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The Prevalence of *Cyclospora cayetanensis* and *Cryptosporidium* spp. in Turkish patients infected with HIV-1

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Abstract

Opportunistic infections such as cryptosporidiosis and cyclosporiasis are commonly encountered in patients with acquired immunodeficiency syndrome (AIDS). We investigated the existence of opportunistic protozoans that significantly affect the quality of life in HIV-1 infected patients using conventional and molecular methods. The study group comprised 115 HIV-1 positive patients. In the identification of *Cyclospora cayetanensis* and *Cryptosporidium*, the formol-ether precipitation method was used and smears were evaluated in optical microscope by staining modified Ziehl–Neelsen (ZN). The primers and probes used for PCR were Heat shock protein 70 for *C. cayetanensis* and the oocysts wall protein for *Cryptosporidium* spp.. *Cyclospora* and *Cryptosporidium* spp. oocysts were detected in one and two patients, respectively, by staining, whereas we detected *C. cayetanensis* in three patients out of 115 (2.6%) by PCR, and *Cryptosporidium* spp. in a further three patients (2.6%). *C. cayetensis* was detected in patients with CD4 counts of 64 cells/µm, 182 cells/µm and 287 cells/µm, respectively. *Cryptosporidium* spp. was detected in patients with CD4 counts of 176 cells/µm, 241 cells/µm and 669 cells/µm. As conclusion, PCR method is faster and more sensitive than microscopic methods and to screen intestinal pathogens routinely in patients infected with HIV should not be neglected in developing countries like Turkey.

Keywords

Cyclospora cayetanensis, *Cryptosporidium* spp, HIV-1

Introduction

Opportunistic infections are commonly encountered in acquired immunodeficiency syndrome (AIDS), wherein the immune system gets progressively more compromised, and the disease follows a systemic and severe course, commonly with relapse despite effective treatment (Mathur *et al.* 2013; Kulkarni *et al.* 2013). In recent years, *Cyclospora* and *Cryptosporidium* spp. have proven to be the most commonly encountered opportunistic protozoan infectious agents (Agholi *et al.* 2013; Chacin-Bonilla *et al.* 2001). The *Cyclospora* genus is currently known to contain 19 species and is an obligate intracellular coccidian. Among these 19 species, the only one that is known to cause disease in humans is *Cyclospora cayetanensis* (Yadav *et al.* 2015). The first case in Turkey was reported in an HIV-positive patient in 1998 (Koc *et al.* 1998). In *Cyclospora* infections, which are commonly referred to as 'tourist's diarrhoea' agents, the oocysts of parasites newly passed via feces are not infectious, but rather the oocysts can sporulate in suitable environmental conditions, thereby gaining infectivity (Chacin-Banilla 2010). Although conventional condensation and acid-fast staining are commonly used in the identification of these types of

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coccidians, the fact that no kind of serological test has been developed so far for this organism has increased the significance of molecular study methods (Eberhard *et al.* 1997). *Cryptosporidium* spp. are intracellular protozoans, and humans are commonly afflicted with *C. parvum* and *C. hominis* infections. These protozoans cause both asymptomatic infections and forms of acute enteritis in humans and, unlike *Cyclospora*, their oocysts are infectious as soon as they are passed from the body, resulting in their ability to be readily transmitted to other people (Bouzid *et al.* 2013). In the identification of *Cryptosporidium*, molecular techniques are used with enzyme immunoassay (EIA) (Chalmers and Katzer 2013). Aside from chronic or long-lasting forms of enteritis that can be lethal in HIV-infected or immunosuppressed individuals, *Cryptosporidium* may also cause gall bladder and biliary, pancreatic and pulmonary infections (Ryan and Hijjawi 2015).

Both protozoa cause mild or moderate diarrhoea in immunocompetent individuals, whereas they result in severe intestinal damage and longer-term diarrhoea in immunocompromised ones. The immune status of the host plays a critical role in the pathogenesis of these parasites, and in immunosuppressed individuals severe clinical complications that can lead up to the death of the patients can be encountered (Helmy *et al.* 2006). In our study, we aimed to investigate the existence of opportunistic protozoans that significantly affect the quality of life in HIV-1 infected patients using conventional microscopical and molecular identification methods.

