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Original Article

Detection of Enteropathogens in Human Immunodeficiency Virus and Non-Human Immunodeficiency Virus-Infected Children with Acute Diarrhea in an Indonesian Tertiary Hospital Using Multiplex Real-Time Polymerase Chain Reaction

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The authors have no financial conflicts of interest.

ABSTRACT

Purpose: Diarrhea is one of the leading causes of mortality in children living in developing countries. The etiology of acute diarrhea in each healthcare center varies depending on place, time, and population. This study aimed to identify pathogen patterns in human immunodeficiency virus (HIV)-infected and non-HIV children suffering from acute diarrhea, using multiplex real time reverse transcriptase polymerase chain reaction (RT-PCR), in an Indonesian tertiary hospital.
Methods: This cross-sectional study was conducted at Dr. Cipto Mangunkusumo National Hospital from March 2019 to April 2020.
Results: The study showed that multiplex RT-PCR results were positive in 58.9% of the specimens, with more positive results in HIV-infected children than in non-HIV-infected children (70% vs. 54.7%). Altogether 72 enteropathogens were detected from all specimens. Enteropathogens in non-HIV children with acute diarrhea consisted of bacteria (70.6%) and viruses (29.4%) with a predominance of enteroaggregative *Escherichia coli* (25.4%), followed by *Campylobacter* spp. (11.8%), enteropathogenic *E. coli* (9.8%), Norovirus GII (7.8%), and *Clostridium difficile* (7.8%). Enteropathogens in HIV-infected children consisted of viruses (57.1%), bacteria (28.6%), and parasites (14.3%) comprising Norovirus GII (24%), *Cryptosporidium* spp. (14.3%), *Campylobacter* spp. (14.3%), Norovirus GI (14.3%), and Astrovirus (14.3%). *Cryptosporidium* spp. was the only parasite found in this study and was found only in HIV-infected children. In non-HIV children with acute diarrhea, most pathogens were invasive bacteria, while in HIV-infected children, more viral and parasite infections occurred, primarily caused by opportunistic pathogens.

Conclusion: The pattern of enteropathogens can help clinicians determine further examinations and appropriate empirical antimicrobial therapy for the patient.

Keywords: Child; Diarrhea; HIV; Multiplex polymerase chain reaction; Tertiary care centers

INTRODUCTION

Diarrhea is one of the most common causes of mortality in children living in developing countries, accounting for two to three million deaths every year. Children <3 years of age reportedly experience 1.3 to 2.3 episodes of diarrhea every year. Diarrhea-related hospitalization in children is estimated to cost 2 billion dollars annually [1]. Diarrhea in human immunodeficiency virus (HIV)-infected children however remains a challenging problem. The World Health Organization in 2011 recorded that 3.4 million children <15 years of age were infected with HIV [2]. The Joint United Nations Program on HIV/acquired immunodeficiency syndrome (AIDS) showed that the incidence and mortality of HIV-infected children aged 0–14 years in Indonesia increased throughout the year in 2018 with 3,100 new cases and 2,200 deaths due to AIDS [3]. Diarrhea is one of the symptoms in HIV- infected children with an incidence rate between 9.9% and 24.5% [4,5]. HIV-infected children are 5.6 times more likely to experience diarrhea than non-HIV children [6]. Hence proper epidemiological studies are required to provide guidelines for empirical therapy.

While stool culture remains the gold standard for identifying the etiology of diarrhea in HIV- and non-HIV-infected children, low culture yield [7] and numerous pathogens including opportunistic pathogens [8] in HIV-infected children pose great diagnostic challenges. This study aimed to identify the enteropathogen pattern in HIV- and non-HIV-infected children with acute diarrhea using multiplex reverse transcriptase polymerase chain reaction (RT-PCR) in an Indonesian tertiary hospital.

