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Use of multiplex PCR in diagnosis of childhood acute viral diarrhoea caused by rotavirus, norovirus, astrovirus and adenovirus in Upper Egypt

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Abstract

Background: Diarrhoea is still a major public health issue in developing countries, and it is one of the leading causes of morbidity and mortality in children. We aimed to assess the use of a multiplex reverse transcription polymerase chain reaction (RT-PCR) assay for *the detection of five viruses, including* rotavirus, norovirus (genogroups 1 and 2), astrovirus, and adenovirus, responsible for gastroenteritis in children under 5 years old in primary care centres in Upper Egypt.

Subjects and methods: A total of 500 stool samples were collected. Fifty samples were randomly selected for viral examination using multiplex RT-PCR *for the detection of rotavirus*, norovirus (genogroups 1 and 2), astrovirus, and adenovirus, causing diarrhoea.

Results: Viruses were detected in 45 (90%) of the 50 stool samples. The most frequently identified virus was norovirus G2, followed by Group A rotavirus, astrovirus and adenovirus. Mixed infection by two and three viruses was observed in 7/50 cases (14%) and 2/50 cases (4%), respectively. Norovirus G1 was not detected in the samples examined.

Conclusion: Our study reveals that multiplex PCR allows for the detection of multiple viral targets in only one reaction, rendering the assay easier to perform compared to existing testing methodologies (RT-PCR and electron microscopy). Additionally, most of the viruses were detected in summer, and the highest prevalence was in the age group less than 1 year. Norovirus G2 and rotavirus were the most frequent agents and the most common coinfections responsible for gastroenteritis in children.

Keywords: Gastroenteritis, Rotavirus, Norovirus, Astrovirus, Multiplex PCR

Background

Diarrhoea is the second leading cause of death in children under the age of five and is responsible for approximately 525,000 deaths per year [1]. Diarrhoea is still a major public health issue in developing countries, and it is one of the leading causes of morbidity and mortality in children, especially in middle- and low-income countries [2]. Although there was a 60% decrease in the incidence of diarrhoea in children worldwide from 2000 to 2016, its incidence showed a relatively moderate decline to 13% in these middle- and low-income countries [3]. A previous study reported that 801,000 deaths worldwide in children under the age of five with gastroenteritis were associated

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with diarrhoea, with higher incidence and mortality rates of diarrhoea in middle- and low-income countries [4]. At least 25 bacteria and protozoa can cause diarrhoea in children, and viruses are responsible for more than 75% of cases; the most common viral agents of acute gastroenteritis are rotavirus, norovirus, enteric adenovirus, human astrovirus, and sapovirus [5].

These five viruses can cause gastroenteritis outbreaks, which may be severe due to the need for hospitalization [6]. However, the diagnosis of infectious diarrhoea in developing countries is based primarily on clinical symptoms, which are not always representative of a particular pathogen [7].

In developing countries, few laboratories in referral hospitals can regularly conduct culture methods, enzyme immunoassays, latex agglutination tests, or immunochromatography (ICT) techniques, whereas laboratories in rural areas are restricted to light microscopy detection of intestinal parasites [8]. In the absence of diagnostic tests, infections are treated with empirical antibiotic regimens, which are often linked to broad-spectrum antibiotic overuse and the production of bacterial resistance [9].

In developed countries, real-time polymerase chain reaction (PCR) assays are increasingly used to diagnose gastrointestinal infections. Multiplex PCR systems have been shown to allow for rapid and simultaneous amplification of multiple targets, with high sensitivity and specificity. In tropical countries, a large number of coinfections as well as asymptomatic carriage of bacterial and parasitic pathogens are widespread among infants, as these countries are endemic for various viral infections and tropical diseases. In general, the use of multiplex PCR for the diagnosis of gastrointestinal infections has yet to be evaluated in these regions. Nevertheless, multiplex PCR can decrease the time requirement and cover multiple targets of infective agents compared with other methods used in the diagnosis of diarrhoea, such as stool culture, enzyme-linked immunoassay (ELISA) or electron microscopy (EM) [10]. To our knowledge, this is the first study to diagnose viral gastroenteritis in Upper Egypt using multiplex RT–PCR.

