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Evaluation of the detection of diarrhoea-associated RNA viruses in immunocompromised children in Iran

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SUMMARY

Background: Gastroenteritis accounts for about 10% of the deaths among children, especially in immunocompromised children. Few studies on the prevalence of gastrointestinal infections caused by RNA viruses have been done in Iran. The aim of the study was to evaluate the detection of RNA viruses causing diarrhoea using a multiplex PCR.

Methods: Stool samples were collected from 130 paediatric patients with diarrhoea who had acute lymphocytic leukaemia, non-Hodgkin lymphoma, and retinoblastoma. After RNA extraction and synthesis of cDNA, multiplex PCR was done to evaluate the presence of rotavirus, norovirus, astrovirus, and enterovirus.

Results: There were 9 (6.9%), 7 (5.4%), 3 (2.3%), and 6 (4.6%) cases of rotavirus, norovirus, astrovirus, and enterovirus detected, respectively. One case of co-infection with astrovirus and norovirus was observed.

Conclusions: This is the first report from Iran which identified the presence of common RNA viruses causing diarrhoea in immunocompromised children. Increased awareness of these viruses will enable healthcare professionals to improve strategies and policies to control spread and infection caused by these viruses.

Introduction

Gastroenteritis accounts for about 10% of deaths among children [1]. Diarrhoea is a common symptom in immunocompromised patients and in acute cases may cause severe disease [2]. RNA viruses such as rotavirus, norovirus, astrovirus, enterovirus, and enteric adenoviruses are common pathogens which cause gastroenteritis in these patients, especially in children [3,4].

Rotavirus is the main cause of paediatric gastroenteritis worldwide and accounts for about 20% of diarrhoea-related deaths in children under 5 years of age [5]. Studies have been shown that rotavirus infection in immunocompetent and immunocompromised children can cause chronic diarrhoea. [6,7].

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Norovirus is highly contagious, has a low infectious dose, short-term immunity against re-infection, and high stability in the environment. These characteristics have made this disease widespread. Long-term or chronic infection with norovirus can occur in patients with primary or secondary immunodeficiency [8]. There are 10 genogroups among norovirus strains, with genotypes GI, GII, GIV, VIII, and IX mainly causing infection in humans [9]. Acute gastroenteritis caused by a norovirus usually resolves spontaneously in 2—3 days, whereas chronic infection may occur in immunocompromised and can last for weeks to years [10,11].

Astrovirus is an important cause of diarrhoea and vomiting in children, adults, and immunocompromised patients, but there are fewer reports compared with other viruses [12].

Enterovirus in immunocompromised hosts causes a wide range of diseases such as respiratory infections, gastroenteritis, myocarditis, meningitis, and gastroenteritis [13]. In a recent report most patients with primary immunodeficiencies including MHC (major histocompatibility complex) defects had prolonged infection with enterovirus [14].

In Iran, there have been few studies on viral diarrhoeal infections in immunocompromised patients, especially children, and the study of multiple microorganisms in these patients has not been performed simultaneously or individually. Gastroenteritis is rarely investigated for all likely pathogens, and in up to 40—50% of cases of diarrhoea associated with gastroenteritis, the pathogen remains unknown. Recent studies using molecular methods to detect multiple intestinal viruses have addressed this diagnostic gap. In addition, it is important to identify and to prevent the spread of infections caused by these viruses in paediatric oncology units [15]. The aim of this study was to evaluate the detection of RNA viruses (rotavirus, norovirus, astrovirus, and enterovirus) causing diarrhoea in immunocompromised children by using a multiplex PCR.

Methods

Stool sampling and microbiology investigations

Stool samples were collected after receiving informed consent from the patients or their parents. For one year, 130 stool samples were collected from immunocompromised paediatric patients who with infectious diarrhoea and who had been referred to the children's hospital in Qom. Most of these children were undergoing chemotherapy at the time of the study. Approximately 2 ml of stool were collected into sterile containers from each patient and then sent to the university research laboratory. A questionnaire including age, gender, and clinical symptoms was also completed by the patient or their parents. Stool examination and bacterial culture, as well as multiplex PCR were performed on each sample.

Preparation of samples

Stool samples were pre-treated by adding 1g stool to 1 ml of phosphate buffered saline (PBS) then adding 1 ml of the suspension to 9 ml PBS (dilution 1:10) or used 10% stool specimen in the case of watery diarrhoea (entirely liquid with no solid pieces). Pre-treated stool samples for PCR were divided into aliquots and stored frozen at −80 °C until testing.

