Original Research Paper

Volume - 14 | Issue - 06 | June - 2024 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Microbiology

UTILITY OF CONVENTIONAL PCR AND MULTIPLEX RT-PCR IN DETECTION OF VIRUSES CAUSING DIARRHEA IN UNDER5 YEAR CHILDREN

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ABSTRACT **Background And Objective:** Diarrhea is a major public health problem in developing countries and despite of high morbidity and mortality in under 5 year children, the laboratory diagnosis is largely neglected and cause of infective diarrhea remains undiagnosed. This study aims to assess the use of Multiplex PCR and Conventional PCR assay for detection of six viruses, including rotavirus, norovirus, adenovirus, astrovirus, sapovirus and enterovirus, responsible for acute diarrhea in children. **Method:** Stool samples obtained from 150 cases of under 5 year children presented with acute diarrhea at Bundelkhand Medical College, Associated hospital, Sagar (M.P) in between July 2021 and July 2022. The identification of causative agents was carried using Conventional PCR and Multiplex PCR. **Result:** Out of 150 samples, 28 samples were positive for viral pathogens by Multiplex RT-PCR with prevalence of 18.7%. Single viral infection was present in 57% (16/28) and mixed infection in 43% (12/28). Whereas, in conventional PCR single infection was detected in 39% (11/28). The highest number of positive samples was seen in infants (1month- <12 months) with 53.6% (15/28) and least in early childhood with 21.4%. **Conclusion:** Multiplex PCR systems allow simultaneous, expeditious amplification of several targets with good sensitivity and specificity along with detection of co-infections. Multiplex molecular assays can give quick results and powerful approach for detection of enteric viral pathogens from clinical specimens, and minimize the subjective errors in interpretation. This helps in avoidance of unnecessary antibiotic intake, targeted and early treatment initiation especially in pediatric cases.

KEYWORDS : Diarrhea, Under5 children, Conventional PCR, Multiplex RT-PCR, Enteric viruses.

INTRODUCTION

Infectious diarrhea remains a global health problem, responsible for 1 in 9 deaths according to Centers for Disease Control and Prevention (CDC) report[1,2]. Infections with enteric pathogens also been associated with substantial additional morbidity in form of intestinal barrier dysfunction, malnutrition and cognitive impairment[1]. A wide range of pathogens, including viruses, bacteria, protozoa, and fungi, can cause infectious diarrhea. Common viruses associated with acute gastroenteritis (AGE) are rotavirus, adenovirus, enterovirus, norovirus, astrovirus, sapovirus. Due to their similar clinical presentation, etiological diagnosis on clinical basis is challenging[3].

Although most episodes of diarrheal illness are self limiting, determining the etiology is important in ensuring administration of antibiotics where required and avoidance of inappropriate antibiotic use in viral etiologies, which may promote drug resistance and impede antimicrobial stewardships effort[4]. A cornerstone of management includes prompt diagnosis and appropriate treatment. Timely diagnosis can impove patient care, assist in infection control, and prevent disease outbreak[4].

Traditional laboratory methods are time consuming , incur increased labor and longer turn around times[4] Multiplex molecular methods rapidly detect a wide range of pathogens simultaneously reducing turn around time and allowing detection of coinfections[5]. The study aimed to assess the use of Multiplex PCR and Conventional PCR for detection of six viruses, including rotavirus, norovirus, adenovirus, astrovirus, sapovirus and enterovirus, responsible for acute diarrhea in under 5 year children.

MATERIALAND METHOD

Stool samples were obtained from 150 cases of under 5 year children presented with diarrhea with three episodes within 24 hour with or without fever and vomiting at Bundelkhand Medical College, Associated hospital, Sagar (M.P) in between July 2021 and July 2022. The identification of causative agents was carried out using Conventional PCR and Multiplex PCR.

Ethical clearance

The study was approved by Institutional Ethics Committee (IEC) of Bundelkhand Medical College, Sagar, MP. Informed consent was obtained from parents/guardian prior sample collection and case registration.

