Investigation of cryptosporidiosis by enzyme-linked immunosorbent assay and microscopy in children with diarrhea

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ABSTRACT

الأهداف: التحقق من تكرار داء البويغات الجريبية بواسطة معايرة أنزيم سوربينت المناعي (ELISA) والتنظير المجهري وعلاقته مع الإسهال.

الطريقة: أجريت هذه الدراسة التطلعية في مختبر علم الطفيليات وعيادة الأطفال الخارجية بمستشفى الأبحاث بجامعة يوزونكو يل بمدينة فان بتركيا، في الفترة ما بين عام 2004م وعام 2006م. تم الحصول على عينات البراز من إجمالي 2000 طفل مصاباً بالإسهال، 870 أنثى و1130 ذكر، تراوحت أعمارهم ما بين 0– 15 عاماً مثلوا مجموعة الدراسة، ومائة طفل من نفس العمر تم اختيارهم بشكل عشوائي كمجموعة التحكم. في بادئ الأمر تم تطبيق طريقة فولتاتين لجميع عينات البراز في محلول سولفيت الزنك المشبع، ثم باستعمال طريقة الاصطباغ بواسطة الاصطباغ السريع بالحمض. تم اختبار جميع العينات أيضاً من أجل مستضد داء البويغات الجريبية بارفيوم بواسطة طريقة (ELISA). كما تم استعمال طريقة محلول لوغو السالب والاصطباغ الثلاثي الألوان لتحديد الطفيليات المعوية الأخرى.

النتيجة: تم تحديد المستضد في 97 طفل (4.9%) من بين 2000 طفل بواسطة طريقة (ELISA)، ولكن تبين وجود البيضات فقط لدى 39 طفل (1.95%) بواسطة التنظير المجهري. لم يتم اكتشاف داء البويغات الجريبية لدى مجموعة التحكم بواسطة طريقة (ELISA) أو بالتنظير المجهري. وجدنا صلة ملحوظة بين الإسهال وداء البويغات الجريبية ($p<0.001$). واكتشفت طفيليات معوية أخرى في 713 طفل (35.7%) من بين 2000 طفل مصاب بالإسهال.

خاتمة: يجب أن يشمل الفحص على بحث المستضد بواسطة طريقة (ELISA) في عينات البراز من أجل تشخيص المرض في جميع المستشفيات.

Objective: To investigate the frequency of cryptosporidiosis by enzyme-linked immunosorbent assay (ELISA) and microscopy and its relationship with diarrhea.

Methods: The study was prospectively performed in the Parasitology Laboratory and Pediatric Outpatient Clinic of the Research Hospital, Yuzuncu Yil University, Van, Turkey between 2004 and 2006. Stool samples were obtained from a total of 2000 children with diarrhea, 870 females, and 1130 males aging 0-15 years as study group, and 100 children of the same age were randomly selected as a control group. The flotation method was firstly carried out for all stool samples in saturated zinc sulfate solution, then staining process by modified acid-fast staining. All samples were also tested for *Cryptosporidium parvum* antigen by ELISA. Native-Lugol and trichrome staining were used to identify other intestinal parasites.

Results: The antigen was determined in 97 (4.9%) of 2000 children by ELISA, however, the oocysts were only seen in 39 children (1.95%) by microscopy. *Cryptosporidium* spp. were not detected in the control group either by ELISA or by microscopy. We found a significant ($p<0.001$) relationship between diarrhea and cryptosporidiosis. Other intestinal parasites were detected in 713 (35.7%) of 2000 diarrheic children.

Conclusion: *Cryptosporidium* spp. antigen searching by ELISA in stool samples should be included for diagnosis of the disease in all hospitals.

Saudi Med J 2008; Vol. 29 (4): 526-529

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Received 16th November 2007. Accepted 5th March 2008.

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Disclosure. This study was supported by the Scientific Research Projects Fund of Yuzuncu Yil University, Van, Turkey with a project number 2003-TTR-001.

Species belonging to the genus *Cryptosporidium* are identified in humans and many vertebrates. The first case of human cryptosporidiosis was reported in 1976.[1-3] Towards the end of the twentieth century, *Cryptosporidium* spp. emerged as an important etiologic agent of diarrheal disease worldwide affecting mostly immunocompromised individuals and children. Children who are close with animals infected with this parasite are at risk.[2,4] Staining methods were used in various research to identify cryptosporidiosis,[5-11] however, in some of the studies, it has been determined that enzyme-linked immunosorbent assay (ELISA) proved more than the staining methods in many ways. Hassan et al[12] carried out ELISA to detect antigen of *Cryptosporidium* spp. in 60 children suffering from diarrhea and the antigen of the parasite was determined in 55 (91.7%) of these children. According to the researchers, it was determined that the detection of *Cryptosporidium* spp. antigens by ELISA in the stool samples has a high sensitivity (91.7%) and specificity (85%). El-Shazly et al[13] stated that the Ziehl-Neelsen staining showed the lowest sensitivity in relation to ELISA and polymerase chain reaction (PCR) for diagnosing *Cryptosporidium parvum (C. parvum).* Ungar[14] determined the antigen of the parasite by ELISA in 51 of 62 patients infected with *Cryptosporidium* spp. In Turkey, most of the previous studies in pediatric diarrhea from different parts of the country were based on microscopic examination of stool samples. *Cryptosporidium* spp. have often infected humans due to being a zoonotic agent and its transmission from person-to-person with direct or indirect contact in Van province, where people live close to animals in some settlement units without adequate sewer systems and water networks and adequate respected hygiene rules. This study aimed to determine the prevalence of *Cryptosporidium* spp. and its relationship with diarrhea in 0-15 age group children in and around Van, and the importance of antigen detection by ELISA.

