

The prevalence of cryptosporidiosis in Turkish children, and genotyping of isolates by nested polymerase chain reaction-restriction fragment length polymorphism

Gulden S. Tamer, MD, PhD, Meral Turk, MD, Hande Dagci, MD, Bayram Pektas, MD, Edward C. Guy, PhD, Adnan Y. Guruz, MD, PhD, Ahmet Uner, PhD.

ABSTRACT

Objective: To verify the incidence of cryptosporidiosis among Turkish elementary school students.

Methods: This study was conducted in the Department of Parasitology, Faculty of Medicine, Ege University, Turkey during a 3-month period in 2006. We assessed the fecal samples of 707 children using modified acid-fast and phenol-auramine staining followed by modified Ritchie concentration method. All *Cryptosporidium* species isolates were analyzed by nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) to differentiate the genotypes of the isolates. After the coprological examination, 4 samples were found to be positive for *Cryptosporidium* species oocysts.

Results: In the present study, all 4 oocysts were of zoonotic origin and belonged to *Cryptosporidium parvum* genotype 2 indicating that in Turkey the potential sources of human cryptosporidiosis is from animals.

Conclusion: The application of genotyping to clinical isolates of *Cryptosporidium* has significantly increased our knowledge and understanding of the distribution and epidemiology of this parasite. The PCR and RFLP techniques represent a more rapid and simple method of genotyping to support epidemiological and clinical investigations than conventional analytical DNA techniques.

Saudi Med J 2007; Vol. 28 (8): 1243-1246

From the Department of Microbiology (Tamer), Faculty of Medicine, Kocaeli University, Kocaeli, Atatürk Training and Research Hospital (Turk), Yesilyurt, Department of Parasitology (Dagci, Pektas, Guruz, Uner), Faculty of Medicine, Ege University, Izmir, Turkey, and the PHLS *Cryptosporidium* Reference Unit (Guy), Swansea Public Health Laboratory, Singleton Hospital, Swansea, United Kingdom.

Received 10th December 2006. Accepted 14th March 2007.

Address correspondence and reprint request to: Dr. Gulden S. Tamer, Department of Microbiology, Faculty of Medicine, Kocaeli University, Umuttepe Kampusu, Kocaeli, Turkey. Tel. +90 (262) 3037540. Fax. +90 (262) 3037003. E-Mail: guldensonmez@hotmail.com

Cryptosporidium is a small coccidian parasite that causes diarrhea in livestock, and humans worldwide. It commonly affects children, the elderly, and immunosuppressed patients, especially patients infected with human immunodeficiency virus.¹ Due to its zoonotic nature, livestock-breeders, tourists, and immunocompetent individuals may encounter cryptosporidiosis. Researchers, who study epidemiology and investigate illnesses associated with diarrhea, have shown that *Cryptosporidium* species are the causative agent in 1-5% of all of the cases. The infection may spread in a number of ways: a) from person to person, b) from animals, c) via food, and water.^{2,3} In immunocompetent individuals, cryptosporidiosis presents itself as a self-limited diarrhea, with symptoms lasting less than 20 days. In immunodeficient patients, *Cryptosporidium* species causes severe diarrhea that may even be life-threatening. A correlation between malnutrition and cryptosporidiosis was reported in children.^{1,2,4} *Cryptosporidium parvum* (*C. parvum*) and *Cryptosporidium hominis* (*C. hominis*) are 2 morphologically identical species of *Apicomplexan* protozoa infecting humans. Although the genomes of these species are 97% identical, their host range is strikingly different. *Cryptosporidium parvum* infects humans and animals and is primarily a zoonotic infection, whereas *C. hominis* is typically not detected in animals.⁵ Differentiation of these species is determined by genotyping. The most common genotype analyses are based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or sequencing of the small subunit (SSU) rRNA,⁶ 70-kDa heat shock protein,⁷ beta-tubulin,⁸ *Cryptosporidium* oocyst wall protein (COWP)⁷ or thrombospondin-related adhesive protein *Cryptosporidium*-1 (TRAP-C1) or TRAP-*Cryptosporidium*-2 (C2) genes.^{7,9-11} In a recent study, *C. parvum* TU502, a genotype 1 isolate of human origin passed through 3 different mammalian

