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Journal of Veterinary Medicine and Research

Research Article

The Research of *Cryptosporidium* spp. on Children in Central Anatolia, Turkey and Their Detection with Different Methods

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Submitted: 09 May 2018

Accepted: 17 May 2018

Published: 18 May 2018

ISSN: 2378-931X

Keywords

- *Cryptosporidium* spp., diagnostic methods, children, multiplex PCR, ELISA, Microscopy

Abstract

Background: Enteric protozoon infections in children are related to morbidity and mortality in the worldwide. *Cryptosporidium* spp. is a zoonotic infection, now being recognized as a significant cause of diarrhea in both immunocompetent and immunocompromised hosts. Current modes of cryptosporidiosis diagnosis involve procedures which are costly and require both a well-equipped laboratory and technical expertise. Our aim was to evaluate the performances of the unidentified *Cryptosporidium* spp. presence during routine parasitological examinations and diagnostic methods.

Methods: 1050 stool samples were collected in children who visited the University hospital with abdominal pain and diarrhea complaints, and additionally selected from seven different regions in primary school students. All stool specimens were examined macroscopically and microscopically by direct microscopic examination, and also were examined by Modified ZiehlNeelsen [mZN] staining method. Enzyme Immuno Assay [EIA] and Multiplex PCR methods could only be used in 450 stool specimens selected from samples showing suspect cyst structures and watery stool specimens on direct microscopic examinations [to the extent that is possible].

Results: We detected, that of the 450 stool specimens examined by mZN stain with microscopic examination 39 [3.7%] are defined *Cryptosporidium*. Spp *oocysts*. Working on 450 stool samples with ELISA and multiplex PCR results respectively; in 28 [7.5%] by ELISA, and in 2 [0.4 %] by Multiplex PCR is defined positive *Cryptosporidium* spp ..Only 2 cases with positive results were detected with 3 methods used in the diagnosis.

Conclusions: Microscopy is the reference standard method for routine diagnosis in stool intestinal parasites, but it requires experience. There are growing interests in the alternative methods due to the limitations of microscopic examination since it requires more time and experienced users. At least two methods must be used together for the diagnosis and attention should be paid to the selection and implementation of the methods.

ABBREVIATIONS

mZN: Modified Ziehl Neelsen; MPCR: Multiplex Polymerase Chain Reaction; ELISA: Enzyme-Linked Immunosorbent Assays

INTRODUCTION

Cryptosporidium spp. is an intracellular, extra cytoplasmic protozoa and infected microvilli of the gastrointestinal epithelium in vertebrates. Clinical appearance varies depending on the immunity status and age of the site [1]. It is an important diarrheal agent in children under 5 years in developing countries. Transmission is especially easier in public places such as schools, nurseries, nursing homes [2-4]. In the world, it is often coming up with waterborne infections [6-10]. In the United States, about 300,000 *Cryptosporidium* spp. cases are reported, with an average of 66 deaths [6].Sensitive methods are needed to identify parasites and it has been suggested that immunodeficient patients should be used in molecular methods together with traditional methods [11-13]. In countries such as the USA and Australia, cryptosporidiosis is highly ranked among waterborne outbreaks, but the prevalence and frequency of infection in Europe vary widely. Outbreaks have been reported in Europe, where a maximum of 27-575 people have been affected, while USA reports numbers exceeding even a single epidemic [6-10]. The prevalence of *Cryptosporidium* spp. among childhood diarrhea has been determined in 5.3-14.7% from Bangladesh and Mozambique [6,14,15].

Several studies in Anatolia conducted on the variousincidence of according to the selected groups.In Turkey, there is a need for healthy, standardized and reliable epidemiological data to determine the frequency of food and waterborne infections [16-20].

Methods based on microscopic examination are being replaced with techniques which rely on the molecular recognition that specific for a target pathogen. PCR techniques have the advantages of improved sensitivity and specificity;however, these methods have limited applicability at point-of-care or low-resource settings due to their high-costs, the need for infrastructure, and a high-level laboratory experience required

[21-28].In this study, we tried to evaluate the performances of the unidentified *Cryptosporidium*spp. presence during routine parasitological examinations and diagnostic methods.

