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Original Article

PCR-based detection of intestinal protozoa in cancer and organ transplant recipient patients compared to a healthy control group

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Abstract: We aimed to determine the prevalence of five intestinal protozoa in cancer and organ transplant recipient patients with molecular methods. This case-control study in a university hospital examined stool samples with microscopy-based conventional and polymerase chain reaction (PCR)-based molecular techniques to determine the existence of five principal protozoa (*Cryptosporidium* spp., *Giardia* spp., *Entamoeba histolytica*, *Blastocystis* spp. and *Dientamoeba* spp.) among 57 cancer patients (CP), 33 organ transplant recipient patients (OTP), in comparison with 90 healthy individuals (HI) from Turkey. The overall frequency of intestinal protozoa was 17.2% (31/180) using microscopy and 51.7% (93/180) using PCR. Because of its high sensitivity, PCR was compared with microscopy in terms of the accuracy of detecting intestinal protozoa, and the agreement was found to be inadequate (κ=0.217; P<0.001). According to the protozoa species, distribution of multiparasitism (68.2%; 22.7%), *Cryptosporidium* spp. (53.8%; 30.8%) and *Giardia* spp. (55.6%; 18.5%) were found in CP and OTP, respectively (P<0.001). Depending on the patient groups, multiparasitism (26.3%; 15.2%), *Giardia* spp. (26.3%; 15.2%) and *Cryptosporidium* spp. (24.6%; 24.2%) were the most frequent agents in CP and OTP, respectively (P<0.001). In accordance with literature review, this is the first study conducted in Turkey clarifying the prevalence of five intestinal protozoa with PCR techniques among these groups, and tries to ensure a ground for further research. Comprehensive consultation and periodic fecal examinations are recommended especially among patients with cancer undergoing chemotherapy using molecular methods in reference laboratories, oncology and/or transplantation departments of hospitals.

Keywords: Intestinal protozoa, cancer, transplantation, PCR, microscopy

Introduction

Human intestinal parasites are still a considerable health issue worldwide, particularly in developing countries. Intestinal protozoa 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 protozoa 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 are generally self-limiting, but they may still cause severe complications (such as persistent diarrhea and/or malabsorption) in patients with immunocompromising conditions (such as, undergoing chemotherapy, organ transplantation and AIDS) [3]. Routinely, the detection of intestinal parasites has been performed by microscopic examination. However, many authors in current studies suggest utilizing molecular methods, such as PCR (Polymerase chain reaction) to increase the efficacy of diagnosis of intestinal parasites especially in immunocompromised patients [4]. Nowadays, the status of parasites in the

gastrointestinal symptom is uncertain, partially because the existence of the protozoa varies importantly between research due to differences in diagnostic techniques, insufficient specimen sizes, and absence of control groups [5].

Worldwide, various groups of immunocompetent people have been studied regarding intestinal parasites. On the other hand, immunocompromised patients including cancer and/or organ transplantation patients are still poorly evaluated. Hence, the primary aim of this case-control study was to evaluate the human intestinal protozoa (*Cryptosporidium* spp., *Giardia* spp., *Entamoeba histolytica*, *Blastocystis* spp. and *Dientamoeba* spp.) with molecular methods among an immunocompromised group consisting of cancer patients (CP) and organ transplant recipient patients (OTP) in comparison with healthy individuals (HI) in Turkey.

Material and methods

Participants

The present cross-sectional study was conducted among 90 HI and different groups of immunocompromised patients, including 57 CP and 33 OTP in Istanbul, Turkey from 2016 to 2017. A total of 180 individuals were included in this study and all participants were negative for human immunodeficiency virus-1 (HIV-1). The eligibility criteria for CP included histologically confirmed adenocarcinoma of stomach and non-small cell carcinoma of the lung; as stage III and IV according to the American joint committee on cancer TNM staging classification manual [6]. A total of 29 lung cancer patients (9 were stage III; 20 were stage IV) and 28 stomach cancer (5 were stage III; 23 were stage IV) patients were included and receiving supportive treatment due to neutropenic status caused by chemotherapy (CT) and radiotherapy (RT) or terminal status of disease. The 33 immunocompromised OTP patients were selected through the transplant recipients and those were unable to tolerate immunosuppressive therapy. All 33 OTP patients included in this study underwent renal transplantation with living-donor kidney transplantation surgery.

