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Molecular Identification of *Encephalitazoon intestinalis* and the Prevalence of Renal Microsporidiosis in Renal Transplant Recipients in Türkiye

Renal Transplant Alıcılarında Renal Microsporidiosis Prevalansı ve Encephalitazoon intestinalis Moleküler Karakterizasyonu

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ABSTRACT

Objective: In patients with end-stage kidney disease, kidney transplantation is the kidney replacement therapy option that provides the most successful survival. However, immunosuppression agents administered after kidney transplantation can increase the risk of opportunistic infections. Microsporidia are obligate intracellular pathogens that can be fatal in immunosuppressed patients. The present study aimed to determine the prevalence of microsporidia in kidney transplantation recipients and the molecular characterization of the detected species.

Methods: To evaluate the prevalence of renal microsporidiosis in kidney transplant recipients, the urine samples from a total of 325 patients were analyzed by real-time and nested polymerase chain reaction for *Encephalitozoon* spp. and *Enterocytozoon bieneusi*.

Results: Only one (0.4%) sample from the adult patient was positive for the *Encephalitozoon* species, while no positivity was found in pediatric patients. It was determined as *Encephalitozoon intestinalis* by *ITS rRNA* gene region sequence analysis. A microsporidia species obtained from humans in Türkiye has been characterized for the first time and registered in GenBank.

Conclusion: Our epidemiological results show that the prevalence of renal microsporidiosis in kidney transplant recipients is very low. In addition, as a result of the phylogenetic analysis of the detected isolate, it was observed that it was 100% identical to the isolates reported from dogs in Kayseri, Türkiye. This situation provided essential data regarding the zoonotic transmission dynamics of microsporidia.

Keywords: Renal microsporidiosis, *Encephalitozoon intestinalis*, zoonotic transmission, kidney transplantation, Türkiye

ÖZ

Amaç: Böbrek nakli, son dönem böbrek yetmezliği olan hastalarda en başarılı sağkalım sağlayan renal replasman tedavi seçeneğidir. Ancak böbrek nakli sonrasında uygulanan immün baskılayıcı ajanlar fırsatçı enfeksiyon riskini artırmaktadır. Microsporidialar, immün sistemi baskılanmış hastalarda ölümcül olabilen zorunlu hücre içi patojenlerdir. Bu çalışmada böbrek nakil hastalarında microsporidia prevalansının belirlenmesi ve tespit edilen türlerin moleküler karakterizasyonunun yapılması amaçlandı.

Yöntemler: Böbrek nakli hastalarında renal microsporidiosis prevalansını değerlendirmek için toplam 325 hastadan alınan idrar örnekleri *Encephalitozoon* spp. ve *Enterocytozoon bieneusi* açısından gerçek zamanlı ve nested polimeraz zincir reaksiyonu ile analiz edildi.

Bulgular: Erişkin hastalardan sadece biri (%0,4) *Encephalitozoon* türleri yönünden pozitif belirlendi, çocuk hastalarda ise pozitiflik saptanmadı. *ITS rRNA* gen bölgesi sekans analizi sonucunda tespit edilen türün *Encephalitozoon intestinalis* olduğu görüldü. Bu çalışma ile Türkiye'de ilk kez insanlardan izole edilen bir microsporidia türü karakterize edilerek GenBank'a kaydedildi.

Sonuç: Elde edilen epidemiyolojik sonuçlar, renal transplant hastalarında renal microsiporidiosis prevalansının çok düşük olduğunu göstermektedir. Ayrıca tespit edilen izolatın filogenetik analizi sonucunda Kayseri'de köpeklerden bildirilen izolatlarla %100 benzer olduğu görüldü. Bu çalışma microsporidiaların zoonotik bulaşma dinamikleri açısından önemli bir veri sağlamaktadır.

Anahtar Kelimeler: Renal microsporidiosis, *Encephalitozoon intestinalis*, zoonotik bulaş, böbrek nakli, Türkiye

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Microsporidia are spore-forming, single-celled obligate intracellular pathogens that can infect many vertebrates and invertebrate hosts. There are approximately 200 genera and over 1200 species described to date. Fourteen species of eight genera are known to cause infections in humans. *Enterocytozoon* (*E.*) *bieneusi*, *Encephalitozoon* (*E.*) *cuniculi*, *E. hellem* and *E. intestinalis* are the most important species that infect humans and have zoonotic characteristics (1-3). Although these pathogens can also be found in immunocompetent individuals, they are considered one of the most important opportunistic pathogens that cause life-threatening infections, especially in immunocompromised patients (3,4). The first target of the parasite during infection is the small intestinal enterocytes. Therefore, it causes gastrointestinal tract infections and persistent, life-threatening diarrhea and malabsorption, especially in immunocompromised patients. However, infections caused by species of *Encephalitozoon* are not limited by intestinal system diseases. They can also be found in lamina propria macrophages, fibroblasts, and endothelial cells that cause disseminated infections (3,5,6). Microsporidian pathogens are a group that cannot be detected by routine parasitological examinations and require unique methods for diagnosis (3). For this reason, it can easily be missed in routine laboratory tests.

