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Epidemiology of Cryptosporidiosis in North American Travelers to Mexico

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Abstract

We studied 1,179 North American travelers who visited Mexico from 2005 to 2007. Travelers' diarrhea (TD) was reported by 521 (44%) participants. Among subjects with TD, 218 cases were examined for cryptosporidiosis by polymerase chain reaction (PCR) and enzyme-linked immunoassays (ELISA). There were 14 (6%) cases of cryptosporidiosis and 141 cases (64%) of bacterial diarrhea. Compared with bacterial diarrhea, a longer stay in Mexico was a risk factor for cryptosporidiosis. Additionally, *Cryptosporidium* cases passed greater number of watery stools ($P < 0.05$), suffered more episodes of diarrhea ($P \leq 0.05$), and were more likely to experience tenesmus ($P \leq 0.05$) compared with bacterial causes of TD. ELISA detected seven (3%) cases of *Cryptosporidium*, whereas PCR identified an additional seven cases (6%). Speciation by 18SrRNA sequencing showed that 13 cases were caused by *C. parvum* and only 1 case was caused by *C. hominis*. ELISA showed a sensitivity of 50% and specificity of 100% compared with PCR.

Introduction

Travelers' diarrhea (TD) affects 20–50% of persons from developed nations who visit developing countries.¹ Although bacterial pathogens as a group are the most common cause of acute diarrhea in travelers, parasites have been associated with persistent and chronic diarrhea. Common causal organisms of parasitic diarrhea in travelers are *Cryptosporidium* spp., *Giardia* spp., and *Entamoeba* spp.^{1–3} Travel acquired parasitic infections are often insidious in onset, and because of long incubation periods, are often diagnosed on return to the original country of departure.

In adults, seropositivity to *Cryptosporidium* is an indicator of past or recent infection and is also a marker of partial protection against the parasite.^{4,5} The rate of seropositivity in US adults is ~25% in contrast to 64% among the Latin American urban population.^{4,5}

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Over the past two decades, the methods for *Cryptosporidium* detection have advanced from simple acid-fast staining to enzyme-linked immunoassays (ELISA), and immunofluorescent assays (IFA) to molecular techniques based on polymerase chain reaction (PCR) amplification of specific gene loci.⁴ Fecal ELISA is a quick, easy, and convenient method to test large numbers of stools specimens that has found wide application in clinical settings.^{6,7} Several studies have documented a high sensitivity for DNA PCR-based methods in detecting *Cryptosporidium* in fecal samples.^{8–13} Although technically more demanding, the nested PCR based on the 18S rRNA gene followed by direct sequencing offers the added advantage of characterizing the infecting organism at the species level.¹³

Cryptosporidiosis is a common disease in Mexican children, as evidenced by a study done in infants with severe diarrhea, where 41% were found to be infected with *Cryptosporidium*.¹⁴ In many instances, the infection is asymptomatic, particularly in older children.^{15,16} In a previous study conducted by our group on 127 US travelers to Mexico and 183 Mexican adults with diarrhea, cryptosporidiosis as determined by ELISA was more commonly seen in US travelers than in Mexican residents (3% versus < 1%).¹⁷ Another study found a 7% *Cryptosporidium* isolation rate among 87 adult travelers to Mexico.¹⁸ Although epidemiologic features of *Cryptosporidium* in human outbreaks has been well described,^{3,19} there are few studies on the clinical characteristics, epidemiology, and genotypes of *Cryptosporidium* that affect international travelers.²⁰

The aim of our study was to characterize the clinical features and risk factors associated with cryptosporidiosis among US travelers to Mexico. We compared the detection rate of *Cryptosporidium* with ELISA to the detection rate with PCR followed by direct sequencing.

Materials and Methods

Human subjects

Participants were travelers from North America who visited the Mexican cities of Cuernavaca and Guadalajara between June 2005 and January 2007 to learn the Spanish language. The eligibility for participation entailed a minimum age of 16 years after parental consent or being an adult with informed consent, good health, and a stay of at least 5 days in Mexico. Subjects with known pre-existing irritable bowel syndrome, lactose intolerance, or pregnancy or those taking antibiotic prophylaxis for TD were excluded from the study.