MATERIALS AND METHODS

This cross-sectional study was performed in Cipto Mangunkusumo National Hospital Jakarta between March 2019 and April 2020, with collaboration between Clinical Pathology Department and Pediatric Department. The inclusion criteria were children 0–18 years of age admitted to the hospital with acute diarrhea. The symptoms of acute diarrhea were assessed clinically by pediatricians and defined as disturbances in bowel movements resulting in soft, loose, bloody, or watery stools occurring with an increasing frequency of ≥3 times per day in less than 14 days. Exclusion criteria were patients without complete medical records and those who refused to participate. Verbal and written informed consent were obtained from all patients who agreed to participate in the study. This research received ethical approval from the Ethical Committee of the Faculty of Medicine, University of Indonesia with approval number: KET-533/UN2.F1/ETIK/PPM.00.02/2019.

Sample collection

Fresh fecal samples were collected from all patients (minimum of 25 mg) within 48 hours after hospital admission, and placed into a sterile container. For children who wore diapers, the diapers were covered with plastic, and the obtained samples were placed into a sterile container; samples were collected within 4 hours after defecation. Routine stool analysis was

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carried out, including macroscopic and microscopic examinations such as color, consistency, mucus, blood, pus, oil, foam, fecal leukocyte count, fecal erythrocyte count, fungi, bacteria, fat, plant fiber, muscle fiber, and starch. The analysis was performed by two experienced and certified laboratory analysts and confirmed by a clinical pathologist within 1 hours after the sample arrived in the laboratory. After routine stool analysis, the feces were frozen at −20°C for 30 days before the multiplex RT-PCR test was carried out.

HIV status

The HIV status of patients was confirmed when HIV viral load examination showed positive results in children <18 months of age or when HIV serological examination showed positive results in children ≥18 months. To determine the viral load of patients, HIV viral load examination was performed on children with positive serological tests. A minimum of 3 mL of ethylenediaminetetraacetic acid blood was taken for HIV tests from all patients. Serological examinations were performed immediately using the following three reagents: Architect HIV Ag/Ab Combo Assay, Vironostika HIV Uni-Form II Ag/Ab, and Murex HIV Ag/Ab Combination. HIV viral load examination was performed immediately using Abbott RealTime HIV-1 m2000sp. CD4 counts were performed only for HIV-positive children using BD FACSCount™ CD4 assay. The assay was conducted and interpreted by a trained medical analyst according to the manufacturer's instructions.

Multiplex RT-PCR

The sample was allowed to thaw at room temperature and a minimum of 25–100 mg of the sample was then transferred to Cary Blair transport medium (Copan FecalSwab™; COPAN Italia). After homogenization, 200 μL of the mixture was manually transferred using a pipette into DiagCORE® (QIAGEN) cartridge. The DiagCORE® cartridge was scanned by the system, and loaded into the analyzer. Extraction, amplification, and detection of nucleic acids in the samples were carried out automatically by the DiagCORE® Analyzer.

QIAstat-Dx® (QIAGEN) Gastrointestinal Panel analyzed the 24 most common diarrhea-causing pathogens, which were *Entamoeba histolytica*, *Cryptosporidium* spp., *Giardia lamblia*, *Cyclospora cayetanensis*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Campylobacter* spp. (*Campylobacter jejuni, C. upsaliensis, C. coli*), *Salmonella* spp., *Clostridium difficile* (tcdA/tcdB), *Yersinia enterocolitica*, enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), Shiga-like toxin *E. coli* (STEC), Shiga-toxin *E. coli* (STEC) serotype O157:H7, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC)/*Shigella*, *Plesiomonas shigelloides*, Human Adenovirus F40/F41, Norovirus GI, Norovirus GII, Rotavirus A, Astrovirus, and Sapovirus GI, GII, GIV, GV. Error in processing resulted in no negative or positive results. These samples (n=10/73) were reprocessed by centrifuging the transport medium containing the sample at 3,000 rpm for 30 second and pipetting the supernatant of the mixture to a new DiagCORE® cartridge.

