ORIGINAL ARTICLE

Rotavirus Samples processing using Indigenously Developed Molecular Transport Medium (IMTM)

Tayyab Un Nisa[1*], Muhammad Mujtaba Babar[2], Ashaq Ali[2], Urooj Zafar[3], Aqeel Ahmad[4], Saifullah Khan[2]

ABSTRACT

Objective: To perform comparative diagnostic study with rotavirus infected stool samples for efficacy of indigenously prepared molecular transport medium (IMTM).

Study Design: It was an experimental study.

Place and Duration of Study: The study was conducted at Department of Microbiology of University of Karachi, Karachi Pakistan from June to July 2021.

Materials and Methods: The current study was conducted based on hospital based surveillance of rotavirus gastroenteritis among children <5 years of age, stool samples were collected in commercially available viral transport medium. Samples were known rotavirus positive /negative through PREMIER Rota clone Enzyme-Linked Immunosorbent Assay (ELISA) kit diagnostic test. Total 64 stool samples were selected to evaluate the efficacy of indigenous molecular transport medium (IMTM). Stool samples were spiked in IMTM and commercial available viral transport medium (CVTM). RNA extraction and Real-time PCR were performed by QIAmp and in house assay respectively as per manufacturer' instructions. Similar method of RNA extraction and Real-Time PCR for both type of spiked samples was adopted.

Results: Comparative analysis of Cycle threshold (Ct) values of paired samples (IMTM vs CVTM) was performed. Real-Time (RT-PCR) for rotavirus positive and negative results were consistent with each pair (p<0.0001).

Conclusion: The indigenously formulated IMTM found capable for stool sample collection, transportation and processing for rotavirus detection through Real-time PCR.

Keywords: *Molecular Transport Medium, Rotavirus, Real-time PCR, Stool Samples.*

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Introduction

The viral diagnostics through virus culture or PCR depends on the proper collection and transportation of the samples to the lab. Collection through a swab or the excreted sample both need to be immediately transferred to a suitable medium to transport to the laboratory. Transport media that are required to carry virus culture specimens may contain Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS) or fraction V Bovine Serum albumin, antibacterial and antifungal agents. Moreover, these media containing samples need refrigerated samples transportation to the lab. Restricted temperature requirements and additional viral stability ingredients make these media suitable for the said purpose. Transport Media which are required only for the diagnostic purpose through nucleic acid molecular detection usually do not require the stringent condition of temperature and stability of infective virus particles during sample transport.[1,2] These are usually called molecular

[1]*Department of Microbiology*
University of Karachi, Karachi/Center of Excellence
in Science and Applied Technologies Islamabad, Pakistan
[2]*Center of Excellence in Science and Applied Technologies*
Islamabad Pakistan
[3]*Department of Microbiology*
University of Karachi, Karachi, Pakistan
[4]*Department of Microbiology*
University of Karachi, Karachi/Department of Biosciences
Salim Habib University, Karachi, Pakistan

Correspondence:
Tayyab Un Nisa
Department of Microbiology
University of Karachi, Karachi/Center of Excellence
in Science and Applied Technologies Islamabad, Pakistan
E-mail: tayyabunnisa@yahoo.com

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transport medium (MTM). Few sample transport media comprise only isotonic solution of 0.85 or 0.9% saline, phosphate buffer saline, Hank's Balanced Salt Solution, or Eagle Minimal Essential Medium (EMEM). While selecting such a medium the risk of spread of pathogens and a safe medium for sample transportation purpose always exist. Processing of lethal pathogens for diagnostics needs a higher biosafety level. Such samples needs to be inactivated just after collection to prevent spread of infection. People used cell lysis reagents as sample transport medium.[3] Caotrophic salts are the basic lysing agent. Among caotrophic salts guanidine thiocyanide is well-known. Longhorn PrimeStore® is an approved molecular transport medium from Center for Disease Control CDC, USA having guanidine thiocyanide as basic ingredient which is utilized to collect and transport good quality nucleic acid for PCR based molecular diagnostics. It is also proven to be used for genomic applications other than PCRs.[4,5,6,7,8,9] A new molecular transport medium was developed in our lab based on similar principle, which comprised of guanidine isothiocynide (Lysis agent), L-lauryl sarcosine (Foaming agent) and sodium acetate (Buffering Agent).[3] Probable use and process flow of collection, transportation of samples is defined in Figure 1.