Nucleic acid extraction

RNA was extracted from stool samples using a RNX-plus kit (Cinnacolon, Tehran, Iran) and according to the manufacturer's instructions. All samples were extracted in a class 2 slow flow dedicated hood to prevent aerosol formation. Chloroform aliquots of 0.1× volume were added, and following mixing, the samples were centrifuged at 8,000 × g for 10 min. The aqueous layer (stool extract) was removed to a fresh tube and stored at −80°C, or the extraction was completed before freezing. Total nucleic acids were eluted from the spin columns using 50 μl of DNase- and RNase-free water. Extracted nucleic acids were stored at −80°C.

cDNA synthesis and multiplex PCR

The complementary DNA (cDNA) was synthesised using BioFACTTM RT Kit [(M-MLV, RNase H, BioFACT, Daejeon, Korea] according to the manufacturer's instructions. All stool samples were tested for the presence of rotavirus, norovirus, astrovirus and enterovirus. The final volume of each reaction was 25 μL, including 10 μL of 1X Master mix (Ampliqon, Denmark), 1 μL of each primer (10 pmol/μL) (Table I), 3 μL of cDNA, and 4 μL of distilled water. Multiplex PCRs were performed in one cycle at 94 °C for 15 min for denaturation, and followed by 40 cycles for amplification, 94 °C for 5 min, 60 °C for 15 min, and 72 °C for 15 min, and a final extension at 72 °C for 10 min. Detection of the amplified products was performed using gel electrophoresis and ultraviolet light trans illumination.

Results

There were 130 patients included in the study, with 62.3% of the patients aged 5 years and under. The mean ±standard deviation of age was 56.72 ±40.49 months. The gender of the patients comprised 79(60.8%) males and 51(39.2%) females. According to the medical records of the patients, 101 had acute lymphoblastic leukemia (ALL), 19 had non-Hodgkin lymphoma

Table I
List of primers used

Virus	Forward primer (5'→3')	Reverse primer (5'→3')	Gene	Size (bp)	References
Rotavirus	AAAGGATGGCCAACAGGATCAT	GTATARAAHACTTGCCACCAT	VP7	569	[16]
Norovirus GI	CTGCCCGAATTYGTAAATGA	CCAACCCARCCATTRTACA	ORF2	330	
Norovirus GII	CARGARBCNATGTTYAGRTGGATGAG	CCRCCNGCATRHCCRTTRTACAT	ORF2	388	
Astrovirus	TCTYATAGACCGYATTATTGG	TCAAATTCTACATCATCACCAA	ORF1a	114	[17]
Enterovirus	CCCTGAATGCGGCTAATCC	ATTGTCACCATAAGCAGCCA	5′UTR	146	[18]

(NHL), and 10 had retinoblastoma. The clinical symptoms recorded in the questionnaire indicated that most patients had bloody diarrhoea (128 cases), fever (120 cases), anorexia (115 cases), and vomiting (98 cases). The results of hospital laboratory evaluations showed that all stool samples were negative for routine bacteria including *Salmonella* spp., *Shigella* spp., and pathogenic *Escherichia coli*.

Based on PCR results, a total of 25 patients (19.2%) were positive for RNA viruses. These comprised 9 (6.9%) with rotavirus, 7 (5.4%) with norovirus, 3 (2.3%) with astrovirus and 6 (4.6%) with enterovirus. All positive cases of norovirus belonged to the genogroup GII. Among the 25 patients, 1 patient (4.0%) had co-infection between astrovirus and norovirus. Most patients who were positive for all viruses had ALL. No patient with retinoblastoma tested positive for any of the viruses in the study. Bloody diarrhoea and fever were observed in all virus positive cases. Except for astrovirus, a higher rate of virus infection was detected in males than females. The prevalence of gastroenteritis in patients was higher in autumn and winter. The data are summarised in Table II.

Discussion

Diarrhoeal disease is an important cause of illness in people of all age groups and especially in children [19]. Various risk factors for diarrhoeal disease in children have been identified including the pathogens, age, sex, seasonal patterns, parental information level, limited access to piped water, poor hygiene and poor waste disposal [20,21]. Among these, a range of pathogens which cause diarrhoeal illness especially in developing countries [22—26]. Patients who are immunocompromised from a range of causes including certain viral infections, transplantations, blood disorders, and malignancies, are at higher risk for severe gastrointestinal infections [27]. In addition, immunosuppressive therapies predispose patients to opportunistic bacterial or viral infections [28].

Enteric viruses, especially those investigated in this study, have been shown to be common pathogens causing acute diarrhoea. There is little information about the prevalence of the viruses investigated in this study in immunocompromised children in Iran. As viruses are not routinely detected in clinical laboratories lacking molecular equipment, this study aimed to investigate four pathogenic viruses including rotavirus, norovirus, astrovirus, and enterovirus.