Data analysis

The data were recorded in Microsoft Excel 2007 (Microsoft) and processed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Co.). The participants' characteristics were displayed in a table and analyzed. Numerical data distribution was analyzed using the Kolmogorov–Smirnov normality test with significance at $p>0.05$. Numerical data with normal distribution are presented as mean±standard deviation and non-normal distribution data are reported as median, minimum, and maximum. Statistical analysis of numerical data was performed using the *t*-test if the data distribution was normal and the Mann–Whitney test if the distribution was not normal. Categorical data are reported in terms of frequency and

percentage. Statistical analysis of categorical data was performed using the Chi-square test with Yates correction or Fisher-Exact test if there was any exception.

RESULTS

Seventy-three specimens were included in the study, and none of the specimens were excluded. The participants' characteristics are displayed in **Table 1**. The majority of the subjects were female, n=38/73, 52.1%) in the age group of 1–6 years, with a median age of 3.13 (0.06–17.8) years. HIV-infected children with acute diarrhea accounted for 27.4% of the specimens (n=20) with a median viral load of 105.568 (428–1.475.292) copy/mL, median CD4 count of 125 (3–567) cell/mm^3, and median CD4 percentage of 7.5 (1-35)%. Of the participants, 68.8% were HIV-positive children with CD4 count <200 cells/mm^3, 18.8% with CD4 count 200–499 cells/mm^3, and 12.5% with CD4 count ≥500 cells/mm^3; 55% of the HIV-infected children had received antiretroviral therapy. The majority of patients (n=68/73, 93.1%) had received antibiotics prior to multiplex RT-PCR, of which cephalosporins (63.2%) was the most common, followed by carbapenem (14.7%), nitroimidazole (10.3%), aminoglycoside (7.3%), penicillin (2.5%), and macrolide (2.0%). All HIV-positive children received additional cotrimoxazole as prophylaxis.

Routine fecal analysis is presented in **Table 2**. Both pus and grease were not found macroscopically in all the specimens. Similarly, muscle fiber was not detected microscopically in all the specimens. There was a statistically significant difference in the fecal consistency between HIV- and non-HIV-infected children, p<0.05. Watery (n=5/20) and loose (n=7/20) consistencies occurred more in the stools of HIV-infected children, while the stool of non-HIV children were mostly soft (n=38/53).

Table 1. Participants' characteristics

Characteristics	Total	HIV-negative subjects	HIV-positive subjects	p-values
Age (yr)	3.13 (0.06–17.8)	2.6 (0.06–17.8)	7.4 (0.07–17.6)	0.251‡
<1	14/73 (19.2)	12/53 (22.6)	2/20 (10.0)	<0.05*
1–6	36/73 (49.3)	29/53 (54.7)	7/20 (35.0)	
≥6	23/73 (31.5)	12/53 (22.6)	11/20 (55.0)	
Sex				0.205*
Male	35/73 (47.9)	23/53 (43.4)	12/20 (60.0)	
Female	38/73 (52.1)	30/53 (56.6)	8/20 (40.0)	
Positive results on multiplex RT-PCR	43/73 (58.9)	29/53 (54.7)	14/20 (70.0)	0.237*
Multiple infections	21/43 (48.8)	15/29 (51.7)	6/20 (30.0)	0.131*
Single infection	22/43 (51.2)	14/29 (48.3)	8/20 (70.0)	0.567*
Bristol score				<0.05*
5	46/73 (63.0)	38/53 (71.7)	8/20 (40.0)	
6	17/73 (23.3)	10/53 (18.9)	7/20 (35.0)	
7	10/73 (13.7)	5/53 (9.4)	5/20 (25.0)	
History of antibiotics therapy	68/73 (93.2)	51/53 (96.2)	17/20 (85.0)	0.09*
Duration of antibiotics therapy before multiplex RT-PCR (d)	2 (0–27)	2 (0–27)	3 (0–9)	0.026‡
Number of pathogens detected	72/72 (100.0)	51/72 (70.1)	21/72 (29.9)	<0.01*
Bacteria	42/72 (58.3)	36/51 (70.6)	6/21 (28.6)	<0.01*
Virus	27/72 (37.5)	15/51 (29.4)	12/21 (57.1)	<0.05*
Parasite	3/72 (4.2)	0/51 (0.0)	3/21 (14.3)	<0.01†