Recruitment and management

Students were enrolled within 72 hours of arrival at Mexico. The cohort was observed for the incidence, severity, and etiology of diarrhea while in Mexico. Participants who had soft unformed or watery stools at a frequency of three or more times in a 24-hour period were asked to contact the study personnel and report to the clinic within 72 hours for stool sample collection. Fecal samples from travelers matched for age, sex, and duration of stay were studied as a control group. This study was approved by the University of Texas Committee for the Protection of Human Subjects.

Laboratory procedures

For detection of enteric pathogens, stools were cultured for *Shigella* spp., *Salmonella* spp., *Providencia* spp., *Plesiomonas* spp., *Serratia* spp., and *Campylobacter* spp. Enterotoxigenic *Escherichia coli* was identified by colony PCR or colony hybridization, whereas enteroaggregative *E. coli* were detected by HEp2 cell adherence assay or by colony PCR. Stool samples were examined for the presence of cryptosporidiosis by a commercially available microplate ELISA kit (Remel ProSpecT, Lenexa, KS) following the manufacturer's instructions.

PCR for cryptosporidiosis

An aliquot of stools was frozen for PCR studies. DNA extraction from stools (2 mg of frozen stools or 200 µL of liquid stools) was carried out with QIA amp DNA stool mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The small subunit of the *C. parvum* 18S rRNA gene was amplified by a two-step nested PCR protocol. Amplification of a PCR product of 833 bp (GenBank accession no. X64341) was done with the forward primer CryrRNAF3 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3'), and the reverse primer CryrRNAR3 (5'-AAGGAGTAAGGAACAACCTCCA-3') for the first PCR.¹³ The first PCR was performed in 1:10 reaction volume of 1× Hot Star Taq master mix buffer using 0.5 µmol/L of each primer, 1:2 dilution of 0.05 µg/µL bovine serum albumin, and 2 µL of 1:2 dilution of stool DNA as a template. The first PCR cycling steps consisted 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and a final extension step at 72°C for 4 minutes. For the second amplification step, the PCR nested primers CryNestF1 (5'-TTCCAGCTCCAATAGCGTATA-3'), and CryNestR1 (5'-TCACCTCTGACTGTAAATAC-3') were used. An amplicon of 308 bp was produced in this reaction. The same quantities of reagents were used during the second PCR as were used in the first PCR reaction, except that a 1:2 dilution of the primary PCR mixture was used as a template. The nested reaction consisted of 35 cycles that were run after an initial denaturation step at 94°C for 1 minute, primer annealing at 58°C for 1 minute, and DNA extension at 72°C for 1 minute; these cycles were followed by a final incubation at 72°C for 4 minutes. The amplified product was separated by electrophoresis on a 1.5% agarose gel and visualized under a UV transilluminator after incubation with ethidium bromide. PCR products from the nested reaction used CryNestF1 (5'-TTCCAGCTCCAATAGCGTATA-3') and CryNestR1 (5'-TCACCTCTGACTGTAAATAC-3') primers, which allow for the amplification of polymorphic regions at nucleotide position 639–656, and 689–699. An ABI 3130x1 Genetic Analyzer (Applied Biosystems, Foster City, CA) was used for sequencing.

Statistical analysis

Univariate analyses were performed using SPSS software, version 15 (SPSS, Chicago, IL). The χ^2 test was used to determine association between socio-demographic, clinical, and microbiological data. The cases of cryptosporidiosis with bacterial co-infection were analyzed as cryptosporidiosis. Sensitivity and specificity were used to determine the diagnostic performance of ELISA using PCR followed by sequencing as the gold standard.

