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A prospective cohort study of enteric pathogens in human immunodeficiency virus-infected Indian children and their relationship with diarrheal recurrence

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ABSTRACT

Background: Opportunistic intestinal infections can increase the risk of death 11- fold in Human immunodeficiency virus (HIV) infected children presenting with diarrhea. Understanding the etiology of diarrhea and its predictors can help strategize a targeted approach to reduce child mortality due to diarrhea in this vulnerable group. Authors aim was to compare the enteric pathogens in HIV-infected children with and without acute diarrhea, to assess the association between carriage of enteric pathogens in HIV-infected children and the occurrence of diarrhea within the next 3 months and to ascertain the relationship between enteric pathogens in HIV-infected children with their immunological and nutritional status.

Methods: Stool samples were collected from HIV-infected children with acute diarrhea (n=41) and without diarrhea (n=52). All samples were subjected to microscopic examination, modified acid-fast and Trichrome staining, hanging drop examination, and bacterial culture. Serology for Cryptosporidium parvum was determined. Children who had received any antimicrobial therapy within the previous 2 weeks were excluded. Participants were followed up for three months for occurrence of diarrhea.

Results: Intestinal pathogens were isolated in 48.8% and 42% of children in the diarrheal and non-diarrheal group respectively. The most common pathogens isolated in the diarrheal and non-diarrheal group were Cryptosporidium parvum and *Escherichia coli* (29.3% vs. 17.3%). During follow up, 8 children in each group had diarrheal occurrence. The pathogen isolated in subsequent episodes matched with the initial isolate in 3 children in each group.

Conclusions: HIV-infected children without diarrhea also harbour enteric pathogens in comparable proportions to symptomatic children, which can predispose them to diarrheal occurrence in future, hence indicating need for assessing the need for preventive screening and prophylactic antibiotic regimens in this vulnerable group.

Keywords: Child, Diarrhea, Enteric, Human Immunodeficiency Virus, Pathogen

INTRODUCTION

In 2000, the Millennium Declaration was adopted by the United Nations and it aimed at reducing child mortality and combating HIV/AIDS infection.¹ Despite all the efforts, the Global Burden of Disease Study 2013 (GBD 2013) highlighted that diarrhea continues to remain a

leading cause of childhood mortality globally.² Children infected with human immunodeficiency virus (HIV) are particularly predisposed to higher diarrheal deaths, especially in tropical countries.³ Infants with HIV infection have an 11-fold increased risk of death from diarrhea, largely persistent diarrhea, which is often preceded by recurrent episodes of acute diarrhea,

malnutrition, or immunosuppression.⁴ Understanding the etiology of diarrheal episodes in this vulnerable group will provide us with some insights into methods to be undertaken to curtail the morbidity and mortality due to diarrheal illnesses in these children.

Several enteric pathogens have been associated with diarrhea in HIV-infected patients including bacteria, parasites, fungi, and viruses and coccidian parasites. 5-7 Opportunistic microorganisms like Cryptosporidium parvum, Cyclospora cavetanensis, Isospora belli, and Microsporidium spp. have been associated with diarrhea in patients with AIDS.⁶ Diarrhea caused by Cryptosporidium and Isospora have been included in AIDS-defining illnesses especially amongst those with low CD4 counts.8-10 Despite the use of appropriate antimicrobials, intestinal pathogens can be difficult to eradicate in HIV-infected individuals predisposing this group to chronic infection and acute diarrheal recurrences.³ The therapeutic failure can be attributed to the immune dysfunction, increased parasite load, and malabsorption of orally administered drugs.

Authors conducted study to determine the enteric pathogens in HIV-infected children with and without acute diarrhea. The study intended to determine the relationship between etiological agent for acute diarrhea and the immunological and nutritional status of the child. Authors also wanted to ascertain the diarrheal recurrence in HIV-infected children with acute diarrhea when followed-up for 3 months. They also wished to ascertain if asymptomatic carriage of enteric pathogens in HIV-infected children predisposes them to diarrhea.

METHODS

This prospective cohort study was conducted amongst HIV-infected children aged 18 months to 12 years registered for treatment at the Pediatric Anti-Retroviral Therapy (ART) Centre at University College of Medical Sciences and Guru Teg Bahadur Hospital, a tertiary hospital in Delhi, India, catering to the urban slum population of east Delhi and neighboring districts of Uttar Pradesh.

