

Figure 1. *Encephalitozoon intestinalis*-specific primers and probe.

2.4. Statistical analysis

The chi-square exact test was applied for the comparison of categorical variables. Multiple comparisons of chi-square tests were made with the Bonferroni correction Z-test. The normal distribution of numerical variables was inspected with the Shapiro–Wilk normality test. For two groups a comparison with independent samples was performed with the t-test and two-group comparisons were performed with one-way variance analysis. Turkey's HDS test was used for a multiple comparison test and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Specificity and sensitivity of probe-based real-time PCR

The sensitivity of the probe-based real-time PCR reaction was tested by using serial dilutions prepared from plasmids containing the gene region of *E. intestinalis* 16S SSU rRNA (Figure 2). No amplification (Ct > 40) was obtained with plasmids that contained the gene region of *E. cuniculi*, *E. hellem*, and *E. bieneusi* 16S SSU rRNA; from genomic DNA samples that were positive for *G. intestinalis*, *C. parvum*, *B. hominis*, *T. gondii*, and *Leishmania* spp.; or from sterile distilled water.

3.2. Prevalence of *Encephalitozoon intestinalis*

The positivity rate of *E. intestinalis* on the basis of the results obtained with probe-based real-time PCR was identified. The distribution of *E. intestinalis* was investigated according to sex, age, research groups, and diarrhea (Table 2).

As can be seen in Table 2, no significant differences were found regarding *E. intestinalis* infection between sexes ($P = 0.459$) and diarrhea presence ($P = 0.241$). There was a statistically significant relationship between *E. intestinalis* infection and age groups ($P = 0.007$). *E. intestinalis* infection was found higher in the age groups of 0–17, 41–50, and 51–60 but it was not found in the 31–40 age group. There was also a relationship between *E. intestinalis* infection and the research groups ($P = 0.043$). The lowest rate of *E. intestinalis* infection was in control group II, namely the healthy volunteers. The highest rate of *E. intestinalis* infections was in the oncology patients and children with growth retardation.

3.3. *Encephalitozoon intestinalis* load in stool samples

Parasitic load was calculated based on the crossing point (Cp) values that were obtained as a result of real-time PCR analysis. The Cp values according to sex, age, research groups, and diarrhea and statistical analysis are given in Table 3.

As can be seen in Table 3, no significant differences were found regarding the parasitic load among sexes ($P = 0.346$), age ($P = 0.619$), and diarrhea condition ($P = 0.163$). There was also a relationship between the parasitic load and research groups ($P = 0.000$). The highest parasitic load was in control group I.

3.4. Evaluation of the sensitivity and specificity of IFA-MAbs and nested PCR for detection of *Encephalitozoon intestinalis*

According to the results obtained with probe-based real-time PCR, the sensitivity and specificity of IFA-MAbs and nested PCR for the detection of *E. intestinalis* were determined (Table 4). Twelve (8%) and 29 (19%) of 150 patients were evaluated as positive by nested PCR (Figure 3) and IFA-MAbs (Figure 4), respectively.