Values are presented as mean (range) or number (%).
HIV: human immunodeficiency virus, RT-PCR: reverse transcriptase polymerase chain reaction.
*Chi square test with Yates correction.
†Fisher-Exact test.
‡Mann Whitney test.

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Table 2. Routine stool analysis

Test	Total (n=73)	HIV-negative subjects (n=53)	HIV-positive subjects (n=20)	*p*-value
Macroscopic				
Color				0.137*
Yellow	44 (60.3)	30 (56.6)	14 (70.0)	
Brown	19 (26.0)	13 (24.5)	6 (30.0)	
Green	7 (9.6)	7 (13.2)	0 (0.0)	
Red	2 (2.7)	2 (3.8)	0 (0.0)	
Black	1 (1.4)	1 (1.9)	0 (0.0)	
Consistency				<0.05*
Soft	46 (63.0)	38 (71.7)	8 (40.0)	
Loose	17 (23.3)	10 (18.9)	7 (35.0)	
Watery	10 (13.7)	5 (9.4)	5 (25.0)	
Mucus	39 (53.4)	30 (56.6)	9 (45.0)	0.375*
Foam	2 (2.7)	0 (0.0)	2 (10.0)	0.072†
Blood	1 (1.4)	1 (1.9)	0 (11.1)	1†
Microscopic				
Stool leucocytes				0.479†
>5 cells/HPF	11 (15.1)	7 (13.2)	4 (20.0)	
≤5 cells/HPF	62 (84.9)	46 (86.8)	16 (80.0)	
Stool erythrocytes				0.525*
>1 cell/HPF	48 (65.8)	36 (67.9)	12 (60.0)	
≤1 cell/HPF	25 (34.2)	17 (32.1)	8 (40.0)	
Fungal budding	5 (6.8)	3 (5.7)	2 (10.0)	0.61†
Bacteria	69 (94.5)	49 (92.5)	20 (100.0)	0.57†
Fat	17 (23.3)	14 (26.4)	3 (15.0)	0.368†
Plant fiber	4 (5.5)	1 (1.9)	3 (15.0)	0.06†
Amylum	1 (1.4)	0 (0.0)	1 (5.0)	0.274†

Values are presented as number (%).
HIV: human immunodeficiency virus, HPF: high-power field.
*Chi-square test with Yates correction.
†Fisher-Exact test.

Of the collected specimens, 58.9% tested positive in multiplex RT-PCR. A total of 72 enteropathogens were detected from all specimens. The majority were bacteria (n=42/72) followed by viruses (n=27/72). Parasites (n=3/72) were only found in the stools of HIV-infected children, $p<0.05$. More HIV-infected children tested positive for viral infections (n=12/21) compared to non-HIV children (n=15/51), $p<0.01$. More non-HIV-infected children had bacterial infections (n=36/51 vs. n=6/21), $p<0.01$. The enteropathogen pattern of HIV-infected and non-HIV-infected children is shown in **Fig. 1**. Enteroaggregative *E. coli* was the most common enteropathogen bacteria found in non-HIV children (n=13/72) and was statistically significant when compared to HIV-infected children, $p<0.05$. While Norovirus GII was the most frequent enteral virus detected in HIV-infected children (n=5/21), Norovirus GI was a statistically significant pathogen in HIV-infected children (n=3/21), $p<0.05$. The parasites detected in HIV-infected patients were *Cryptosporidium* spp. (n=3/21), $p<0.05$.

