Microsporidia in HIV-Positive and HIV-Negative Pediatric Patients with Diarrhea at a Tertiary Care Hospital

Dinesh Kumar[1] Varun Goel[2] Suneeta Meena[1] Purva Mathur[3] Sushil Kumar Kabra[4] Rakesh Lodha[4] Sarman Singh[1,5]

[1] Division of Clinical Microbiology & Molecular Medicine, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India
[2] Department of Microbiology, Government Institute of Medical Sciences, Greater Noida, Uttar Pradesh, India
[3] Department of Laboratory Medicine, Jai Prakash Narain Apex Trauma Centre, All India Institute of Medical Sciences, New Delhi, India
[4] Department of Paediatrics, All India Institute of Medical Sciences, New Delhi, India

Address for correspondence Sarman Singh, MD, FRCP, FRSC, Mentor, Medical Sciences and Engineering Research (MEDSER) Centre, Indian Institute of Science Education and Research, Bhopal-462066, Madhya Pradesh, India (e-mail: sarman_singh@yahoo.com).

[5] Medical Sciences and Engineering Research (MEDSER) Centre, Indian Institute of Science Education and Research, Bhopal-462066, Madhya Pradesh, India

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Abstract

Background Human microsporidiosis presents as an important and rapidly emerging opportunistic infection. However, the exact burden of this infection especially in the pediatric population of Northern India remains unknown. In this study, we investigated the prevalence of microsporidia among human immunodeficiency virus (HIV)-positive and HIV-negative pediatric patients who presented with diarrhea.

Methods A total of 263 children were recruited consisting of 98 HIV seropositive with diarrhea and 165 HIV seronegative but with diarrhea. Morning stool samples were collected and both direct and formol ether concentrated samples were examined for the presence of intestinal parasites. The modified acid-fast staining was done for coccidian parasites and trichrome stain for microsporidia detection. Further, the species were detected using a real-time polymerase chain reaction (PCR) targeting a conserved region of the small ribosomal subunit rRNA gene of *Enterocytozoon bieneusi*, *Encephalitozoon hellem*, *Encephalitozoon intestinalis*, and *Encephalitozoon cuniculi*.

Results Overall, one or more parasites were detected in 52.04% (51/98) of HIV seropositive and 53.33% (88/165) of seronegative children ($p = 0.8391$). However, coccidian parasites were detected in a significantly huge number of HIV seropositive children (21.43% [21/98]) as compared with HIV seronegative children (4.24% [7/165]). Microsporidial DNA could be detected in HIV seropositive with diarrhea children (17.35% [17/98]) by PCR. A significant correlation between low CD4 count ($\leq$ 200/µL) and intestinal parasite positivity could be established.

Conclusion Microsporidia is a significant cause of diarrhea in HIV seropositive pediatric patients and should be kept in mind as one of the differential diagnoses in such patients.

Keywords
- CD4 count
- *Cryptosporidium*
- *Cyclospora*
- *Cystoisospora*
- HIV/AIDS

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Introduction

Diarrhea is a leading cause of death among all ages as well as a leading cause of diarrhea disability-adjusted life-years because of its disproportionate impact on young children.[1] Parasitic infection is a foremost global public health problem, mostly in developing countries that are contributing to the greatest cause of illness and disease.[2] It is well-established fact that immunocompromised children are predisposed to many infections including parasitic infections.[3]

Globally, it is estimated that 2.78 million children aged between 0 and 19 years are living with human immunodeficiency virus (HIV) with daily mortality of 330 children from acquired immunodeficiency syndrome (AIDS)-related causes.[4] However, in India it is estimated around 81,000 children are living with HIV/AIDS less than 15 years.[5] As of 2020, 54% of children with HIV received antiretroviral therapy.[6] In the absence of antiretroviral therapy, approximately 30% of untreated HIV-positive children die before their first birthday and more than 50% die before they reach 2 years of age.[7] Diarrhea cases among children with HIV have more severe, longer-lasting, and more comorbidities and higher case-fatality rates.[8] It has been observed that 10 to 50% of untreated HIV infected patients get diarrhea.[9] Several studies have been conducted on the prevalence of parasitic infections in children where high incidence and prevalence rates of parasites like *Giardia lamblia*, *Entamoeba histolytica/Entamoeba dispar*, *Hymenolepis nana*, and *Ascaris lumbricoides* have been seen in school-going children.[10,11] However, opportunistic infections like *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Cystoisospora belli*, and *Blastocystis hominis* are frequently detected in immunocompromised children especially those infected with HIV.