Ethics and consent

A prior approval from the Institutional Ethics Committee was obtained. A written informed consent was also obtained from the parent/guardian for participation of their child in the study.

Enrolment

All consecutive HIV-infected children aged 18 months to 12 years, presenting with acute diarrhea between November 1, 2015 and January 31, 2017 were considered eligible for recruitment. Acute diarrhea was defined as the passage of three or more unformed stools in 24 hours and of less than 14 days' duration. HIV-infected

children without diarrhea were regarded as controls. Children who had received any antibiotic /antiparasitic treatment in the preceding two weeks were excluded.

Assessment

A base-line assessment including detailed history and physical examination of all participants was done. The key historical information was included duration of diarrhea, number of stools, and consistency of stool, presence of vomiting and fever, and presence of any blood or mucus in the stool. Dehydration was assessed as per WHO guidelines. 11 Anthropometrical measurements (weight, height, mid-upper arm circumference) were done using standard techniques. 12 For dehydrated children; the anthropometry (weight) was re-measured after hydration. Weight following rehydration was recorded finally, for the purpose of the study. Weight for height Z score, weight for age Z score, height for age Z score and body mass index Z score was computed using WHO anthroplus software and Anthrocal software based upon WHO reference standards for growth. 13-15 Hemoglobin level was also used as a surrogate marker to ascertain nutritional status and anaemia was defined as per the UNICEF guidelines.16 The CD4 lymphocyte count was served as a marker to determine the immune status. The CD4 lymphocyte count was determined by automated flow-cytometry analyser (FACS Calibre, Becton Dickinson, New Jersey, USA) and the subjects were categorized as per their immune status. WHO-defined CD4 cut-offs for age-related staging immunodeficiency and WHO clinical staging of HIV for infants and children were used. HIV-infected children were administered HAART and co-trimoxazole as per the latest Guidelines of the National AIDS Control Organization.¹⁷

Stool examination

Stool samples were collected from each subject into a clean, sterile, screw capped wide-mouthed disposable plastic containers for laboratory assessment. All the stool samples were observed macroscopically for colour, consistency, presence of blood or mucus and visible parasites.

A part of the sample was concentrated by formol-ether technique and used further for direct microscopy (saline and iodine wet mount for parasitic ova and cysts), modified acid-fast staining for coccidian parasite and modified trichrome staining for Microsporidia.

For bacterial pathogens, fresh stool sample was also inoculated on appropriate culture media (MacConkey agar, XLD (Xylose Lysine Deoxycholate) medium, and Bile Salt Agar (BSA) and identified by the conventional standard biochemical test. Yeast was identified by standard techniques. Figure 1 depicts the algorithm for processing of stool sample.

Serological examination

Sera were analysed for antibodies to C. Parvum using ELISA based kit.

All biological samples remaining at the end of the study were discarded while observing guidelines for biomedical waste disposal.

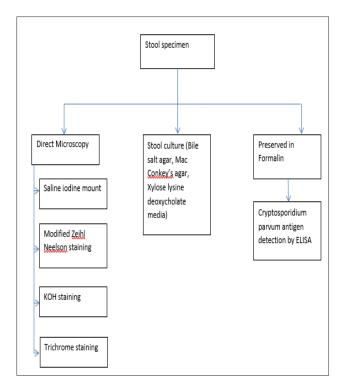


Figure 1: Outline for processing of stool sample from the participants.

Treatment of diarrheal episode

Dehydration was corrected as per the standard WHO protocol.¹¹ Specific antimicrobial therapy was given based upon the pathogen isolated from the stool specimen.¹⁷ Children with stool specimens positive for trophozoites of Entamoeba histolytica were treated with oral metronidazole 35-50 mg/kg/day for seven days' duration and those positive for trophozoites of Giardia received oral metronidazole 15mg/kg/day for seven days' duration. Cryptosporidiosis or Microsporidiosis were treated with oral Nitazoxanide in doses of 100 mg twice daily for children aged 1-3 years, and 200mg twice daily for children aged 4-11 years, for three days' duration. Isospora/cyclopspora infection was treated with oral Trimethoprim/Sulfamethoxazole 20 mg/kg/day of TMP in two divided doses for seven days' duration. Infections with Ascarias lumbricoides, Ancyclostoma duodenale, Trichuris trichuira and Enterobius vermicularis were treated with a single oral dose of albendazole 400 mg. Children with dysentery were administered oral Ciprofloxacin (15 mg/kg/day X 3 days) if the child was not sick or intravenous ceftriaxone (75mg/kg/day) for a sick child requiring hospitalization. If no parasite was detected in stool and ≥10 leucocytes/high power field were seen in the stool microscopy then a possibility of infection with Shigella, *E. coli*, Campylobacter, or Salmonella was kept and the child received intravenous ceftriaxone (75mg/kg/day). Oral zinc supplements were administered twice a day for 14 days at 20 mg/day for children aged 18-60 months. All children were reevaluated after two days, for the signs of improvement i.e., absence of dehydration, weight gain, improved appetite, no fever and no blood in stool or passage of fewer stools. If there was no improvement or clinical worsening, the antibiotics were revised depending upon clinical condition/co-morbidities like pneumonia, etc.

