

Detection of Five Parasites in Renal Transplantation Patients by Molecular Methods

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Abstract

Objective: Intestinal parasitic infections are among the prominent public health concerns in patients with suppressed immune function.

Material and methods: Here we examined stool specimens by microscopy and polymerase chain reaction (PCR) to evaluate the infection of five principal protozoans (*Cryptosporidium* spp., *Giardia* spp., *Entamoeba histolytica*, *Blastocystis* spp. and *Dientamoeba* spp.) among 90 renal transplant recipient patients (RTP) in comparison with 90 healthy individuals (HI) from Turkey.

Results: The overall frequency of any parasites was 17.2% (31/180) with microscopy and 51.7% (93/180) with PCR. Because of its high sensitivity, PCR was compared with microscopy in terms of the accuracy of detecting intestinal parasites, and the agreement was found to be inadequate ($\kappa= 0.217$; $p<0.001$). Multiparasitism (90.9%), *Cryptosporidium* spp. (84.6%) and *Giardia* spp. (74.1%) were the most frequent agents in RTP, respectively ($p<0.001$).

Conclusions: This is the first study performed in Turkish reporting the prevalence of five intestinal parasites with PCR techniques among this group and seeks to provide a basis for future studies.

Keywords: Intestinal parasites, Transplantation, PCR, Microscopy

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Renal Transplantasyon Hastalarında Beş Parazitin Moleküler Yöntemler ile Tespiti

Öz

Amaç: Paraziter intestinal infeksiyonlar, immün sistemi baskılanmış hastalardaki halk sağlığı sorunları arasındadır.

Gereç ve yöntem: Bu çalışmada, Türkiyedeki 90 renal transplantasyon (RTP) hastasından alınan dışkı örnekleri beş önemli protozoan (*Cryptosporidium* spp., *Giardia* spp., *Entamoeba histolytica*, *Blastocystis* spp. ve *Dientamoeba* spp.) varlığı açısından mikroskopi ve polimeraz zincir reaksiyonu (PCR) ile 90 sağlıklı bireyle (HI) karşılaştırmalı olarak incelenmiştir.

Bulgular: Çalışmada saptanan genel parazit prevalansı mikroskopi yöntemi ile % 17.2 (31/180); PCR yöntemi ile % 51.7 (93/180) olarak belirlenmiştir. Sahip olduğu yüksek duyarlılık oranı nedeniyle PCR, intestinal parazit saptama doğruluğu açısından mikroskopiyle karşılaştırılmış ve iki yöntem arası uyum yetersiz bulunmuştur ($\kappa = 0.217$; $p < 0.001$). RTP grubunda sırasıyla, multiparazitizm (% 90.9), *Cryptosporidium* spp. (% 84.6) ve *Giardia* spp. (% 74.1) en sık saptanan etken olarak belirlenmiştir ($p < 0.001$).

Sonuç: Bu çalışma, Türkiyedeki RTP grubunda PCR yöntemi ile beş intestinal parazit prevalansını bildiren ve yapılacak ileri çalışmalar için temel oluşturmayı amaçlayan ilk araştırma niteliğindedir.

Anahtar Kelimeler: İntestinal Parazitler, Transplantasyon, PCR, Mikroskopi

Introduction

Intestinal parasitic infections are still major health problem in worldwide, especially in developing countries. Intestinal parasites could be transmitted by personal contact, fecal contamination of food, water or environmental surfaces. These infections represent the socioeconomic and hygiene status of a society (1). Moreover, intestinal protozoan infection agents (especially *Cryptosporidium* spp., *Giardia* spp. and *Entamoeba* spp.) are among the major cause of gastrointestinal conditions in developing countries (2). In healthy individuals, intestinal parasitic infections generally self-limiting, but it may cause severe complications (such as persistent diarrhea and/or malabsorption) in patients with immunocompromising conditions (such as, undergoing chemotherapy, renal transplantation and AIDS) (3). Routinely, the detection of intestinal parasites has performed by microscopic examination. However, many authors in current studies suggest that the use of molecular methods, such as PCR (Polymerase chain reaction) to increase the efficacy in diagnosis of intestinal parasites especially in immunocompromised patients (4). Currently, the role of the parasites in gastrointestinal symptom is unclear, partly because the prevalence of the parasites varies considerably

between studies owing to differences in diagnostic approaches, small sample sizes, and lack of control groups (5).