Microsporidia is another emerging pathogen in immunocompromised patients, although now reclassified as fungus, clinically can present as the above parasitic organism. Microsporidia is a group of organisms, among which, 14 spp. of 8 genera widely infect humans with *Enterocytozoon bieneusi* and the *Encephalitozoon* spp among the most common microsporidia causing diarrhea and systemic diseases.[12–14] Intestinal microsporidiosis was prevalent among 14.2% of HIV/AIDS patients and was significantly associated with diarrhea (odds ration [OR] 3.4).[12] The prevalence of microsporidia in humans ranges from 1 to over 50%, depending on the geographic region, the methods applied for diagnosis, and demographic characteristics of the studied population.[13] At a national level, in India there is scanty data on microsporidial infections in HIV-positive pediatric patients.[14–16] Therefore, this study was undertaken to find the prevalence of microsporidia in HIV seropositive and HIV seronegative pediatric patients seen at a tertiary care North Indian health setting.

Materials and Methods

This prospective study was performed at an apex tertiary care teaching hospital from 2013 to 2017. The study protocol was approved by the Institutional Ethics Committee (Ref. no. IESC/T-315/02.08.2013).

Pediatric patients under 15 years of age with diarrhea attending the pediatric outpatient department and Antiretroviral therapy (ART) clinic were recruited for this study. Those subjects who had not taken any specific antidiarrheal therapy in the last 15 days were enrolled after obtaining the written consent from their guardians. Age and sex-matched randomly selected HIV seronegative children with symptoms of diarrhea were also enrolled as a control group who were advised to get their stool samples examined for ova and parasites. A total of 263 patients were recruited consisting of 98 HIV seropositive with diarrhea and 165 with diarrheal only children. Morning stool samples from each subject on three consecutive days were collected. Clinical and demographic information, including personal identification, age, the stage of disease, CD4 count, and other clinical symptoms, were recorded. One part of the stool sample was used for wet mount and modified Z-N stained smear microscopy was used and the other part of the sample was stored at −80°C in normal saline for further processing.

Diarrhea was defined as the passage of three or more loose or watery bowel movements in 24 hours. Acute diarrhea was defined as diarrhea that lasted 7 days or less at the time of presentation. Persistent diarrhea was defined as diarrhea that lasted for more than 7 days but less than 14 days at presentation. Diarrhea was called chronic if it lasted for more than 14 days.[17] Unconcentrated stool samples and those after formol ether concentration were subjected to wet mount examination and modified acid-fast staining for the demonstration of ova, cysts, and coccidian intestinal parasites.[3] From the concentrated samples, three to four smears were made and modified trichrome staining was done using the method of Kokoskin et al[18] to visualize the spores of microsporidia. Briefly, the dried smear was chemically fixed by dipping the slide 4 to 5 times in methanol. Thereafter, slide was kept in a coupling jar containing trichrome stain for 90 minutes at room temperature. After that, the slide was dipped in acid alcohol for 1 minute. Thereafter, the slide was kept in two batches of 95% ethanol for 2 minutes. each. Further, the slide was kept in 100% ethanol for 2 minutes. Finally, the slides were dipped in xylene, dried and examined under 1000X magnification in oil immersion.

To confirm the microsporidial spores electron microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were also done as a gold standard.

One aliquote of the stool samples from patients and controls collected on three consecutive days were pooled to economize the cost of nucleic acid amplification. The pooled samples were used for the DNA extraction by QIAamp DNA stool mini kit (QAIGEN Inc., Valencia, California, United States) and processed according to the manufacturer's instructions. The extracted fecal DNA was subjected to amplify the conserved region of the small subunit ribosomal rRNA (SSU rRNA) gene that was used for amplification of the entire SSU-rRNA gene of *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, and *Encephalitozoon hellem* using forward (5′-CACCAGGTTGATTCTGCCTGAC-3′) and reverse (5′-GGTTACCTTGTTACGACTT-3′) primers described by Franzen