Follow-up

All participants were followed up every 2 weeks. At each contact, an interval history for any new diarrheal episode was taken. Caregivers were asked to bring the child to the hospital in the emergency whenever a diarrheal episode occurs. All diarrheal episodes in study population were investigated to determine the inciting pathogen. Recurrent diarrhea was defined as any new episode occurring in a child during follow-up. (Figure 2) depicts the flow diagram for participants in the study.

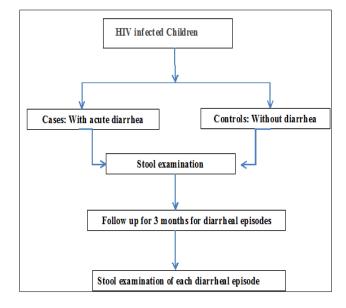


Figure 2: Participants in the study.

Sample size estimation

Based on a study by Uppal et al. from Delhi, wherein the prevalence of enteric parasites amongst HIV-infected patients with diarrhea and without diarrhea was 20% and 2% respectively, a sample size of 41 patients per group was needed, to compare the prevalence of enteric parasites in both groups at 80% power and 5% alpha error (one-tailed hypothesis). In the same study, the prevalence of enteric microbial pathogens was 80% and 26% amongst the HIV-infected patients with and without

diarrhea respectively. Therefore, a sample size of 15 patients per group was needed to compare the prevalence of enteric microbial parasites in HIV-infected children with and without diarrhea, at 80% power and 5% alpha error (one-tailed hypothesis).

Statistical analysis

The analysis was carried out using SPSS software package version 20 (Chicago, IL). Baseline demographic characteristics like age, haemoglobin, total leucocyte count, platelet count, mean CD4 count, time to resolution of diarrhea, and duration of hospital stay were compared between the two groups by student's t test.

Differences in proportion (for categorical variables) like male sex, proportion of severely immunosuppressed children, and orphans between the groups were ascertained by using Chi-square test or Fisher's exact test. Proportions of different enteric pathogens in HIV-infected with and without diarrhea were compared by Chi-square test.

Predictors (age<5y, sex, HAART, co-trimoxazole therapy, presence of anaemia, severe immunodeficiency, severe thinning) for acute diarrhea were determined by logistic regression analysis and the odds ratio (95% CI) were calculated.

RESULTS

Authors enrolled 41 HIV-infected children with acute diarrhea and 52 HIV-infected children without diarrhea. Stool samples were collected and processed from all the 93 participants. The mean age of the study population was 7.5 (±3.3; range 18 months - 12 years) years, 29 (31.1%) participants were aged below 5 years. Out of 93 participants, the mode of acquisition of HIV was vertical in 73 participants (78.5%). Two participants (2.2%) had acquired the HIV infection through blood transmission and the source remained unknown in 18 participants (19. 4%). All participants had domestic supply of piped drinking water and did not need to access to tankers/wells for the same.

Table 1: Baseline characteristics of participants in both groups.