This study aimed to assess the use of a multiplex RT–PCR assay in the detection of five viruses, i.e. rotavirus, norovirus (genogroups 1 and 2), astrovirus, and adenovirus, responsible for gastroenteritis in children under 5 years old in primary care centres in Qena and Aswan.

Methods

Study population

This study is a cross-sectional observational study that was approved by the ethical committee at the National Research Centre (number 13037). The participants were 500 children who presented with diarrhoea in the Primary Health Care Centers of Qena and Alkhatara in Aswan in Upper Egypt from August 2015 to March 2017; verbal consent was obtained from the children's parents.

Inclusion criteria under 5 years with acute diarrhoea (less than 14 days' duration), whereby diarrhoea is defined as the passage of three or more loose or liquid stools per day or more frequent passage than normal for each individual.

Exclusion criteria older than 5 years old, persistent or chronic diarrhoea (more than or equal 14 days' duration), malabsorption syndrome, previous antibiotic intake and suspected contaminated sample, e.g. specimens collected from bedpans.

Sample collection

Stool samples were collected from patients enrolled in this study on the same day of presentation using a sterile leak-proof container that was labelled with the patient's name, date and time of collection (can be stored up to 24 h at 2–8 °C before culturing). For multiplex PCR, stool samples were pre-treated treated (by adding 1 g stool to 1 ml of phosphate buffer saline (PBS) then add 1 ml of the suspension to 9 ml PBS (dilution 1:10) or 10% faecal 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 −70 °C until testing.

Laboratory method

Microbiological examination of all faecal specimens

- Wet preparation with eosin and saline to exclude *Entamoeba histolytica*, *Giardia lamblia* and other ova or cysts of parasites.
- Gram stained film to detect bacteria.
- Culture on MacConkey agar, xylose lysine deoxycholate, sorbitol MacConkey agar, Salmonella Shigella agar, alkaline peptone and thiosulfate citrate bile sucrose (TCBS) media for detection of bacterial growth.

Multiplex PCR

Fifty cases were randomly selected from among the 500 cases (40 cases with negative bacterial culture and with no parasitic infection and 10 cases with parasitic infections): 50% in warm weather and 50% in cold weather. The age of patients from whom the 50 samples were collected was ranged from 4 to 41 months, with an average of 16.6 months.

The samples were subjected to multiplex RT-PCR to detect five viruses: astrovirus; Group A rotavirus; enteric adenovirus; norovirus G1; and norovirus G2.

The Seeplex® Diarrhoea-V ACE Detection kit was used (Seegene, Seoul, Korea, catalogue number: DR6411Y), which is a multiplex assay that permits simultaneous amplification of target deoxyribonucleic acid (DNA)/complementary deoxyribonucleic acid (cDNA) of human enteric adenovirus, Group A rotavirus, norovirus GI/G2, and astrovirus. Seegene uses a new-concept oligo technology, dual priming oligonucleotide (DPOTM) technology, which provides freedom in primer design and PCR optimization and maximizes PCR specificity and sensitivity by fundamentally blocking non-specific priming. Seeplex® Diarrhoea-V ACE detection is based on four major processes: nucleic acid extraction; reverse transcription; PCR amplification of target DNA utilizing DPOTM primers (Table 1); and detection by agarose gel electrophoresis.

The steps were as follows:

- *Nucleic acid Extraction*
- A GeneAll Ribo spin vRD nucleic acid isolation kit (Seoul, South Korea, catalogue number: SG1701) was used for extraction of RNA according to the manufacturer's instructions.
- *Reverse Transcription*
- A RevertAidTM First Strand cDNA Synthesis kit (Fermentas, catalogue number: SG1300) was used according to the manufacturer's instructions:
- We added 8 µl total RNA to 1 µl random hexamer (0.2 µg/µl) and then 3 µl diethyl polycarbonate (DEPC)-treated water. The tube was incubated at 80 °C for 3 min. The tube was chilled on ice for 2 min and then centrifuged briefly. The following reagents were added to the tube: 4 µl 5X RT buffer, 2 µl 10 mM dNTPs, and 1 µl RNase inhibitor (20 µg/µl). Finally, we added 1 µl Reverse Transcriptase (200 µg/µl) and incubated the tube at 37 °C for 90 min. After that, the tube was heated at 94 °C for 2 min and chilled on ice for 2 min. The tube was centrifuged briefly. All cDNA samples were stored at -20 °C until use.
- *Amplification by the Seeplex® Diarrhoea-V ACE Detection kit*
- We followed the manufacturer's instructions:

A reaction mixture was prepared by adding 17 µl of master mix to 3 µl of each sample's nucleic acid added. For the negative control, we added 3 µl of diarrhoea ACE negative control; the diarrhoea ACE positive control was also used. The tubes were placed in a preheated (94 °C) thermal cycler, and the following cycles were performed: one cycle at 94 °C for 15 min for denaturation 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 PCR amplification products

Detection of the amplified products was performed using gel electrophoresis and ultraviolet light trans illumination.