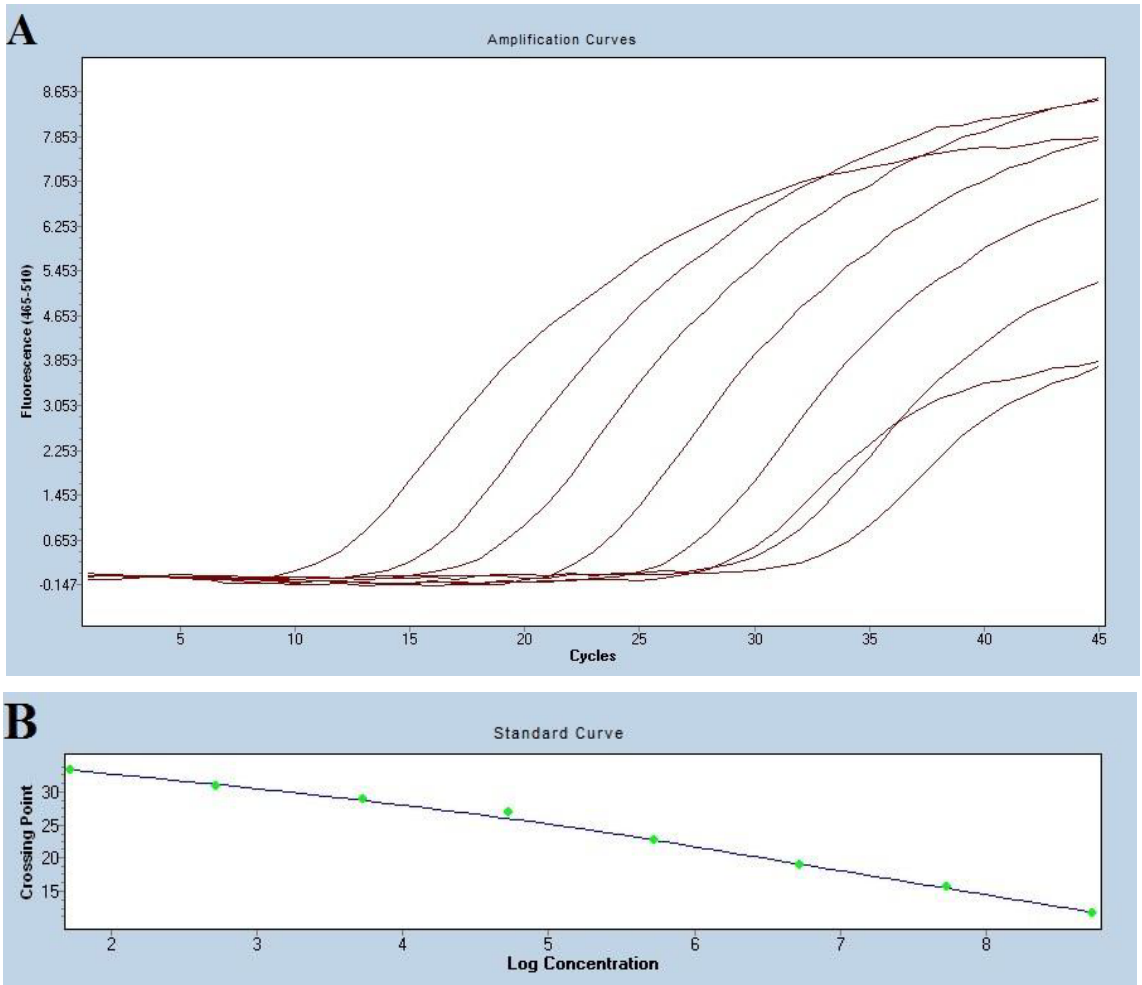

Figure 2. Analysis by probe-based real-time PCR of serial dilutions prepared from plasmids that contain the gene region of *E. intestinalis* 16S SSU rRNA. A) Amplification curve. B) Standard curve.

Twelve (8%) patients were found to be positive by both nested PCR and real-time PCR, while 27 (10%) patients were found to be positive only by real-time PCR. When the probe-based real-time PCR test was accepted as a reference, it was found that nested PCR had a positive predictive value of 100%, a negative predictive value of 80.4%, sensitivity of 30.8%, and specificity of 100%.

Twenty-two (14.7%) patients were found to be positive by both IFA-MAbs and real-time PCR, while 17 (11.3%) patients were found to be positive by only real-time PCR. When the probe-based real-time PCR test was accepted as a reference, it was found that IFA-MAbs had a positive predictive value of 75.9%, a negative predictive value of 86%, sensitivity of 56.4%, and specificity of 93.7%.

