

Detection of *Rotavirus*, *Norovirus*, and *Astrovirus* Causing Acute Diarrhoea in Children by Multiplex PCR in a Tertiary Care Hospital

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ABSTRACT

Objective: To evaluate the use of a multiplex real-time polymerase chain reaction (RT-PCR) test in detecting three viruses namely *Rotavirus*, *Norovirus* (genotypes 1 and 2), and *Astrovirus* that cause gastroenteritis in children under the age of five years.

Study Design: A cross-sectional study.

Place and Duration of the Study: Department of Virology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, from January to July 2023.

Methodology: A total of 87 children having acute diarrhoea and less than 5 years of age were included in this study from the outpatient clinic. Multiplex PCR was performed for the detection of three viruses: *Rotavirus*, *Norovirus*, and *Astrovirus* in stool samples of patients using a commercially available PCR kit. The data were analysed using Statistical Package for Social Sciences (SPSS) version 27:00.

Results: Out of total 87 children, aged 2.5 ± 1.5 years, 56 (64.3%) were positive for multiplex RT-PCR and 31 (35.6%) were negative. *Rotavirus* was identified in 24 (27.5%) children as the most common cause of acute diarrhoea, followed by *Norovirus* in 20 (22.9%), and *Astrovirus* in 7 (8%) patients, while co-infection with multiple viruses occurred in 5 (5.7%) of the cases.

Conclusion: This study revealed viral aetiology as a significant cause of acute diarrhoea in children. Multiplex PCR in the healthcare system can make it easier to identify, treat, and control the upsurge of diarrhoea. Prompt diagnosis of viral causes can lead to the prevention of unnecessary use of antibiotics.

Key Words: Multiplex polymerase chain reaction, *Rotavirus*, *Norovirus*, *Astroviridae*, *Diarrhoea*.

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INTRODUCTION

Acute diarrhoea in children is defined as an increase in stool frequency (more than three loose or watery stools per day or more than twice the child's typical daily bowel movements).¹ Acute diarrhoea is a common health problem among children under five years, especially in low-income countries. It is one of the leading causes of infant death in underdeveloped countries, contributing to over 63% of the world's diarrhoea cases.² Lack of proper sanitation, little awareness of personal hygiene, and shortages of well-trained healthcare workers increase the risk of diarrhoea among children in developing regions as compared to those in the developed nations.³

Viruses play a significant role in causing diarrhoea across different countries regardless of the income levels of people.⁴ Among children below the age of five years, *Rotavirus* is the primary cause of severe diarrhoea. Other viruses that are commonly associated with diarrhoeal episodes include *Norovirus*, *Astrovirus*, *Sapovirus*, and human *Adenovirus* F (types 40/41).⁵ These viruses are generally transmitted by faecal-oral route.⁶ Viral gastroenteritis typically presents with watery diarrhoea and vomiting.⁷ *Rotavirus* infections tend to occur in children below the age of five years and lead to severe diarrhoea and higher mortality rates compared to other viruses associated with gastroenteritis.⁸ A *Norovirus* can cause gastroenteritis in individuals of any age. This highly contagious virus is the main cause behind gastroenteritis outbreaks.⁹ The majority of those affected by *Sapovirus* are infants and toddlers. It causes a less severe clinical disease than *Rotavirus*.¹⁰ People of all ages can contract *Astrovirus*, which can potentially lead to outbreaks.¹¹ It is less frequently isolated from stool samples as compared to *Rotavirus* and *Norovirus*. *Adenovirus* gastroenteritis is usually caused by serotypes 40 and 41 and it predominantly affects children below four years of age.¹² Although coin-

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fection with multiple virus infections can also occur, their detection does not seem to have an impact on the severity of the illness or its clinical manifestation.¹³