Table 1 Oligonucleotide primers sequence from 5′ - 3′ used for the amplification of 5 viruses causing diarrhoea

Virus and primer sequence	Amplicon size (bp)
Astrovirus	
PreCAP1 GGA CTG CAA AGC TTC GTG 82b GTG AGC CAC CAG CCA TCC CT-	719
Group A rotavirus	
VP7 1_(F) AAA GGA TGG CCA ACA GGA TCA GT END9(S) GTA TAR AAH ACT TGC CAC CAT-	569
Adenovirus	
Ad1 TTC CCC ATG GCI CAY AAC AC + Ad2 CCC TGG TAK CCR ATR TTG AT-	482
Norovirus G2	
COG2F CAR GAR BCN ATG TTY AGR TGG ATG AG + G2SKR CCR CCN GCA TRH CCR TTR TAC AT-	387
Norovirus G1	
G1SKF CTG CCC GAA TTY GTA AAT GA + G1SKR CCA ACC CAR CCA TTR TAC A-	330

bp base pair

Statistical methods

Recorded data were analysed using the Statistical Package for Social Sciences, version 20.0 (SPSS Inc, Chicago, Illinois, USA). Quantitative data are expressed as the mean ± standard deviation (SD). Qualitative data are expressed as the frequency and percentage. An independent-samples t test of significance was used when comparing two means. One-way analysis of variance (ANOVA) was used to compare more than two means. The chi-square test of significance was used to compare proportions between qualitative parameters. The confidence interval was set to 95%, and the margin of error accepted was set to 5%. Therefore, the p value of 0.05 was considered significant.

Results

Demographic data for the studied groups are shown in Table 2. We included 500 samples: 299 males and 201 females. In total, 204 samples were collected in summer, and 296 were collected in winter. The mean of age of the groups was 16.6 months. Of 50 examined samples, we detected 45 positive cases of viral infection using multiplex PCR. Norovirus G2 was the most prevalent virus, as detected in 26/50 patients (52.0%), followed by Group A rotavirus in 12/50 patients (24%), astrovirus in 4/50 patients (8.0%) and enteric adenovirus in 3/50 patients

Table 2 Demographic data of the studied groups

Demographic data	Total ($N=500$)
Sex	
Male	299 (59.8%)
Female	201 (40.2%)
Age (months)	
Mean ± SD	16.62 ± 1.323
Season	
Summer	204 (40.8%)
Winter	296 (59%)
Governorate	
Aswan	327 (65.4%)
Qena	173 (34.6%)

Table 3 Incidence of coinfection in the studied groups

	No	%
Two infections		
Rotavirus and norovirus	6/50	12
Norovirus G2 with astrovirus	2/50	4
Norovirus G2 with adenovirus	1/50	2%
Triple mixed infection		
Rotavirus with norovirus G2 and adenovirus	1/50	2
Rotavirus with norovirus G2 and astrovirus	1/50	2

(6.0%). However, norovirus GI was not detected in the studied population. Regarding coinfection, mixed infection by two viruses was observed in 9/50 cases (18%). The most prevalent coinfection was rotavirus and norovirus G2, which represented 6/50 cases (12%); norovirus G2 and astrovirus coinfection was observed in 2/50 cases (4%), followed by coinfection of norovirus G2 and adenovirus, which was 1/50 (2%). Regarding mixed infection by 3 viruses, there were 2/50 cases (4%). Rotavirus, norovirus G2 and adenovirus were detected in 1/50 cases (2%); rotavirus, norovirus G2 and astrovirus were also detected in 1/50 cases (2%) (Table 3 and Fig. 1).