Ethics statement

This study was approved by Istanbul University Clinical Research Ethics Committee 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 profiles were recorded in patients and control group by interview.

Sample collection and microscopic examination

Two stool samples were collected from each of the 180 cases included in the study. Each sample of 250 mg of feces was stored at -80°C for DNA extraction and the rest of specimen was handled by formol ethyl-acetate concentration technique (FECT) to determine the existence for *Giardia* spp., *Blastocystis* spp. and *Entamoeba histolytica*. Ziehl-Neelsen and trichrome staining were implemented to allow the detection of *Cryptosporidium* spp. and *Dientamoeba* spp., Obtained fecal concentrates were independently examined in two copies (with and without iodine) for ova, oocysts, larvae, also Ziehl-Neelsen and trichrome preparations by two skilled microscopists.

DNA extraction and molecular examination

The second sample was used for molecular detection of these 5 intestinal protozoa. For this purpose, the DNA of protozoa 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 protozoa was evaluated according to Cp (Crossing Point) values. For the PCR assay primers, GenBank entries were searched for the selected parasites sequences including *Cryptosporidium* spp., *Giardia* spp., Entamoeba *histolytica*, *Blastocystis* spp. and *Dientamoeba* spp. The primers were designed by using Primer-BLAST software, NCBI National Center for Biotechnology Information. The overall sensitivity and specificity rates of PCR performed with the selected primers vary between 67-100% and 95-100%, respectively [7]. Designed primers were obtained from Eurofins Genomics (Louisville, Kentucky, USA).

Statistical analysis

Compliance with the normal distribution of variables was checked with Shapiro-Wilk test.

Table 1. Demographic information of the study participants

Variable		Immunocompromised patients		Healthy individuals (HI)	Total	*p* value
		Cancer patients (CP)	Organ transplant recipient (OTP)			
Sex n (%)	Male	31 (54.4%)	15 (45.5%)	57 (63.3%)	103 (57.2%)	0.180
	Female	26 (45.6%)	18 (54.5%)	33 (36.7%)	77 (42.8%)	
Age Mean ± SD		30.9 ± 19.4	25.1 ± 20.1	31.3 ± 11.9	30 ± 16.5	0.159

Homogeneity of groups' variances was checked by Levene's test. Parametric test assumptions were available so Student's t test was used for comparison of the two gender groups' age means. Immunocompromised patient groups and healthy individual groups' age means were compared by one-way ANOVA. Chi-squared test was used to analyze distributions of protozoa detection rate between the immunocompromised patients and healthy individuals. When the expected frequency was less than 5, Likelihood ratio test was applied instead of chi-square test. Cohen's Kappa statistics was 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 [8]. A *p*-value of ≤0.05 was considered statistically significant.

Results

Table 1 shows the demographic characteristics of the case and control groups. A total of 180 people were recruited, including 57 cancer patients (CP), 33 organ transplant recipient patients (OTP) and 90 healthy individuals (HI). Stool samples were collected from all participants. The 57 CP group was comprised of 31 (54.4%) males and 26 (45.6%) females and their mean (± SD) age was 30.9 (± 19.4) years. Among the 33 OTP, 15 (45.5%) were male and 18 (54.5%) were female and their mean (± SD) age was 25.1 (± 20.1) years. Among the 90 HI, 57 (63.3%) were male and 33 (36.7%) were female and their mean (± SD) age was 31.3 (± 11.9) years. There was no statistically significant difference with regard to gender distribution (P=0.180) and age means between the gender groups (P=0.159) (**Table 1**).

The accuracy of the techniques was analyzed based on the parasites that showed the highest detection frequency. It's well known that the use of conventional techniques alone for common and routine parasitological examinations in Turkey has unsatisfactory diagnostic value. Thus, in the detection of protozoa, the accuracy of the microscopy technique was analyzed 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 protozoa detection showed a below average to poor agreement between the microscopy and PCR techniques (P<0.001) (**Table 2**).