MATERIALS AND METHODS

The Stool specimens were collected from children between 2 – 11 ages between 2011 and 2013 years, who either consulted to Eskisehir Osmangazi University Hospital due to various complaints or they were selected from 7 elementary schools in the province. For stool specimens to be collected from schools, the children who have agreed upon participating in the study has given a proclamation form and stool collection container on the day before the collection of samples. All stool specimens were examined with four different using methods for the diagnosis of *Cryptosporidium* spp.

Firstly, all stool specimens were examined macroscopically then microscopically by direct microscopic examination and then, all of them were examined by Modified ZiehlNeelsen [mZN] staining method. Two main groups of samples, altogether 450 samples, were selected from 1050 samples after this examination: the watery stool specimens and the suspected cyst structures during the direct microscopic examinations, and they were later examined by Enzyme Immuno Assay [EIA] and Multiplex PCR methods.An important fact to mention here is, that it might be expected, that more samples could have been examined on the research, but due to the lack of financial aid in this project, the research team selected the 450 most plausible samples to process further.

After the samples from the laboratory were prepared by sedimentation technique, each of the samples was run through 3 main examination methodologies, namely mZN, ELISA and MPCR respectively. The first step of the examination was performed with wet mouth microscopic slide and mZN stain. Before the second stage of examination, the samples were kept at -20 ° C until the study for ELISA and PCRtechniques.In the second step, namely the ELISA test, GIARDIA / CRYPTOSPORIDIUM CHEK ELISA kit manufactured by TECHLAB [Blacksburg, VA 24060, USA] was used . There are monoclonal and polyclonal antibodies against the *Giardia* and *Cryptosporidium* spp. oocyst antigens in the wells,which is rabbit polyclonal antibody against *Giardia* cell-surface antigen and polyclonal antibody against *Cryptosporidium* spp. cell-surface antigen.Before the MPCR, DNAsfrom the samples were extracted from stool samples using QIAamp DNA Stool Mini Kit [QIAGEN, GmbH, Germany] as it is given in the manufacturer's instruction manual. DNA extracts were then stored at −20°C until their examination. The third step was the Multiplex Tandem Real-Time PCR.The principle of Multiplex-Tandem PCR [AusDiagnostics Pty Ltd], that is used for diagnosis; consists of two sequential PCR steps. Preamplification was performed with the primers in all target regions in the first step. At this stage, target regions of RNA [ribonucleic acid] were formed by reverse transcriptase enzyme. All theseweredone automatically by the Easy-Plex ™system. ThePCR reactionwas monitored with Rotor-Gene Q. Specific DNA amplification occurs in the presence of DNA, during which the amount of DNA becomes measurable by fluorescence with 'Eva-Green ™dye'.

Multiplex Tandem Real-Time PCR method that was used as a molecular method for *Cryptosporidium* spp., detects the 18S ribosomal RNA gene region.

Table 1: Distribution of direct microscopic examination results of stool specimens by parasite species and leucocyte-erythrocyte rate.*

Parasite	n	%
Suspected amoeba/ cyst	100	9.5
Leukocyte	100	9.5
Erythrocyte	57	5.4
Blastocystishominis	132	12.6
Giardia intestinalis	20	1.9
Endolimax nana	8	0.8
Entamoeba coli	21	2.0
Enterobius vermicularis	20	1.9
Ascaris lumbricoides	6	0.6
Strongyloidesstercoralis	1	0.1
Pentatrichomonashominis	1	0.1
*Toxocara*spp.	2	0.2
Negative	267	73.0
Total	1050	100

*This table shows the importance of direct microscopic examination to compare stained mZN and non-stained mZN, which could lead to false positives/negatives, namely that Leukocyte and Erythrocyte could appear to be parasites in non-stained examinations.

RESULTS

In the first step, the samples were studied with the direct microscopic examination. The results of this examination of 1050 [538 female, 512 male] stool samples are given in Table 1.

In the second step, all samples were studied with mZN stain method and examined at x100 magnification in the microscope. We identified by mZN stain 39 [3.7%] samples as *Cryptosporidium* spp. oocystsand in 201 [19.2%] samples as *Microsporidium* spp.

In the third step, 450 selected stool specimens were evaluated by ELISA method. In 28 [7.5%] samples by ELISA were defined as positive, *Giardia lamblia* and/or *Cryptosporidium* spp.

In the fourth step, 450 samples were studied by multiplex tandem real-time PCR and 2 [0.4%] *Cryptosporidium* spp. presence has been identified. The frequency of *Cryptosporidium* spp. and identification techniquesare shownin Table 2.