Materials and Methods

In our study, we aimed to evaluate the role of CD4 counts in individuals infected with parasites and investigate the prevalence of opportunistic protozoans that significantly affect the quality of life in HIV-1 infected patients by conventional and molecular identification methods. Our study was planned as a prospective randomized study and was undertaken as a cross-sectional prevalence study.

Study Group

The study group comprised 115 HIV-1 positive patients who had applied to the Okmeydani University and Research Hospital, Ministry of Health of the Turkish Republic. In these individuals, the existence of opportunistic protozoans was evaluated in the Istanbul University Department of Medical Microbiology.

Conventional Methods

In the identification of *Cyclospora cayetanensis* and *Cryptosporidium*, the formol-ether precipitation method was applied as a multiplication method for the evaluation of the feces samples of HIV-positive patients and the prepared smears were evaluated in optical microscope by staining modified Ziehl–Neelsen (ZN) and Kinyoun acid-fast staining. *Cyclospora* and *Cryptosporidium* oocytes were seen in variable staining (from pale to red).

Molecular Methods

A two-step system was preferred for the process of DNA isolation in the application of the molecular method. First, an InhibitEX tablet was used with a manual classical isolation method, and when the inhibitory substances were dispersed, the samples were loaded to a QIAcube machine. In our study, we preferred the QIAGEN Q1 Aamp DNA Stool Mini Kit, which is compatible with the QIAcube machine. For the Real-Time PCR application, we used the Genesig Primerdesign Ltd. Quantification of *Cyclospora cayetanensis* Heat Shock Protein 70 (HSP70) gene kit (UK). For the *Cryptosporidium* genus, we used the Genesig Primerdesign Ltd Quantification of *Cryptosporidium* (Crypto) Genomes gene kit (UK). Our study was undertaken using a Qiagen Rotor Q Real Time PCR machine.

The primers and probes used for the TagMan probe-based Real-Time PCR application amplify the specific regions targeting the Heat shock protein 70 (HSP70) region for *C. cayetanensis* and the oocysts wall protein (COWP) region for *Cryptosporidium*. The positive, negative and internal DNA extraction controls and the pathogen identifying mixtures were prepared in accordance to the contents of their respective kits. Some 15 µl worth of mixture and 5 µl worth of extracted DNA (extracted from the samples) were pipetted into each of the 0.2 mL PCR tubes. In the end, all the tubes contained 20 µl of fluid. After the PCR tubes were loaded into the Rotor Gene Q machine, the amplification protocol specified on the Real-Time PCR kit was calibrated in the computer software (Rotor Gene Q Series Software) and the reaction was started.

The enzymatic activation occurred in the first cycle, and the denaturation and data gathering processes were continued for 50 cycles. The increase in fluorescence at the end of each cycle was detected by the Real Time PCR machine. The collection of the fluorogenic data took place on the FAM and VIC channels.

Results

Patients Characteristics

The sex, age, duration of treatment, CD4 T cell counts, HIV-RNA levels and the distributions according to seasonal isolation in HIV-1 positive patients with opportunistic infections are shown in Table I.

Microscopy and PCR Results

In our study, we detected *Cyclospora* and *Cryptosporidium* spp. oocysts in one and two patients by the modified ZN method on samples using the formol-ether precipitation method, respectively, whereas we detected *C. cayetanensis*

Table I. The sex, age, duration of treatment, CD4 T cell counts, HIV-RNA levels in HIV-1 positive patients who have opportunistic protozoan and other microorganism infections

Gender	Age	Oppurtunistic Infectious Agents	Duration of Treatment for HIV-1 (Years)	CD4 Count	HIV-RNA Levels	Seasonal Isolation
Male	46	*T. pallidum*	0	260	8.290	–
Male	44	*T. pallidum*	4	360	Negative	–
Male	50	*T. pallidum*	3	315	Negative	–
Male	36	*M. tuberculosis*	7	785	Negative	–
Female	33	*M. tuberculosis*	1	403	107.170	–
Male	44	*M. tuberculosis*, *C. cayetenensis*	3	287	115	Ağustos
Male	61	*T. pallidum*	3	669	Negative	–
Male	28	Toxoplasma, *Epstein-Barr* virus, *Cytomegalovirus*	0	422	Negative	–
Male	36	*Epstein-Barr* virus	2	401	25.250	–
Male	47	Toxo	1	128	Negative	–
Female	47	*M. tuberculosis*	8	736	Negative	–
Male	30	*Epstein-Barr* virus	6	289	20.065	–
Male	22	*M. tuberculosis*	1	297	66.715	–
Male	59	*T. pallidum*	1	737	Negative	–
Male	45	*T. pallidum*	2	692	430.000	–
Male	34	*C. cayetenensis*	7	65	129.959	Agust
Male	28	*M. tuberculosis*	2	170	98.081	–
Male	49	*C. cayetenensis*	2	182	82.235	July
Male	44	*Epstein-Barr* virus	3	243	Negative	–
Male	42	*Epstein-Barr* virus	2	296	18.174	–
Female	32	*Epstein-Barr* virus	0.8	193	Negative	–
Male	61	*T.pallidum*, *Cryptosporidium* spp.	3	669	Negative	Agust
Male	37	*Cryptosporidium* spp.	3	176	350.000	July
Male	41	*Cryptosporidium* spp.	2	241	110.201	Agust