Preparation of stool suspension:

A part of the stool sample was pre-processed by preparing stool suspension. Stool samples were suspended in 10% phosphate-buffered saline(PBS) (pH7.4) in a 30% w/v ratio, vortex for 3 min, and then centrifuged at 13,000 rpm for 15 min to separate the solid stool debris. The supernatant was collected as the stool suspension, transferred to a sterile 1.5 ml Eppendorf centrifuge tube, and stored at-20ºC. These suspensions were hence forth used for RNA extraction.

RNA Extraction:

RNA extraction was carried out using a commercially available MagMax™Viral/Pathogen Nucleic Acid Isolation kit by Thermo Fischer Scientific, Applied Biosystems. 200µl of the sample was added to 280 µl of lysis buffer, RNA was extracted according to kit protocol, and RNA was eluted into a final volume of 70 µl of elution buffer. This RNA was stored at-80ºC for further testing by Conventional PCR and Multiplex PCR.

Primers

Published primers of 4 different enteric viruses were used for the amplification of the genomes for rotavirus, enterovirus, norovirus II, astrovirus [6]

Table 1. Primers of enteric viruses

Viruses and primers	Amplicon size (bp)	Target region	Sequence 5-3	Sense	Position
Rotavirus VP6-F VP6-R	379 bp	VP6	GACGGVGCRACTACATGGT GTCCAATTCATNCCTGGTGG	+ _	747-766 1106-1126
Enterovirus 160 F 597 R	440 bp	5'NCR	CAAGCACTTCTGTTTCCCCGG ATTGTCACCATAAGCAGCCA	+ _	160-180 599-580
Norovirus SR46-F GR12-R	125 bp	RdRp	TGGAATTCCATCGCCCACTGG AGTTGTCAGATCTCATCATCATCACC	+ _	4754-4775 4879-4853
Astrovirus Mon340-F Mon340-R	289 bp	ORF 1a	CGTCATTATTTGTTGTCATACT ACATGTGCTGCTGTTAACTATG	+ _	1182-1204 1470-1449

Conventional RT-PCR

Conventional RT-PCR was carried out on extracted RNA of stool samples by cDNA synthesis. cDNA synthesis was carried out by preparing a Reaction Mix containing MasterMix (2XMM) of 12.5µl,

Enzyme Mix 1µl, and forward, reverse primers of all 4 enteric viruses along with sample RNA. This Reaction Mix was incubated for initial denaturation at 50ºC for 30min, 35 amplification cycles of 95ºC for 15 min, 94ºC for 30sec, 48ºC for 30sec, and 72ºC for 80 sec followed by a final extension of 72ºC for 7 min.

Gel electrophoresis

RT-PCR amplicons were analyzed by gel electrophoresis on 2% agarose gel using 1x Tris-acetate EDTA (TAE) buffer and ethidium bromide(EtBr) and loaded with 10 µl PCR product. All PCR products were determined by molecular weight and compared with a 1000 bp DNA ladder.

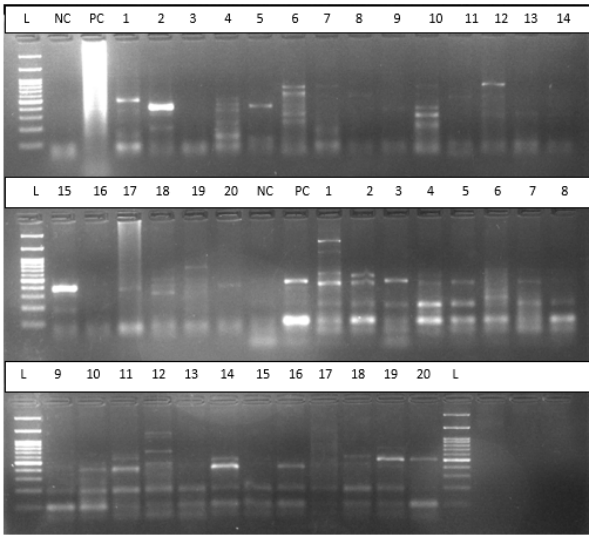


Figure1. Result of conventional RT-PCR on gel electrophophoresis

Multiplex RT-PCR

Detection of five different enteric viruses Norovirus GI/GII, RotavirusA, Adenovirus F (serotype 40/41), Astrovirus, and Sapovirus (GenogroupsG1, 2, 4, 5) in 150 stool specimens was carried out using Allplex™GI-Virus Assay, Multiplex real-time one-step RT-PCR on CFX96™ Real-time PCR System (Bio-Rad).