	Diarrheal group (n=41)	Non-diarrheal group (n=52)	p- value
Age* (years)	6.9(±3.4)	8(±3.2)	0.11
Under-5 children (n, %)	15(36.5%)	14 (26.9%)	0.39
Male sex (n, %)	21(51.2%)	36 (69.2%)	0.07
Mode of transmission (n, %)			
Vertical	31(75.6%)	42(80.8%)	0.83
Parenteral/Needle stick	1(2.4%)	1(1.9%)	
Unidentified	9(22%)	9(17.5%)	
Orphan (n, %)	7(17.1%)	5(9.6%)	0.2
Piped water supply (n, %)	41, 100%	52, 100	>0.05
Anthropometry			
Weight for age Z score (WAZ)\$	-2.4(±1.5)	$-1.6(\pm 0.89)$	0.008
Height for age Z score (HAZ)	-1.9(±1.5)	$-2.0(\pm 1.6)$	0.59
Mid upper arm circumference for age Z	-2.8(±1.1)	-2.3(±0.8)	0.16
score (MUACZ)#			
Weight for height Z score (WHZ)#	-2.0(±1.7)	$-2.0(\pm 1.7)$	0.06
Body mass index Z score (BMIZ)	-2.4(±2.6)	$-1.0(\pm 1.4)$	0.003
WHO Clinical Staging			
Stage I	27(65.9%)	44(84.6%)	
Stage II	0	2(3.8%)	0.06
Stage III	4(9.8%)	5(9.6%)	
Stage IV	10(24.4%)	1(1.9%)	
Laboratory Characteristics			
Hemoglobin*	10.2(±1.38)	$10.7(\pm 1.05)$	0.06
Anemia	29(70.7%)	31(59.6%)	0.26
Total leucocyte count*	7625(±2656)	7836(±2516)	0.69
CD4 count *	604(±528)	719(±481	0.27
Severe immunodeficiency*	18(43.9%)	5(9.6%)	< 0.001

^{*}Expressed as mean (± SD), # For children aged <60 months, \$ For children aged <10 years

The mean height for age Z score (HAZ), mid upper arm circumference Z score (MUACZ) and weight for height Z score (WHZ) were similar in participants in both groups but the body mass index Z score (BMIZ) and weight for age Z score (WAZ) were significantly lower in the diarrheal group. The proportion of children with severe

underweight (WAZ<-3) or severe thinning (BMIZ<-3) was significantly higher in the diarrheal group. The proportion of children with severe immunodeficiency in the diarrheal group was significantly more than the non-diarrheal group, (Table 1) depicts the comparative baseline characteristics of participants in both groups.

Table 2: Enteric pathogens in HIV-infected children.

	Total (n=93)	Diarrheal group (n=41)	Non-diarrheal group (n=52)	p-value
Bacterial pathogen	25/93 (26.9%)	12/41 (29.3%)	13/52 (25%)	0.64
Escherischia coli	21/93 (22.6%)	12/41 (29.3%)	9/52 (17.3%)	0.17
Proteus	1/93 (1.1%)	1/41 (2.4%)	0	0.44
Pseudomonas	3/93 (3.2%)	0	3/52 (5.8%)	0.25
Citrobacter	1/93 (1.1%)	0	1/52 (1.9%)	>0.05
Coccidian parasite	22/93 (23.7%)	13/41 (31.7%)	9/52 (17.3%)	0.1
Cryptosporidium	21/93 (22.6%)	12/41 (29.3%)	9/52 (17.3%)	0.17
Modified AFB stain	10/93 (10.8%)	7/41 (17.1%)	3/52 (5.2%)	>0.05
Antigen detection	18/93 (19.4%)	11/41 (26.8%)	7/52 (13.5%)	0.1
Cyclospora	1/93 (1.1%)	1/41 (2.4%)	0	0.44
Isospora	1/93 (1.1%)	1/41 (2.4%)	0	0.44
Microsporidium	0	0	0	-
Yeast	2/93 (2.1%)	2/41 (4.8%)	0	-
Protozoan parasite				
H nana	1/93 (1.1%)	1/41 (2.4%)	0	>0.05
E. histolytica	1/93 (1.1%)	0	1/52 (1.9%)	>0.05
Giardia	9/93 (9.7%)	6/41 (14.6%)	3/52 (5.8%)	0.18

Table 3: Recurrence of diarrheal in HIV-infected children with acute diarrhea during follow up.

Patient	CD4 count (cells/mm ³), Immunological Staging	Pathogen isolated in episode 1	Pathogen isolated in episode 2	Pathogen isolated in episode 3
8 years/ Female	12, severe immunodeficiency	E. coli, Giardia, Cryptosporidium	Giardia, Cryptosporidium	X
12 years/ Male	140, severe immunodeficiency	E. coli, Proteus, Giardia, Cryptosporidium, Isospora	No pathogen isolated	X
7 years/ Female	123, severe immunodeficiency	Cryptosporidium, Yeast	Yeast	No pathogen isolated
4 years/ Male	872, no immunodeficiency	No pathogen isolated	No pathogen isolated	X
4 years/ Male	155, severe immunodeficiency	No pathogen isolated	No pathogen isolated	X
3 years/ Female	418, moderate immunodeficiency	E coli	E coli	X
10 years/ Female	107, severe immunodeficiency	Giardia	No pathogen isolated	No pathogen isolated
7 years/ Male	434, mild immunodeficiency	E coli, Cryptosporidium	No pathogen isolated	X