in three patients out of 115 (2.6%) by EIA and Real-Time PCR, and Cryptosporidium spp. in a further three patients (2.6%).

CD4 Counts

C. cayetensis was detected in three patients with CD4 counts of 64 cells/µm, 182 cells/µm and 287 cells/µm, respectively. *Cryptosporidium* spp. was detected in three patients with CD4 counts of 176 cells/µm, 241 cells/µm and 669 cells/µm. The sex distribution of the HIV-1 positive patients according to their CD4 counts is shown in Table II.

Upon the macroscopic analysis of the patients' fecal materials, six patients were observed to have soft stools. Microscopically, red-stained *C. cayetanensis* oocysts upon a blue surface were encountered in the ZN-stained preparation taken from patient numbered 52 (Fig. 1A). *Cryptosporidium* spp. oocysts were encountered in two patients (Fig. 1B).

Table II. The patient distribution between sex and positivity according to CD4 counts

Gender	<200 cells/µm (11)	200–349 cells/µm (33)	350–500 cells/µm (25)	>500 cells/µm (46)
Male	7[a,c]	24[b,c]	18	38[c]
Female	4	9	7	8

[a]*C. cayetanensis* positive 2 patients
[b]*C. cayetanensis* positive 1 patient
[c]*Cryptosporidium* spp. positive 1 patient

