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RESEARCH ARTICLE

Investigation of Cryptosporidium spp. in Immunosuppressive and Immunocompetent Cases with Diarrhea by Microscopic, Serological and Molecular Methods

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Abstract

Objective: In this study, our aim was to compare the diagnostic methods with each other and show the presence of Cryptosporidium oocysts by using molecular, serological and microscopic methods in stool samples which were collected from immunosuppressive and immunocompetent patients with diarrhea

Methods: Total 172 stool samples were collected from 80 immunosuppressive patients and 92 immunocompetent patients (between 0-94 years) with diarrhea. These stool samples were obtained from the different clinics of Ataturk University, Yakutiye Research Hospital between January 2014 and July 2014. Patient group composed of 49 persons between 0-14 years and 123 persons between 15-94 years. On the other hand, 141 patients were using tap water while 41 of them were using well water. Modified acid-fast staining, ELISA and DFA techniques were applied to detect the Cryptosporidium parasite positivity. Nested PCR method was performed to the samples which were detected positive with one of the above methods.

Results: The positivity was detected in 5.8%, 4.1% and 3.5% by ELISA, DFA technique and Modified acid-fast staining method, respectively. Cryptosporidium DNA was detected in only 1.2% by PCR method. The rates of positivity were 6.3% and 5.4% in immunosuppressive and immunocompetent patients, respectively. The positivity was detected in 10.2% and 4.1% in 0-14 age group and 15-94 age group patients, respectively. On the other hand, 4.3% and 12.9% positivity rates were detected in tap water and well water users respectively.

Conclusion: Our study pointed out that the investigation of Cryptosporidium oocysts as diarrhea agents in especially immunosuppressive patients, individuals in childhood and well water users may be useful. Because cryptosporidiosis is a common disease in children and immunosuppressive individuals. Additionally, we think that ELISA method can be preferred to other methods in terms of high sensitivity and ease of application.

Key words: Cryptosporidium, DFA, ELISA, immunosuppressive, immunocompetent, modified acid-fast staining, PCR

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Introduction

Susceptibility for parasitic infections increased along with elevated number of cancer patients, raising use of immunosuppressive agents, aging of the population, and as a consequence of malnutrition. Among them, cryptosporidiosis has become world-wide prevalent, which creates health problems both in immunosuppressive and immunocompetent individuals. Cryptosporidium spp. is a zoonotic protozoon, located intracellular and extracytoplasmic on the microvilli of digestive and respiratory epithelia of a broad spectrum of vertebrates, including human (Xiao et al., 2004). Infectious transmission is by contaminated food and water through fecal-oral route upon human-human and animal-human contacts (Fayer, 2004; Xiao et al., 2004). Currently, 26 different species of Cryptosporidium have been reported (Galvan et al., 2014). Severity and duration of the infections caused by Cryptosporidium spp. varies on the immune system and the age of the host (Alves et al., 2006; Fayer, 2010; Ekinci, 2012;). Causing agent was known to be associated with altered intestinal epithelial function, to affect intestinal epithelium and nervous system, and lead to microvillus dysfunction if parasitic infection is extensive (Kar, 2007).

Routine diagnosis of cryptosporidiosis is based on stool screening and direct inspection of the causative agent (Sears & Kirckpatrick, 2001). Oocysts are seen through such staining methods as Kinyoun's acid-fast, modified acid-fast, Giemsa, nigrosin, safranin methylene blue, and carbol-fuchsin (MacPherson and McQueen, 1993; Starling and Arrowood, 1993; Sears and Kirckpatrick, 2001). Antibodies developed in cryptosporidiosis could be detected via IFAT (indirect immunofluorescent antibody test), ELISA, and Western Blot assays. Monoclonal antibody-based DFA method is a valuable test for detection of surface antigens of the organisms isolated from the stool (Elgün, 2009). In addition, molecular research has provided important insights for the taxonomy and distinction of Cryptosporidium species (Xiao et al., 2002).