Results

Among the 1,179 eligible students who traveled to Mexico between June 2005 to January 2007, 521 (44.19%) developed TD (Table 1). There was no difference in the proportion of TD by sex. US travelers with Asian ancestry displayed a lesser predisposition to develop diarrhea in Mexico. The risk for TD was associated to traveler's age, length of stay, and season of travel. There was a 2% decrease in risk of diarrhea for every 1-year increase in age (odds ratio [OR], 0.98; 95% confidence interval [CI], 0.97–0.99; $P < 0.001$) and a 3% increase in risk for diarrhea with every additional day of stay in Mexico (OR, 1.03; 95% CI, 1.02–1.05; $P < 0.001$). We also noted that travel during months of March to September was associated with an increased frequency of diarrhea compared with travel during October to February (OR, 2.19; 95% CI, 1.55–3.07; $P < 0.001$).

A subset of 218 travelers with TD, in whom *Cryptosporidium* ELISA and fecal PCR for *Cryptosporidium* were done, was analyzed further in this study. Overall, *Cryptosporidium* was detected in 14 (6%) stools examined. ELISA detected seven (3%) cases of cryptosporidiosis. In contrast, an additional seven cases were identified by PCR ($P = 0.12$; Table 2). Bacterial pathogens were isolated from 141 (66%) cases. Enterotoxigenic *E. coli* ($N = 102$; 47%) was

the predominant pathogen, followed by enterotoxigenic *E. coli* ($N = 62$; 28%). Other bacterial pathogens isolated were *Salmonella* spp. ($N = 7$; 3%), *Campylobacter* spp. ($N = 2$; <1%), and *Plesiomonas* spp. ($N = 1$; 0.5%). Multiple bacterial pathogens were identified from 33 (15.1%) of stools examined.

No significant differences were noted in terms of age, race, ethnicity, or sex between bacterial cases and *Cryptosporidium* diarrhea. Of interest, the proportion of men with cryptosporidiosis was higher in the bacterial subgroup (43% versus 28%; $P = 0.23$). A longer period of stay in Mexico was significantly associated with an increased risk of TD caused by *Cryptosporidium* (OR, 1.07; 95% CI, 1.03–1.13; $P = 0.008$) compared with bacterial diarrhea. Most cases of cryptosporidiosis and bacterial TD occurred between June and August.

The clinical characteristics of the TD cases categorized by cause of diarrhea are depicted in Table 3. Students infected with *Cryptosporidium* had greater numbers of episodes ($P = 0.05$) and passed more unformed ($P = 0.07$) and watery stools ($P = 0.02$) than TD caused by bacterial agents. The time from day of arrival in Mexico to the onset of diarrhea after arrival in Mexico was similar in both groups. Among cryptosporidiosis cases, abdominal pain ($N = 14$; 100%) was the most common complaint. Other frequently experienced clinical features were flatulence ($N = 12$; 86%), urgency ($N = 11$; 79%), nausea ($N = 8$; 57%), and tenesmus ($N = 7$; 50%). Vomiting ($N = 2$; 14%) and fever ($N = 3$; 21%) were relatively infrequent symptoms. Presenting symptoms were similar for cases with *Cryptosporidium* and bacterial infection except for tenesmus ($P = 0.05$), which was more common in cases of *Cryptosporidium*.

Results of the DNA sequencing of the 18S rRNA gene showed that 13 of 14 (93%) cases were infected with *Cryptosporidium parvum* and only one with *Cryptosporidium hominis*. Using PCR followed by genetic sequencing as the gold standard, the sensitivity of ELISA was 50% and specificity was 100% (Table 3). The positive predictive value of ELISA was 100%, and the negative predictive value was 96.7%. Thirty-one matched controls were studied by ELISA and PCR; none of the control samples were positive by ELISA. Three *C. parvum* cases were identified among healthy controls by PCR.

Discussion

In this prospective study done in US travelers to Mexico, the incidence of cryptosporidiosis was found to be 6.4%, which was similar to rate of cryptosporidiosis in Mexican children^{14–16} and comparable to the previous series of travelers with similar characteristics.^{17,18} Consistent with prior observations, we found that TD was more frequent among subjects who had a longer length of stay, were younger in age, and traveled during the summer season. Our data are consistent with existing literature that describes the above factors as known risk factors for TD.^{1,2,21}

Cryptosporidiosis manifested with greater numbers of episodes and watery stools compared with bacterial diarrhea. This may be explained by the use of empiric antibiotics to treat travelers with diarrhea. Symptoms experienced included abdominal pain, flatulence, and nausea in addition to diarrhea in frequencies that correspond with observations in other studies.^{20,23} In our study, 10 of the 14 cases of *Cryptosporidium* diarrhea (71.4%) presented with bacterial co-infection. The common bacterial agents co-infecting being enteroaggregative *E. coli* and enterotoxigenic *E. coli*.