DISCUSSION

Molecular diagnosis is progressively replacing traditional diagnostic methods;however, their clinical usefulness remains to be better defined.Within the development of diagnostic methods, the studies on *Cryptosporidium* are increased and also the incidence of Cryptosporidium has increased accordingly [3,5,7,9,27,28]. The incidence of infection was reported to be 1-3% in Europe and America in 1997, and 5-10% in developing countries; In 2005, seropositivity was reported to be 25-35% in Europe and America, and 65% in Venezuela and Peru [2,4,6-8]. The studies that were conducted in different groups in Turkey reported the frequency of incidence of *Cryptosporidium* spp. between 0.13 and 35.5% [16-20].

Table 2: Comparison of the diagnostic methods for the detection of *Cryptosporidium* spp.

	mZN stain		ELISA*		Multiplex PCR	
	n	%	n	%	n	%
mZN stain	30	8.4	1	0.3	0	0.0
ELISA	1	0.3	28	7.8	1	0.3
PCR	0	0.0	1	0.3	2	0.6

*ELISA results together with Cryptosporidium/Giardia

On behalf of the variety on the frequency of incidence, it is significant to mention the variety of the diagnostic methods used [2.11,13,21]. In this study, it is aimed to identify*Cryptosporidium* spp., which is an important diarrheal agent that can easily be overlooked during routine parasitological examinations, and also to research efficient and easy-to-use methodologies for its detection; to identify the prevalence of parasites in children's diarrhea in the surrounding region and to compare them with similar studies.

Through staining with modified ARB,*Cryptosporidium* spp.,is detected as 8.4% positive in 1050 stool samples, whereas within the ELISA Test[Cryptosporidium/Giardia]. it is detected as 7.8% in the selected 450 stool samples.by PCR method, *Cryptosporidium* spp. was detected in 2 samples [0.6%]. PCR, ELISA, and modified ARB found only one common positive sample.

The prevalence of the parasite in Turkey is 3.1% [18] by mZNin asymptomatic persons engaged in the foodbusiness in the province of Mersin [17]. However, inthe city center of Sivas, 6.2% *Cryptosporidium* spp. Antigen was detected in asymptomatic people [18]. In 115 HIV-positive patients, it was detected as 2-6% with ARB and PCR methods [17,21].

Eskisehir is located in the center of Turkey, with a continental climate. The city is well-developed, most of the citizens visited high-education and have a high income. The cases that are found in Eskisehir are lower than other provinces. In the university's hospital records, the frequency of occurrence of *Cryptosporidium* spp.is defined as 0.01% and the 90% of these cases are in children and immunocompetent patients [16].

Nazeer et al., collected stool specimens from 396 diarrhea and 202 healthy control groups. They applied direct microscopy and multiplex PCR method and they detected in 1% [with direct microscopy] and 3% [with PCR Method] of the patients *Cryptosporidium* spp. [22]. Tamer et al. used Aist fast and ELISA method in fecal samples collected from 80 patients with diarrhea and 65 healthy patients and detected the presence of *Cryptosporidium* spp. as 3.75% and 6.25%, respectively. Only three of the samples were positively associated [19].

Kaushik et al., investigated the presence of *Cryptosporidium* spp. in fecal samples from 206 HIV-positive, 153 HIV-negative patients with modified Ziehl-Neelsen [ZN], safranin methylene blue [SM], ELISA and nested PCR methods. The results of the study in HIV positive patients are respectively 10 [4.9%], 9 [4.4%], 39 [18.9%] and 27 [13.1%]. In HIV-negative patients, respectively: 7 [4.6%], 6 [3.9%], 21 [13.7%] and 17 [11.1%] *Cryptosporidium* spp. were found [23].

The antigen detection Immunochromatographic method is used for the detection of *Cryptosporidium/Giardia* specific antigens. Agnamey et al., compared four commercials rapid immunochromatographic assays and found that the sensitivity and specificity depend on the kit and species. They found sensitivities for all *Cryptosporidium* spp. Species were 47.2%, 62.4%, 68.8%, and 70.6%, respectively, although it is a rapid method, the kit cost is higher [24].