In patients with chronic kidney disease (CKD), kidney replacement therapy requirements can onset since the glomerular filtration rate decreases below 15 mL/min/1.73 m^2 (7). Two treatment options are available at this stage; dialysis or kidney transplantation. Kidney transplantation is the preferred treatment modality due to providing more prolonged survival, a better quality of life, and cost-effectiveness (7). Although the immunosuppressive drugs in kidney transplant recipients (KTR) ensure minimizing acute rejection or chronic allograft nephropathy, they can lead to unfavorable side effects and increase the risk of opportunistic infections. Therefore, KTR should be closely monitored regarding the exceptionally infectious complications in the post-transplant period (8,9).

Because of their lifetime immune suppression, solid organ transplant recipients represent the group of patients at the highest risk for microsporidia infections. Undiagnosed kidney infections can cause proteinuria or deterioration in graft function. In addition, spores are transported from the kidney to the ureters and bladder in the urine, where they can infect the transitional epithelium and cause micro- or macro-hematuria (10). In small case series, it was shown that these pathogens infect the kidney and urinary tract (11,12). However, there is still no large-scale study evaluating the risk factors and clinical features of renal microsporidiosis in KTR.

The present study aimed to determine the prevalence of microsporidia, which can be easily missed in routine follow-ups, in KTR and the molecular characterization of the detected species. It also aimed to evaluate the clinical findings of positive cases to reveal possible conditions that the parasite may cause.

METHODS

Ethics Statement

This study has been approved by the Ethics Committee of the Faculty of Medicine Erciyes University Türkiye (application deadline: 03.03.2021, no: 2021/172). Written informed consent was obtained from every patient before the examination.

Patients and DNA Extractions

Between May and December 2021, urine samples were collected from 325 KTR, 276 (84.9%) adults, and 49 (15.1%) children in the kidney transplantation outpatient clinic. Thirty-four (69.4%) of the pediatric patients were male, 15 (30.6%) were female, and their mean age was 11.7 (±4.64). Of the adult patients, 165 (59.5%) were male, 111 (40.5%) were female, and the mean age was 43.7 (±13.04). The characteristics and symptoms of patients included in the study are shown in Table 1.

When the patients applied to the hospital for their routine follow-up, approximately 50 mL of urine samples were taken and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded, while the pellet at the bottom was washed twice with 1 mL of sterile PBS following the same centrifugation conditions. After washing, the supernatant was removed, and the pellet was used for DNA isolation. DNA was isolated using the GeneAll® Exgene Cell SV Mini Kit (GeneAll Biotechnology, Seoul, South Korea) according to the manufacturer's recommendation. The DNA isolation was performed with a negative control and a positive control containing ATCC 50506 *E. intestinalis* spores. Acquired DNA was stored at -20 °C.

Molecular Methods

Real-time polymerase chain reaction (PCR) and Nested PCR analyses were performed on all samples. For real-time PCR, primers MsRTf1 and MsRTr1 amplifying the 258-319 bp region of SSU-rRNA were used as previously reported by Polley et al. (13). Amplifications were performed in 20 µL of total volume, containing 8 µL of distilled water, 0.5 µL of both reverse and forward primers (20 µM), 10 µL 2x SYBR-Green Master Mix (Roche Diagnostic, Germany) and 1 µL of genomic DNA. The thermal profile for real-time PCR amplification consists of the following steps: Initial incubation at 98 °C for 3 min., 45 cycles of 98 °C for 15 s., 55 °C for 20 s., and 72 °C for 10 s. followed by 0.5 °C increase in every 2nd between 65-95 °C for melting curve analysis.