Methods. The study was prospectively performed in the Parasitology Laboratory and Pediatric Outpatients Clinic of the Research Hospital, Yuzuncu Yil University, Van, Turkey between 2004 and 2006. The approval from the Clinical and Laboratory Research Ethic Committee was obtained, and an informed patient consent was received from all of the parents. Stool samples were obtained from a total of 2000 children, 870 females and 1130 males with diarrhea aging 0-15 years as study group, and 100 children of the same age were randomly selected as control group. Children with chronic diseases such as metabolic disorders, chronic renal insufficiency, chronic hepatitis, chronic infectious diseases, primary and secondary immunodeficiency such as use of corticosteroids and immunosuppressive therapy, leukemia, and lymphoma were not included in the study. However, it was then realized that 2 patients with leukemia were unintentionally included in study. The flotation method was firstly carried out on all stool samples in saturated zinc sulfate solution, then staining process by modified acid-fast staining. All samples were also tested for *C. parvum* antigen by ELISA (R-Biopharm, Darmstadt, Germany). In addition, native-Lugol and trichrome staining were also used to identify other intestinal parasites. Preparations were examined by bright field microscopy by screening 100 oil immersion fields for oocysts of *Cryptosporidium* spp. All stool samples were also examined for detection of various ova and cysts by microscopy after native-Lugol, trichrome staining, by concentration methods (by saturated zinc sulfate solution flotation method and formalin-ether sedimentation method).

In statistical analysis, Z test was used to evaluate the importance of the relation between cryptosporidiosis and diarrhea. Minitab version 14 was used for statistical analysis.

Results. Out of 2000 stool samples examined, 97 (4.9%) samples were positive for *C. parvum* (39 by microscopy and ELISA, 58 by ELISA). Namely, *C. parvum* oocysts were found only in 39 of 97 (40.2%) cryptosporidiosis cases by microscopy (modified acid-fast staining), and the antigen was detected in 97 patients by ELISA. *Cryptosporidium* spp. were not detected in the stool samples of the control group either with ELISA or by microscopy. Two of the 97 patients with *C. parvum* infection detected with ELISA had leukemia. It has been detected that *C. parvum* is the agent in all samples in the study. The age distribution of patients with cryptosporidiosis is shown in **Table 1.** The highest prevalence was in the age group of 1-12 months (6.7%). The youngest child to be infected in our study was 2 months old. The other patients infected with the parasite came to our hospital generally with diarrhea complaint. Of the 97 patients with *C. parvum,* 9 were diagnosed with *Giardia intestinalis*, 3 with plentiful *Blastocystis hominis*, 2 with *Entamoeba coli*,

Table 1 - Age-wise distribution of patients with cryptosporidiosis.

Age	Number of patients with diarrhea	*Cryptosporidium* positive cases n (%)
1-12 months	238	16 (6.7)
1-5 years	825	40 (4.8)
6-10 years	598	25 (4.2)
11-15 years	339	16 (4.7)
Total	**2000**	**97 (4.9)**

Table 2 - Distribution of the other intestinal parasites detected in the study.

Parasites	Positive cases n (%)
Giardia intestinalis	245 (12.3)
Blastocystis hominis	201 (10.1)
Entamoeba coli	51 (2.6)
Chilomastix mesnili	20 (1)
Iodamoeba butschlii	11 (0.6)
Dientamoeba fragilis	7 (0.4)
Entamoeba histolytica / Entamoeba dispar	5 (0.3)
Endolimax nana	5 (0.3)
Entamoeba hartmanni	2 (0.1)
Enteromonas hominis	2 (0.1)
Ascaris lumbricoides	36 (1.8)
Hymenolepis nana	26 (1.3)
Enterobius vermicularis	5 (0.3)

2 with *Ascaris lumbricoides*, and one with *Chilomastix mesnili.* Other intestinal parasites were detected in 713 (35.7%) of 2000 diarrheic children whose stool samples were examined. Of these, 275 (31.6%) were girls, and 438 (38.8%) were boys. One species of the parasites was detected in 610 (30.5%), 2 parasite species in 85 (4.3%), 3 parasite species in 14 (0.7%) and 4 parasite species in 4 (0.2%) of 2000 patients. Number and rates of the parasites detected in the study are presented in **Table 2.** The association between diarrhea and cryptosporidiosis was found to be highly significant on statistical analysis (p<0.001).