Our findings are also consistent with a previous report on travel-associated cryptosporidiosis in German travelers, which found cryptosporidiosis to be associated with longer length of stay abroad.²⁰ In our study, all cases of *Cryptosporidium* occurred in travelers who remained in Mexico for at least 2 weeks ($P = 0.02$). This likely reflects the longer incubation period needed for *Cryptosporidium* compared with bacterial causes of diarrhea. However, we also found that

the mean time to onset for diarrhea after arrival in Mexico was comparable in both groups. A possible reason could be the high proportion of concomitant bacterial infections among the *Cryptosporidium* cases. The mean incubation period of *Cryptosporidium* is ~9 days,^{22–24} and some participants may have acquired the parasite on their last days of the visit. Hence, our study may underestimate the true number of cases of travel related cryptosporidiosis.

The distribution of the infecting species of *Cryptosporidium* varies according to the type of population studied and the geographic location.^{25–40} Studies have noted that differences in predominant infecting *Cryptosporidium* species relate to the level of urbanization, season studied, the immune status, and age of subjects considered.^{26,39,40} Studies in Europe and New Zealand have reported a predominance of the *C. parvum* species among human infections.^{26–17,33} In other series, a greater proportion of *C. hominis* has been reported from Thailand, the United States, and Japan.^{26,28–32} *C. hominis* has been frequently detected among children and HIV-positive adults in other Latin American studies.^{34–38} The high proportion of *C. parvum* compared with *C. hominis* seen in our study suggests that the source of infection may have been zoonotic in origin, although the possibility of person to person transmission cannot be ruled out. As far as we know, this is the first study on the speciation of infecting *Cryptosporidium* species acquired by US travelers to Mexico. Our subjects were healthy adult travelers, whereas previous Latin American studies focused on children and HIV-positive men.^{26,34,36} It is possible that the risk factors and modes of transmission are different among these study populations. There is a need for more extensive molecular studies with a larger number of cases to unravel the relationship between the distribution, epidemiology, and transmission routes of *Cryptosporidium* in travelers.

Cryptosporidium ELISA showed a higher specificity and sensitivity compared with conventional staining techniques.^{6,7,10} Because oocyst wall antigens are well conserved among most species in the *Cryptosporidium* genus, a limitation of the ELISA is its inability to identify the different infecting species.^{13,40} Although previous studies have noted high sensitivity rates for fecal PCR,^{13–16} the detection of *Cryptosporidium* spp. in environmental samples has a low sensitivity.^{41–43} The presence of bilirubin, bile salts, and other contaminants in fecal samples can inhibit the DNA extension in PCR reactions, making the detection less accurate.¹³ We minimized this by adopting a DNA extraction protocol that remove DNases and facilitates the downstream enzymatic polymerase reaction (QIAamp; Qiagen). We also negated the likelihood of contamination in our study by the use of multiple negative controls during the reactions. The probability of underdetection was also decreased by the use of nested PCR, a method sensitive enough to detect < 1 pg of *C. parvum* DNA in human stool.¹²

Our study confirmed that the infection rates of cryptosporidiosis are underestimated by *Cryptosporidium* fecal ELISA. ELISA detected only 50% of the positive cases that were identified by PCR followed by genetic sequencing. A possible explanation could be that the parasite burden was lower in PCR-only positive cases, because the threshold for detection by PCR is thought to be ~10–50 oocysts/g of stool in contrast to the 10³–10⁴ parasites needed for ELISA.^{7–10,44}

Being a highly infectious and ubiquitous parasite that can cause large outbreaks in healthy, immunocompromised persons and the elderly, a quick and reliable diagnosis along with knowledge of the infecting species are of great importance in the management of cryptosporidiosis. The use of molecular tools for the diagnosis of cryptosporidiosis coupled with the knowledge of the parasite's epidemiology in travelers will promote the understanding of the factors influencing species transmission pathways and host susceptibility factors, which can aid in more targeted and successful interventions for prevention and control of cryptosporidiosis.