Molecular analyses are progressively replacing traditional diagnostic methods but their clinical usefulness remains to be better defined. Polymerase chain reaction [PCR] is the most sensitive of all the methods for *Cryptosporidium* spp. detection in both clinical and environmental samples. A comparative study was made by Beena Uppal et al., and they found that nested PCR was able to detect 17.78% more positives than ZN, microscopy, and ELISA. Although it is sensitive and specific, the main drawback is that the species/genotypes can be identified only by sequencing or RFLP analysis and it takes a long time to complete the procedure [25].

In our study, the lowest number of incidences is identified through the use of PCR method, though in the references, it is remarked that the molecular method is superior to PCR method.Real-time PCR is sensitive, specific, reproducible, and improved laboratory workflow and turnaround time. It is a real-time detection of DNA using hybridization probes [24-28]. The equipment being expensive is the only limitation to the utilization of this method. The main drawback of these entire PCR-based assays is the difficulty in DNA extraction from fecal sample and their possibility of contamination. Furthermore, this multiplex assay combines different targets into one assay. The only problem is that technical expertise is required [5,24,28]

Diagnosis of the *Cryptosporidium* spp.oocyst in stool samples by conventional microscopy is labor-intensive and time-consuming. The advantages of modified acid-fast staining method are that it is a low-costmethod, an aid in screening a large number of samples, and the samples are permanent stain [28]. The limitations include time-consuming procedure, require intensive training, and experience to interpret the results, for interpretation 50,000–5,00,000 oocysts/g of the stool. They have a low sensitivity of 83.7% and specificity of 98.9% [27]. *Cryptosporidium* spp. Antigen-based diagnostic method [ELISA] is a more rapid method than microscopy and does not require experts, but itsspecificity is very low. Molecular diagnostic tests are successfully applied in routine practice and new algorithm organized. Molecular tests are quite successful in diagnostics, butcannot be performed everywhere because special equipment is required. Investigators should apply at least two different methods together in order to reduce the mistakes to a minimum level [13,22,23,25].

The bile salts in the stool, inhibitor substances such as hemoglobin degradation products, and the difficulty to extract DNA from the stool are the disadvantages of molecular methods. Furthermore, the possibility of the absenceof the parasite or less parasitic load in the stool samples, that we are aiming to extract from, results in false negative results. Storing stool specimens at -70 ° C after collection and not studying with the fresh samples has a significant effect on the occurrence of false negative

results. Due to these reasons, there is a need for studies in order toovercome the problems during the extraction of parasite DNA from stooland to obtain a standard by studyingdifferent gene regions of the parasite.

CONCLUSION

Enteric protozoa continue to be the most commonly encountered parasitic diseases, resulting in significant morbidity and mortality all around the world, affecting millions of people in developing and developed countries. The microscopic investigation is standard methods for intestinal protozoans. The direct investigation of the stools by the experienced laboratory persons is very important. Due to the numerous substances in stool, the Protozoan cysts/trophozoites can easily cause fallacies. The research we conducted is a screening study and we tried to compare it with the molecular method that is reported as 100% sensitive and specific in the literature with mZN used in routine diagnosis, but we could not find any harmony between the methods used. It is necessary to make the procedures of the commercial tests easy and to use two different methods in the diagnosis. In addition, it should be kept in mind, that the microbiological diagnosis is only a parameter in the diagnosis of infectious disease, and, therefore, the patientshould be diagnosed together with other adjuvant data. Seroepidemiologic studies on the diagnosis are usually based on the idea that serological and molecular methods are easier and more successful.

The ZN staining technique was less sensitive for the detection of *Cryptosporidium* spp.in the study population;however, it has the advantage of being the only technique that only indicates active infections, unlike the ELISA and PCR techniques which may not distinguish between active and non-active infections. Furthermore, less expertise and financial aidare needed for the application of the ZN staining technique although the use of this technique means that several cases of cryptosporidiosis will go undiagnosed as it less sensitive.

A combination of the ZN staining technique with ELISA and also PCR techniques would be a “gold standard” as specificity and sensitivity would be very high, thus ensuring *Cryptosporidium* spp. infections do not go undiagnosed.

The study was approved by the Institutional Ethics Committee, and informed consent was obtained from the parents/guardians/ person attending the study subject after a description of the study was given. This study was supported by Osmangazi University as a scientific research project “Parasitic infections transmitted by water”.

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Cite this article

Dogan N (2018) The Research of Cryptosporidium spp. on Children in Central Anatolia, Turkey and Their Detection with Different Methods. J Vet Med Res 5(4): 1135.