4. Discussion

The spores of *E. intestinalis* cannot be identified by conventional stool examination methods because of their small size; therefore, there is a need for different diagnostic methods (1,2,7). Monoclonal antibodies are one of the methods used for diagnosis. This method is accepted as the gold standard by some researchers for the diagnosis of *E. intestinalis* because it is faster and cheaper than PCR (8,9). However, some researchers assert that samples evaluated as positive with IFA-MAbs need to be confirmed by other methods (10,11). In recent years, many researchers have preferred to use molecular methods for diagnosis (12–15). In our study, nested PCR and real-time PCR methods were used. According to the obtained results, we determined that there were no false negative results by nested PCR. However, when compared with real-time PCR, the sensitivity of nested PCR was quite low. Many researchers have reported that probe-based real-time PCR methods have 100% sensitivity and specificity. It was reported that 10^2 spores/mL in stool can be detected by probe-based real-time PCR methods (12–16). Therefore, in the present

Table 2. The distribution of *E. intestinalis* according to sex, age, research groups, and diarrhea.

Group		Positive	Negative	Total
		n (%)	n (%)	n (%)
Sex	Male	18 (22.8)	61 (77.2)	79 (52.7)
	Female	21 (29.6)	50 (70.4)	71 (47.3)
	Total	39 (26)	111 (74)	150 (100)
Age	0–17 (n: 25)	10 (40)	15 (60)	25 (16.7)
	18–30 (n: 37)	7 (18.9)	30 (81.1)	37 (24.7)
	31–40 (n: 23)	-	23 (100)	23 (15.3)
	41–50 (n: 22)	7 (31.8)	15 (68.2)	22 (14.7)
	51–60 (n: 21)	10 (47.6)	11 (52.4)	21 (14)
	>61 (n: 22)	5 (22.7)	17 (77.3)	22 (14.7)
	Total	39 (26)	111 (74)	150 (100)
Diarrhea	Positive	18 (33.3)	36 (66.7)	54 (36)
	Negative	21 (21.9)	75 (78.1)	96 (64)
	Total	39 (26)	111 (74)	150 (100)
Groups	Oncology (n: 25)	10 (40)	15 (60)	25 (16.7)
	*BMT (n: 25)	6 (24)	19 (76)	25 (16.7)
	Urticaria (n: 25)	5 (20)	20 (80)	25 (16.7)
	Growth retardation (n: 25)	10 (40)	15 (60)	25 (16.7)
	Control 1 (n: 25)	7 (28)	18 (72)	25 (16.7)
	Control 2 (n: 25)	1 (4)	24 (96)	25 (16.7)
	Total	39 (26)	111 (74)	150 (100)

*BMT: Bone marrow transplantation, Control 1: Patients with acute gastrointestinal complaints but without any chronic disease, Control 2: Healthy volunteers.

study, the results were achieved with probe-based real-time PCR.

Microsporidian pathogens are opportunistic pathogens and are most commonly found in immunocompromised patients (1,3). They can also cause serious infections in patients receiving chemotherapy and in bone marrow transplant patients and may have an adverse effect on the treatment process (17–20). In this study, it was found that *E. intestinalis* was prevalent in patients receiving chemotherapy (40%) and in bone marrow transplant patients admitted with acute gastrointestinal complaints (24%). It should be remembered that *E. intestinalis* appears to have a primary infection site in the small bowel, but it has also been found in the duodenum, jejunum, ileum, colon, kidney, liver, and gallbladder and other sites, including the lower respiratory tract (1–3). This situation can adversely affect both the patient's quality of life and the treatment process. We think that patients receiving chemotherapy and bone marrow transplant patients, especially those with gastrointestinal complaints, should be regularly evaluated in terms of *E. intestinalis* to improve the quality of life and to protect them from parasitic infections.

One of our research groups included patients with urticaria. Urticaria is a skin disease that can appear as red, itchy, swollen areas of the skin that can range in size; it can occur anywhere on the body but is mainly found on the trunk, arms, or legs. Urticaria is defined as chronic when it persists for longer than 6 weeks. The etiology is unknown in approximately 70% of chronic urticaria cases. Sensitivity to nutrients takes first place among the causes identified (21,22). Studies on the role of parasites in the etiology of urticaria have shown that the most responsible protozoa are *G. intestinalis* and *B. hominis* (23,24). The number of studies on the role of microsporidian pathogens in the etiology of urticaria is quite low. We found only one study conducted so far, in which the incidence of microsporidian pathogens was reported as 19.7%, but it did not specify species (25). No data have been found

Table 3. Crossing point values according to sex, age, research groups, and diarrhea and statistical analysis.