Enteric pathogens

Stool examination revealed an enteric pathogen in 20 out of 41 children with acute diarrhea compared to 22 out of

52 children without diarrhea, 14 cases and 4 controls showed coinfection with more than one enteric pathogen. The most common pathogen isolated in both the diarrheal and non-diarrheal group was Cryptosporidium parvum and *Escherischia coli*. The isolation rate of enteric

pathogens in the non-diarrheal group was comparable to that in the diarrheal group, as shown in (Table 2). One child with acute dehydrating diarrhea due to cryptosporidium parvum expired within 6 hours of admission

Table 4: Occurrence of diarrhea in HIV-infected children without acute diarrhea during follow up.\

Patient	CD4 (cells/mm³)	Immunological staging	Pathogen isolated while asymptomatic	Pathogen isolated while having diarrhea
11 years/ Female	572	Normal, no immunodeficiency	Cryptosporidium	Cryptosporidium
12 years/ Male	53	Severe immunodeficiency	No pathogen isolated	No pathogen isolated
4 years/ Male	155	Severe Immunodeficiency	No pathogen isolated	E. coli
2 years/ Male	1231	Advanced immunodeficiency	E coli, Pseudomonas	E coli
3 years/ Male	557	Mild immunodeficiency	No pathogen isolated	E coli
4 years/ Male	301	Severe immunodeficiency	$E\ coli$	E coli
12 years/ Female	585	Normal, no immunodeficiency	No pathogen isolated	No pathogen isolated
10 years/ Male	820	Normal, no immunodeficiency	No pathogen isolated	No pathogen isolated

Table 5: Predictors for acute diarrhea in HIV-infected children.

	Diarrheal group (n=41) n (%)	Non-diarrheal group (n=52) n (%)	p- value	Odds ratio (95% CI)
WAZ<-3	13/32(40.6%)	3/34(8.8%)	0.004	7.0(1.8-27.7)
HAZ<-3	14/41 (34.1%)	17/52 (32.7%)	1.000	1.06(0.44 - 2.54)
BMIZ<-3	14/41 (34.1%)	3/52(5.8%)	0.001	8.5(2.2 - 32.3)
MUACZ<-3	7/15 (46.7%)	3/14(21.4%)	0.24	3.2(0.6-16.3)
WHZ<-3	4/15 (26.7%)	1 /14(7.1%)	0.16	4.7(0.45-47.6)
Proportion with anaemia	29 (70.7%)	31(59.6%)	0.26	1.6(0.7 - 3.9)
Proportion with severe immunodeficiency	18 (43.9%)	5(9.6%)	< 0.001	7.3(2.4 - 22.2)
Receiving Anti-retroviral therapy	41 (97.6%)	50(98%)	0.86	
Receiving Co-trimoxazole prophylaxis	29 (70.7%)	27(51.9%)	0.07	2.2(0.9-5.3)

Follow up

Ninety two participants were followed up for 3 months' duration to assess diarrheal recurrence/occurrence (40 cases and 52 controls). Eight cases (20%) had recurrence of acute diarrhea during the follow up period; two of them had two recurrent episodes and six had one recurrent episode. Out of the 10 repeat episodes, the pathogen isolated in 3 matched with the previous organism. Eight controls (15.3%) developed acute diarrheal episodes during follow up of 3 months' duration. Of these, in three participants the pathogen isolated in subsequent episodes matched with the isolate in asymptomatic stage see (Tables 3 and 4).

Predictors of acute diarrhea

The odds for developing acute diarrhea were significantly more in children with WAZ<-3 and BMIZ<-3 as is shown in (Table 5). Children with severe immunodeficiency were 7.3 times more likely to have acute diarrhea (P<0.001). Receipt of anti-retroviral therapy, co-trimoxazole prophylaxis and anaemia were not found to determine occurrence of acute diarrhea.

DISCUSSION

We found a high prevalence rate of intestinal pathogens of 48.8% and 45.1%, respectively, in HIV-infected children with and without acute diarrhea. Surprisingly,

the colonization rates in both groups were comparable. Few studies have evaluated intestinal pathogen infestation rates in HIV-infected children with diarrhea in India with rates ranging from 20% to 73% (18-20). None of these studies have been done exclusively in children.