The nested PCR protocol reported by Katzwinkel-Wladarsch et al. (14) and Buckholt et al. (15) amplifying the ITS region of *E. bieneusi* and *Encephalitozoon* spp. using microsporidia-specific primers was performed. The tubes were placed in the thermal cycler. The thermal profile was for the first PCR steps at 95 °C for 4 min, 35 cycles at 95 °C for 30 seconds, at 40 °C (*Encephalitozoon* species) at 47 °C (*E. bieneusi*) for 30 seconds, and at 72 °C for 1 minute; it was then set at 72 °C for 10 minutes. The second PCR steps are the same as the first, and amplification will is performed only by setting the annealing degree to 46 °C for the *Encephalitozoon* species and 57 °C for *E. bieneusi* (14,15).

DNA Sequencing and Phylogenetic Analysis

ITS gene regions were sequenced on an Applied Biosystems (ABI) 3500 Sanger sequencing platform using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). Geneious11.0.2 software was used to analyze the sequence reads (16). Sequences were aligned with previously published sequences using the BLASTn. Multiple sequence alignments were performed using the MAFFT server. The DNA sequence of the variant reported in this study was submitted to GenBank with the accession number ON182064. The phylogenetic tree was generated using the ultrafast bootstrap (UFBoot) IQ-TREE web server (17). According to BIC (Bayesian Information Criterion), the most suitable model was F81+F+G4. A bootstrap

Table 1. Demographic, clinical and laboratory parameters of kidney transplantation recipients involved in the study

	Adults (n=276)	Children (n=49)
Age, years, average (range)	43.7 (19-80)	11.7 (2-18)
Sex		
Male	165 (59.5%)	34 (69.4%)
Female	111 (40.5%)	15 (30.6%)
Allograft survival, months, average (range)	84.5 (2-336)	42.6 (4-156)
Etiology of CKD		
Hypertension	105 (38.1%)	-
Diabetes mellitus	26 (9.4%)	-
Primary glomerular disease	31 (11.2%)	11 (22.5%)
Polycystic kidney disease	6 (2.2%)	3 (6.1%)
Nephrolithiasis	10 (3.6%)	1 (2%)
Congenital anomalies of urinary tract	30 (10.9%)	21 (42.9%)
Others	28 (10.1%)	7 (14.3%)
Unknown	40 (14.5%)	6 (12.2)
Dialysis history		
HD	149 (54%)	6 (12.2%)
PD	44 (15.9%)	19 (38.8%)
HD+PD	24 (8.7%)	1 (2%)
Transplantation preempitive	59 (21.4%)	23 (47%)
Comorbidity		
Yes	94 (34.1%)	10 (20.4%)
No	182 (65.9%)	39 (79.6%)
Donor		
Living	238 (86.2%)	42 (85.7%)
Deceased	38 (13.8%)	7 (14.3%)
Immunosuppressive regimen		
CNI+MMF+steroid	247 (89.5%)	33 (67.3%)
CNI+mTORi+steroid	10 (3.6%)	8 (16.3%)
MMF+mTORi+steroid	3 (1.1%)	1 (2%)
CNI+steroid	16 (5.8%)	1 (2%)
CNI+MMF	-	4 (8.2%)
MMF+mTORi	-	2 (4.1%)
Laboratory parameters		
Proteinuria	30 (10.9%)	3 (6.1%)
Hematuria	36 (13%)	3 (6.1%)
Hematuria+proteinuria	20 (7.2%)	1 (2%)
BUN (average, range, mg/dL)	19.6 (7-39.7)	18.6 (5.5-58.5)
Creatinine (average, range, mg/dL)	1.3 (0.6-7.3)	0.9 (0.28-1.84)
Hemoglobin (average, range g/dL)	13.1 (7.2-17.3)	11.93 (8.2-16.7)
Leukocyte (average, range, per µL)	8182 (1390-14000)	8513 (3950-20030)
Lymphocyte (average, range, per µL)	2050 (130-13000)	3674 (660-28400)

CKD: Chronic kidney disease, CNI: Calcineurin inhibitors, MMF: Mycophenolate mofetil, mTORi: Mammalian Target of Rapamycin inhibitors, BUN: Blood urea nitrogen, HD: Hemodialysis, PD: Peritoneal dialysis

test with 1000 repetitions was used to determine the reliability of the trees created by maximum likelihood (ML) analysis.

Statistical Analysis

Since the number of positive patients was only one, statistical analysis could not be performed according to patient characteristics and clinical findings.