Group		Number of positive patients	Crossing point values				
			Min–max	Average	SD	F	P
Sex	Male (n: 79)	18	29.78–38.29	33.88	1.78	33.818	0.346
	Female (n: 71)	21	27.63–36.86	33.21	2.82		
Age	0–17 (n: 25)	10	29.27–36.31	34.35	2.07	0.667	0.619
	18–30 (n: 37)	7	30.54–36.86	33.79	2.21		
	31–40 (n: 23)	-	-	-	-		
	41–50 (n: 22)	7	29.78–38.29	33.14	2.87		
	51–60 (n: 21)	10	28.65–36.22	33.40	2.37		
	>61 (n: 22)	5	27.63–34.98	32.25	3.39		
Diarrhea	Positive (n: 54)	18	27.63–34.95	32.9	2.45	36	0.163
	Negative (n: 96)	21	28.65–38.29	34	2.45		
Groups	Oncology (n: 25)	10	33.55–34.95	34.55	0.9	13.501	0.000
	*BMT (n: 25)	6	31.77–34.8	33.25	1.21		
	Urticaria (n: 25)	5	32.51–38.29	35.23	2.42		
	Growth retardation (n: 25)	10	29.27–36.31	34.35	2.07		
	Control 1 (n: 25)	7	27.63–30.94	29.56	1.12		
	Control 2 (n: 25)	1	-	-	-		

*BMT: Bone marrow transplantation, Control 1: Patients with acute gastrointestinal complaints but without any chronic disease, Control 2: Healthy volunteers.

Table 4. Sensitivity and specificity of IFA-MAbs and nested PCR for detection of *Encephalitozoon intestinalis*.

Methods		Real-time PCR		Sensitivity	Specificity
		Positive	Negative		
IFA-MAbs	Positive	22	7	56.4%	93.7%
	Negative	17	104		
Nested PCR	Positive	12	-	30.8%	100%
	Negative	27	111		

in the literature concerning the relationship between *E. intestinalis* and urticaria. In this study, it was found that *E. intestinalis* was prevalent in patients with urticaria (20%). It is very important to determine the cause of urticaria, which adversely affects the activities of daily living and quality of life of patients, in order to give the correct therapy. Intestinal parasites also cause various symptoms in the nervous system, the urogenital tract, and the skin, in addition to gastrointestinal complaints. At the same time, it should be remembered that *E. intestinalis* can also cause infections in immunocompetent individuals (1–3), and patients with urticaria, especially of unknown cause, should be regularly evaluated in terms of *E. intestinalis*.

Another of our research groups included children with growth retardation. Growth and developmental retardation occur due to various reasons such as eating disorders, malabsorption, intestinal parasites, hormonal reasons, family reasons, and chronic diseases. Intestinal parasites can cause malnutrition in addition to gastrointestinal disorders such as vomiting, diarrhea, and appetite changes and may lead to failure in the physical and mental development of the child (26). Although