This study aimed to compare diagnostic values of molecular, serological, and microscopic methods to detect presence of Cryptosporidium parasite in stool samples that were collected from immunosuppressive and immunocompetent patients with diarrhea.,

Methods

Samples: A total of 172 diarrheic stool samples of 80 immunosuppressive and 92 immunocompetent patients from different clinics of Erzurum Yakutiye Research Hospital between January and July 2014 were included to the study. Immunosuppressive patients mostly consisted of patients with oncology department, patients with organ transplantation, patients with chronic renal failure, and patients with terminal age. The study was approved by the Ethics Committee for Clinical and Laboratory Trials of Ataturk University School of Medicine (Approval Date: 26.12.2013, Decision No: 18).

Modified Acid-Fast (MAF) Staining: Stool samples of diarrheic patients were prepared by MAF staining method, and preparations were assessed under light microscope at 40x and 100x magnification (Garcia, Bruckner, Brewer, & Shimizu, 1983; Usluca, 2009).

ELISA Method: Cryptosporidium 2nd Generation ELISA kit (Diagnostic Automation Lot: Daln1082) was used to determine Cryptosporidium spp. antigens in patient samples. Stool samples at -200C and reactants stored at +40C were brought into room temperature. Reaction findings obtained by using blank, positive, and negative controls per test's manufacturer recommendations were read at 450-630 nm wavelength. Absorbance values indicating ≥0.15 or <15 optimal density (OD) on ELISA reader were considered as positive result or negative result, respectively.

DFA method: MERIFLUOR Cryptosporidium / Giardia (Made in USA) kit was used in this study. Results obtained by the use of positive and negative controls per test's manufacturer recommendations were assessed such that each well was screened completely at 100-200x magnification. Slides where fluorescence was observed were confirmed with further magnification. Any specimen with one or more apple-green fluorescence of characteristic oocyst morphology was regarded as positive for the presence of Cryptosporidium spp.

DNA extraction and Nested PCR amplification: Using i-genomic stool, DNA Extraction Mini Kit (lot no: 14210146) (Intron Biotechnology, Inc. South Korea) per manufacturer's protocol, DNA was obtained from stool specimens that stored at -800C without any added preservative. Primers that were reported to have successful results and targeted at SSU rRNA f of Cryptosporidium were used (Lihua Xiao et al., 1999; Yu, Lee, & Park, 2009). Nested PCR method was performed in this study. At the first step of PCR, rRNA (5'-TTC TAG AGC TAA TAC

ATG CG-3) and rRNA R (5'-CCC TAA TCC TTC GAA ACA GGA-3') primers were used to obtain 1325 bp PCR product. At the second step of PCR, nest rRNA F (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3) and nest rRNA R (5'-AAG GAG TAA GGA ACA ACC TCC A-3') primers were used to obtain approximately 826 bp PCR product. Reaction volume was prepared to make a total volume of 50 µl. Pre-denaturation for 5 minutes at 940C and a final elongation for 10 minutes at 720C was performed at first and second step of PCR. During these two steps, amplification was performed by 35 turns for 50 seconds at 940C, for 30 seconds at 550C, and for 50 seconds at 720C through thermal cycle device. A positive and a negative control sample were used at every PCR test. Obtained PCR product was advanced in 1% agarose gel electrophoresis using 100 bp DNA marker, upon which DNA bands were visualized, and sizes of the bands were compared and recorded.

Statistical analysis: The association between variables such as patient groups, age groups, and source of used drinking water were determined through statistical analyses that were performed via SPSS software (version 17.0, SPSS Inc.). A p value below 0.05 was regarded as statistically significant according to Pearson's chi-square test results.