The stools of non-HIV children were further analyzed by age group (**Fig. 2**). Bacterial infections were higher in all age groups especially in children >6 years old. Children aged 1–6 years had the highest number of viral infections (37%). The majority of infections were found in children between the ages of 1 and 6, with enteroaggregative *E. coli* being the most common cause of infections. *Salmonella* spp. were detected more frequently in children <1 year of age than in any other age group, $p<0.05$.

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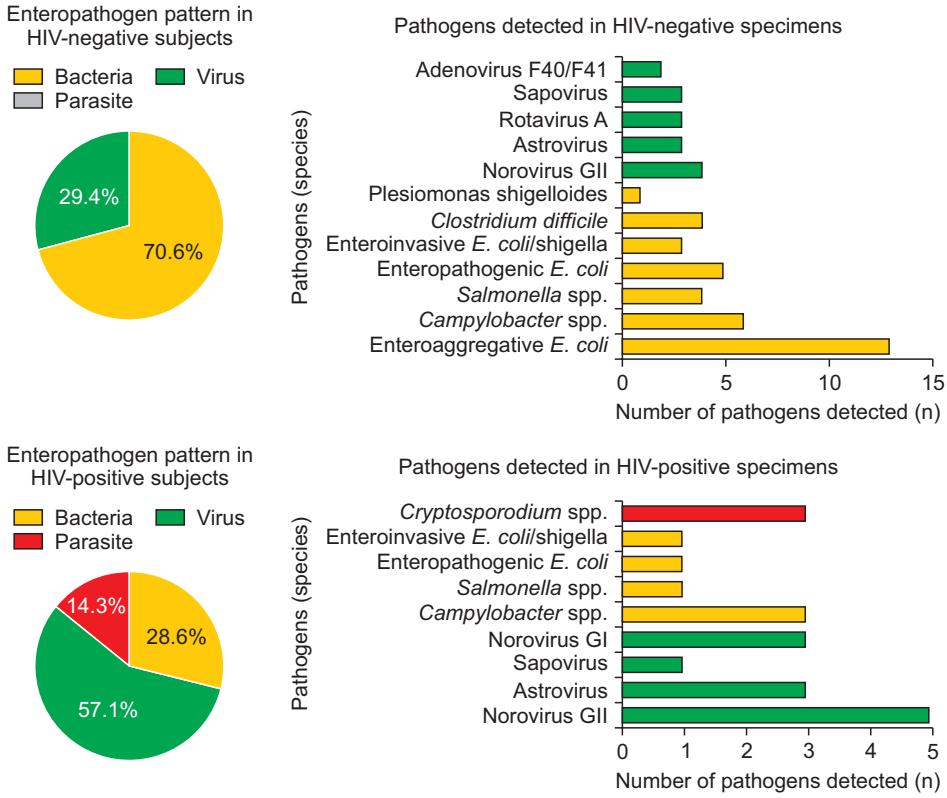

Fig. 1. Enteropathogen pattern between HIV- and non-HIV-infected children with acute diarrhea.
HIV: human immunodeficiency virus, *E. coli*: *Escherichia coli*.

DISCUSSION

This study is one of the first studies in Indonesia to utilize multiplex RT-PCR for determining the types of enteropathogens in children with acute diarrhea. A total of 73 specimens were acquired, and 27.4% of the specimens came from HIV-infected children. The prevalence of diarrhea among HIV-infected children in our study is slightly higher compared to that in a similar study in Cipto Mangunkusumo by Putra et al. [9]; it might be due to differences in the study population, in which Putra et al. [9] mainly focused on persistent diarrhea. HIV-infected children in this study are mostly older, which shows that, at an age where normal children already have sufficient resistance toward enteropathogens, children with HIV are still susceptible to acute diarrhea due to their immunodeficiency. This is supported by a low CD4 count and only half of the subjects received antiretroviral therapy in the study.