RESULTS

According to the results of SYBR green real-time PCR analysis of urine samples, all 49 pediatric patients were negative for microsporidian pathogens. Only one (0.4%) of 276 adult patients was found positive. Also, according to the ITS rRNA PCR analysis result, all the pediatric patients were negative. Amplicons of

approximately 300 bp were detected in an adult patient (real-time PCR positive). The sequence analyses of the PCR product showed 100% identity to *E. intestinalis*. In this study, no *E. hellem*, *E. bieneusi* or *E. cuniculi* infection was found. A 40-year-old female patient with a positive PCR test had developed renal failure after Lupus Nephritis. Peritoneal dialysis was first applied for approximately four years as renal replacement therapy, and then a kidney was transplanted from a living donor eight years ago. She did not experience any rejection attacks in her follow-up. She still has a functional allograft and was on tacrolimus, mycophenolate mofetil, and prednisolone at the time of urine sampling for the study. No additional leukopenia was detected in the hemogram examination. In addition, there is no known further disease other than hypertension.

The DNA sequence of the variant reported in this study was submitted to GenBank with accession number ON182064. In our study, phylogenetic analysis was performed, including our molecularly characterized as an *E. intestinalis* genotype and isolates of various genotypes reported from multiple regions worldwide, as well as some genotypes isolated from animals in Türkiye. Figure 1 shows the results of the phylogenetic analysis of the ITS sequence of *E. intestinalis* isolate from the recovered renal transplant recipients in Kayseri, Türkiye. Multiple alignments of nucleotide sequences of the same isolate are shown in Figure 2. In our study, it was determined that the isolate belonging to the genotype named ERU-RT-Eint, which was first identified from humans in Türkiye, was closely related (99.6-100%) to the isolates of the genotype "ERU-Eint1-3" reported from Kayseri and "DP-3-Samsun" reported from Samsun.

DISCUSSION

In the present study, the prevalence of renal microsporidiosis in KTR was very low. Considering the genetic characteristics of the microorganism, it suggests a zoonotic infection with dogs as a possible source.

It has been known for many years that microsporidia can infect many vertebrates and invertebrates. It can cause severe complications by causing opportunistic infections in HIV-infected and immunocompromised patients for other reasons, such as children, tourists, contact lens wearers, and the elderly (3,4). However, in the last 30 years, it has gained significant importance with reporting many AIDS-related cases and detection in 70% of HIV-infected patients.

Since the first successful human kidney transplant was performed in 1954, solid organ transplantation has increased worldwide. In patients with CKD stage 5, kidney transplantation is the best treatment option for long-term survival. Using lifelong immunosuppressive treatment regimens in the postoperative period has become more potent. Although graft survival has increased after these successful treatment regimens, infections have become an obstacle to disease-free survival, especially in the post-transplant period. In particular, an increase in the incidence and spectrum of opportunistic infections has been observed. Many species of viruses, bacteria, and parasites are among the causative pathogens. Prophylactic treatments are essential for transplantation since these pathogens must be recognized beforehand (8,18).

Some prospective and retrospective studies worldwide have reported microsporidian infections in transplant recipients. Liguory et al. (19) detected *E. bieneusi* in eight transplant patients, and six of these patients were KTR. Similarly, Rabodonirina et al. (20) detected *E. bieneusi* in 23 organ transplant recipients, five of whom were KTR, and Bednarska et al. (21) in 17% of immunocompromised patients, Ghoshal et al. (22) reported that they detected microsporidian pathogens in 5.8% of 272 KTR. In a study conducted in Türkiye, Çetinkaya et al. (23) reported that they detected positivity in 39% of bone marrow transplant patients. The prevalence of intestinal microsporidiosis was investigated in these studies, but the presence of disseminated infections or renal involvement was not investigated.

There are several case reports evaluating renal microsporidiosis, and the common symptoms in these cases seem to be fever and an increase in serum creatinine levels (8,12,24-27). Kicia et al. (11) evaluated renal microsporidiosis in 86 KTR and reported that microsporidian pathogens were detected in 25.5% of the patients.