Results

Our study was performed with 172 patients, including immunosuppressive (n=80) and immunocompetent (n=92) subjects who applied to Research Hospital of Ataturk University Faculty of Medicine with the complaint of diarrhea. Forty-nine patients were at 0-14 age group and 123 patients were ≥15 years old (range: 0-94 years). While 141 subjects (82%) were using tap water, 31 subjects (18%) were using well water as the drinking source. In our study, six out of 172 specimens (3.5%) had oocysts that were though to belong to Cryptosporidium parasite by MAF staining method. In serological studies, 10 (5.8%) and 7 (4.1%) specimens elicited positive results in ELISA and DFA methods, respectively. These results were investigated via Nested PCR, where DNA's of Cryptosporidium spp. were confirmed in 2 (1.2%) specimens.

According to MAF staining, positivity was demonstrated in 3.8% and 3.3% of immunosuppressive and immunocompetent patients, respectively (n=6) (Figure 1).

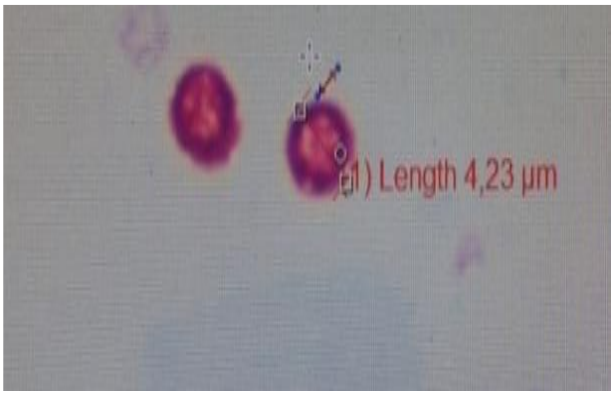

Figure 1. Appearances of *Cryptosporidium* oocysts in MAF staining (100x)

This staining method showed positivity in 8.2% of the subjects below 15 years old and in 1.6% of the subjects ≥15 years old, where the difference was statistically significant. When patients were stratified according to source of drinking water, positivity was found in 2.1% and 9.7% of tap water and well water users, respectively (Table 1).

According to ELISA method, positivity was detected in 6.3% and 5.4% of immunosuppressive and immunocompetent subjects, respectively (n=10) (Figure 2). This method showed positivity in 10.2% of the patients below 15 years old and in 4.1% of the subjects ≥15 years old. Stratification by utilization of water showed that positivity of Cryptosporidium antigen was found in 4.3% and 12.9% of tap water and well water users, respectively (Table 1).

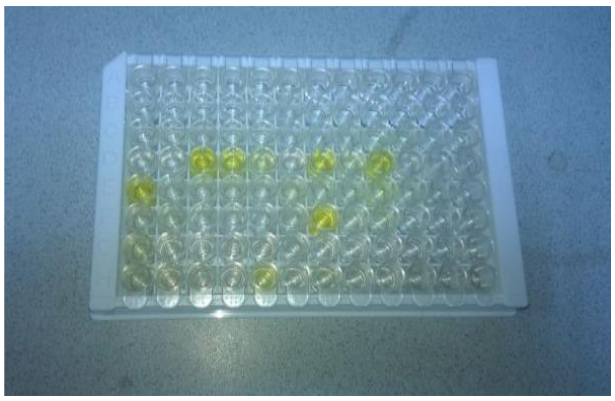

Figure 2. Appearance of preparations of 81 patients in ELISA plate

Positive results in DFA method (n=7) was found in 5.0% and 3.3% of immunosuppressive and immunocompetent patients, respectively (Figure 3). DFA method revealed positivity in 8.2% of the subjects below 15 years old and in 2.4% of the subjects ≥15 years old. Stratification by utilization of water showed that positivity of Cryptosporidium

antigen was detected in 2.8% and 9.7% of tap water and well water users, respectively (Table 1).