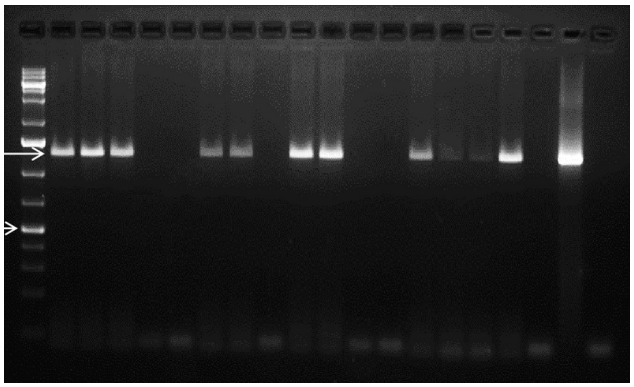

Fig. 1 Polymerase chain reaction results for the detection of microsporidia. First lane small arrow denotes 1 kb DNA ladder; Big arrow denotes positive band at 1.2 kb; positive samples lane: 2, 3, 4, 7, 8, 10, 11, 14, and 17; positive control lane: 19; negative control lane: 20.

et al.[19] The polymerase chain reaction (PCR) was performed in a 20 µL of a total volume containing 200 mM each de-oxynucleoside-triphosphates, 12.5 pmol of each primer, and 1 unit of Taq DNA polymerase. PCR conditions were standardized as follows: initial denaturation of template DNA at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute and elongation at 72°C for 1.5 minute for 30 cycles, and a final extension at 72°C for 10 minutes. Template-free sterile water was used as a negative control. The samples positive for microsporidia yielded amplicons of 1200 bp. The PCR amplified DNA was analyzed using 1% agarose gel electrophoresis. The DNA of samples positive for microsporidia was 1.2kb. The band of 1.2kb fragment of the conserved region of SSU rRNA)gene of microsporidia using forward (5′-CACCAGGTTGATTCTGCCTGAC-3′) and reverse (5′- GGTTACCTTGTTACGACTT-3′) primers were amplified from the fecal specimen of all seropositive patients (►**Fig. 1**). The performance of the standardized PCR was 100% concordant with microscopic results.

Statistical Analysis

Categorical, nonparametric continuous data were presented as proportion, mean, and standard deviation, respectively. Chi-squared and Mann–Whitney U tests were used to compare categorical and continuous nonparametric data, respectively. The fisher Chi-squared test and the difference between two variables were considered significant if the *p*-value was less than 0.05. Statistical analysis was done using SPSS version 15 (SPSS, Inc., Chicago, Illinois, United States).

Results

A total of 263 pediatric patients were enrolled in the study, which included 98 (37.26%) HIV seropositive cases with diarrhea and 165 (62.74%) HIV-negative cases but with diarrhea.

Of the 98 HIV-positive patients, the majority of cases (61 [62.24%]) were boys with a mean age of 9.7 ± 3.9 years. The mean age of girls was 9.94 ± 3.7 years ($p = 0.7641$). In HIV-negative patients, 94 (56.97%) were boys with a mean age of 7.8 ± 4.7 years and the girls had a mean age of 8.35 ± 5.1 years ($p = 0.4721$). The majority of children were school going, while only 21.3% of boys and 16.2% of girls were in preschool or not going to any school.

Of the study subjects, chronic diarrhea was significantly more in the HIV seropositive than the HIV seronegative (►**Table 1**). Abdominal pain, weight loss, fever, and night sweats were the most common symptoms in HIV seropositive diarrheal cases with Microsporidiosis. Other manifestations in these patients were dehydration, flatulence, and oral thrush (►**Table 2**).

Overall, in 52.04% (51/98) of HIV seropositive individuals and 53.33% (88/165) of seronegative individuals one or more intestinal parasite was detected. This difference was not statistically significant ($p = 0.8391$). However, coccidian parasites were detected mainly in HIV seropositive children (21.43%; 21/98) as compared with seronegative children (4.24%; 7/165) that were statistically highly significant ($p = 0.0001$). *Cryptosporidium* was detected in both groups. Only three children had coinfection with *Cryptosporidium* and *Microsporidium*. However, five cases of coinfection with *Entamoeba histolytica/Entamoeba dispar* and *Giardia lamblia* were seen (►**Table 3**).

Microsporidia were detected in 15 (15.31%) by modified trichrome staining and 17 (17.35%) by PCR in HIV seropositive children with chronic diarrhea (►**Table 4**).