Stool specimen and rectal swabs are two types of preferred samples for rotavirus detection. Since long, Carry Blair transport medium has been the choice of transport medium for the rectal swab transportation to the laboratory at refrigerated temperature.[10,11,12,13] Viral transport medium (VTM), Hanks Balanced salt solution containing media and Universal transport medium (UTM) utilization has also been reported for rotavirus suspected stool sample collection, transportation and archiving.[14,15,16,17,18,19] Fomite transmission of rotavirus in general and during sample transportation in special lead to utilize the IMTM for rotavirus samples and its efficacy for providing good quality viral genomic RNA for Real-time PCR.[20,21,22] Rotavirus suspected stool samples were selected and parallel spiked in a commercially available medium (CMTM) and indigenously prepared medium (IMTM) for efficacy check and for comparative analysis through Real-time PCR. All other parameters of sample processing like sample storage temperature (Room Temperature), RNA extraction method, Real-time PCR conditions along with PCR platform were kept constant.

Materials and Methods

As a part of hospital based surveillance study of rotavirus gastroenteritis among children <5 years of age, stool samples were collected in commercially available viral transport medium. The confirmatory analysis for presence of Rotavirus in stool samples were carried through Rotaclone Kit (Rotavirus Antigen detection) using ELISA. An indigenously developed molecular transport medium was used in parallel to monitor its efficacy for collecting and transporting stool samples for rotavirus diagnosis using molecular PCR.

An equal volume of one pea sized stool sample or approximately 500 µl in case of watery stool was inoculated in commercial medium and IMTM. Samples were transported to lab as per defined criteria of commercial kit. Samples collected in indigenous medium were transported and kept at room temperature till extraction of viral RNA. RNA extraction was performed from both sets of samples by using QiaGen (QIAmp) Viral RNA Mini Kit 52906 as per manufacturer's instructions. Real time RT PCR was performed by using Super script III Platinum one-step qRT-PCR Kit Cat No. 11732-020. Reaction mix was prepared with 2X reaction mix, primers and probe concentration (10µM each), ROX was used as reference dye. Primer and probe used were: Primer ROVF 5'ACCATCTWCACRTRACCC TC3', Primer ROVR 5' GGTCACATAACGCCCCTATA3' and Probe ROVP FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-BHQ1. Rotavirus Real Time RT PCR Cycling Condition used were: 50°C for 15 minutes hold, 95°Cfor 02 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Fluorescent signal acquisition was performed on ABI 7500 system.

Bacterial Spiked MTM analyzed for 16S Ribosomal DNA Amplification

Single colony of each bacterium spiked to 3 ml of IMTM and samples were kept on bench overnight night. Next day nucleic acid (DNA) of MTM samples spiked with bacterial cultures were extracted by QIAamp® Viral RNA Mini Kit (Cat#52906), Qiagen, Germany using kit instruction manual except the omission of addition of carrier RNA at the lysis step. PCR for the amplification of bacterial 16s rDNA gene[23]

was carried out in 25µl total volume, with 12.5µl Bioron 2x Taq Master Mix (Cat#S101605), 0.5µl each of both primers V1-6F and V1-6R (10pmol/µl), 2.5µl of cDNA template and 9µl of nuclease free water. PCR for the amplification of expected 1073bp amplified product was carried out in FlexCycler2 analytikjena, Biometra GmbH. Thermal cycling involved initial denaturation at 94°C for 2 minutes followed by 35 cycles of 94°C for 20 seconds, 55°C for 25 seconds and 72°C for 90 seconds.