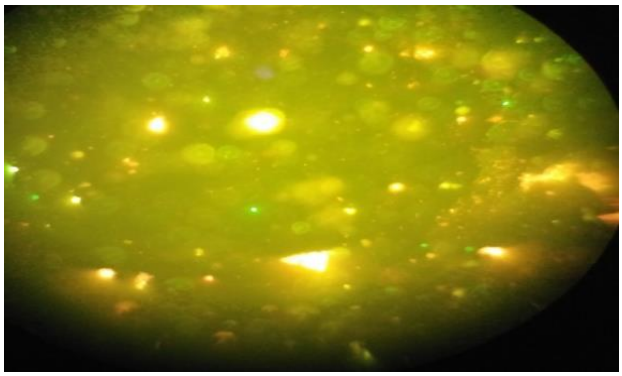

Figure 3. A specimen where a *Cryptosporidium* spp. oocyst was detected by DFA method (40x)

Among specimens stored at -800C, those stool specimens documented as positive by microscopic and serological methods were analyzed with nested PCR methods, and two of them showed Cryptosporidium spp. specific bands. In this method, positivity was demonstrated in 0.0% and 2.2% of immunosuppressive and immunocompetent subjects, respectively. This method showed positivity in 4.1% of the patients below 15 years old and in none of the subjects ≥15 years old. Positivity was found in 0.7% and 3.2% of tap water and well water users, respectively (Table 1).

Images of positive bands under UV system upon advancing of nested PCR-positive Cryptosporidium spp. specimens in 1% agarose gel were shown at Figure 4.

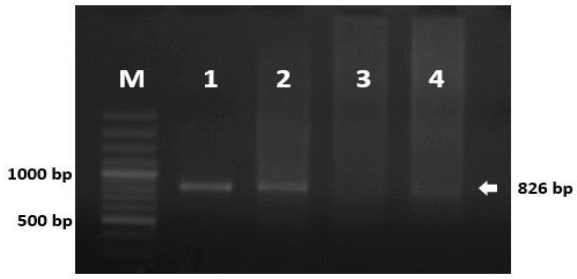

Figure 4. PCR image of cases in which *Cryptosporidium* spp. where detected. M: Marker (100 bp ladder) 1,2: positive result, 3: negative control, 4: positive control (826 bp).

Figure 4. PCR image of cases in which Cryptosporidium spp. where detected. M: Marker (100 bp ladder) 1,2: positive result, 3: negative control, 4: positive control (826 bp).Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of ELISA, MAF staining, and PCR methods were determined in reference to DFA method (Table 2).

Table 1. Distribution of *Cryptosporidium* spp. positivity detected by different methods in immunosuppressive and immunocompetent patients

Method / Patient group	MAF Staining N (%)	ELISA N (%)	DFA N (%)	NESTED PCR N (%)
Immunosuppressive	3 (3.8)	5 (6.3)	4 (5.0)	0(0.0)
Immunocompetent	3 (3.3)	5 (%5.4)	4 (%3.3)	2 (2.2)
P value	0.60	0.54	0.42	0.29
0-14 years old	4 (8.2)	5 (10.2)	4 (8.2)	2 (4.1)
≥15 years old	2 (1.6)	5 (4.1)	3 (2.4)	0 (0.0)
P value	0.05	0.12	0.10	0.08
Well water	3 (9.7)	4 (12.9)	3 (9.7)	1 (3.2)
Tap water	3 (2.1)	6 (4.3)	4 (2.8)	1 (0.7)
P value	0.07	0.08	0.11	0.33

Table 2. Diagnostic values of other methods in reference to DFA method

	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)
ELISA	%100.0	%98.2	%70.0	%100.0
MAF Staining	%85.7	%100.0	%100.0	%99.4
PCR	%28.6	%100.0	%100.0	%97.1

Discussion

Increasing prevalence of some compromising diseases, e.g. malignancies, has uncovered immune system problems affecting every age groups. Being more common in these patient groups, many causative agents that are refractory to treatment and often overlooked during diagnostic tests have become increasingly observed among population, especially in these patient groups. One of these agents is the parasite, Cryptosporidium spp., which was well recognized in veterinary medicine by leading to fatal diarrhea cases in newborn calves with consequent economic losses, and it may now also cause fatal events in humans. In fact, it is of paramount importance that Cryptosporidium is the 3rd most common cause (7.2%) of nosocomial diarrhea (Ramratnam and Flanigan, 1997; Koturoglu et al., 2004; Eren, 2011). It is thought that Cryptosporidium spp. was responsible for 12% and 7% of diarrhea cases in developing and developed countries, respectively (Koturoglu et al., 2004; Alabdeen, 2014). The severity of human Cryptosporidium infections are correlate the host's immune status. İn immunocompetent people, the disease is self-limiting, while in immunosuppressed patients, especially those with T-cell deficiency, cryptosporidiosis is frequently chronic and severe with risks of extra-intestinal disease development (Brunet et al., 2016).