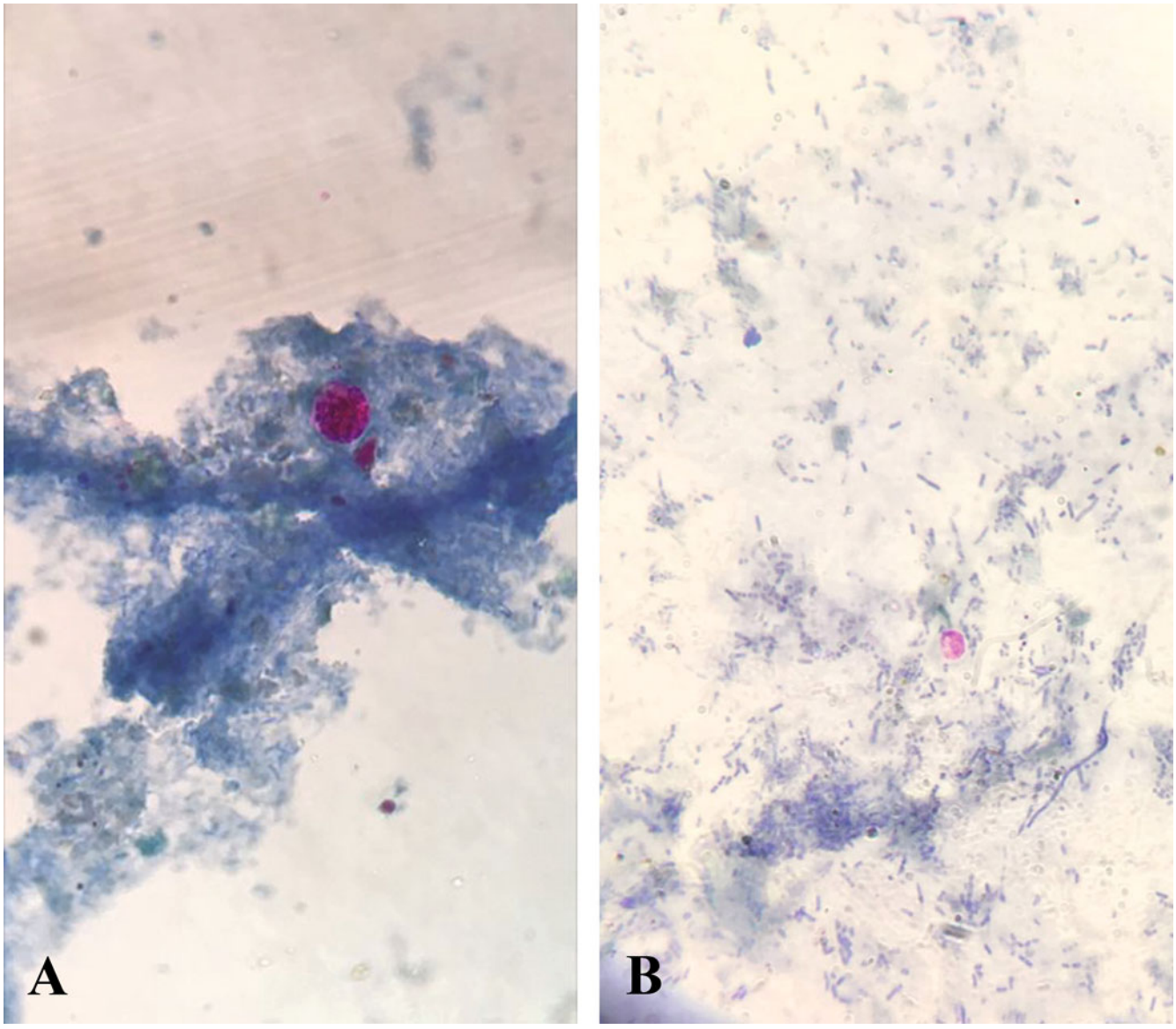

Fig. 1. A – *Cyclospora. cayetanensis* oocysts; Microscopically, red-stained *C. cayetanensis* oocysts upon a blue surface were encountered in the ZN stained preparation taken from patient numbered 52. **B –** *Cryptosporidium* spp. oocysts; Microscopically, red-stained *Cryptosporidium* spp. oocysts upon a blue surface were encountered in the ZN stained preparation taken from patient numbered 103

Discussion

The coccidian parasites (*Cryptosporidium*, *Cyclospora*, *Isospora*) along with microsporidia) are responsible from persistent diarrhoea in more than 50% of HIV-infected patients in all over the world (Goodgame 1996, Farthing *et al.* 1996). *C. cayetanensis* is usually responsible from traveler's diarrhoea, and it is not routinely used in laboratory diagnosis for immunocompetent patients, unless prolonged diarrhoea or a history of travel to an endemic region (Ozdamar *et al.* 2010). There are limited Turkish studies related to *C. cayetanensis* and *Cryptosporidium* spp. in HIV positive patients. In Turkey, there were 6 cases between 1996 and 2002, 2 of them were AIDS patients (Yazar *et al.* 2004). Koc *et al*. (1998) reported first *Cyclospora* infection in a HIV patient with chronic diarrhoea in Turkey. On the other hand, Akin *et al.* (2012) detected *Cryptosporidium* spp. in 3 cases infected with HIV between 1996-2012. In other studies with patient groups not infected with HIV; 4 (%1.6), 4(5.5%) and 8 (1.6%) of 250, 72 and 500 patient samples were detected positive for *Cryptosporidium* oocysts by Fındık *et al.(*1994), Otag *et al.* (2007) and Atambay *et al.* (2003), respectively.

However, in this study where 115 HIV-1 positive patients were studied, *Cyclospora* and *Cryptosporidium* spp. oocysts were detected in one and two patients by the modified ZN method, respectively, whereas *C. cayetanensis* was detected

in three patients (2.6%) by Real-Time PCR and *Cryptosporidium* spp. in a further three patients (2.6%). In a study conducted in Turkey on immunocompetent patients with enteritis between the years 2006 and 2009; *C. cayetanensis* was detected in two patients in 2006, in 17 patients in 2007 and in one patient in 2009 (Ozdamar *et al.* 2010).

In other international studies with HIV positive patients; Rivero-Rodríguez *et al.* (2013), reported that 2 *C. cayetanensis* (3.92%) and 4 *Cryptosporidium* spp. (7.84%) were detected in faecal samples of 56 HIV patients. Prevalence of intestinal parasitic infections among 346 HIV-infected individuals in Malaysia was determined as 37.9% (131 of 346) with protozoa infections (*Cryptosporidium parvum*, 12.4%, *C. cayetanensis*, 4.9%) in the study of Asma *et al.* (2011). They concluded that intestinal parasitic infections are ubiquitous among HIV-infected patients, especially with low CD4 T cells counts.