The overall frequency of intestinal protozoa was 17.2% (31/180) with microscopy and 51.7% (93/180) with PCR technique. The presence of intestinal protozoa in the CP was 24.6% (14/57) and 80.7% (46/57), in the OTP it was 18.2% (6/33) and 57.6% (19/33), in the HI was 12.2% (11/90) and 31.1% (28/90) with microscopy and PCR techniques, respectively. Microscopy based conventional methods are still the most frequent procedures in routine parasitological diagnosis. **Table 3** shows the detection data of intestinal protozoa with microscopy. The relationship with intestinal protozoa and study groups was not found as being statistically significant in terms detection with microscopy-based technique (P=0.191). When only the PCR technique was considered, an absence of intestinal protozoa was 19.3% (11/87), 42.4% (14/87), 68.9% (62/87); the presence for *Blastocystis* spp. was 1.8% (1/6), 0.0% (0/6), 5.6% (5/6); for *Cryptosporidium* spp. was 24.6% (14/26), 24.2% (8/26), 4.4% (4/26); for *Dientamoeba* spp. was 0.0% (0/5), 0.0% (0/5), 5.6% (5/5); for *Entamoeba histolytica* was 1.8% (1/7), 3.0% (1/7), 5.6% (5/7); for *Giardia* spp. was 26.3% (15/27), 15.2% (5/27), 5.8% (7/27) in CP, OTP and HI groups, respectively. The multiparasitism (infected with two or more species concurrently) was detected in 26.3% (15/22) in CP, 15.2% (5/22) in OTP and 2.2% (2/22) in HI groups. The obtained data differences between the study groups absence and presence and species of intestinal protozoa was statistically significant in

Table 2. Comparison of PCR and microscopy for detection of intestinal protozoa 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%)			

Table 3. Intestinal protozoa infections of CP, OTP and HI study participants with microscopy

Protozoa species	Immunocompromised patients n (%)		Healthy individuals n (%)	*p* value
	Cancer	Organ transplant recipient		
Absence	43 (75.4)	43 (81.8)	79 (87.8)	0.191
Blastocystis spp.	7 (12.3)	3 (9.1)	2 (2.2)	
Cryptosporidium spp.	0 (0.0)	0 (0.0)	2 (2.2)	
Dientamoeba spp.	1 (1.8)	1 (3.0)	3 (3.3)	
Entamoeba histolytica	2 (3.5)	2 (6.1)	2 (2.2)	
Giardia spp.	3 (5.3)	0 (0.0)	2 (2.2)	
Multiparasitism	1 (1.8)	0 (0.0)	0 (0.0)	
Total	57 (100.0)	33 (100.0)	90 (100.0)	

Table 4. Intestinal protozoa infections of CP, OTP and HI study participants with PCR

Protozoa species	Immunocompromised patients n (%)		Healthy individuals n (%)	*p* value
	Cancer	Organ transplant recipient		
Absence	11 (19.3)	14 (42.4)	62 (68.9)	<0.001
Blastocystis spp.	1 (1.8)	0 (0.0)	5 (5.6)	
Cryptosporidium spp.	14 (24.6)	8 (24.2)	4 (4.4)	
Dientamoeba spp.	0 (0.0)	0 (0.0)	5 (5.6)	
Entamoeba histolytica	1 (1.8)	1 (3.0)	5 (5.6)	
Giardia spp.	15 (26.3)	5 (15.2)	7 (5.8)	
Multiparasitism	15 (26.3)	5 (15.2)	2 (2.2)	
Total	57 (100.0)	33 (100.0)	90 (100.0)	

terms of detection with PCR techniques (P< 0.001) (**Table 4**).

Discussion

The data herein indicates that intestinal parasitic infections (especially *Cryptosporidium* spp., multiparasitism and *Giardia* spp.) 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 protozoa were found in 50% of the population with microscopic examinations. Furthermore, the authors indicate that *Cryptosporidium* spp. was found as the main cause of intestinal infection and special methods are needed for identification of intestinal protozoa in immunocompromised patients with diarrhea [9]. In a study from Iran, which is the geographical neighbor of Turkey, the authors note that the importance of routine fecal examination for scanning of intestinal parasitic infections should be implemented as a part of medical practice in this patient group [10].