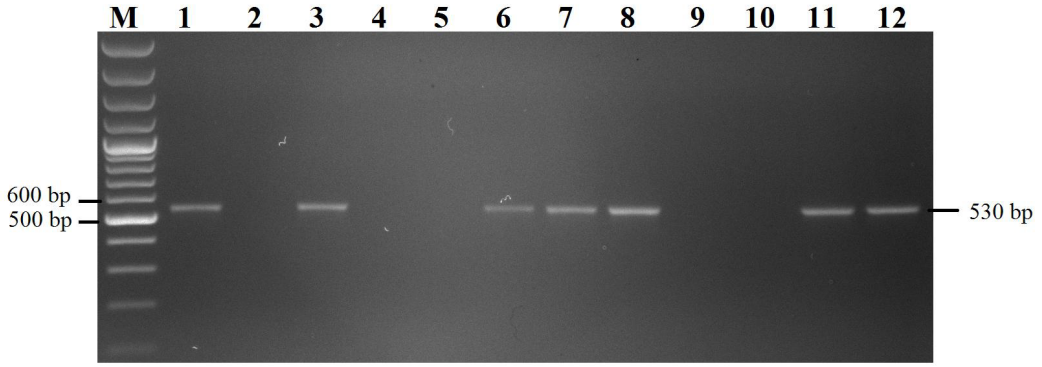

Figure 3. Agarose gel showing amplifications of nested PCR of *E. intestinalis.* M: 100-bp Plus DNA Ladder; lane 1: positive control, lane 2: negative control; lanes 3, 6–8, 11, 12: *E. intestinalis*-positive samples; 4, 5, 9, 10: *E. intestinalis*-negative samples.

microsporidian pathogens have been reported in children in previous studies (27–29), to the best of our knowledge, this is the first report to assess the relationship between *E. intestinalis* and growth retardation. In our study, it was found that *E. intestinalis* was prevalent in children with growth retardation (40%). We think that it is necessary to evaluate children with growth retardation in terms of *E. intestinalis* regardless of the presence of gastrointestinal complaints.

Another of our research groups included patients with acute gastrointestinal complaints but without any chronic disease. The most typical symptoms of intestinal microsporidiosis are watery diarrhea (without blood and leukocytes), loss of appetite, and weight loss. As a result of the development of diagnostic methods and increasing awareness, it has been established that *E. intestinalis* may cause acute and self-limiting diarrhea not only in immunocompromised patients but also in immunocompetent individuals. Therefore, many studies concerning *E. intestinalis* have focused on patients with gastrointestinal complaints (1–3). Microsporidian pathogens were reported to have a prevalence ranging from 6.5% to 83% in studies in patients with diarrhea (30–34). In the present study, it was found that *E. intestinalis* was prevalent in patients with acute gastrointestinal complaints but without any chronic disease (28%). Diarrhea is a clinical condition that adversely affects people's daily activities and causes a large amount of fluid and electrolyte loss. Determining the cause of diarrhea is very important for proper treatment. We think that patients with acute and chronic gastrointestinal complaints but without any chronic disease should be evaluated in terms of *E. intestinalis*.

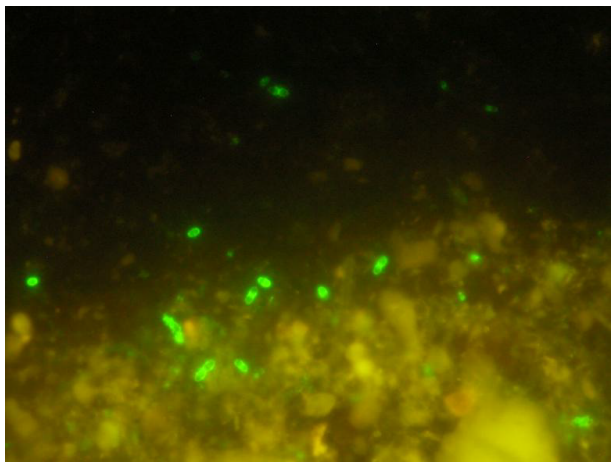

Figure 4. *E. intestinalis* spores in positive sample with IFA-MAbs (1000×).

In conclusion, *E. intestinalis* was found to be common not only in immunocompromised patients but also in children with growth failure, in patients with urticaria, and in patients with acute gastrointestinal complaints. In nested PCR, which has low sensitivity, the probability of false-negative results is high, while in IFA-MAbs, which has low sensitivity and specificity, the probability of false-negative or false-positive results is high. One of the most important limitations of this study is that each study group was composed of only 25 patients. At the same time, the relationships between sociodemographic factors/patients' symptoms and the presence of parasites/parasite load were not demonstrated in detail. In future studies the relationship between these factors and *E. intestinalis* should be investigated in detail. We think that *E. intestinalis* should be investigated in other diseases, especially in diseases of unknown cause.

Acknowledgment

The work was supported by the Erciyes University Scientific Research Projects Unit, Turkey (Project No. TDK - 2013 - 4511).