Table 4 shows the distribution of the viruses based on age categories; 25 of 45 (55.6%) positive patients were younger than 1 year; 15 of 45 (33.3%) cases occurred between the ages of 13 and 16 months and 5 of 34 cases (11.1%) between 37 and 60 months. Thirteen (50%) patients were infected with norovirus G2, and 8 (66.7%) were infected with rotavirus; 2 of 4 patients (50%) were infected with astrovirus, and 2 of 3 (66%) in the age group less than 1 year were infected with adenovirus (Table 4 and Fig. 2).

Regarding seasonal variation, 15 (57.6%) of 26 patients positive for norovirus G2, 8 (66.7%) of 12 positive for rotavirus, 3 (75%) of 4 positive for astrovirus and 3 (100%) positive for adenovirus occurred in summer (Fig. 3).

Discussion

Viral gastroenteritis is a major health problem with significant morbidity and economic consequences. Viral gastroenteritis is caused by a number of viruses, including norovirus, rotavirus, adenovirus, astrovirus, and sapovirus [11].

Our detection rate was exactly equal to the detection rate in Seoul, South Korea, using the same multiplex PCR

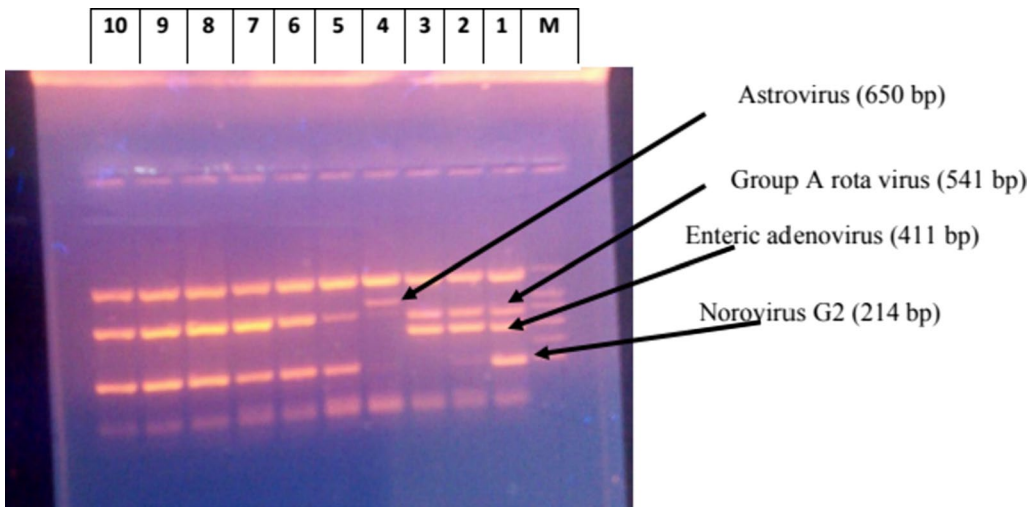

Fig. 1 PCR results using Seeplex® Diarrhoea-V ACE detection. Lane M: Ladder (100 bp). Lane 1: Positive for rotavirus, enteric adenovirus and norovirus G2. Lanes 2, 3: Positive for rotavirus and enteric adenovirus. Lane 4: Positive for astrovirus. Lanes 5–10: Positive for rotavirus and norovirus G2

Table 4 Age distribution of patients with virus infection

Subject	No	Age	Age group months			*P* value
			< 12 months	13–16 months	37–60 months	
Total	50	30.50 ± 8.24 (1–60)	30	15	5	< 0.001**
Virus-positive	45	22.50 ± 6.08 (4–41)	25 (55.6%)	15 (33.3%)	5 (11.1%)	< 0.001**
Norovirus-G2	26	22.50 ± 6.08 (4–41)	13 (50%)	10 (38.5%)	3 (11.5%)	0.048*
Rotaviruses	12	17.50 ± 4.73 (5–30)	8 (66.7%)	3 (25%)	1 (8.3%)	0.039*
Astroviruses	4	24.50 ± 6.62 (8–41)	2 (50%)	1(25%)	1 (25%)	0.002*
Adenovirus	3	15.00 ± 4.05 (6–24)	2 (66.7%)	1 (33.3%)	0	0.564
Norovirus-G1	0	0.00 ± 0.00 (0–0)	0	0	0	–