Routine stool analysis of HIV-positive children shows that it has several differences from that of non-HIV children. The stool of HIV-infected children is mostly loose and watery in consistency. This is consistently observed because viruses are the most common infection in HIV-infected children in our study. Amylum and plant fiber, observed microscopically, were more frequently found in HIV-infected children, which indicates malabsorption. This shows that in HIV-positive children, acute diarrhea can occur due to infectious/non-infectious causes, or both. Infection can cause the destruction of enterocyte cells and reduce brush border enzymes resulting in maldigestion. Non-infectious etiologies of acute diarrhea in HIV-infected children in the study might be HIV enteropathy or the side effects of antiretroviral therapy [10], although both could not be excluded. In both the groups,

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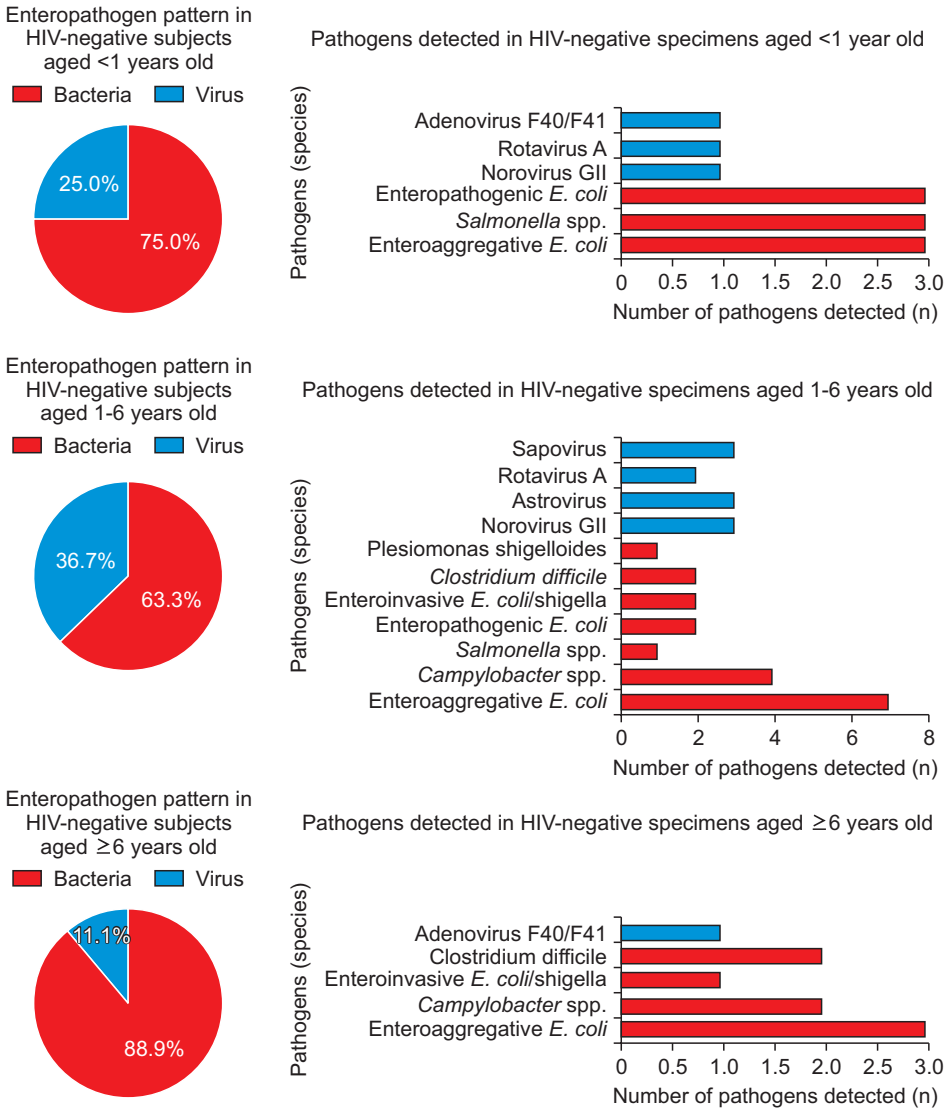