hosts, including humans, pigs, and calves.¹² Recently, several single-nucleotide polymorphisms (SNPs) have been described, together with methods for their determination, and analysis that have indicated the genetic relationship of isolates of *C. parvum*.¹³ The majority of isolates belong to one of 2 broad genotypes: *C. hominis*, previously known as *C. parvum* human genotype or genotype 1 (or H), generally restricted to humans and nonhuman primates,⁶ and *C. parvum*, previously known as *C. parvum* bovine genotype 2 (or C), found in both humans, and animals.^{9,14}

The PCR-RFLP and rapid nested PCR are simple methods to use for identification of type-specific polymorphisms in a *C. parvum* TRAP-C2 gene. These methods are based on simple restriction enzyme analysis.^{9,10} The TRAP-C2 gene is an example of a well-characterized gene, which demonstrates the polymorphic nature of this parasite genome, and it is not often used as an epidemiological tool.¹¹ In this study, we aim to investigate the incidence of cryptosporidiosis in elementary school students in Beyda, a rural region of Izmir, Turkey, where livestock-breeding is a major income source for the inhabitants, and to differentiate the genotypes of the isolates by nested PCR-RFLP method to show the sources of transmission in the study area.

Methods. Following ethical committee approval, 707 fecal samples obtained from elementary school students, ages between 7 and 15 years (median, 11 years) were examined between January and March 2006. All subjects were from Beydag, Izmir, Turkey, which is a town mostly populated by farmers, and stock breeders. Among 707 students, 390 (55.2%) were male and 317 (44.8%) were female. There was only one elementary school in the region. To easily reach all the children in the region, students in the school were included to this study (Cluster sampling). Questionnaires were distributed to the students and students were verbally informed and instructed on how they should fill the questionnaires, which contained questions about the socioeconomic status of their families, and any parasitic symptoms.

All fecal samples were freshly collected from students and transported to the coprology laboratory at the Department of Parasitology, Ege University Medical School, Izmir, Turkey, for examination. All samples were concentrated by modified Ritchie method, and smears were stained with modified acid-fast, and phenol-auramine stains.⁴ Smears were evaluated under both light and fluorescent microscopes for the presence of *Cryptosporidium species* oocysts. Fresh patient stool samples containing *Cryptosporidium species* (detected by microscopy in primary testing laboratories) were

stored at 4°C prior to oocyst preparation by floatation using 36% (w/v) sodium chloride (NaCl) solution. Oocyst suspensions were also stored at 4°C prior to DNA extraction.¹¹ A 200 ml sample of prepared oocyst suspension was incubated at 100°C for 60 minutes, and DNA was extracted using a QIAMP DNA mini kit (QIAGEN Ltd., Crawley, United Kingdom). Purified DNA was stored at -20°C until used.¹¹ Positive fecal samples of *Cryptosporidium species* oocyst were genotyped at Swansea Public Health Laboratory, Singleton Hospital, PHLS Swansea, United Kingdom using nested PCR-RFLP. The reason why we used TRAP-C2 was because our preliminary experiments (acid-fast stain) revealed the presence of *Cryptosporidium species*. For amplification of the TRAP-C2 gene, nested PCR was used based on a single PCR method.⁹ The TRAP-C2 assay is largely specific for *C. parvum*.¹¹ The nested PCR products were subjected to digestion with HaeIII and BstEII at 37°C for 12-18 hours. Each enzyme used has a different recognition sequence, present only in one of the 2 genotypes under investigation. At position 42 within the *C. parvum* and TRAP-C2 gene, type 1 DNA has the nucleotide cytosine, which is part of the recognition sequence for HaeIII: GGCC. At the same position, type 2 DNA has within the sequence GGTCACC the nucleotide thymine, which is recognized and digested by BstEII.¹¹ Digested PCR products were analyzed using agarose (2% wt/vol) gel electrophoresis (Phorecus; Biogene, Cambridge, United Kingdom) as previously described.¹¹ Product size was confirmed by comparison with a DNA molecular-weight-standard marker (Life Technologies, United Kingdom). The digested products were visualized using ethidium bromide (0.1mg/100ml) and recorded using a digital camera and KDSID analysis software (Kodak, Rochester, New York).¹¹