Chacin-Bonilla *et al.* (2001) have reported that they detected *C. cayetanensis* in 9.8% among 71 HIV positive patients aged 22–45 and Sherchan *et al.* (2012) have reported that they detected it in six (4.1%) patients among 146 HIV positive patients aged 22–45. Alakpa *et al.* (2002) have detected *C. cayetanensis* in 11 patients (seven of which were HIV positive) aged 22–45, of which eight were females and three were males. In a North Indian study, cyclosporiasis was reported in 3.3% of the 120 HIV positive subjects (Kulkarni *et al.* 2009). Lim *et al*. (2005) indicated two (3.0%) were positive for *Cryptosporidium* in a total of 66 faecal specimens obtained from patients infected with HIV by modified ZN staining and confirmed with immunofluorescence stain. Silva *et al.* (2003) reported that parasitological staining techniques and EIA were both positive for *Cryptosporidium* spp. infection in 3/52 (5.8%) samples of patients with HIV, while 4/52 (7.7%) samples were positive in EIA. Therefore, they concluded that EIA may be an alternative method for detecting *Cryptosporidium*-specific coproantigen in HIV/AIDS patients. The prevalence of *Cryptosporidium* in 156 HIV-infected Thai patients was reported as 12.8% (10.0% in males and 19.1% in females) by Saksirisampant *et al.* (2002). Adjei *et al.* (2003) revealed *C. parvum* in six (28.6%) of 21 Ghanaian AIDS patients. Cranendonk *et al.* (2003) assessed the importance of *C. parvum* as a cause of diarrhoea among patients. Thirteen (11%) of them had *C. parvum*. Two hundred and four (84%) patients were HIV positive. They showed the importance of *Cryptosporidium* infections in the HIV positive patients as the cause of diarrhoea.

In studies where the relationship between HIV infection and CD4-T cells were studied, Gupta *et al.* (2008) reported the mean CD4 count of HIV-positive patients with detected coccidian infections as 186.3 cells/µm and those with other detected protozoan infections as 201.4 cells/µm, whereas Velasquez *et al.* (2004) have determined the mean CD4 count of HIV positive patients having chronic enteritis with detected *C. cayetanensis* infections to be 100 cells/µm. Similarly, Kurniawan *et al.* (2009) have observed that 4.5% among 268 HIV – positive patients had *C. cayetanensis* and that their CD4 counts were below 200. In the study of Tumwine *et al.* (2005), mostly HIV positive children with counts < 25% CD4 cells were more likely to have *Cryptosporidium* (odds ratio = 6.45; 95% CI = 3.28 to 12.76) than those with higher CD4 percentages. Agholi *et al.* (2013) reported that 23, 8 and 3 *Cryptosporidium* spp. were detected in patients with CD4 < 200 cells/ml, CD4 200–500 cells/ml and CD4 > 500 cells/ml, respectively and in one patient with *C. cayetanensis*,CD4 count was < 200 cells/ml. They concluded that a CD4 count < 200 cells/ll was significantly associated with the presence of opportunistic parasites and diarrhoea ($p < 0.05$).

However, in this study, the three patients detected with *C. cayetanensis* were determined to have CD4 counts of 64 cells/µm, 182 cells/µm and 287 cells/µm. The drop in the CD4 T-lymphocyte counts in HIV positive patients (especially below 200 cells/µm) increases the risk for protozoan infections drastically. In HIV positive patients whose CD 4 T-cell counts are below 200 cells/µm, the prevalence of *Cryptosporidium* was increased compared to patients whose counts are above 200 cells/µm (Hunter and Nichols, 2002). Assefa *et al.* (2009) have reported the *Cryptosporidium* prevalence among 214 HIV positive patients to be 50.8% when the count was below 200 cells/µm, 12.1% when the count was between 200 and 349 cells/µm, 8.7% when the count was between 350 and 499 cells/µm, and 2% when the count is at or above 500 cells/µm. In this study, however, the three patients in which we had detected *Cryptosporidium* spp. had CD4 counts of 176 cells/µm, 241 cells/µm and 669 cells/µm respectively. The results of our study were thus consistent with other studies, except for the *Cryptosporidium* positive patient with the CD4 T-cell count above 669 cells/µm. The prevalence of microsporidial infections has declined in countries with use of HAART. In an Australian study, the incidence of intestinal microsporidiosis in HIV-infected patients decreased from 11% in 1995 to 0% in 2004. On the other hand, in the incidence of coccidiosis and microsporidiosis still remains high in developing countries because of limited HAART usage (van Hal *et al.* 2007).Even though the viral load is high, in the situations where the immune system remains competent, it is known that the development of opportunistic diseases is rare (Swindells *et al.* 2002). However, 22 patients among our sample size of 115 HIV – positive patients have contracted opportunistic infections.