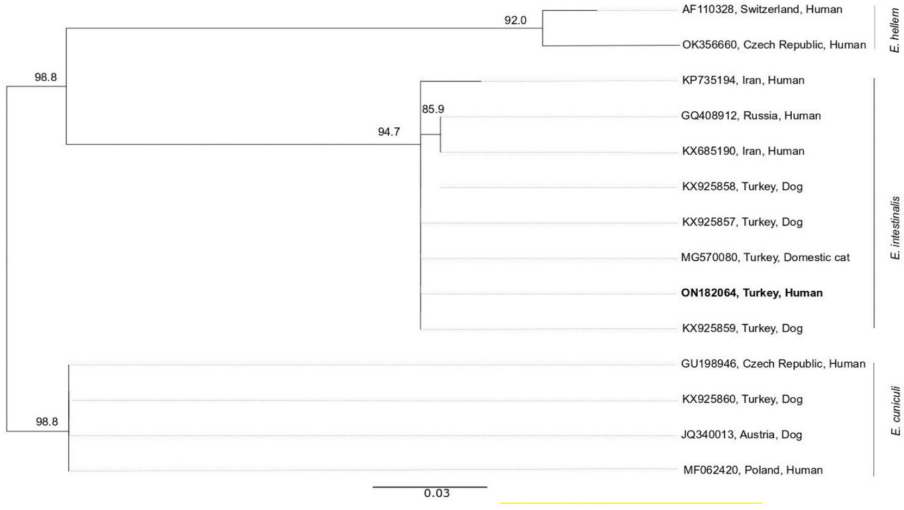

Figure 1. Phylogenetic relationship of ITS sequences of *Encephalitozoon intestinalis* isolate (ON182064, Türkiye, Human) identified from humans in this study and other isolates from people in various regions worldwide and some animals in Türkiye previously deposited in GenBank. Accession number, host, country, and species identified the isolates

They also emphasized that the prevalence is higher in urine samples and that fever and diarrhea are frequently seen in positive patients. In our study, all the renal transplant recipients who applied to the outpatient clinic for routine follow-up, regardless of whether or not they had any clinical complaints, were included in the study, and only urine samples were collected. If we had formed the cohort over the symptomatic patient group instead of randomized selection, our probability of detecting pathogens would have been higher. The clinical findings most associated with microsporidia infections are fever and diarrhea. Kicia et al. (11) reported that 81.8% of positive KTR had a fever, and 59.1% had diarrhea. In this study, only one adult patient had fever and diarrhea (0.3%), while two of the pediatric patients had a fever (0.6%), and two (0.6%) had diarrhea. However, these five patients were negative for microsporidian pathogens. *E. intestinalis* was also detected in a 40-year-old female patient who underwent kidney tranplantation due to Lupus Nephritis.

It is known that spore excretion from infected individuals is not regular and examining only one sample will not reflect the true prevalence. The probability of detecting microsporidia increases with the number of samples tested (28,29). Since the patients who came for their routine follow-ups were included in our study, we did not have the chance to obtain a second sample from many of them. This is thought to be one of the reasons for the low prevalence obtained in our study.

The most detected species in studies with different patient groups on humans are *E. bieneusi, E. intestinalis*, *E. hellem* and *E. cuniculi*. Among these species, *E. bieneusi* and *E. intestinalis* are most commonly associated with intestinal tract infections. *E. intestinalis* is the most frequently detected species in our country. In urinary tract infections, Kicia et al. (11) found that 59% (13/22) of microsporidia-positive patients only had *E. cuniculi* (genotype II), and 23% (5/22) had *E. bieneusi* (genotype D). They also reported that 18% (4/22) were infected with both species simultaneously. They emphasized that they did not detect *E. hellem* and *E. intestinalis* infection in the study (11). Hernández-Rodríguez et al. reported that four (57%) of seven patients with renal involvement had *E. cuniculi*. In other patients, it was reported that the species could not be identified, but it was the genus Encephalitozoon (26). Our study determined that the species isolated from the patient, which was positive due to sequence analysis, was *E. intestinalis*.

Much epidemiological information, such as hosts, life cycle, host specificity, and transmission routes of microsporidia, remains unclear. Recently, the increasing use of molecular methods in diagnosis, especially the sequence analysis of the *ITS* gene region, has contributed to obtaining more information about the genetic diversity, transmission routes, and sources of microsporidia (30,31). It has been reported that four microsporidia species that frequently cause human infection are zoonotic. Various domestic and wild animals can act as reservoir hosts in transmission (31,32). Recent molecular studies of *E. intestinalis* from humans and animals have thus far shown no or very low levels of intraspecific variation in the *ITS* gene. Therefore, it is thought that there is no barrier of transmission between species defined as hosts for *E. intestinalis* (19,33). The isolate we identified as a result of sequence and phylogenetic analysis in our study was genetically close to the isolates reported in dogs and cats in Türkiye.