References

1. Curry A. Microsporidiosis. In: Cox FEG, Wakelin D, Gillespie SH, Despommier DD, editors. Topley and Wilson's Microbiology and Microbial Infections; Vol. 5: Parasitology. 10th ed. Washington, DC, USA: ASM Press; 2005. pp. 529-555.
2. Franzen C, Muller A. Microsporidiosis: human diseases and diagnosis. Microbes Infect 2001; 3: 389-400.
3. Kotler DP, Orenstein JM. Clinical syndromes associated with microsporidiosis. Adv Parasitol 1998; 40: 321-349.
4. Franzen C, Müller A. Molecular techniques for detection, species differentiation and analysis of microsporidia. Clin Microbiol 1999; 12: 243-285.
5. Visvesvara GS, da Silva AJ, Croppo GP, Pieniazek NJ, Leitch GJ, Ferguson D, de Moura H, Wallace S, Slemenda SB, Tyrrell I. In vitro culture and serologic and molecular identification of *Septata intestinalis* isolated from urine of a patient with AIDS. J Clin Microbiol 1995; 33: 930-936.
6. Da Silva AJ, Slemenda SB, Visvesvara GS, Schwartz DA, Wilcox CM, Wallace S, Pieniazek NJ. Detection of *Septata intestinalis* (Microsporidia) Cali et al. 1993 using polymerase chain reaction primers targeting the small submit subunit ribosomal RNA coding region. Mol Diagn 1997; 2: 47-52.
7. Weber R, Bryan RT, Schwartz DA, Owen RL. Human microsporidial infections. Clin Microbiol 1994; 7: 426-461.
8. Alfa Cisse O, Ouattara A, Thellier M, Accoceberry I, Biligui S, Minta D, Doumbo O, Desportes-Livage I, Thera MA, Danis M et al. Evaluation of an immunofluorescent-antibody test using monoclonal antibodies directed against *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* for diagnosis of intestinal microsporidiosis in Bamako (Mali). J Clin Microbiol 2002; 40: 1715-1718.
9. Enriquez FJ, Ditrich O, Palting JD, Smith K. Simple diagnosis of *Encephalitozoon* sp. microsporidial infections by using a panspecific antiexospore monoclonal antibody. J Clin Microbiol 1997; 35: 724-729.
10. Garcia LS, Shimizu RY, Bruckner DA. Detection of microsporidial spores in fecal specimens from patients diagnosed with cryptosporidiosis. J Clin Microbiol 1994; 32: 1739-1741.
11. Didier ES, Orenstein JM, Aldras A, Bertucci D, Rogers, LB, Janney FA. Comparison of three staining methods for detecting microsporidia in fluids. J Clin Microbiol 1995; 33: 3138-3145.
12. Hester JD, Varma M, Bobst AM, Ware MW, Lindquist HD, Schaefer FW. Species-specific detection of three human-pathogenic microsporidial species from the genus Encephalitozoon via fluorogenic 5' nuclease PCR assays. Mol Cell Probes 2002; 16: 435-444.
13. Menotti J, Cassinat B, Sarfati C, Liguory O, Derouin F, Molina JM. Development of a real-time PCR assay for quantitative detection of *Encephalitozoon intestinalis* DNA. J Clin Microbiol 2003; 41: 1410-1413.
14. Verweij JJ, Ten Hove R, Brienen EA, van Lieshout L. Multiplex detection of *Enterocytozoon bieneusi* and *Encephalitozoon* spp. in fecal samples using real-time PCR. Diagn Microbiol Infect Dis 2007; 57: 163-167.
15. Wolk DM, Schneider SK, Wengenack NL, Sloan LM, Rosenblatt JE. real-time PCR method for detection of *Encephalitozoon intestinalis* from stool specimens. J Clin Microbiol 2002; 40: 3922-3928.
16. Rinder H, Janitschke K, Aspöck H, Da Silva AJ, Deplazes P, Fedorko DP, Franzen C, Futh U, Hünger F, Lehmacher A et al. Blinded, externally controlled multicenter evaluation of light microscopy and PCR for detection of microsporidia in stool specimens. J Clin Microbiol 1998; 36: 1814-1818.
17. Lono AR, Kumar S, Chye TT. Incidence of microsporidia in cancer patients. J Gastrointest Cancer 2008; 39: 124-129.
18. Karaman Ü, Atambay M, Daldal N, Çolak C. Kanser tanısı almış hastalarda microsporidium görülme sıklığı. Türkiye Parazitol Derg 2008; 32: 109-112 (in Turkish).
19. Hernández-Rodríguez OX, Alvarez-Torres O, Ofelia Uribe-Uribe N. Microsporidia infection in a Mexican kidney transplant recipient. Case Rep Nephrol 2012; 2012: 928083.
20. Hamamci B, Çetinkaya Ü, Berk V, Kaynar L, Kuk S, Yazar S. Kemoterapi alan kanserli hastalarda *Encephalitozoon intestinalis* ve *Enterocytozoon bieneusi* prevalansı. Mikrobiyol Bul 2015; 49: 105-113 (in Turkish).
21. Wai YC, Sussman GL. Evaluating chronic urticaria patients for allergies, infections, or autoimmune disorders. Clin Rev Allergy Immunol 2002; 23: 185-193.
22. Sicherer SH, Leung DY. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects. J Allergy Clin Immunol 2006; 118: 170-177.
23. Verma R, Delfanian K. Blastocystis hominis associated acute urticaria. Am J Med Sci 2013; 346: 80-81.
24. Doğruman Al F, Adişen E, Kuştimur S, Gürer MA. The role of protozoan parasites in etiology of urticaria. Turkiye Parazito Derg 2009; 33: 136-139.
25. Karaman Ü, Şener S, Çalık S, Şaşmaz S. Investigation of microsporidia in patients with acute and chronic urticaria. Mikrobiyol Bul 2011; 45: 168-173.
26. Bhandari N, Kausaph V, Neupane GP. Intestinal parasitic infection among school age children. J Nepal Health Res Counc 2011; 9: 30-32.
27. Wanachiwanawin D, Chokephaibulkit K, Lertlaituan P, Ongrotchanakun J, Chinabut P, Thakerngpol K. Intestinal microsporidiosis in HIV-infected children with diarrhea. Southeast Asian J Trop Med Public Health 2002; 33: 241-245.
28. Termmathurapoj S, Engkanun K, Naaglor T, Taamsri P, Wirote A, Leelayoova S, Mungthin M. Cross-sectional study of intestinal protozoan infections in orphans and childcare workers at the Phayathai babies' home, Bangkok Thailand. J Trop Med Parasitol 2000; 51: 345-349.