In developing countries, only limited referral laboratories have the capability to detect viral causes of gastroenteritis by using the latex agglutination tests, immunochromatography (ICT) techniques, serology, and polymerase chain reaction (PCR). When diagnostic tests are not available, infections are usually considered to be bacterial in nature and are treated with empirical antibiotic treatments, contributing to the widespread use of broad-spectrum antibiotics with the emergence of antimicrobial resistance.¹⁴ Conversely, RT-PCR assays are being used more often in resource-rich countries to diagnose gastrointestinal diseases. Multiplex PCR has emerged as a sensitive and specific tool for the simultaneous diagnosis of multiple pathogens in a single sample.¹⁵ It offers advantages such as high throughput, rapid turnaround time, and the ability to detect low viral loads although at a higher cost.

There are no specific antivirals for viral diarrhoea, necessitating management through fluid and electrolyte replenishment.¹⁶ The introduction of licensed *Rotavirus* vaccines in numerous countries globally has substantially decreased *Rotavirus*-related illness and death rates, marking a notable achievement in disease control.

This study aimed to find out frequent causes of viral (*Rotavirus*, *Norovirus*, and *Astrovirus*) gastroenteritis in children using multiplex PCR. Prompt and precise diagnosis of viral causes of diarrhoea can guide appropriate treatment and help prevent the spread of the infection. It can also provide valuable information for the development of effective strategies to control and prevent diarrhoeal diseases.

METHODOLOGY

This study was conducted at the Department of Virology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. Permission from the Institutional Review Board (FC-VIR21-8/READ-IRB/23/1781) was obtained before conducting this study. Prior to conducting the study, written informed consent was obtained from the parents of the participants. A sample size of 87 was calculated using the WHO sample size calculator with a prevalence of 6% and a margin of error 0.05.¹⁷ A total of 87 stool samples were taken from the children presenting in the emergency or from outdoor clinics in tertiary care hospitals. Children below the age of five years with acute diarrhoea (less than 14 days' duration) were included in this study. The study eliminated children with chronic diarrhoea malabsorption syndrome, a history of antibiotic use, parasitic infections, or suspected tainted samples, such as those taken from bedpans.

A predesigned proforma was used to gather demographic data and the history of the participants regarding clinical features (number of stools per day, duration of symptoms, vomiting, fever, and abdominal pain). A sterile, leak-proof container labelled with the patient's name, the collection date, and the

time was used to gather the stool samples. Faeces suspension was prepared by adding 4 ml of normal saline and 1 ml of faeces. The mixture was vortexed to prepare a homogenous suspension and centrifuged at 7000-12000g for 5 minutes. Nucleic acid extraction was performed by using the automated extractor, Tanbead. Qualitative multiplex PCR was performed for the detection of *Rotavirus A*, *Norovirus*, and *Astrovirus* using commercially available PCR kit. Primers for three different enteric viruses were used for the amplification of different target genomic regions such as the VP6 gene for *Rotavirus*, ORF-1 for *Astrovirus* and RdRp for *Norovirus*.¹⁸ For amplification, each PCR mix was prepared by adding 10 µl of RT-PCR-mix-1 *Rotavirus/Astrovirus/Norovirus* / IC, 5 µl of RT-PCR-mix-2, 0.5 µl of polymerase, 0.25 µl of RT-G-mix-2, and 0.25 µl of MMIV. The mixture was vortexed and centrifuged. About 15 µl of the prepared reaction mix and 10 µl of the extracted sample were added to the appropriate tube. The tests were run along with positive and negative controls. The results were interpreted by device software. Internal control was detected at FAM channel, *Norovirus* on JOE, *Rotavirus* on HEX, and *Astrovirus* on Cy3 channel.

The Statistical Package for Social Sciences (SPSS) version 27:00 was used to analyse the data. Percentages and frequencies were calculated for qualitative variables (gender, viral cause, and clinical signs and symptoms) while the quantitative variables (age) were analysed by calculating mean and standard deviation.