In worldwide, various groups of immunocompetent people have been studied regarding intestinal parasites. On the other hand, immunocompromised patients including having cancer and/or renal transplantation are still poorly evaluated. Hence, the main aim of this case-control study was to detect the intestinal parasites (*Cryptosporidium* spp., *Giardia* spp., *Entamoeba histolytica*, *Blastocystis* spp. and *Dientamoeba* spp.) with molecular methods in renal transplant recipient patients (RTP) in comparison with healthy individuals (HI) in Turkey.

Material and Methods

The present cross-sectional study was conducted among 90 HI and group of immunocompromised patients, including 90 RTP in Istanbul University Istanbul Medical Faculty from 2016 to 2017. Total of 180 individuals were included in this study and all participants were negative for human immunodeficiency virus – 1 (HIV – 1). Immunocompromised 90 RTP patients were selected through the transplantation patients and those are unable to tolerate immunostimulant treatment.

This study was approved by Istanbul University Istanbul Medical Faculty Clinical Research Ethics Committee with 1160 protocol number in terms of the study methods and protocols. Moreover, data collection was started after an informed consent form was signed by each patient. Demographic data and socioeconomic profile were recorded in patient and control group by interview.

Two stool samples were collected from each of the 180 cases that included in the study. From each specimen, 250 mg of feces was stored at -20°C for subsequent DNA extraction and the remainder of each specimen was processed by an in-house formol ethyl-acetate concentration technique (FECT) to determine the existence for *Giardia* spp., *Blastocystis* spp. and *Entamoeba histolytica*. Ziehl-Neelsen staining was performed to enable the detection of *Cryptosporidium* spp. and trichrome staining was performed to enable the evaluate the presence of *Dientamoeba* spp. Fecal concentrates obtained by FECT were independently evaluated in duplicates (with and without iodine) for ova, (oo) cysts, larvae, also Ziehl-Neelsen and trichrome preparations by two skilled microscopists.

Second sample was used for molecular detection of these 5 intestinal parasites. For this purpose, the DNA of parasites was extracted using the QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany). The extracted DNA was quantified by a spectrophotometer by Nanodrop. The appropriate extracts were performed by LightCycler® 480 II multiplex PCR (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' instructions and the presence of parasites was evaluated according to Cp (Crossing Point) values.

Compliance with the normal distribution of age variable has checked with Shapiro-Wilk test. Homogeneity of groups' variances has checked by Levene's test. Parametric test assumptions

were available so Student's t test was used for comparison of two gender groups' age means. Immunocompromised patients groups and healthy individuals groups' age means were compared by oneway ANOVA. Chi-squared test has used to analyze distributions of parasite detection rate between the immunocompromised patients and healthy individuals. When the expected frequency was less than 5, Likelihood ratio test has applied instead of chi-square test. Cohen's Kappa statistics has used as a measure of agreement between the PCR and microscopy methods. Data analyses were performed using the Statistical Package for the Social Sciences, version 19.0 (6). A p value of ≤ 0.05 was considered statistically significant.

Results

A total of 180 people were recruited, including 90 renal transplant recipient patients and 90 healthy individuals. Stool samples were collected all participants. The 90 RTP comprised 46 males and 44 females and their mean (\pm SD) age was 30.9 (\pm 19.4) years. Among the 90 HI, 57 were male and 33 were female and their mean (\pm SD) age was 31.3 (\pm 11.9) years. There were no statistically significant difference with regard to gender distribution ($p=0.180$) and age means between the gender groups ($p=0.159$).

The accuracy of the techniques was analysed based on the parasites that showed the frequency. It is well known that the use of FECT-microscopy alone for general, routine parasitological diagnosis in Turkey has limited diagnostic value. Thus, in the detection of parasites, the accuracy of the microscopy technique was analysed in comparison to that of the PCR technique. This analysis revealed that the PCR technique presented the highest accuracy and Kappa statistics (κ) and percent values of parasite detection showed below average to poor agreement between microscopy and PCR techniques ($p<0.001$) (Table 1).