Kehl et al. of USA used two ELISA methods, DFA, and acid-fast staining methods do detect Cryptosporidium oocysts and found a positivity rate of 42.6% in 129 patients (Kehl et.al., 1995). Rosenblatt et al. detected the positivity by ELISA and IFA methods as 33.8% among 296 specimens obtained from patients applied to Mayo Clinics in the USA (Rosenblatt and Sloan, 1993). In Turkey, Elgun examined 154 diarrheal stool specimen by ELISA and MAF staining methods and found the positivity as 24.0% and 5.2%, respectively (Elgun, 2009). In our study, we evaluated 172 diarrheal specimens and found a positivity of 5.8% by ELISA method, 4.1% by DFA, 3.5% by MAF staining, and 1.2% by nested PCR.

In developing countries, cryptosporidiosis cases are more common at the age group of 1-4 years old (Xiao et al., 2001; Pereira et al., 2002; Gatei et al., 2006). While Fathy et al. (2014) reported positivity of 22.4% by PCR in 250 children from Egypt, Shah et al. from India found the positivity as 4.0% by Kinyoun's acid-fast staining and 27.4% by ELISA method from the specimens obtained from 175 diarrheic children (Bera et al., 2014). In Turkey, Yilmaz et al. (2008) reported presence of Cryptosporidium oocyst in 4.9% and 1.95% of specimens of 2000 children, documented by ELISA and staining, respectively. In our study, 49 diarrheal specimens from ≤14-year-old population showed a positivity of 10.2% by ELISA method, 8.2% by DFA, and 8.2% by MAF staining. On the other hand, 123 diarrheal specimens from ≥15-year-old population indicated a positivity of 4.1% by ELISA method, 2.4% by DFA, and 1.6% by MAF staining. Cryptosporidium was more prevalent during childhood in our study. This may be explained by the fact that contamination due to lack of hygiene in this age group may affect the prevalence.

Many studies investigated association of cryptosporidiosis with malignant diseases where the immune system was compromised. The prevalence of Cryptosporidium spp. in patients with malignancies was reported to vary between 1.3-1.7% (Tanyuksel et. al., 1995; Sreedharan et al., 1996). In their study of 111 cases with acute lymphocytic leukemia, chronic lymphocytic leukemia, anti-HIV positivity, or other immune deficiencies, Batero et al. (2003) reported Cryptosporidium as the most commonly encountered parasite. In an Indian study, Jayalakshmi et al. examined stool specimens of 89 diarrheic HIV patients with ELISA and MAF staining method and found 12.4% positivity in this group (Jayalakshmi et. al., 2008). Nwodo et al.

performed a study with stool specimens obtained from 35 HIV-positive diarrheic patients of South Africa and detected a positivity of 74.3% with sad-ELISA method (Omoruyi et. al., 2014). In Turkey, Eren et al. reported Cryptosporidium positivity as 7.4% in 254 immunosuppressed subjects and 3.6% in 55 healthy control subjects, as measured by ELISA in 2011 (Eren, 2011). In our study, while Cryptosporidium spp. positivity in immunosuppressive patients was found as 6.3%, 5.0% and 3.8% with ELISA, DFA, and MAF staining methods, this was 5.4% for ELISA, 3.3% for DFA, and 3.3% for MAF staining method in immunocompetent patients. Therefore, it may suggest that Cryptosporidium spp. should be included to the diagnostic work-up of diarrhea in immunocompromised patients.