In this study, rotavirus infection was detected in 9 cases (6.9%), which was a higher percentage compared with other viruses investigated in the study. According to previous studies, the average annual prevalence of rotavirus in Iran is estimated at 36.5% (range, 15.3%—67.6%), which shows a higher percentage compared with this study [29,30]. However, similar to this study, Bruijning-Verhagen *et al*. in the Netherlands revealed that gastrointestinal infection with rotavirus was identified in 35 (4.1%) hospitalised adults and 259 (6.9%) paediatric patients [6]. In the study of Ribeiro *et al*. on the prevalence of rotavirus infection in immunocompromised subjects with acute gastroenteritis in Portugal, 0.8% positive cases of rotavirus were identified [31]. Studies in non-immunocompromised patients have reported a higher prevalence [32]. The studies suggested that other factors such as the season and diagnostic methods, played a role in the prevalence.

Norovirus is an important pathogen among children worldwide and also in Iran. In the studies conducted by Arashkia

Table II
Gender, age, season, underlying disease and symptoms in patients with positive virus results

Parameters		Patients							
		Rotavirus-positive patients (N=9)		Norovirus-positive patients (N=7)		Astrovirus-positive patients (N=3)		Enterovirus-positive patients (N=6)	
		No.	%	No.	%	No.	%	No.	%
Gender	Female	2	22.2	3	42.9	2	66.7	2	33.3
	Male	7	77.8	4	57.1	1	33.3	4	66.7
Age (Month)	(M±SD)	52.8± 34.1		54.6± 29.0		76.0±34.1		37.7±64.3	
Season	Spring	0	0.0	1	14.3	0	0.0	0	0.0
	Summer	0	0.0	1	14.3	2	66.7	0	0.0
	Autumn	4	44.4	4	57.1	1	33.3	1	16.7
	Winter	5	55.6	1	14.3	0	0.0	5	83.3
Disease	ALL (Male/ Female)	7 (6/1)	77.8 (71.4/28.6)	6 (3/3)	85.7 (50.0/50.0)	2 (0/2)	66.7 (0.0/100.0)	5 (4/1)	83.3 (80.0/20.0)
	NHL (Male/ Female)	2 (1/1)	22.2 50.0/50.0)	1 (1/0)	14.3 (100.0/0.0)	1 (1/0)	33.3 (100.0/0.0)	1 (0/1)	16.7 (0.0/100.0)
Clinical symptoms	Bloody diarrhoea	9	100.0	7	100.0	3	100.0	6	100.0
	Fever	9	100.0	7	100.0	3	100.0	6	100.0
	Anorexia	7	77.8	6	85.7	3	100.0	5	83.3
	Vomiting	9	100.0	4	51.7	1	33.3	4	66.7
	Weight loss	8	88.9	4	51.7	2	66.7	3	50.0
	Abdominal pain	5	55.6	2	28.6	0	0.0	0	0.0

et al. and Eftekhari *et al*. on children under 5 years of age in Iran, the prevalence of norovirus was 17.5% and 20.0%, respectively [33,34]. This virus is an important pathogen in immunocompromised patients where cases of norovirus-associated death have been reported in this group of patients, including in non-Hodgkin lymphoma [35]. The first cases of advanced chronic lymphocytic leukaemia were reported in two patients, a 64-year-old man and a 59-year-old woman, in 2011, who suffered from chronic diarrhoea associated with persistent norovirus excretion [36].

In this study, norovirus was detected in 7 (5.4%) of patients. This rate was lower compared with some studies. Davis *et al*. during 2012—2018 in immunocompromised paediatric patients with haematologic malignancy, brain tumour, haematologic disorder, infectious disease, primary immunodeficiency, and solid tumour, found the prevalence of norovirus at 10.8% during this time period [37]. In 2012, Frange *et al*. reported that norovirus was the most frequent virus (11/24 positive samples) detected in both combined and humoral immunocompromised children [38]. In 2016, a review by Bok *et al*. showed that the prevalence of norovirus was 17—18% in immunocompromised patients [39]. Munir *et al*. also investigated norovirus infection in immunocompromised children and children with hospital-acquired acute gastroenteritis. They reported 23.4% of this group of patients had norovirus infection [40].

In the current study, enterovirus ranked third out of the four viruses investigated with 6 positive cases (4.6%) detected. Few studies of this virus have been conducted on patients with underlying conditions. Moschovi *et al*. in a retrospective study based on the medical records of all patients with malignancy with clinical presentation of a possible enteroviral infection, reported that 55 of 104 children were positive for this virus [41]. Nowak-Wegrzyn *et al*. detected enteroviral RNA in stool and CSF using PCR in two infants with severe combined immunodeficiency [42]. Other cases of infection caused by this virus in immunocompromised patients have been reported in other parts of the world [43,44].