Table 1: Comparison of PCR and microscopy for detection of intestinal parasites in stool samples

		PCR, n (%)		Kappa value (κ)	Asym. SD	p value
		Absence	Presence			
Microscopy, n (%)	Absence	82 (94.3%)	5 (5.7%)	0.217	0.053	<0.001
	Presence	67 (72.0%)	26 (28.0%)			

The overall frequency of any intestinal parasites was 17.2% (31/180) with microscopy and 51.7% (93/180) with PCR technique. The presence of intestinal parasites in RTP was 22.2% (20/90) and 72.2% (65/90), in HI was 12.2% (11/90) and 31.1% (28/90) with microscopy and PCR techniques, respectively. The multiparasitism (infected with

two or more species concurrently) was detected in 90.9% (20/22) in RTP and 9.1% (2/22) in HI groups. The obtained data differences between study groups and absence, presence and species of intestinal parasites was statistically highly significant in terms of detection with PCR technique (p<0.001) (Table 2).

Table 2: Intestinal parasitic infections of RTP and HI study participants

Methods	Parasite species	Renal transplant recipient	Healthy individuals	Total	p value
Microscopy, n (%)	Absence	70 (47%)	79 (53.0%)	149 (100.0%)	0.191
	<i>Blastocystis</i> spp.	10 (83.3%)	2 (16.7%)	12 (100.0%)	
	<i>Cryptosporidium</i> spp.	0 (0.0%)	2 (100.0%)	2 (100.0%)	
	<i>Dientamoeba</i> spp.	2 (40.0%)	3 (60.0%)	5 (100.0%)	
	<i>Entamoeba histolytica</i>	4 (66.6%)	2 (33.3%)	6 (100.0%)	
	<i>Giardia</i> spp.	3 (60.0%)	2 (40.0%)	5 (100.0%)	
	Multiparasitism	1 (100.0%)	0 (0.0%)	1 (100.0%)	
PCR, n (%)	Absence	25 (28.7%)	62 (71.3%)	87 (100.0%)	<0.001
	<i>Blastocystis</i> spp.	1 (16.7%)	5 (83.3%)	6 (100.0%)	
	<i>Cryptosporidium</i> spp.	22 (84.6%)	4 (15.4%)	26 (100.0%)	
	<i>Dientamoeba</i> spp.	0 (0.0%)	5 (100.0%)	5 (100.0%)	
	<i>Entamoeba histolytica</i>	2 (28.6%)	5 (71.4%)	7 (100.0%)	
	<i>Giardia</i> spp.	20 (74.1%)	7 (25.9%)	27 (100.0%)	
	Multiparasitism	20 (90.9%)	2 (9.1%)	22 (100.0%)	

Discussion

The data herein indicates that intestinal parasitic infections (especially *Cryptosporidium* spp. and multiparasitism) were highly prevalent among Turkish immunocompromised patients, and this prevalence was significantly higher compared with the burden of these infections in HI groups. In a previous Turkish retrospective study, 36 patients with common variable immune deficiency were included and intestinal parasites were found in 50% of these populations with microscopic examinations. Furthermore, the authors indicate that *Cryptosporidium* spp. was found as the major cause of parasitic intestinal infection in this patient population and special methods are needed to identification of intestinal parasites in immunocompromised patients with diarrhea (7). In a study from Iran, which is our geographical neighbours, the prevalence of intestinal parasitic infections were determined in different groups of immunocompromised patients, including haemodialysis patients, renal transplant recipients, cancer and AIDS patients in comparison with healthy individuals, the overall infection rate was found as 11.7% (31/265) in patient groups and 0% (0/120) in healthy individuals, and the authors note that the importance of periodic stool examinations for screening of intestinal parasitic infections should be included as a part of routine medical care in these patients (8).

In our study, the prevalence of *Blastocystis* spp., *Dientamoeba* spp., *Entamoeba histolytica* and the absence for intestinal parasitic infections were more common in HI with PCR. An epidemiological study reported a frequency of the overall rate of intestinal parasites was 63.1% in cancer patients. In this study, *Cryptosporidium parvum* was found the major parasite with 30.1% positivity followed by *G. lamblia* with 18.0% and then *Cyclospora cayetanensis* with 5.3%. Besides, *Blastocystis hominis* and *Entamoeba histolytica/dispar* were detected in 4.9% and 2.4% respectively. In this local study, Al-Qobati et al. used microscopic techniques for determining the intestinal parasites and indicated that diarrhea was associated with higher risk of cryptosporidiosis and giardiasis (9).