The reaction tubes were prepared as per the protocol of the test kit. There action tubes were placed in a thermal cycler for single cycle of 50ºC for 20 min and 95ºC for 15 min followed by 45 cycles of 95ºC for 10 sec, 60ºC for 1 min, and 72ºC for 30 sec. The result was interpreted as positive if an exponential fluorescence was observed with ct value <40.

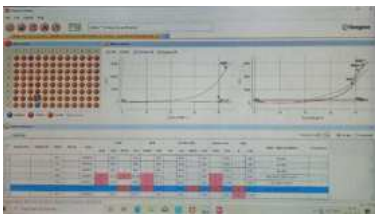


Figure2. Result Of Multiplex Real-time One-step RT-PCR

Data Collection:

A detailed clinical history and demographic profile were obtained from parents of under-5year children admitted to the pediatric ward through a case report form and structured questionnaire. Written informed consent was taken from the parents of respective children presenting with acute diarrhea.

Statatics Analysis:

Data were analyzed using MS Excel 2010 and appropriate statistics were applied. The Chi-square test was used to determine the statistically significant difference between the different categorical variables. A P-value <0.05 was considered statistically significant. The SPSS version 20 was used for data analysis.

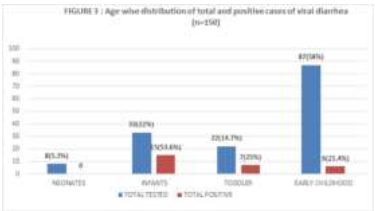
RESULTS

Out of 150 samples, 28 samples were positive for viral pathogens by Multiplex RT-PCR. Single viral infection was present in 57% (16/28) and mixed infection in 43% (12/28). Whereas, in conventional PCR single infection was detected in 39% (11/28). Thus, in our study Multiplex PCR shows 39% (11/28) concordant results with conventional PCR. Multiplex molecular diagnostic panels have greatly enhanced detection of gastrointestinal pathogens. **TABLE 2.** Shows distribution of infection in positive cases of viral diarrhea.

Figure 3. Shows the age- wise distribution of cases of viral diarrhea among under-5 year children which is statistically significant with p-value < 0.05. The highest number of positive samples was seen in **infants (1month- <12 months)** with **53.6%** (15/28), followed by **toddler (1yr-<2yr)** with **25%** (7/28), and early **childhood (2yr- <5yr)** with **21.4%** (6/28). There was no positive case of viral diarrhea in **neonates (birth - <1month).**

Table 2. Distribution Of Infection In Positive Cases Of Viral Diarrhea

Type of Infection	Single Infection 16(57%)			Mixed Infection 12(43%)		
Etiology of infection in viral diarrhea	Rota	Entero	Adeno	Rota + Adeno + NoroI/II	Rota + Adeno + Astro	NoroI/II + Adeno
28	7	6	3	5	4	3
(18.7%)	(43.8%)	(37.5%)	(18.7%)	(41.7%)	(33.3%)	(25%)



DISCUSSION

In our study, Multiplex PCR shows 39% (11/28) concordant results with conventional PCR. Similarly, the study conducted by **Tatte and Gopalkrishna** results showed high concordance between monoplex conventional PCR and multiplex assay with 98.9% [6]. The study done by **Mitra S et al**. also showed relative agreement between conventional RT-PCR and real time RT-PCR [7].

The present study showed a prevalence rate of **18.7%** (28/150)of viral diarrhea in under 5 years of age children. According to the study done by **Stanly AM** et al in south India, the prevalence of diarrhea is **22.5%** [8]. Similarly study done in West Bengal by **Gupta et al**.reported a prevalenceof **22.36%** [9]. According to the study conducted by **Abida et al.** in Telangana, the prevalence ofdiarrhea was found to be**15.7%** [10].