The amplified product mixed with 6X DNA Loading Dye (thermoscientific, Cat# R0611) to a 1:6 ratio was loaded on to 1.5% agarose prepared in 1X Tris Acetate-EDTA buffer (TAE) buffer containing ethidium bromide at a concentration of 0.5µg/ml. GeneRuler™100 bp Plus DNA Ladder (thermoscientific, Cat# SM0321) was used as reference DNA size marker. Electrophoresis was performed in 1X TAE buffer for 60 minutes at 85 volts. Visualization of amplified product and gel documentation were performed in Azure c200 Gel Imaging Workstation, Azure Biosystems Inc.

The one sample paired t-test was applied to calculate the agreement between IMTM and commercial medium by using Graph Pad Prism 8.0.2.

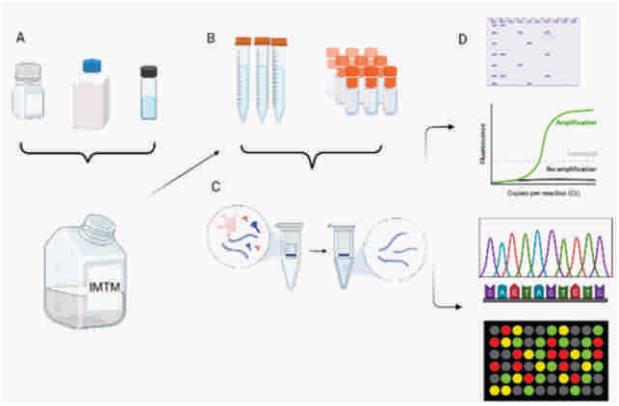

Fig 1: IMTM preparation and use B: for clinical sample collection Room temperature storage, C: RNA extraction and D: utilization of extracted Nucleic acid for PCR, Real-time PCR, sequencing and Nucleic acid based arrays

Results

A total of 161 diarrhea samples were collected from hospitalized children under 5 years of age. Out of these 161, 36 (22.36%) were found rotavirus positive tested through ELISA. Moreover, the results suggest comparative CT values for all the samples in the two media tested (p<0.0001) comparing the PCR results no significant difference was observed between the two media (P<0.0001), suggesting that the local designed transport medium (IMTM) is suitable for rotavirus infected samples diagnosis through PCR in stool samples (Table 2).

IMTM provided excellent results when used with a bacterial spiked range of samples. Indicating the excellent efficiency of IMTM processed samples for conventional PCR.

Discussion

There are many diagnostic tools available for rotavirus diagnostics, Such as Enzyme linked immune sorbent assay (ELISA), Polymerase chain reaction (PCR), Real-time PCR (RT-PCR), Electron microscopy (EM), Immuno-cromatographic strips (ICTs), latex agglutination (LA) and immune-PCR.[24, 25, 26, 27, 28, 29, 30] Moreover, Numerous multiplex Real-time diagnostic panels for gastrointestinal pathogens and nucleic acid based arrays have been developed globally for rapid detection of gastrointestinal pathogens, which needs good quality genomic nucleic acid (DNA/RNA).[31,32] We developed a molecular transport medium that not only fulfill the requirement of sample collection, transportation of stool samples but also inactivates the virus to prevent dissemination of virus during transportation. There were other sample transport media present in the lab, however only IMTM was capable to inactive viruses (Table 1).

Table 1: IMTM Capability Comparison with Other Commercial Sample Transport Media in lab

Name	Company	Ingredient	Purpose	Temperature (°C)
Sofra	Zhejiang sofra life sciences research company ltd. china	Modified Hank's HEPES ,buffer solution, cresol red	Virus isolation and nucleic acid extraction	2-8
Citoswab	Meridian Healthcare, Germany	Modified Hank's-HEPES buffer ,BSA ,Gelatin ,Glucose ,Antibiotic, Phenol red	Virus isolation and nucleic acid extraction	5-25
Sample storage reagent	Sansure biotech, China	Normal saline and RNAse inhibitor	Virus isolation and nucleic acid extraction	2-8
Copan UTM	Copan diagnostic, USA	Buffer, amino acids ,gelatin, anti biotics	Virus isolation and nucleic acid extraction	2-25
IMTM	Center of Excellence in Science & Applied Technologies, Pakistan	Sodium Acetate, Guanidine Isothiocyanide, L-lauryl sarcosine	Nucleic acid inactivation and nucleic acid extraction	2-25

Other commercially available transport media that were in use in laboratory were more costly than IMTM, Secondly they require stringency of refrigeration temperature for sample processing as well as media storage which ultimately increases the cost of sample transportation. Third, the use of guanidine thiocyante made IMTM selectively bio-safe.