There are conflictive findings regarding the association between source of drinking water and occurrence of the infection; while some research suggested an association to the use of unboiled tap water (Baumgartner et. al., 2000), others reported that use of tap water was not the main route of transmission for this infection (Xiao et al., 2001; Hunter et al., 2004; Usluca, 2009). In our study, percentage of Cryptosporidium positivity was higher in well water users than tap water users (Table 1). This is thought to be due to the fact that sources of drinking water in rural areas may be more prone to be contaminated by fecal wastes of both human and animal origin.

Microscopic examination is the most common method in the assessment of stool specimens for Cryptosporidium (Morgan et al., 1998). Modified Kinyoun's acid-fast method is regarded as useful due to several factors such as easy applicability, low cost, persistency, and detailed visualization of inner structures of oocysts. ELISA kits are recommended to detect and monitor Cryptosporidium infection in epidemiological and prospective studies (Moss et. al., 1998). IFAT test was reported to have near 100% sensitivity, being capable of detection even 100 oocysts in a 1 ml fluid (Clark, 1999). Nevertheless, the test also had some disadvantages, such that it may be influenced by oocyst concentration techniques or the composition of the feces (Leng et.al., 1996). More importantly, tests that are based on the demonstration of antigen-antibody formation possess the risk of cross-reaction with other microorganisms to some extent (Fayer et. al., 2000).

PCR as a molecular method allows for differentiation between species by some methods like sequence analysis or RFLP. Despite being a rapid, reliable, and sensitive method, it has some limitations leading to emergence of false positive results due to detection of non-viable microorganisms or laboratory contamination (Fayer et al., 2000). It was also reported that PCR method might elicit less positive results compared to microscopic examination in case that the number of oocysts in the stool specimen is very few and they are not evenly distributed within the specimen (Amar et. al., 2004; Magi et. al., 2006; Usluca, 2009). Moreover, microscopic examination and PCR might give differing results since some oocysts may be damaged before DNA extraction during the latter method (Amar et al., 2004; Magi et al., 2006). In addition, it is reported that nested PCR technique is 4-5 times more sensitive than Simple PCR technique (Kato et. al., 2003).

Compared to demonstration of genomic DNA of oocysts in 2 specimens by nested PCR, we detected oocysts in 10 of specimens by ELISA, in 7 of specimens by DFA, and in 6 of specimens by MAF staining method. In general, specimens detected to have Cryptosporidium were observed to have few oocysts. This might be explained by lesser quantity and uneven distribution of oocysts in the stool. Lower detection of positivity in PCR may also originate from potential damage to oocysts prior to DNA extraction.

In their study of 138 patients below 12 years old, Sirrisena et al. confirmed PCR-positivity of only one of the eight specimens detected as positive by modified Ziehl-Neelsen stain (Sirisena et. al., 2014). In our present study of 172 patients, we found six positive specimens by MAF staining and could confirm only two of them by PCR.

When we considered DFA method as the gold standard, ELISA method had 100% sensitivity and 98.2% specificity, MAF staining method had 85.7% sensitivity and 100% specificity, and PCR method had 28.6% and 100% specificity.

Conclusion

Our study has shown that diagnostic work-up for Cryptosporidium oocysts will be useful in the investigation of diarrhea that is especially seen in immunosuppressive people, or during childhood, or in those using well water as the source of drinking water. Since it has high sensitivity, allows for assessment of multiple specimens, gives rapid results, and provides easy applicability, ELISA method may be preferred over other methods in facilities where great numbers of specimens should be tested.

Ethics Committee Approval: The study was approved by the Ethics Committee for Clinical and Laboratory Trials of Ataturk University Medical Faculty (Approval Date: 26.12.2013, Decision No: 18).
Peer-review: Externally peer-reviewed.
Author Contributions: Concept – AY and HU; Design – AY and HU; Supervision– HU; Materials - AY and OA; Data Collection and/or Processing – AY, OA, HA and EG; Analysis and/or Interpretation – AY, OA and EG; Literature Review - AY; Writing - AY; Critical Review – AY and HU.
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