Results. After the coprologic examination, 4 out of 707 (0.6%) samples were positive for *Cryptosporidium species* oocysts. All 4 students with *Cryptosporidium species* had diarrhea, and their parents were livestock-breeders. Based on the data obtained from the questionnaires; 95 students (13.4%) had pets in their houses, 235 (33.2%) were suffering from abdominal cramps and 82 (11.6%) had diarrhea. *Cryptosporidium species* oocyst positive fecal samples were analyzed at the PHLS, Swansea, Wales using nested PCR and restriction enzymes for genotyping. Amplification was achieved via DNA extract from characterized isolates of *C. parvum*, generating a nested PCR product of the expected size (266 bp). **Figure 1** shows the typical results obtained after restriction enzyme digestion of the TRAP-C2 PCR product generated from characterized

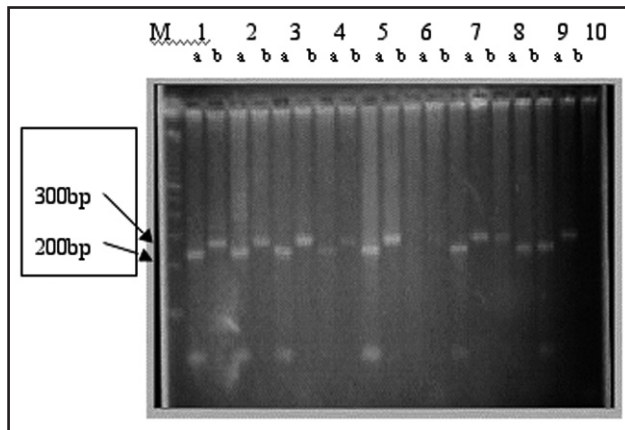


Figure 1 - Agarose gel electrophoresis of BstEII and HaeIII restriction endonuclease cleavage products. M, DNA molecular weight-standard marker; 1, 2, 3, 4, 5 and 7, patient samples; 6 and 10, PCR negative control; 8, positive control, *Cryptosporidium parvum* (human isolate, genotype 1); 9, positive control, *C. parvum* (animal isolate, genotype 2).

genotype 1 and genotype 2 isolates. Within our nested PCR, each genotype contains the recognition sequence for either BstEII or HaeIII. Since the recognition sequence for these enzymes is at the same locus on the PCR product, cleavage with either BstEII (genotype 2) or HaeIII (genotype 1) results in 2 fragments (227 and 39bp). Agarose gel electrophoresis of BstEII and HaeIII restriction endonuclease cleavage products. M, DNA molecular weight-standard marker. 1, 2, 3, 4, 5 and 7, patient samples *C. parvum* (animal isolate, genotype 2); first lane BstEII cleavage products and second lane HaeIII-resistant PCR product. 6 and 10, PCR negative control, 8, positive control, *C. parvum* (human isolate, genotype 1) first lane (8a) BstEII-resistant product, second lane (8b) HaeIII cleavage product, 9, positive control, *C. parvum* (animals isolate, genotype 2) first lane (9a) BstEII-cleavage product, second lane (9b) HaeIII-resistant product. Note: 5: second feces (after 10 days) of patient no. 1, 7: second feces (after 10 days) of patient no. 2 a presents BstEII cleavage products, b presents HaeIII cleavage products. The results indicated that all 4 oocysts belonged to genotype 2 and of zoonotic origin.