RESULTS

A total of 87 children, with a mean age of 2.5 ± 1.5 years (ranging from newborn up to 5 years), were included in the study. Among them, 54 (62%) were males and 33 (38%) were females. *Rotavirus* was detected in 24 (27.5%) patients, followed by *Norovirus* in 20 (22.9%), and *Astrovirus* in 7 (8%) patients. Coinfection with more than one virus was observed in 5 (5.7%) patients, while no viral aetiology was identified in 31 (35.6%) patients, as shown in Figure 1.

Out of the total, 22 (25%) children had a history of febrile illness, while 65 (73.9%) were afebrile. Regarding stool frequency, 9 (10.2%) had more than 10 episodes per day, 51 (58%) had 5-10 episodes per day, and 27 (30.7%) had less than 5. Vomiting and abdominal pain were present in 33 (37.5%) and 34 (38.6%) children, respectively. Vaccination history for *Rotavirus* was present in 24 (27.3%) children. Majority of the children belonged to rural areas as compared to urban areas (60% vs. 40%), as given below in Table I.

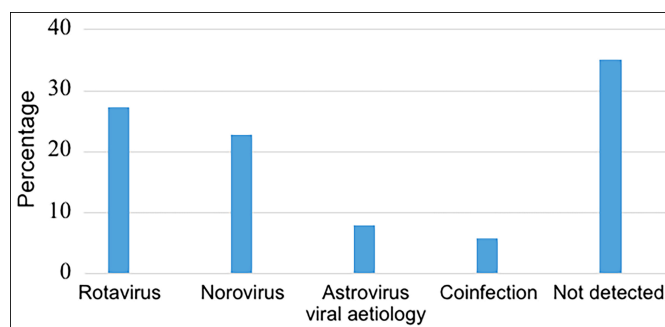


Figure 1: Frequency of different viruses causing acute diarrhoea.

Table I: Frequency and percentages of variables associated with acute diarrhoea.

Demographic data	Variables	Frequency	Percentage (%)
Gender	Male	54	62%
	Female	33	38%
Stool frequency	<5	27	31%
	5-10	51	58.6%
	>10	9	10.3%
Vomiting	Present	33	37.9%
	Absent	54	62%
Abdominal pain	Present	34	39%
	Absent	53	60.9%
Fever	Present	22	28.7%
	Absent	65	74.7%
Vaccination history of <i>Rotavirus</i>	Yes	15	17.2%
	No	72	82.7%
Residence	Urban	36	41.3%
	Rural	51	58.6%

DISCUSSION

In this study, 87 children with a mean age of 2.5 with SD 1.5 years were included. *Rotavirus* 24 (27.3%), *Norovirus* 20 (22.7%), and *Astrovirus* 7 (8%) were identified as common causes of acute diarrhoea. Coinfection with multiple viruses also occurred in 5 (5.7%) of cases, while 31 (35.2%) remained negative for these viruses. Febrile illness was reported in 25% of children, with varying stool frequencies: More than 10 episodes per day (10.2%), 5-10 episodes (58%), and less than 5 episodes (30.7%). Vomiting and abdominal pain were present in 37.5% and 38.6% of children, respectively. *Rotavirus* vaccination history was documented in 27.3% of cases, and the majority of children resided in rural areas (60% vs. 40%) as compared to urban. This study found that viral infections play a significant role in causing acute diarrhoea in children. Most of the infections are caused by *Rotavirus*, reflecting a low vaccine coverage. Moreover, using multiplex PCR in healthcare can make it easier to detect viral causes which can prevent the unnecessary use of antibiotics.

A study was conducted by Li *et al.* in China in 2022, which focused on acute diarrhoea in children with diarrhoea. A notable finding was that 71 samples out of 100 tested positive, resulting in a positivity rate of 44.38%.¹⁹ Among these positive samples, *Norovirus* emerged with the highest positivity rate at 10%, followed by *Adenovirus*, *Rotavirus A*, *Astrovirus*, and *Sapovirus*. Various co-infections involving these viruses were also observed in a small subset of cases. This study's findings are in consistence with the present study's results on acute diarrhoea in children, showing the prevalence of *Norovirus*, *Adenovirus*, *Rotavirus A*, and *Astrovirus* as the significant contributors to this condition.