Diarrhea is a frequent complication in renal transplant recipients. Chronically loose stool is often counted by clinicians and patients to be an unavoidable part of transplant everyday life, accounting for both a lack of attention from clinicians and incomplete reporting by patients ^[10]. Posttransplant diarrhea, abdominal pain and fever are associated with reduced quality of life, accelerated decline of graft function and higher mortality ^[11]. The lack of a clear description of posttransplant diarrhea, a condition typically self-reported by patients, has led to significant confusion in the clinical practice. To improve the consistency of literature and the resulting clinical conclusions, investigators should use the World Health Organization approved definition of diarrhea: the passage of 3 or more loose or liquid stools per day, or more frequently than is normal for the individual. It is usually a symptom of gastrointestinal infection, which can be caused by a variety of bacterial, viral and parasitic organisms ^[12]. A pathogen is easily detected in 20% to 30% of cases of posttransplant diarrhea when assessed with conventional methods and in up to 70% with molecular techniques ^[13]. The burden of infectious causes increases with time posttransplant, whereas drug toxicity dominates early posttransplant period ^[14]. When compared with healthy individuals, renal transplant recipients are in general more susceptible to opportunistic intestinal pathogens ^[15]. In transplanted patients, cryptosporidiosis may lead to profuse and persistent diarrhea sometimes leading to malabsorption, profound dehydration and life-threatening complications ^[16]. The global prevalence of cryptosporidiosis in renal transplant recipients has been reported as 18.8% – 34.8% ^[17]. A study on renal transplant recipients in India determined cryptosporidial diarrhea in 16.6% of cases ^[18]. Cryptosporidiosis has also been reported in pediatric patients with liver transplantation ^[19]. In contrast, in a study carried out on renal transplant recipients in Brazil, *S. stercoralis* (11/16) was the most frequent helminthic infection (20). The diagnosis of *Cryptosporidium* spp. infection is made primarily by the presence of oocysts in a modified stool acid-fast staining. However, standard microscopy based techniques have some limitations to detect intestinal parasites, so advanced and current molecular based methods are required especially in patients at risk for intestinal parasitic infections. In

the present study, the overall presence of intestinal parasites in renal transplant recipient patients was 18.2% (6/33) and 57.6% (19/33) with microscopy and PCR techniques, respectively. When only PCR technique was considered, *Cryptosporidium* spp was found the most detected pathogen with 30.8% followed by multiparasitism with 22.7% in RTP.

Conventional microscopy-based techniques are still the most frequently used diagnostic procedure in routine clinical parasitology laboratories (21). In spite of the effortlessness of the conventional microscopy-based methods for the detection of intestinal parasitic infection, these methods require the observation of intact cysts or trophozoites in fecal specimens and, therefore, the deformed cysts/trophozoites may not be detected. Moreover, the number of cysts in chronic infections are highly low and cysts are excreted discontinuously, therefore, the conventional microscopy-based methods can only detect up to one-third of chronic infections when performed on a single specimen (22). Three consecutive stool microscopy examinations can detect up to 90% of parasites, but it is considered labor intensive and not applicable in areas in which human intestinal parasites are endemic. Furthermore, techniques such as using the duodenal fluid aspirates obtained by esophagogastroduodenoscopy for trophozoites, entero-test or biopsy of the small intestine offer more sensitive methods of diagnosis, but are rarely used because economic situation and invasive in nature (23,24). Therefore, different techniques have been evaluated to overcome limitations of the conventional diagnostic methods in order to achieve more accurate and specific diagnosis. In recent years there has been greater attention given to DNA-based diagnostic approaches, including conventional and real-time PCR-based diagnostic techniques (25). However, stool-based PCR assays require specialized equipment, developed diagnostic laboratories, specialized training and skilled staff. These impediments, combined with higher rates of false positives, make PCR-based assays of limited practicality for basic routine parasitology laboratories, especially in developing and non-developed countries (26).

In conclusion, intestinal parasites are the most prevalent agents that effect numerous patients

who have a suppressed or deficient immune system. Critical digestive and gastrointestinal problems in patients with immunocompromising conditions can occur with these agents. Thus, periodic fecal examinations should be considered in immunocompromised patients via more sensitive and DNA-based diagnostic approaches in reference laboratories and/or transplantation departments of hospitals.

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