*p value < 0.005 is significant

**p value < 0.001 is highly significant

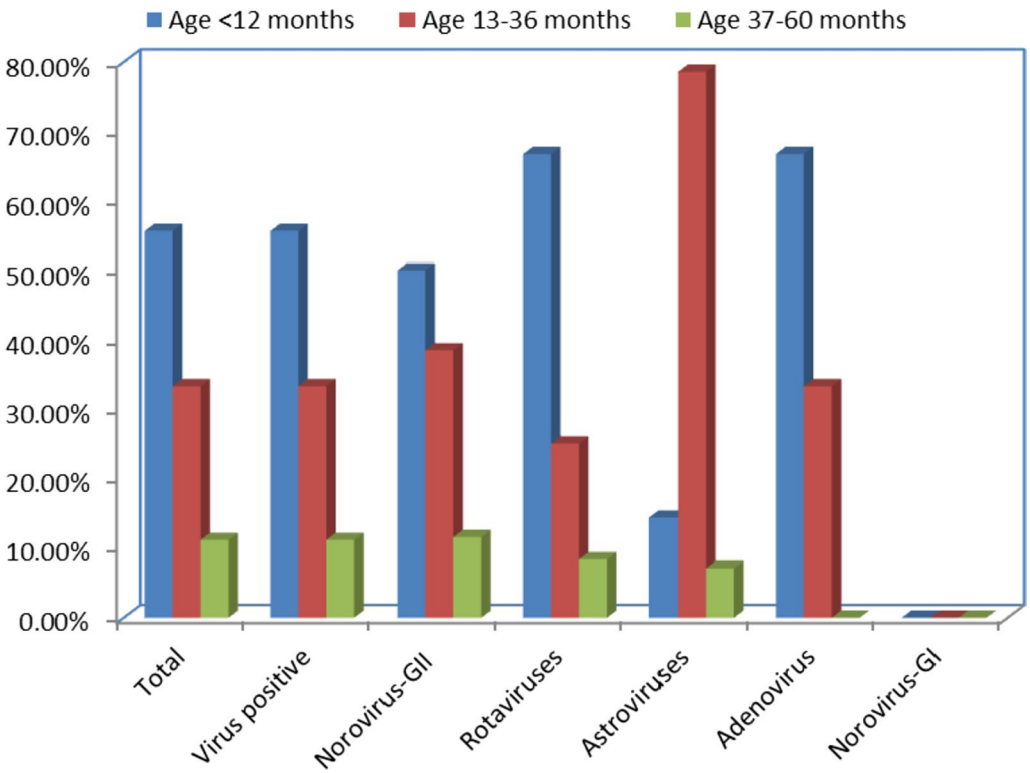

Fig. 2 Distribution of viral pathogens according to age group among 50 children

technique and the same kit [12]. Our results are also in agreement with a study on Egyptian children with gastroenteritis using qualitative PCR, in which viral pathogens were detected in 62%. In another study performed in Egypt, multiplex RT–PCR detected viral pathogens in 57% of samples [13]. In Kyoto, Japan, single-tube multiplex PCR was employed for rapid detection of 10 viruses that cause diarrhoea, with 47.2% of samples being positive for 8 forms of the target viruses. This variation may be attributed to sample size and geographical distribution differences [14].

The most common viruses found in our patients were norovirus G2 and rotavirus, followed by astrovirus and adenovirus. Among norovirus-infected patients, norovirus G2 accounted for 52% (26/50) of the cases, exceeding rotavirus infection. This has been observed in several studies from various countries after implementing rotavirus vaccination [15–17]. Our finding is consistent with

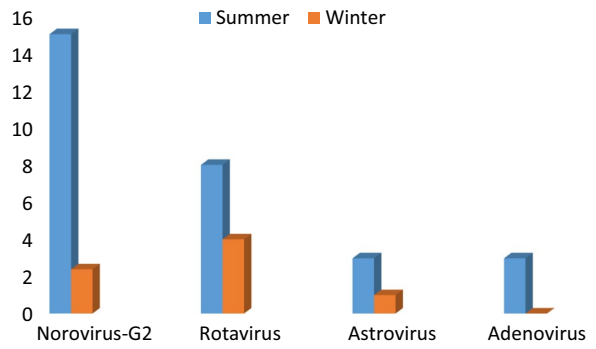

Fig. 3 Seasonal variation of virus detection

a systematic review conducted by Kreidieh et al.'s (2018), which included studies from 24 countries in the Middle East and North Africa (MENA) from 2000 to 2015. This review showed that norovirus was considered the second leading cause of gastroenteritis, with an infection rate that ranged from 0.82 to 36.84% in the MENA region [18]. Similarly, in Japan, 57.8% of samples were positive for norovirus [19]. Lower rates were reported in previous studies in Egypt, in which a Qiagen (Hilden, Germany) one-step RT–PCR kit was used. The norovirus detection rate was 26.0%, and the difference in the results may reflect differences in patient characteristics (age 1 month to 18 years) [20].