Mundaca *et al.* (2008) compared the results of PCR and direct microscopy and reported that PCR is 2.2 times better than microscopy as an identification technique. Lalonde and Gajadhar (2008) have reported that in fluorescent microscopy and modified EZN applications sporulation must take place for the detection of *C. cayetanensis*, and that experienced specialists are needed. The PCR method is preferred in the identification of *C. cayetanensis* due to the fact that it delivers fast and trustworthy results. Ten Hove *et al.* (2009) have reported that, in patients developing post-travel enteritis, the Real-Time PCR method had high sensitivity

and specificity for parasites, moreover, Blans *et al.* (2005) have reported that in an outbreak in Indonesia, the Real-Time PCR method could diagnose infection levels as low as one oocyst and that most patients they had found positive with Real-Time PCR gave negative results using microscopy. Varma *et al.* (2003) have also reported that Real-Time PCR results are faster and more sensitive compared to microscopic results. In this study, three among 115 HIV-positive patients were detected as *Cyclospora* – positive by the Real-Time PCR method, whereas only one patient was detected as having *Cyclospora* oocysts by modified acid fast staining. These results indicate that molecular methods are more sensitive in the detection of *C. cayetanensis*.

Moreover, in this study, when the preparations we had sampled using the formol-ether method were analyzed by the ZN method, one patient was detected as having *Cryptosporidium* oocysts, whereas three patients were detected as having *Cryptosporidium* by Real-Time PCR. Using modified EZN staining, ELISA and PCR methods, Uppal *et al.* (2014) have detected *Cryptosporidium* in respectively 17 (29.4%), 39 (67.3%) and 45 (77.5%) patients among 58 HIV positive patients with enteritis. They additionally remarked that, compared to the others, the PCR method could be a lot more sensitive and that an early diagnosis could reduce morbidity and mortality in patients. PCR is more sensitive, specific, and allows identification of *Cryptosporidium* genotypes, but is more expensive. HAART effectively decreased cryptosporidiosis. The rise in CD4 count shows correlation with the management of diarrhoea (Chawla and Ichhpujani, 2011).

Mtapuri-Zinyowera *et al.* (2014) studied 113 participants who consume untreated water in Zimbabwe and 29 (25.7%) of participants were confirmed HIV positive. 23(22.1%) *C. cayetanensis* and 8 (76%) *Cryptosporidium parvum* were isolated from stool samples. One HIV positive (no diarrhoea) patient and 5 HIV positive patients (2 with diarrhoea and 3 with no diarrhoea) had *C. cayetanensis* and *C. parvum*, respectively. They concluded that the water sources were being used without treatment and were shown to pose a risk for protozoan parasites. Improving sanitation conditions, increasing the regulation of food and ensuring hygiene, especially in the preparation of raw salads and similar foods would help to prevent the occurrence of *Cyclospora* and *Cryptosporidium* outbreaks.

Since Turkey is considered an endemic country for *Cyclospora* and *Cryptosporidium*, these pathogens must be considered, especially in immunocompromised patients and patients with long-lasting enteritis. As conclusion, we also agree with other studies that PCR method is faster and more sensitive than microscopic methods and to screen intestinal pathogens routinely in patients infected with HIV should not be neglected in developing countries like Turkey. In addition, we propose that more reliable data be obtained in developing countries such as Turkey by conducting case studies on *Cyclospora* and *Cryptosporidium* using wider cohorts, both in immunosuppressed and immunocompetent patients.

Conflict of interests

None declared.

Ethics

This study was approved by the Non-Invasive Clinical Research Ethics Committee of Istanbul Medical Faculty (Number: 2012/54-911/, Date: 26 January 2012). All patients provided their informed consent to participate in the study.

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