Since, these molecular diagnostic assay included both DNA/RNA viruses, the same standard protocol of RT-PCR was used for detection of enteric viruses. Subsequently, multiplex PCR using multiple primer pairs were used to detect multiple gastroenteric viral pathogens simultaneously. However, In India, there are few reports available on conventional PCR, monoplex PCR for detection of enteric viruses in acute diarrheal cases. Few such attempts have been made to detect wide range of pathogens using One-Step RT-PCR kit [6]. In contrast to other studies reported, the combinations and panel of viruses selected in multiplex PCR and methodology optimized was completely different as in the current study, conventional PCR was done to detect most common viruses causing diarrhea including Rotavirus, Norovirus, Enterovirus, and, Astrovirus. The sensitivity was increased by multiplex PCR using Seegene one-step RT-PCR detecting viruses Rotavirus, Adenovirus, Astrovius, Norovirus I/II and Sapovirus along with coinfections.

The present study shows the highest prevalence of viral pathogens in the age group of **7-12 months** with **39.3%**(11/28) followed by **toddler (1yr-<2yrn)** with **25%** (7/28), and **early childhood (2yr-<5yr)** with **21.4%** (6/28). There was no positive case of viral diarrhea in neonates (birth--<1month).

Our study shows concordance with the study conducted in South India by **Stanly A.M et al.** Children in the age group 7-12 months had the highest prevalence of diarrhea at 40.7% followed by 32.1% in the age group of 13-24 months and 17% in 0-6 months and 12.8% in the age group above 25 months[8]. In the study of West Bengal by **Gupta A et**

al..The majority (26.97%) ofthe study participants were in the age group of less than 1 year and the prevalence of diarrhea was highest in the age group of 7-12 months (57.69%) followed by13-24 months (25.71%)[9].

A study was done in Bhopal by **Gautam GC , and Vishwakarma R.** This study shows a high prevalence of 27.3% in the age group of 7-12 months, followed by 26.6% in children from 13-24 months, 25% in 0-6 months, and a low prevalence of 21% in 25 months and above children[11]. According to a study done in Lucknow by **Gupta et al.,** the positivity was higher among children less than 2 years of age [12]. And according to **Rohra et al**. study done in South Mumbai, 65.21% of children under 3 years of age had viral diarrhea[13].The study conducted by **Gopalkrishna et al**. in Maharashtra western India, the majority (79%) of acute diarrhea cases were seen in children <2 years of age with **21.4%** (6/28)[14].

The present study shows the prevalence of diarrhea causing viruses in children with the highest positivity of Rotavirus infection in 57.1% (n=16) of cases, followed by Adenovirus 53.6% (n=15), Norovirus 28.6% (n=8), and Enterovirus 21.4% (n=6) with Astrovirus 14.3% (n=4) as the least common pathogen causing diarrhea.

According to study conducted by **Gopalkrishna et al.,** the prevalence of Rotavirus A was 30.5% followed by 14.3% for norovirus, 8.4% for adenovirus, and 5.5% for astrovirus. Among them, 3.9% (n=12) showed mixed infections with the majority of RVA (n = 8) and norovirus (n = 8) followed by astrovirus(n = 6) andadenovirus (n=4) [14]. According to Golder et al. study, single viral infection was seen in33.7% and mixed viral infections in 21.2%, and adenovirus was the most commonly associated virus (33.7%) followed by rotavirus (28.7%). The highest coinfection was seen with adenovirus and norovirus (20.4%),followed by adenovirus and rotavirus (13.6%), sapovirus and rotavirus(4.5%)and norovirus,adenovirus and rotavirus (4.5%) [15].

CONCLUSION

Currently, molecular diagnosis by RT-PCR-based tool is considered to be a convenient, useful and powerful approach for detecting enteric viral pathogens from clinical specimens. A rapid, sensitive, specific and cost-effective multiplex assay system will thus prove to be a useful tool to screen a large number of clinical samples. The availability of affordable viral diagnosis will further help to improve patient care by reducing the unnecessary use of antibiotics and preventing development of resistance. In a developing country such as India, where diarrhea remains as a leading cause of death, identification of the most predominant circulating viral etiological agents will be useful in development of intervention strategies to control disease.

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