Discussion. Cryptosporidiosis is transmitted by the fecal-oral route during the oocyst stage. The *C. parvum* is zoonotic, and has no host specificity among mammals. Risk of infection rises with close human-to-human contact, during care of infected livestock, zoo animals, or pets. Drinking and recreational water sources may play a role in the outbreaks. It has been shown that *C. parvum* is highly prevalent in young

calves worldwide. Dog and cats may serve as hosts to *C. parvum*.^{15,16} Currently, regional studies are carried out with immunocompetent patients in Turkey. In a study with 194 individuals, *Cryptosporidium species* was found in 1%.¹⁷ In a rural region in Eskisehir, Turkey, 7 (2.5%) positive cases in 478 elementary school children were detected by using Ziehl-Nielsen staining.¹⁸ *Cryptosporidium species* were not detected in the stool samples of 148 immunocompetent children with ages ranging from 0-12 years old who lived in an orphanage of an urban area in Izmir.¹⁹ When modified Kinyoun's acid-fast (MAF) staining Giemsa staining, and direct and indirect immunofluorescence antibody methods were used, 7 (3.5%) positive cases in 200 children with diarrhea in Turkey were found.²⁰

Molecular epidemiology of *Cryptosporidium* isolates has proved to be useful in determining the sources of infection.²¹ For example, 5 different species of *Cryptosporidium* were differentiated using PCR-RFLP.²² In another study, the difference between human and animal isolates of *C. parvum* has been demonstrated by using molecular and biological markers.²³ In addition the use of molecular tools has been useful in the assessment of the zoonotic potential of various *Cryptosporidium species* and sources of children, and has played a significant role in the characterization of transmission dynamics in endemic and epidemic areas. The *C. parvum* was detected in 58 of the children (94% of the infected cases), *C. hominis* was detected in 3 of the children (5%) and both *C. parvum* and *C. hominis* were detected in one of the children (1%) in Kuwait.²⁴ Restriction analysis of the SSU, and PCR products has revealed that 41 out of 43 children in Malawi PCR-positive samples had *C. hominis*, and 2 had *C. parvum*.²⁵

Cryptosporidiosis is a major public health concern. The role of water and food in the epidemiology of these diseases is now well recognized. Molecular techniques are available to determine the species and genotypes of *Cryptosporidium* and to distinguish human from non-human pathogens.²⁶ In our analysis, we detected a low rate of infection. All the positive samples were of *C. parvum* cattle type indicating a probable zoonotic source of infection. We believe that attention should be paid in rural areas where farming is the major source of income. Surveys have demonstrated that most sources of drinking water were contaminated with oocysts prior to treatment. Oocysts are more frequent and in higher densities in water contaminated with agricultural, sewage, or urban runoff. Organisms found in water include both *C. parvum* and *C. hominis* bovine genotype. Low-grade contamination has also been documented in 24% of samples of treated water.^{2,3,15} Currently, we do not have convincing data on the

quality of our water sources, and the infection rate in livestock or in pets. A more detailed study should be planned to investigate the major sources of infection and the condition of drinking, and recreational water sources in this region.

In conclusion, our results indicated that cryptosporidiosis was not a common infection for immunocompetent children in Turkey.