Astrovirus accounted for the lowest number of positive cases (3 cases, 2.3%). There have been reports of identifying this virus in immunocompromised patients. In a study conducted in 2001, astrovirus antigen was detected in 12 cases (5.0%) in stool samples of immunodeficient patients hospitalised with diarrhoea [45]. A case report demonstrated an association between astrovirus infection and the appearance of diarrhoea in an immunocompromised patient [46]. In addition, another case of severe astrovirus gastroenteritis in a chronic lymphocytic leukemia (CLL) patient was reported by Coppo *et al*. [47]. Conversely, in another study, although astrovirus infection was found more frequently in HIV-infected children, astroviruses were not significantly associated with diarrhoea in these children [48].

In this study, only 1 case (4.0%) of co-infection between astrovirus and norovirus was detected. Co-infections between viruses/bacteria, parasites/bacteria, and viruses/viruses have been observed in various clinical scenarios [49,50]. In a previous study, co-infection between adenovirus and rotavirus in non-immunocompromised children was found in only 5 cases (21.7%) [24]. In a study in Tehran, Iran, Mousavi Nasab *et al*. reported co-infection of rotavirus and norovirus in only 6 cases (3.5%) [16]. In the research conducted by Koh *et al*. on viral agents in Korean children with acute watery diarrhoea, it was found that co-infection with rotavirus and norovirus was the most common combination (12.9%) [51].

Unlike other viral agents, only norovirus was isolated throughout the year (mostly in autumn). This finding was similar to that reported by Bok *et al*., who showed that seasonal prevalence of gastrointestinal infection caused by norovirus in immunocompromised hosts occurred year-round [39]. Most cases of diarrhoea caused by these viruses have been reported in autumn and winter. For example, Zeng *et al*. in China reported that the peak seasons for rotavirus diarrhoea were in autumn and winter, while norovirus was in winter and spring [52]. Also, Thwiny *et al*. in Iraq described that the prevalence of rotaviruses, adenoviruses, and astroviruses was higher in the cold seasons of the year [53].

Except for astrovirus, the other viruses in the study were more common in males than females. The prevalence of infection in different studies has been predominant in both males and females. Jadali *et al*. in evaluation of rotavirus-associated diarrhoea in children's hospitals in five Iranian cities, showed that 59.2% and 40.8% of positive cases occurred in males and females, respectively [54]. Bucardo *et al*. in Nicaragua indicated that children with diarrhoea caused by norovirus were mostly girls [55]. In a study by Jacobsen *et al*. in Germany from 2010 to 2015, the detection rate of astroviruses in males and females was 44 (4.7%) and 64 (5.5%), respectively [56].

Based on clinical symptoms, the patients in this study mainly had bloody diarrhoea, fever, anorexia, vomiting, and abdominal pain. Since the patients were undergoing chemotherapy during the sampling, it is possible that the bloody diarrhoea reported in all these patients was a side effect of chemotherapy [57] or was caused by the viruses of interest in this study. Bloody diarrhoea and other symptoms caused by these viruses have been reported. For example, Sharma *et al*. demonstrated bloody mucoid stools in rotavirus infection in the term and preterm NICU patients [58]. In a study in Brazil on astrovirus detection in sporadic cases of diarrhoea among children, fever was the main symptom, followed by vomiting and bloody diarrhoea [59]. Zaki and colleagues in Egypt also investigated diarrhoea caused by astrovirus, adenovirus, and norovirus in children, and the main symptoms in the patients were fever, vomiting, abdominal pain, watery diarrhoea, and bloody diarrhoea [60].

There were a number of limitations in this study. There was no control group with patients without immunocompromised conditions, which could have strengthened the findings. Additionally, due to financial limitations, other RNA viruses were not investigated in this study.

Conclusions

This study was the first in Iran to investigate RNA viruses that cause diarrhoea among immunocompromised children. The results showed that these viruses are present in our region and cause disease in immunocompromised children. Increased awareness of these viruses will enable healthcare professionals to improve strategies and policies to control and prevent infection caused by these viruses. Further research examining other infectious agents, including viruses and bacteria, in immunocompromised children with diarrhoea is needed in the future.

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Conflict of interest statement

None.

Author contributions

AF, SDMN, HK, and SS involved in the management of the project and writing up the paper. SS, SY, and SDMN involved in collecting samples and performing the study. SS involved in the analysis of results. All authors read and approved the final manuscript.

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Ethics approval

This research complies with the Declaration of Helsinki and approved by the Ethics Committee of Qom University of Medical Sciences (code No.: IR.MUQ.REC.1396.89). All actions were performed according to the guidelines and regulations of the committee.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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