CONCLUSION

Our results provide current epidemiological data on the prevalence of renal microsporidiosis in pediatric and adult renal transplant recipients. In this study, all KTR who applied to the outpatient clinic for routine follow-up were included, regardless of whether they had any clinical complaints. This study provides the first

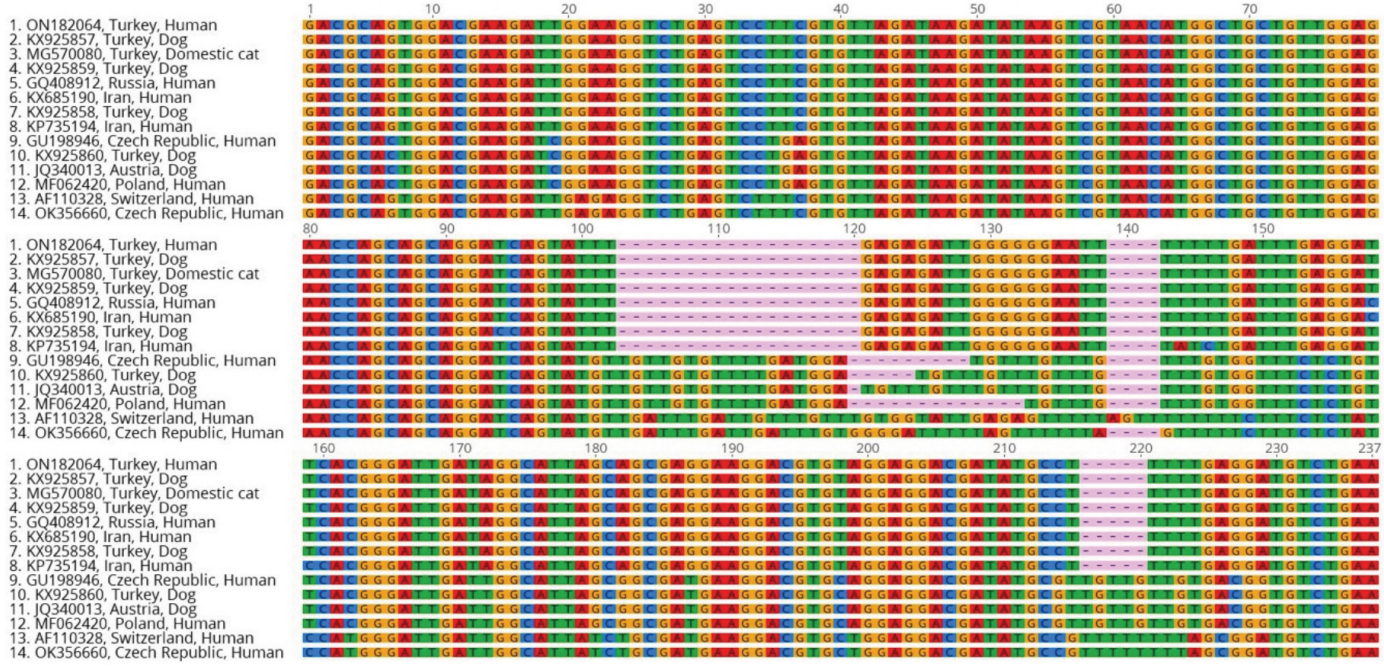

Figure 2. Multiple alignments of ITS sequences of *Encephalitozoon intestinalis* isolate (ON182064, Türkiye, Human) identified from humans in this study and other isolates from people in various regions worldwide and some animals in Türkiye previously deposited in GenBank

genetic data on *E. intestinalis* in humans in GenBank in Türkiye. However, the absence of any clinical complaints in our patients represents the a significant weakness of this study. The genetic similarity between the isolate that we obtained, and the other isolates reported from animals in our region will contribute to developing preventive strategies against microsporidia infections regarding zoonotic transmission.

* Ethics

Ethics Committee Approval: This study has been approved by the Ethics Committee of the Faculty of Medicine Erciyes University Türkiye (application deadline: 03.03.2021, no: 2021/172).

Informed Consent: Written informed consent was obtained from every patient before the examination.

* Authorship Contributions

Concept: Ü.Ç., M.G.Ö., Design: Ü.Ç., M.G.Ö., Data Collection or Processing: Ü.Ç., M.G.Ö., C.U., S.Y., M.B., İ.D., M.H.S., Analysis or Interpretation: Ü.Ç., M.G.Ö., M.B., İ.D., M.H.S., Literature Search: Ü.Ç., M.G.Ö., Writing: Ü.Ç., M.G.Ö., C.U.

Conflict of Interest: No conflict of interest was declared by the authors.

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