References

- Clinton W Jr. Cryptosporidiosis. In: Mandell GL, Benett JE, Dolin R, editors. *Mandell, Douglas and Benett's Principles and Practice of Infectious Diseases*. 6th ed. Philadelphia (PA): Churchill Livingstone; 2005. p. 3215-3228.
- John TJ, Petri AW. Markell and Voge's Medical Parasitology. 9th ed. Philadelphia (PA): WB Saunders; 2006. p. 68-71.
- Rose JB, Huffman DE, Gennaccaro A. Risk and control of waterborne cryptosporidiosis. *FEMS Microbiol Rev* 2002; 26: 113-123.
- Garcia LS. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. *J Clin Microbiol* 1983; 18: 185-190.
- Tanriverdi S, Widmer G. Differential evolution of repetitive sequences in *Cryptosporidium parvum* and *Cryptosporidium hominis*. *Infect Genet Evol* 2006; 6: 113-122.
- Spano F, Putignani K, Cristanti L, Sallicandro P, Morgan UM, Le Blancq SM, et al. Multilocus genotypic analysis of *Cryptosporidium parvum* isolates from different hosts and geographical origins. *J Clin Microbiol* 1998; 36: 3255-3259.
- Sulaiman IM, Morgan UM, Thompson RC, Lal AA, Xiao L. Phylogenetic relationships of *Cryptosporidium* parasites based on the 70-kilodalton heat shock protein (HSP70) gene. *Appl Environ Microbiol* 2000; 66: 2385-2391.
- Sulaiman IM, Lal AA, Arrowood MJ, Xiao L. Biallelic polymorphism in the intron region of beta-tubulin gene of *Cryptosporidium* parasites. *J Parasitol* 1999; 85: 154-157.
- Peng MM, Xiao L, Freeman AR, Arrowood MJ, Escalante AA, Weltman AC et al. Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. *Emerg Infect Dis* 1997; 3: 567-573.
- Sulaiman IM, Xiao L, Yang C, Escalante L, Moore C, Beard B, et al. Differentiating human from animal isolates of *Cryptosporidium parvum*. *Emerg Infect Dis* 1998; 4: 681-685.
- Elwin K, Chalmers RM, Roberts R, Guy EC, Casemore DP. Modification of a rapid method for the identification of gene-specific polymorphisms in *Cryptosporidium parvum* and its application to clinical and epidemiological investigations. *Appl Environ Microbiol* 2001; 67: 5581-5584.
- Akiyoshi DE, Feng X, Buckholt G, Widmer G, Tzipori S. Genetic Analysis of a *Cryptosporidium parvum* human genotype 1 isolate passaged different host species. *Infect Immun* 2002; 70: 5670-5675.
- Clark DP. New insights into human cryptosporidiosis. *Clin Microbiol Rev* 1999; 12: 554-563.
- Tanriverdi S, Arslan MO, Akiyoshi DE, Tzipori S, Widmer G. Identification of genotypically mixed *Cryptosporidium parvum* populations in humans and calves. *Mol Biochem Parasitol* 2003; 130: 13-22.
- Ward PI, Deplazes P, Regli W. Detection of eight *Cryptosporidium* genotypes in surface and waste waters in Europe. *Parasitol* 2002; 124: 359-368.
- Stantic-Pavlinic M, Xiao L, Glaberman S, Lal AA, Orazen T, Rataj-Verglez A, et al. Cryptosporidiosis associated with animal contacts. *Wien Klin Wochenschr* 2003; 115: 125-127.
- Yücel A, Bulut V, Yılmaz M. The study of Intestinal Parasites in Elementary school students of in Elazığ. *Acta Parasitol Turcica* 2000; 24: 126-132.
- Dogan N, Akgun Y. The study of Intestinal Parasites in Elementary school students of Bozan area. *Acta Parasitol Turcica* 1998; 22: 395-398.
- Orhon V, Aksoy U, Akisu C, Inci A, Acikgoz M. The distribution of intestinal parasites in the Karsiyaka Orphanage of Izmir. *Acta Parasitol Turcica* 2000; 24: 283-285.
- Akyon Y, Erguven S, Arıkan S, Yurdakok K, Gunalp A. *Cryptosporidium parvum* prevalence in a group of Turkish children. *Turk J Pediatr* 1999; 41: 189-96.
- McLauchlin J, Amar C, Pedraza-Diaz S, Nichols GL. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1705 fecal samples from humans and 105 fecal samples from livestock animals. *J Clin Microbiol* 2000; 38: 3984-3990.
- Awad El, Kariem FM, Warhurst DC, McDonald V. Detection and species identification of *Cryptosporidium* oocysts using a system based on PCR and endonuclease restriction. *Parasitol* 1994; 109: 19-22.
- Awad El, Kariem FM, Robinson HA, Petry F, McDonald V, Evans D, et al. Differentiation between human and animal isolates of *Cryptosporidium parvum* using molecular and biological markers. *Parasitol Res* 1998; 84: 297-301.
- Sulaiman IM, Hira PR, Zhou L, Al-Ali FM, Al-Shelahi FA, Shweiki HM, et al. Unique endemicity of cryptosporidiosis in children in Kuwait. *J Clin Microbiol* 2005; 43: 2805-2809.
- Peng MM, Meshnick SR, Cunliffe NA, Thindwa BD, Hart CA, Broadhead RL, et al. Molecular epidemiology of cryptosporidiosis in children in Malawi. *J Eukaryot Microbiol* 2003; 50: 557-559.
- Smith HV, Caccio SM, Tait A, McLauchlin J, Thompson RC. Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections in humans. *Trends Parasitol* 2006; 224: 160-167.