Out of 15 samples that were positive for microsporidia on modified trichrome staining and PCR, four representative samples were subjected to the SEM and TEM. In all four samples oval-shaped microsporidial spores measuring approximately 1 to 3um under the SEM. In TEM dense outer layer of the chitin-rich wall (exospores) and the electron-lucent inner layer (endospore; ►**Fig. 2**).

Table 1 Duration of diarrhea in study

Duration	HIV seropositive with diarrhea, *n* (%) (percentage) (*n* = 98)	HIV seronegative with diarrhea, *n* (%) (*n* = 165)	*p*-Value (chi-squared test)
<1 week (acute diarrhea)	45 (45.92)	103 (62.42)	0.0103
1–2 weeks (persistent diarrhea)	20 (20.41)	48 (29.09)	0.1454
>2weeks (chronic diarrhea)	33 (33.67)	14 (8.48)	0.0001

Abbreviation: HIV, human immunodeficiency virus.

Table 2 Associated clinical features on presentation to hospital of HIV-infected diarrhea subjects with/without microsporidia

Clinical feature	Without microsporidia ($n = 81$) *n* (%)	With microsporidia ($n = 17$) *n* (%)	*p*-Value
Abdominal pain	67 (82.72)	17 (100)	0.1199
Weight loss	67 (82.72)	17 (100)	0.1199
Loss of appetite	58 (71.60)	13 (76.47)	0.7739
Dehydration	29 (35.80)	13 (76.47)	0.0028
Flatulence	28 (34.57)	12 (70.59)	0.0127
Fever	78 (96.29)	17 (100)	1.0000
Night sweat	76 (93.83)	17 (100)	0.5835
Respiratory infections	39 (48.15)	13 (76.47)	0.0591
Oral thrush	21 (25.93)	12 (70.59)	0.0011
Dysphonia	4 (4.94)	6 (35.29)	0.0016
Lymphadenopathy	4 (4.94)	1 (5.88)	1.0000
Tuberculosis	7 (8.64)	1 (5.88)	1.0000
Recurrent ear infections	2 (2.47)	0	1.0000
Hepatosplenomegaly	3 (3.70)	1 (5.88)	0.5394
Altered sensorium	6 (7.41)	2 (11.76)	0.6244
Parotid enlargement	5 (6.17)	3 (17.65)	0.1395
Developmental delay	6 (7.41)	4 (23.53)	0.0681
Genital ulcer	2 (2.47)	0	1.0000

Abbreviation: HIV, human immunodeficiency virus.

Table 4 Comparison of PCR and modified trichrome results for microsporidia in retro-positive and negative pediatric patients with diarrhea

Modified Trichrome method	PCR positive	PCR negative	*p*-Value
Positive	15	0	< 0.00001
Negative	2	81	
Total	17	81	

Abbreviation: PCR, polymerase chain reaction.

The CD4 levels were found to be directly associated with the incidence of coccidian parasitic infection and patients with lower CD4+ counts had a higher incidence of coccidian parasitic infection. The *Cryptosporidium* species was detected in 84.61% children who had CD4+ count less than 200 cells/uL. Similarly, *Cyclospora* in 85.71% and *Cystoisospora* in 100% were detected in children who had CD4+ count less than 200 cells/uL. Likewise, in 92.86% of these children microsporidia spores were observed. However, no coccidian parasites and microsporidial spores were detected in the children with CD4+ count more than 301 cells/uL (►**Table 5**).

Discussion

Diarrhea is among the leading complication resulting in hospital visits in patients with HIV.[20] The etiology of diarrhea

Table 3 Parasite positivity in HIV-positive and HIV-negative pediatric patients

Wet mount	HIV positive with diarrhea ($n = 98$), *n* (%)	HIV negative with diarrhea ($n = 165$) , *n* (%)	*p*-Value
Pathogenic species			
Giardia lamblia	11 (11.22%)	41 (24.84%)	0.007
Entamoeba histolytica/ Entamoeba dispar	3 (3.06%)	11 (6.67%)	0.208
Ascaris	3 (3.06%)	9 (5.45%)	0.368
Strongyloides stercoralis	4 (4.08%)	5 (3.03%)	0.650
Hookworm	4 (4.08%)	13 (7.88%)	0.226
Hymenolepis nana	1 (1.02%)	5 (3.03%)	0.291
Modified ZN stain			
Cryptosporidium	9 (9.18%)	7 (4.24%)	0.105
Cyclospora	7 (7.14%)	0	0.000
Cystoisospora	5 (5.10%)	0	0.000
Modified trichrome			
Microsporidia	15 (15.31%)	0	0.000