Fig. 2. Enteropathogen pattern in non-HIV-infected children with acute diarrhea based on age groups.
HIV: human immunodeficiency virus, *E. coli*: *Escherichia coli*.

there was a lack of consistent increase in fecal leukocyte count. The possibility of false low results in the fecal leukocyte count might be caused by the degeneration of leukocytes due to delays in examination or an increase in fecal transit time [11]. The increase in fecal erythrocytes, observed microscopically, is one of the parameters indicating the possibility of invasive diarrhea caused by invasive pathogens. In non-HIV children, the presence of fecal erythrocytes is slightly higher, which is also consistent with the majority of enterobacterial infections detected within the group.

Enteropathogens in non-HIV children with acute diarrhea comprised bacteria (70.6%) and viruses (29.4%) with a predominance of enteroaggregative *E. coli* (25.4%), followed by *Campylobacter* spp. (11.8%), enteropathogenic *E. coli* (9.8%), Norovirus GII (7.8%), and *Clostridium difficile* (7.8%). This is different from previous reports, which identified Rotavirus as the most common pathogen in HIV-negative children with acute diarrhea [12,13]. The prevalence of pediatric diarrhea caused by Rotavirus infection remains high in Indonesia

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particularly in children <5 years old [14]. The absence of a national program for Rotavirus vaccine along with the presence of mostly HIV-negative subjects in this study (under 6 years old) explain the supposedly high prevalence of diarrhea associated with Rotavirus infection. However, the low prevalence of Rotavirus in the study can be attributed to complete resolution of Rotavirus infection in primary or secondary health care centers while patients with severe clinical conditions and comorbidities were referred to tertiary care hospitals.

Enteropathogens in HIV-infected children consisted of viruses (57.1%), bacteria (28.6%), and parasites (14.3%) composed of Norovirus GII (24%), *Cryptosporidium* spp. (14.3%), *Campylobacter* spp. (14.3%), Norovirus GI (14.3%), and Astrovirus (14.3%). The low prevalence of bacterial infection in HIV-infected children may be due to prior use of antibiotics and the use of cotrimoxazole, via a national program for the prevention of *Pneumocystis jiroveci* implemented in Indonesia for HIV patients. The study also found that parasitic infection is only found in HIV-infected children. *Cryptosporidium* spp. was the only parasite found in HIV-positive children in our study, in accordance with several other studies [15,16].

There are several limitations in our study. First, the multiplex RT-PCR kits used were only able to analyze the 24 most common diarrhea-causing pathogens; therefore other pathogens such as fungal infection, *Mycobacterium* spp., Cytomegalovirus, and *Blastocystis hominis* that were previously reported by Idris et al. [17] could have been reported as negative. Second, this study did not report the clinical data of patients. Third, non-HIV children with immunodeficiency or immunocompromised conditions other than HIV were not excluded from the study. Fourth, there is a possibility that certain nosocomial pathogens reported in this study are a result of hospital-acquired diarrhea for patients referred from a primary or secondary hospital. Fifth, patients received antibiotic therapy. Finally, there is potential for selection bias within the study. As this study was conducted in a tertiary health care center within the national referral system, we did not include patients with common diarrhea that is mostly treatable in the primary and secondary health care centers. Therefore, the results of our study cannot be applied to the general population. Nevertheless, this study highlights the need for multiplex RT-PCR in detecting multiple pathogens, including uncommon pathogens in pediatric cases with acute diarrhea.

In non-HIV children admitted to a tertiary hospital with acute diarrhea, enteropathogens mostly consisted of bacteria, whereas in HIV-infected children, viruses and parasite infections were more dominant. Taking these patterns into consideration, further examinations and appropriate empirical antimicrobial therapy should be performed.

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