A study was conducted in Iran by Mohammadi *et al.* in 2020 to find the prevalence of viral causes of gastroenteritis in children. Out of 103 samples, *Rotavirus* was detected in 17 cases (16%), *Norovirus* in 13 (12%), *Astrovirus* in 4 (3.8%), and *Adenovirus* in 3 (2.9%). Two of the samples showed coinfection. This study showed a low prevalence of *Rotavirus* as compared to the present study (27% vs. 16%).²⁰ This difference may be due to seasonal variations, vaccination coverage, and population characteristics.

In Egypt, Othma *et al.* conducted a study exploring the application of multiplex (*Norovirus G2*, *Rotavirus*, *Astrovirus*, and *Adenovirus*) PCR in diagnosing childhood acute diarrhoea attributed to viral

infections. Their findings indicated viral presence in 45 out of 50 stool samples, accounting for 90% positivity. Notably, *Norovirus G2* emerged as the most prevalent virus, followed by Group A *Rotavirus*, *Astrovirus*, and *Adenovirus*.²¹ Additionally, coinfections were identified in 7 out of 50 cases (14%) and 2 out of 50 cases (4%), respectively. The results of both studies highlight the significant burden of viral infections in childhood acute diarrhoea. However, differences in sample sizes, age groups, and geographical locations may contribute to variations in specific virus prevalence and coinfection rates between the two studies.

A study was conducted in Israel by Danino *et al.* in the year 2023, where 2,662 (45.3%) of the 5,879 rectal swabs were examined and tested positive for at least one virus, and 245 (9.2%) showed multiple viral detections. *Rotavirus* became the most common pathogen. Notably, infections involving *Astrovirus* or *Sapovirus* correlated with increased rates of hospital-acquired acute gastroenteritis (AGE) and immunodeficiency, whereas *Rotavirus* infections were associated with heightened severity of dehydration and acute kidney injury.²² The findings from this study aligned with the present study regarding viral causes, particularly highlighting *Rotavirus* as the predominant cause. However, the study by Danino *et al.* demonstrated a higher prevalence rate of viral presence (45.3%) compared to the present study, likely attributed to its larger sample size.

The limitations of this study are that the results may not be as broadly applicable to a bigger population due to the comparatively small sample size. The study's brief duration may have had an impact on the outcomes.

Larger studies are recommended involving diverse population to better understand the epidemiology and risk factors associated with these viral infections. Efforts should be made to enhance *Rotavirus* vaccination coverage among children, particularly in rural areas. Healthcare providers should be vigilant in considering viral infections as potential aetiologies in children presenting with acute diarrhoea by promoting antimicrobial stewardship.

CONCLUSION

Viral infections are a substantial contributor to acute diarrhoea in children. The use of multiplex PCR within healthcare systems can simplify the detection, treatment, and containment of diarrhoea outbreaks. Timely identification of viral causes can help prevent the unnecessary use of antibiotics.

ETHICAL APPROVAL:

This study was conducted at the Virology Department of Armed Forces Institute of Pathology, Rawalpindi. Permission from the Institutional Review Board (FC-VIR21-8/READ-IRB/23/1781) was obtained before conducting the study.

PATIENTS' CONSENT:

Prior to conducting the study, written informed consent was obtained from the parents of the participants.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

KA: Design of the work, acquisition, and data interpretation.

EG: Final approval of the manuscript.
 MAR: Conception, critical revision, and final approval.
 SKN: Critical revision.
 HS: Data analysis.
 MN: Conception of study design.
 All authors approved the final version of the manuscript to be published.

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