Indigenously developed molecular transport medium (IMTM) was found as an efficient sample transport medium for rotavirus suspected stool samples without compromising Real-Time PCR results (Table 2).

Table 2: IMTM Comparison with Commercial Medium via Real-Time RT PCR Results

Number of samples (N)	Mean	t /df	SD	SEM	95% C.I	R^2	*P* Value
64	0.5208	2.296, 63	1.815	0.2268	0.0675 to 0.9741	0.07721	0.0125

We envisaged that IMTM may be utilized for other pathogens as well as other genomic applications. We propose the use of IMTM for other gastrointestinal pathogen detection like norovirus, astrovirus, sapovirus, adenovirus and other enteroviruses. A similar product Longhorn PrimeStore® has been used for Mycobacterium tuberculosis, vibrio, rotavirus and influenza virus detection through Genexpert and next generation sequencing (NGS).[4,5,6,7,8,9] To the best of our knowledge there is only one report available where molecular transport medium was utilized to store RNA containing Rotavirus genome sample not for collection and transportation.[9] Studies are underway to analyze the effect of storage of rotavirus containing samples in IMTM. This is the first time where we made an indigenous formulation and utilized it for stool sample processing for rotavirus detection. Use of IMTM for collection and transportation of rotavirus is of prime importance as the rotavirus transmission is easy through fecal oral route as well as through fomite. An immediate inactivation of rotavirus is a dire need to mitigate the virus transmission through infected samples. Conventional PCR results of bacteria spike samples are also in agreement with real-time PCR results, thus suggesting the efficiency and potential of IMTM for good nucleic acid based laboratory diagnosis (Figure 2).

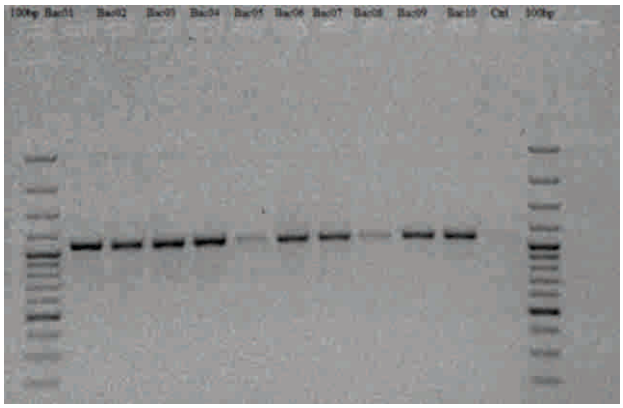

Fig 2: Bacterial Spiked MTM analyzed for 16S Ribosomal DNA Amplification with defined amplified band size 1067bp (Lane 2-Lane 11), Negative Control Lane 12, 100bp ladder Lane 1 and Lane 13

Use of guanidine thiocyante based solution for viral inactivation is well-known, the suitability of guanidine thiocyanate for stool samples is proven where polio virus culture as well as polio infected samples were treated with guanidine thiocyante for inactivation.[33] Guanidine thiocyante base solution was also found suitable to inactivate the virus in brain tissue.[34,35] We may envisaged the use of IMTM for samples other than stool. Furthermore, sample collected in IMTM may be used in sequencing and other nucleic acid based arrays.

Conclusion

Indigenously developed molecular transport medium (IMTM) is found efficient sample transport medium and may be utilized for rotavirus infected stool samples collection, transportation at room temperature. The nucleic acid quantity, purity, and quality remain same even after storage at room temperature for longer duration. Moreover, it is envisaged that IMTM may efficiently be used for detection of other gastrointestinal pathogens in stool specimens. However, further improvement and investigations is needed to make the IMTM as universal transport medium. Efficacy of IMTM need to be established for other type of samples and viruses other than rotavirus.

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