Abbreviation: HIV, human immunodeficiency virus.

in HIV is multifactorial. There are various studies on the etiological agents of diarrhea in HIV but very few reports are published on the microsporidia etiology in HIV pediatric cases in relation to manifestations, and CD4 T lymphocyte counts.[15,20–23] This study highlights microsporidia as one of the etiological agents of diarrhea. This study also looked into the correlation of coccidian parasites isolated with HIV seropositivity status and CD4 T lymphocyte counts.

Of the study subjects, chronic diarrhea was significantly more (33.67%, $p = 0.0001$) in the HIV seropositive than the HIV seronegative. Other studies from North India had earlier reported chronic diarrhea in 60 to 68% HIV-positive patients than the HIV-negative patients.[16,24] This decrease in recent years may be because of better preventive prophylaxis safe drinking water, reduction in the open defecation, promotion of hand-washing, micronutrients, and awareness about HIV/AIDS in the general public. Stool parasites other than microsporidia were detected in 52% of HIV seropositive and 72% of HIV seronegative pediatric patients that were statistically significant ($p = 0.0008$). This result contrasts with other studies where parasite positivity was higher in the seropositive group.[25–27] This reversal can also be explained by the awareness and caution taken by HIV-positive families/parents of HIV-positive children.

In our study, most HIV seropositive diarrheal cases with demonstrable microsporidia spores had abdominal pain, weight loss, fever, and night sweats. Dehydration, flatulence, and oral thrush were significantly associated with HIV seropositive diarrheal cases with microsporidia in comparison to subjects without microsporidia. None of the previous studies as per the literature review has analyzed these features.

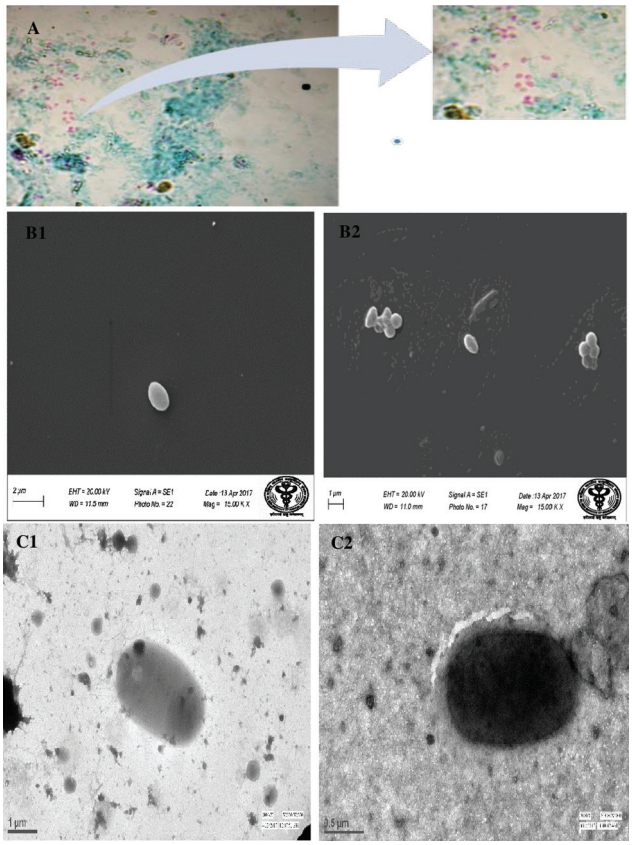

Fig. 2 (**A**) Pictorial presentation of spores of microsporidia observed in modified tri-chrome stain in stool samples (magnification, ×1,000). (**B1**) Scanning electron microscope micrograph (SEM) of microsporidia from a stool specimen of a human immunodeficiency virus (HIV)-infected patient with chronic diarrhea with scale bar of 2 µm. (**B2**) SEM of microsporidia from a stool specimen of an HIV-infected patient with chronic diarrhea with scale bar of 1 µm. (**C1**) Transmission electron microscope micrograph (TEM) of microsporidia from a stool specimen of an HIV-infected patient with chronic diarrhea with scale bar of 1µm. (**C2**) TEM of microsporidia from a stool specimen of an HIV-infected patient with chronic diarrhea with scale bar of 0.5 µm.