Group A rotavirus was the second most prevalent virus detected in our study, accounting for 24%. Two Korean studies reported similar detection rates of 24.8% and 26.9% for rotavirus using the same kit [21]; in Iran, rotavirus was detected in 27.0% of cases using RT-PCR [22]. However, higher results were reported in three other studies using RT-PCR for the detection of viruses causing gastroenteritis in Egyptian children, in which detection rates of rotavirus were 37.0%, 39.0% and 57.4%, respectively [13, 20, 23]. This difference in detection rate can be explained by the difference in geographical distribution, as the two studies were performed in Cairo and Banha, in contrast to our study, which was performed in Upper Egypt in Qena and Aswan. The difference in patient characteristics may also be responsible, as the age group of the studied population was from 1 month to 18 years old in the first study and from 1 month to 2 years old in the second. Additionally, there were different numbers of patients in the studies than in our study. Samples were collected from 100 patients in the first study, from 130 patients in the second study, and from 500 patients in the last study. Other studies performed on children admitted to Abo El Reesh Hospital in Cairo University showed higher results, with a 31.0% detection rate for rotavirus with different techniques (enzyme immunoassay kits RIDASCREEN® viralantigen, RBiopharm AG, Germany) and different sample sizes (119 cases) [24].

Adenovirus was detected in 6.0% of cases. Our results are in agreement with results that 6.7% of children with gastroenteritis in Cairo are positive for adenovirus [24]. Other studies used a qualitative test based on using ICT as a screening test for the detection of rotavirus and adenovirus and reported rates of 28.3% and 19.3% [25], with 22% and 3% by another study in Iraq and Sulaimani [26].

Astrovirus was detected in 8.0% of the patients in our study. Our results are in accordance with those of Ahmed et al., 2011, who detected a rate of 6.3% in Abo Homos, Egypt, using RT–PCR [27]. Lower figures were reported in other studies: 5.5% in Lebanon [28], 3.3% by So et al., 2013, in South Korea [21], 3% by El Mohamady et al., 2006, in Al Fayoum, Egypt [29], 1.7% by Rahouma et al., 2011, in Libya [30], and 1.6% by Maham et al., 2013, in Iran [31]; these differences may be related to the larger sample size compared to our study and different geographical distributions.

On the other hand, higher figures were reported in Nigeria by Ayolabi et al., 2012, who reported a rate of 40.4% [32].

Norovirus G1 was not detected in our study, which is in accordance with Kittigul et al., 2009, who reported a rate of 0.8% in Thailand using the same technique [33].

In our study, coinfection with more than one virus was observed in 9/50 (18%) of cases. Lower figures for the rate of coinfection were reported by Khamrin et al., 2011, in Japan (12.8%) [14] and Zaghloul et al., 2013 (10%) [23]. In our study, norovirus G2 was the most prevalent virus associated with coinfection. A similar finding was documented by Kim et al., 2017, in Korea in their study on children with acute gastroenteritis after the introduction of rotavirus vaccination [12]. Coinfection with rotavirus and norovirus was the most common type of coinfection and occurred in 6/9 (66.7%) of cases with mixed infection. Similarly, Zaghloul et al., 2013, reported that coinfection with rotavirus and norovirus in Egypt was most common and occurred in 62.2% of cases in their study [23].