In our study, the prevalence of *Blastocystis* spp., *Dientamoeba* spp., *Entamoeba histolytica* and the absence of intestinal parasitic infections were more common in HI with PCR. It is well known that the intestinal parasites are detected more frequently in AIDS patients related to their decreased immune status, although little information is available about the presence of intestinal protozoa in patients diagnosed with cancer. An epidemiological study reported the frequency of the overall rate of intestinal parasites as 63.1% in cancer patients. In this study, *Cryptosporidium parvum* was found the major protozoa (30.1%) followed by *G. lamblia* (18.0%) and *Cyclospora cayetanensis* (5.3%). Besides, *Blastocystis hominis* and *Entamoeba histolytica/dispar* were detected in 4.9% and 2.4% of patients, respectively. In this local

study, researchers used microscopic techniques for determining the intestinal parasites and indicated that diarrhea was associated with higher risk of cryptosporidiosis and giardiasis [11].

Diarrhea after transplantation, abdominal pain and fever are related with decreased life quality, accelerated reduction of graft function and elevated mortality [12-14]. The lack of an obvious description of diarrhea seen after the transplantation, a situation mostly noticed by patients, has caused an important chaos in the medical sciences. To evolve the consistency of the literature and the resulting clinical outcomes, investigators should use the World Health Organization approved description of diarrhea: the passage of three or more liquid stools per day, or more usual than it is for a normal person. It is generally a finding of gastrointestinal infection, which can be caused by a variety of microorganisms [15]. A pathogen is easily detected in 20% to 30% of cases of diarrhea seen after transplantation when evaluated with microscopic techniques and in up to 70% with molecular methods [16]. In the post-transplantation period, the presence of infectious agents enhances with time, while drug toxicity predominates the early period [17]. When compared with healthy people, organ transplant recipients are commonly more prone to opportunistic intestinal agents [18]. In transplanted patients, cryptosporidiosis may cause persistent diarrhea occasionally leading to malabsorption, vigorous dehydration and life-threatening situations [19]. The global prevalence of cryptosporidiosis in organ transplant recipients has been reported as 18.8%-34.8% [13, 19]. A study on renal transplant recipients in India determined cryptosporidial diarrhea in 16.6% of cases [21]. Cryptosporidiosis has also been reported in pediatric patients with liver transplantation [22, 23]. 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 protozoa in organ transplant recipients was 18.2% (6/33) and 57.6% (19/33) with microscopy and PCR techniques, respectively. When only PCR technique was considered, *Cryptosporidum* spp. was found to be the most detected pathogen (30.8%) followed by multiparasitism (22.7%) in OTP.

Conventional techniques are still the most frequently-used diagnostic procedures in routine medical parasitology departments [24]. Regardless of the effortlessness of the conventional techniques for the detection of intestinal parasites, these techniques need the examination of intact cysts or trophozoites in feces and, therefore, the deformed cysts or trophozoites might not be identified. Moreover, the count of cysts in chronic infections are highly decreased and cysts are excreted discontinuously, therefore, the conventional techniques can only identify up to one-third of chronic infections when implemented on one specimen [25]. At least 3 sequential fecal microscopic analysis can detect up to 90% of parasites, but it is regarded as labor-intensive and not practicable in regions in which human intestinal parasites are endemic [26]. Furthermore, techniques such as using the duodenal fluid aspirates for trophozoites, and biopsy of the intestine present more sensitive ways of diagnosis, but are hardly used because of economic reasons and its invasive nature [27, 28]. Therefore, different methods have been evaluated to eliminate the limitations of the microscopy-based techniques in order to obtain more proper and specific results. Currently, there has been considerable affinity focused on DNA-based diagnostic approaches, including real-time PCR [29]. Nevertheless, PCR analysis using stool samples need sophisticated equipment, developed in medical laboratories and highly skilled staff. These impediments, combined with higher false positive rates, make molecular analysis of limited applicability for basic routine medical parasitology laboratories, especially in developing and non-developed geographical regions [30]. Microscopy is a method that should be implemented by experienced specialists. If this condition is not met, fecal residues, fibers and other artifacts would be considered as protozoa, leading to an increase in false positive rates. In the present study, *Blastocystis* spp. was more detected by microscopy (17.4%) than PCR (1.8%) in cancer and organ transplant patients and this could be an example or a limitation of this study.

In conclusion, intestinal protozoa are the most prevalent agents that affect 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 implemented in immunocompromised patients via sensitive methods like DNA-based diagnostic approaches in reference laboratories, oncology and/or transplantation departments of hospitals especially if routine diagnosis reveals negative results.

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Disclosure of conflict of interest

None.

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