29. Calik S, Karaman U, Colak C. Prevalence of microsporidium and other intestinal parasites in children from Malatya, Turkey. Indian J Microbiol 2011; 51: 345-349.

30. Field AS, Hing MC, Milliken ST, Marriott DJ. Microsporidia in the small intestine of HIV-infected patients. A new diagnostic technique and a new species. Med J Aust 1993; 158: 390-394.

31. Rabeneck L, Gyorkey F, Genta RM, Gyorkey P, Foote LW, Risser JM. The role of Microsporidia in the pathogenesis of HIV-related chronic diarrhea. Ann Intern Med 1993; 19: 895-899.

32. Türk S, Doğruman Al F, Karaman U, Kuştimur S. Investigation of Microsporidia prevalence by different staining methods in cases of diarrhea. Mikrobiyol Bul 2012; 46: 85-92.

33. Atambay M, Karaman Ü, Daldal N, Çolak C. İnönü Üniversitesi Turgut Özal Tıp Merkezi Parazitoloji Laboratuvarına gelen erişkin hastalarda microsporidium görülme sıklığı. Türkiye Parazitol Derg 2008. 32: 113-115 (in Turkish).

34. Karaman U, Daldal N, Atambay M, Çolak C. The epidemiology of microsporidias in humans (Malatya sample). Turk J Med Sci 2009; 39: 281-288.§