All pathogens identified in our study showed the highest prevalence among children younger than 2 years of age. Faecal-oral contact, ingestion of contaminated water or food and person-to-person contact, the most common modes of transmission, may be the cause, as most of our patients live in rural and urban areas of Qena and Aswan. Additionally, the decreased prevalence of viral gastroenteritis in older children may be due to developed immunity against these viruses causing diarrhoea; the difference may also be due to the behaviour of older children. Norovirus G2 was detected in 50% of the age group less than 1 year, 38.5% of the age group between

13 and 16 months and 11.5% of the age group between 37 and 60 months. This is similar to the distribution in Egypt according to Zaghloul et al., 2013, who reported 35.8% of children infected with norovirus younger than 1 year of age, 38.2% from 1 to 3 years and 13.6% from 3 to 5 years in outpatient clinics at Ain Shams University of Egypt [23]. Additionally, a study in Delhi performed by Gupta et al., 2018, showed that most norovirus-infected children were younger than 1 year old [34]. In our study, 66.7% of rotavirus-infected children were less than 1 year old, 25% were between 13 and 16 months old, and 8.3% were between 37 and 60 months old. Our finding is in accordance with Gupta et al., 2018, in Delhi and Ferreira et al., 2012, in southern Brazil, who showed that most children infected with rotavirus were younger than 2 years old [34, 35]. Fifty percent of astrovirus-infected patients in this study were younger than 1 year old, 25% were between 13 and 16 months old, and 25% were between 37 and 60 months old. This finding was similar to a study in Iran by Maham et al., 2013, who reported that most astrovirus-infected patients were younger than 2 years old [31]. A total of 66.7% of children in our study were infected by adenovirus in the age group less than 1 year, and 33.3% of them were in the group between 13 and 16 months. This finding is in agreement with Filho et al., 2007, who detected 79% of adenovirus-infected cases in children less than 2 years of age [36].

The prevalence of all detected viruses was higher in summer (May to October) than in winter, which is in agreement with other Egyptian studies that found that norovirus, rotavirus, astrovirus and adenovirus were present in 26.5%, 19%, 11.7% and 47% of cases as a single pathogen or co-pathogen, respectively, with seasonal distribution from May to October [37]. In our study, the prevalence of norovirus G2 in warm weather was 57.7% compared to 42.3% in cold weather. Similarly, in Indonesia [38], Thailand [33] and Morocco, norovirus G2 had a peak in summer months [39].

In our study, positive cases of rotavirus were 66.7% in warm weather compared to 33.3% in cold weather. These findings are in agreement with Zagazig University hospitals in Egypt [40]. Although two other studies in Egypt showed that there was no seasonal variation for rotavirus infection, the first study was performed on children in rural areas [41], while the children in the second study were from the Nile River Delta and showed that the peak of rotavirus causing diarrhoea was in late summer and early winter [42]. This difference may be explained by the seasonal nature of rotavirus infections being not universal: in developed countries, the peak season of viral infection is winter in a temperate climate; in developing countries with tropical and subtropical weather, rotavirus is present throughout the year [43].

In our study, all positive cases of adenovirus were reported in summer weather. Modarres et al., 2006, documented that in most parts of the world, adenovirus is present throughout the year [44], regarding astrovirus, most positive cases were reported in warm weather, which is in agreement with El-Mohammady et al., 2012, who detected astrovirus mainly in warm weather y[37].

Conclusion

Our study revealed that multiplex PCR permits the detection of multiple viral targets in only one reaction. This makes the assay easier to perform compared to existing testing methodologies (RT-PCR and EM). Additionally, most viruses in this study were detected in summer, and the highest prevalence was in the age group less than 1 year. Norovirus G2 and rotavirus were the most frequent agents and the most common coinfections responsible for gastroenteritis in children.

Abbreviations

Bp: Base pair; cDNA: Complementary deoxyribonucleic acid; DEPC: Diethyl polycarbonate; DNA: Deoxyribonucleic acid; dNTPs: Deoxynucleotide triphosphates; DPOTM: Dual-priming oligonucleotide; ELISA: Enzyme-linked immunoassay; EM: Electron microscope; ICT: Immunochromatographic technique; MENA: Middle East North Africa; NRC: National Research Centre; PBS: Phosphate-buffered saline; RT–PCR: Reverse transcription polymerase chain reaction; SD: Standard deviation.

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Authors' contributions

GH, AE, EM, AE and SM designed the study and edited the manuscript. AA collected the samples and shared in the analysis of data. AA helped in collection of samples and availability of patients' data. HE wrote, revised and submitted the manuscript. All authors have approved the submitted version of this article.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

This work was approved by the ethical committee (number: 13037) at the National Research Centre, Cairo, Egypt.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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