Discussion. In developing countries, the association of *Cryptosporidium* spp. with diarrhea, especially in children is striking. The prevalence was found generally between 1-2% in Europe and, non-outbreak-associated prevalence rates ranged from 0.6-4.3% in North America. In contrast, it was detected that prevalence rates in Asia, Australia, Africa, and Central and South America generally began at 3-4% and reached 10-20%.[2,4] It was reported that the prevalence of cryptosporidiosis was determined in pediatric patients with diarrhea in Argentina as 90%,[10] India as 18.9%,[7] West Africa as 7.4%,[9] Italy as 7.2%,[5] Iran as 7%,[6] and Iraq[8] as 9.7%. In Turkey, most of the previous studies in pediatric diarrhea and other patient groups were based on microscopic examination of stool samples prepared with modified acid-fast staining. The conducted studies encountered *Cryptosporidium* spp. oocysts between 0.4-30.4% in different patient groups.[15-24] The studies conducted by different researchers and mentioned above expressed that only the study made by Ok et al[23] determined 6.7% oocysts of parasite in the control group, however, other studies did not observe any parasite oocyst in control groups. In our study, it was determined that there were oocysts of the parasite in only 1.95% of 2000 patients with diarrhea by microscope, and it was established that there was antigen of *C. parvum* in 4.85% of the same patients by ELISA. No *Cryptosporidium* spp. was determined in the control group. This result has indicated that diarrhea is an important symptom for cryptosporidiosis. The studies conducted by Godekmerdan et al[15] and Dogan and Akgun,[16] constituted of children with diarrhea as a patient group, established agent of oocysts in the rates of 4.55%, and 3.6% in the patients. However, ELISA was not used in these 2 studies. In our study, it was detected by microscope only in 40.2% of cryptosporidiosis cases, which were determined by ELISA. Therefore, this showed that results of studies without using ELISA in Turkey are lower than the actual prevalence.

In the present study, the obtained results were low when we consider that the people are in close contact with animals in some settlement units, and there are no adequate sewer systems and water networks, and people do not show adequate consideration for the hygienic rules. The main reason for the lower cryptosporidiosis rate than our prediction might be the long winter period in the Van province. The other studies indicated that *Cryptosporidium* spp. are widespread in other countries and has been frequently encountered.[5-12] In addition to *C. parvum*, the determination of 13 different intestinal parasites species in 35.7% of 2000 children with diarrhea has shown that there is a rich variety of intestinal parasitosis in Van province. Staining methods were used in various research carried out to identify cryptosporidiosis.[5-11] However, in some of the studies, it has been determined that ELISA proved more accurate than the staining methods in many ways. Hassan et al[12] detected fecal antigen in 91.7% of 60 infected with *Cryptosporidium* children suffering from diarrhea by ELISA. According to the researchers, the detection of *Cryptosporidium* spp. antigens by ELISA in the stool samples has a high sensitivity (91.7%) and specificity (85%). El-Shazly et al[13] diagnosed *C. parvum* in stool samples by Ziehl-Neelsen staining as 5.3%, ELISA as 8.3%, and PCR as 9.6%. They stated that the Ziehl-Neelsen staining showed the lowest sensitivity in relation to ELISA and PCR. Ungar[14] determined the antigen of parasite by ELISA in 51 of 62 patients infected with *Cryptosporidium* sp, the sensitivity of the assay was 82.3%, and specificity was 96.7%. It has been stated that the acid-fast staining method is the most reliable and specific, and has a high diagnostic value among staining methods used for identification of *Cryptosporidium* spp. oocysts. However, ELISA has been

preferred in the laboratories of the developed countries due to its high specificity and sensitivity, easy usage, fast application and scoring, and easy standardization for determination of *Cryptosporidium* spp. antigens in stool samples. It has been also stated that ELISA must undoubtedly be carried out together with one of the the staining methods due to the reason such as be of *Cryptosporidium* agents small amount in the stool samples or not determination in the staining methods used or overlooking *Cryptosporidium* oocysts.[15,16,23,25]

In our study, modified acid-fast staining method was also used due to its easy application and the permanency of preparations, its low cost, easy differentiation of oocysts painted red color in blue background and easy observation of interior structure of oocysts. There are some limitations to our study such as; a) roles of bacterial and viral agents were not investigated in children with diarrhea, b) PCR was not used for the diagnosis of *Cryptosporidium* due to lack of laboratory facilities in our hospital (higher prevalence could be found, if we used PCR), c) after specific treatment, we could not perform laboratory investigation and follow-up of children with cryptosporidiosis.

In conclusion, we believed that *Cryptosporidium* antigen searching by ELISA in stool samples should be included in laboratories where no PCR application is possible for routine examination of cryptosporidiosis coursed with diarrhea in childhood. In addition, we found a 35.7% prevalence of intestinal parasitosis in children with diarrhea in the 0-15 age group in our study, which showed that there is still the parasitosis problem in Van province.

Acknowledgments. *We extend our sincere thanks to Scientific Research Projects Fund of Yuzuncu Yil University for its support.*

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