The intestinal pathogen infestation rates in the nondiarrheal group were much higher in our study than that reported from elsewhere in India 18-20 ranging from 0% 19 to 30.5%. 20 These results are despite a much higher use of ART (97.8%) which may have been due to different endemicity, or socio-economic conditions or cultural practices of the populations. Though, all participants in our study were receiving piped drinking water supply, majority of them consumed raw water without boiling/additional water treatment like filtration or reverse osmosis. The poor nutritional standards seen in our children could also predispose them to high intestinal pathogen infestation, as has been reported previously.²¹ Children with severe thinning and severe underweight seemed to incur 8.5 times and 7 times higher risk for diarrhea in our study. Anemia was not found to be a significant predictor for acute diarrhea.

Though, about two-thirds of our study participants were receiving co-trimoxazole prophylactic treatment (CPT), 22 (23.7%) children still housed coccidian parasites; the prevalence being comparable in those with (31.7%) or without diarrhea (17.3%). Cryptosporidium parvum was the most common coccidian parasite isolated in 22.6% of participants, followed by Isospora (1.1%) and Cyclospora (1.1%). Co-trimoxazole prophylaxis did not seem to offer any protective role for occurrence of acute diarrhea [OR 2.2 (0.9-5.3), P=0.07]. The infestation rates of infection with C. parvum in diarrheal subjects (29.6%) in our study were higher compared to non-diarrheal subjects (17.3%) but were statistically comparable. Similar results have been reported from Delhi by Uppal, et al. 18 However, the infestation rates of infection with C. parvum in nondiarrheal subjects (17.3%) in our study were much higher compared to those reported by Uppal et al, and Ram Mohan, et al, but similar to those by Kaur, et al, from Haryana (21.4%).¹⁸⁻²² The variation in results could be due differing population characteristics, viz., age and type of diarrhea. The extremely low isolation of Isospora belli and Cyclospora in our study may be due to endemicity of the pathogens and protection due to CPT. No case of Microsporidia was isolated in our study; this may have been due to difficulty in its detection by conventional staining methods and its small size.²³

The CD4 count in the diarrheal group was slightly lower than the non-diarrheal group, although the proportion of severely immunodeficient children in the diarrheal group was much higher. Children with severe immunodeficiency were 7.3 times more likely to suffer from acute diarrhea [OR 7.3 (2.4 - 22.2)]. The prevalence rates of intestinal parasites have been shown to be inversely related with CD4 counts.^{22,24} A negative

correlation has also been shown between CD4 counts and duration of diarrhea. ^{25,26}

During follow up, 8 children with diarrhea had recurrence of acute diarrhea. The pathogen isolated in subsequent episodes matched with previous pathogen suggesting that pathogens colonizing HIV-infected children may have been drug-resistant or may require a longer duration of anti-microbial treatment. Similar hypothesis has been proposed by Amadi, et al, though they did not report any benefit of using higher doses of Nitazoxanide in treating Cryptosporidiosis in HIV-infected children.³ However, more such studies are needed. Out of 22 children without diarrhea who harbored one or more enteric pathogen, 8 children (36.3%) suffered from acute diarrhea during follow up with the organism matching the previous isolate in 3 cases. This suggests the need for exploring pre-emptive treatment in severely malnourished and severely immunodeficient HIV-infected children following active screening.

Some of the limitations of our study are the lack of assessment for viral aetiology of diarrhea. Our study did not involve tests like colonoscopy and intestinal biopsy which could be preferred to ascertain prevalence of pathogens like Microsporidia. The major strength of our study includes a homogenous sample comprising of children aged 18 mo. - 12 years with acute diarrhea. Another, strength is the follow up of participants for diarrheal recurrence.

CONCLUSION

Authors conclude that the most common enteric pathogens in HIV-infected children with and without acute diarrhea in Delhi are Cryptosporidium parvum and coli. Severe malnutrition and severe immunodeficiency are significant predictors development of acute diarrhea in these children. It may be relevant to screen HIV-infected children with severe immunodeficiency or severe thinning for opportunistic enteric pathogens and treat them pre-emptively. The current guidelines for treatment of diarrhea in HIVinfected children need revamping. More studies evaluating the role of longer duration and varying doses of anti-microbial therapy, including higher doses of Nitazoxanide, in HIV-infected children with diarrhea are needed.

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Ethical approval: The study was approved by the

Institutional Ethics Committee

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