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Table 1
Demographic features of US travelers to Mexico and risk factors for acquiring travelers' diarrhea

Characteristics	Healthy (N = 658)		Diarrhea (N = 521)		OR (95% CI)	P value
	N	Percent	N	Percent		
Sex						
Females (N = 861)	491	74.6	370	71.0	(Ref)	
Males (N = 318)	167	25.4	151	29.0	1.2 (0.9–1.6)	0.19
Ethnicity						
Hispanics (N = 133)	76	11.6	57	10.9	(Ref)	
Non-Hispanics (N = 1046)	582	88.5	464	89.1	1.1 (0.7–1.5)	0.74
Race						
Whites (N = 1056)	581	88.3	475	91.2	(Ref)	
African Americans (N = 71)	44	6.7	27	5.2	0.8 (0.5–1.2)	0.26
Asians (N = 29)	21	3.2	8	1.5	0.5 (0.2–1.1)	0.07
Others (N = 23)	12	1.8	11	2.1	1.1 (0.5–2.5)	0.78
Season						
October–February (N = 187)	133	20.2	54	10.4	(Ref)	
March–September (N = 992)	525	79.8	467	89.6	2.2 (1.6–3.1)	< 0.001
Length of stay (days)	21.4 ± 9.1 *		24.3 ± 9.6 *		1.03 (1.02–1.05)	< 0.001
Age (years)	32.5 ± 14.3 *		29.6 ± 12.8 *		0.98 (0.97–0.99)	< 0.001

* Student t test; means ± SD.

Table 2
Comparison of PCR followed by genotyping versus ELISA for *Cryptosporidium* detection

Method	No of samples examined	No of positives detected	Sensitivity (%) [†]	Specificity (%) [‡]	Positive predictive value (%) [§]	Negative predictive value (%) [¶]
PCR [*]	218	14	100	100	100	100
ELISA	218	7	50	100	100	97.6

^{*} PCR followed by genotype sequencing.
[†] Calculated as [number of true positives/(number of true positives + number of false negatives)].
[‡] Calculated as [number of true negatives/(number of true negatives + number of false positives)].
[§] Calculated as [number of true positives/(number of true positives + number of false positives)].
[¶] Calculated as [number of true negatives/(number of true negatives + number of false negatives)].

Table 3
Epidemiologic and clinical features of bacterial TD compared with *Cryptosporidium* TD

Variable	Bacterial diarrhea (N = 141)		Cryptosporidiosis* (N = 14)		P value
	N	Percent	N	Percent	
Clinical features					
Episodes					
Single (N = 114)	107	76.4	7	57.1	0.23
Multiple (N = 41)	34	23.6	7	42.9	
Total unformed stools/100 days of stay	84.3 (72.2–96.4) [†]		121.1 (86.2–155.9) [†]		0.07
Total watery stools/100 days of stay	40.8 (31.6–50.0) [†]		76.4 (39.4–113.3) [†]		0.02
Onset after arrival in Mexico (days)	10.6 (9.3–11.8) [†]		9.3 (5.4–13.16) [†]		0.53
Symptoms					
Abdominal pain (N = 130)	116	82.3	14	100.0	
Flatulence (N = 118)	106	75.2	12	85.7	0.75
Nausea (N = 92)	84	59.6	8	57.1	1
Vomiting (N = 33)	31	22.0	2	14.3	0.73
Urgency (N = 116)	105	74.5	11	78.6	1
Tenismus (N = 40)	33	23.4	7	50.0	0.05
Fever (N = 15)	12	8.5	3	7.1	1

* Includes the cases of cryptosporidiosis only and cryptosporidiosis with bacterial co-infection. Cryptosporidiosis cases in which bacterial entero-pathogens were identified were counted once in the cryptosporidiosis group for analysis and were not included in the bacterial infection subgroup.

[†] Student t test; mean (95% CI).