The prevalence of microsporidia infection in pediatric patients with diarrhea was 15.3% by microscopy and 17.4% by PCR. All microscopy positive specimens were also positive by PCR. Interestingly, among all the parasites studied in the HIV-positive patients with diarrhea, microsporidia were the most common parasite (17.4%) followed by *Giardia lamblia* (11.2%), when analyzed irrespective of HIV status and CD4 counts. This may be due to the unique cohort we have taken of HIV-positive pediatric patients with diarrhea, while most other studies have a majority of the adult population in the study group.[21–28]

In our study, no microsporidia were observed in HIV-negative patients. It is in contrast with other studies from India and other countries that have reported a very low prevalence (1.2–5%) of microsporidia in the stool samples of HIV-negative/immunocompetent patients.[6,29] Similar rate of prevalence has been reported in another study from northern India in hematological malignancy patients.[30] But none of these studies has been done exclusively in a pediatric population. On comparing the two diagnostic modalities, PCR was found more sensitive than modified trichrome stain ($p < 0.00001$). Similarly, the sensitivity of modified trichrome stain has been reported to vary from 64 to 94% in two studies published in North India.[17,29] The technical expertise is essential to identify the small (1–3 µ) spores of this organism. Another disadvantage of this stain is a short shelf-life stain and recurrent eye-straining during the screening. The above-mentioned studies from India were based on the detection of microsporidia by microscopy, which is lesser sensitive than PCR.

In this study, PCR could also detect microsporidia in two additional pediatric patients that were microscopically negative. This shows that phenotypic methods of diagnosis are not so sensitive and therefore, the detection rates will vary according to the tests applied. The variation in the rate of prevalence in different countries could also be explained by different techniques used for detection. However, the role of geographical and ethnic differences in the prevalence of microsporidia needs further investigation. Also, microsporidia like other parasites are shaded intermittently in stools, so a single sample and low organism load may have inadvertently affected the prevalence and outcome of the many other studies.

We also selected a few samples randomly to be viewed in SEM and TEM to identify and confirm the presence of microsporidia. Evidence of the polar filament or other ultrastructural features unique to the phylum is considered uncontroversial proof of microsporidiosis. Microsporidia showed a dense outer layer of the chitin-rich wall electron-lucent inner layer (endospore). But negative staining was not performed so characteristic polar tubules of microsporidia were not visualized.

Among other opportunistic parasites, *Cryptosporidium* was seen in seronegative patients as well that is in

Table 5 Correlation of CD4+ cell counts in coccidian parasites and *Microsporidium* (number of cases [%])

CD4+ count in range[a]	No. of cases	*Cryptosporidium*	*Cyclospora*	*Cystoisospora*	*Microsporidium*	*Microsporidium*+ *Cryptosporidium*[b]
< 100 cells/µL	14	4 (30.77)	1 (14.28)	1 (20)	6 (42.86)	2 (66.67)
101–200 cells/µL	24	7 (53.85)	5 (71.43)	4 (80)	7 (50)	1 (33.33)
201–300 cells/µL	4	2 (15.38)	1 (14.28)	0	1 (7.14)	0
Total cases	42	13	7	5	14	3

[a]No of cases observed with CD4+ count more than 301 cells/µL.
[b]Three children had coinfection with *Cryptosporidium* and Microsporidium.

concordance with other studies.[31,32] The prevalence rate of 10.9 and 9.8% was reported in immunocompetent patients from India and Turkey.[33,34] As far as correlation with CD4+ count is concerned, in our study also parasite positivity increased significantly as the count decreased that is in concordance with other studies. Both coccidian parasites and microsporidia were significantly higher in children with CD4+ count less than 200 cells/uL. In this study, PCR was performed using genus-specific primers so speciation could not be done that is an apparent limitation of the study.

This is the first study of its kind to determine prevalence of microsporidia in HIV-negative and HIV-positive pediatric patients with diarrhea in Northern India. Microsporidia is a significant cause of diarrhea in HIV seropositive pediatric patients and should be kept in mind as one of the differential diagnoses in such patients as routine microscopic methods used in most laboratories may miss them.

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Conflict of Interest
None declared.

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