Original Article

Development of Poly (A)-Tailed Universal Reverse Transcription PCR Method for Sequence-Independent Amplification of Rearranged Rotavirus

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Abstract

Background: Group A Rotaviruses (GARV) are the main viral cause of acute gastroenteritis, leading to 870,000 deaths annually in the developing world and representing a major health problem. Therefore, diagnosis and treatment of this disease are crucial. Gene rearrangement within segmented viruses as well as rotavirus is seen throughout chronic rotavirus infection in immunodeficient young children and through serial passage of rotavirus in cell culture at a high multiplicity of infection. Detailed knowledge of rotavirus biology allows design of a vaccine against rotavirus by engineered antigens. The aim of this study was to develop Poly (A) -Tailed universal Reverse Transcription Polymerase Chain Reaction (RT-PCR) method and compare the efficacy of this procedure with specific multiplex PCR protocol for detecting normal and rearranged segments.

Methods: Virus was propagated on confluent monolayer of MA-104 cells and aliquots of each passage were kept frozen for further RNA genomic profiles analysis by polyacrylamide gel electrophoresis. Purified Rota virus RNA was polyadenylated and used for the amplification and detection of normal and rearranged segments of rotavirus using RT-PCR.

Results: The generation of gene rearrangement through multiple serial passages of rotavirus was shown using MOI ≥ 1. The rearranged RNA segments of *NSP1* and *NSP3* genes with different migration pattern in PAGE were detected by poly(A)RT-PCR.

Conclusion: In the current research, a novel Poly(A) -Tailed Universal Reverse Transcription PCR method was introduced for the high throughput amplification and analysis of the informative untranslated regions of the rotavirus genome.

Keywords: Gastroenteritis, gene amplification, gene rearrangement, rotavirus

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Introduction

Group A rotaviruses (GARV), a members of the Reoviridae family, are a major cause of infantile gastroenteritis. Serial passage of rotavirus in cell culture at a high multiplicity of infection (MOI) or chronic infection in immunodeficient individual cause gene rearrangement within segmented viruses and alter the rotavirus evolution, ensuing in costly pediatric hospitalizations and an estimated 18 million severe cases, leading to 870,000 deaths annually in the developing world in human.[1,2] The development of a rotavirus vaccine has been a main public health concern. The methods used for detection and characterization of rearranged rotaviruses evolve and are refined continuously.[3,4] Considerations for successful implementation of rotavirus studies and significant impact of rotavirus vaccine development, data collections and the first-line tests used to detect rotaviruses are necessary for understanding the molecular and antigenic features of the virus. Based on the patterns of both *in vivo* and *in vitro* biological properties, rotavirus comprises seven distinct groups (A to G) and mature particles contains 11 segments of double-stranded RNA (dsRNA) genes which provide a suitable option for recombination such as effective intergroup reassortment or rearrangement of rotaviruses. Analysis of genomic electropherotypes is a relatively easy, rapid and popular technique for direct visualization of rotavirus genome and to monitor virus outbreaks and transmission in molecular epidemiology studies. In most cases, the electrophoretic pattern of group A rotaviruses genome is composed of four high-molecular-weight dsRNA segments (1 to 4), two middle-sized segments (5 and 6), a distinctive set of three segments (7 to 9), and two smaller segments (10 and 11) which are readily observed in polyacrylamide gel electrophoresis (PAGE) analysis on 10% gels to 17.5%. These patterns can change by three different mechanisms: mutation, rearrangement and reassortment, because these reactions can alter segment electrophoretic mobility, depending on their secondary structures.[5] The recognition of loci of genetic divergence between rotaviruses and the identification of altered genes, has been defined by particular feature of biological properties of rotavirus such as rearrangement. Rearrangement is explained by deletions, and often as random duplications within individual genome segments.[1,6] In the current study, we try to develop an efficient protocol for development of a sequence independent amplification strategy on various types of dsRNA viruses, and optimize this method as a general and cost effective technique for obtaining full genome segments of normal/rearranged rotavirus genome. This approach takes into consideration the sequence-independent

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single primer amplification (SISPA) methodology.[7] The majority of sequencing methods depend on reverse transcription polymerase chain reaction (RT-PCR) amplification conditions using anticipated primers from sufficient sequence data. Theoretical backgrounds show that SISPA based methods facilitate amplification of different gene fragments in rotavirus and other segmented viruses, therefore, parallel to multiplex PCR, using one primer set (anchor primer) for all segments in the one reaction. A further significant characteristic of this procedure is that both RNA strands are used as a template for reverse transcription polymerase chain reaction (RT-PCR) reaction and DNA synthesis, similar behavior could be seen as DNA replication process. Thus, in order to increase the coverage of the 3'-5' ends of normal and rearranged rotavirus double strand genomes, heat-treatment is followed by polyadenylation. Poly(A) tail is added to single strand RNA (ssRNA) at the 3'end of the target genome that can provide priming sites for the synthesis of double- strand cDNA , then poly-A tailed molecules are converted into cDNA during reverse transcription reaction using poly(T) anchor primers. Double stranded DNA is amplified by PCR using the anchor primer (Figure 1), and the products are separated by electrophoresis.

Materials and Methods

Viruses and Cell culture

Rotavirus (RF strain) was propagated in confluent monolayer fetal rhesus monkey kidney cells (MA-104) (Pasture, Iran). MA-104 cells were cultured in a-25 cm^2 flask containing DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C in 5% CO_2.[8] In order to optimize and obtain high titers and rearrangment of rotavirus, confluent monolayers of MA104 cells were infected with rotavirus at diffrernt MOI. For virus activation, trypsin (porcine pancreatic type IX: (Sigma Aldrich, Germany) to a final concentration of 10 µg/mL was added and incubated for 1 hour at 37°C.[9] The infected cells were collected after two days and were subjected to freeze-thaw cycles. The cellular debris was removed by centrifugation at 4,000 × g for 5 min, and the virus present in the supernatant fluid was used for RNA extraction.

RNA extraction

Rotavirus RNA was used for the development of this technique and viral RNA was extracted from the infected cell supernatant. To prepare viral RNA, the QIAampViral RNA Mini kit (Qiagen, Germany) was used according to the manufacturer's instructions. The aliquots of purified RNA were eluted with 50 µL of nuclease-free water and stored at -70°C. The RNA integrity and electeropherotyping of viral RNA was confirmed using 10% polyacrylamide gels electrophoresis and silver staining.

Preparation of dsRNA

Purified ligated dsRNA was denatured by heating in a thermal cycler at 95°C for 5 – 7 min. The mixture was immediately quenched in an ice-water bath to prevent the re-annealing of the dsRNA. RNA integrity was confirmed using electrophoresis.

Polyadenylation reaction

Poly(A) -Tailed Universal Reverse Transcription PCR was done using extracted RNA following preparation of dsRNA. In the first step, the poly(A) polymerase (PAP) is used to synthesize poly(A) tails at the 3' termini of ssRNA of purified normal and rearranged rotavirus dsRNA molecules, as the primary annealing site for a first poly(T) oligonucleotide anchor primer. Approximately 23 µL (15ng) of RNA was added as a substrate to a 30 µL reaction mixture containing 3 µL ATP (1 mM), 3 µL poly(A) polymerase buffer (10X), 0.5 µL of poly(A) polymerase (M0276S New England Biolabs (NEB) (5,000 units/mL)). The reaction mixture was incubated at 37°C for 10 min. The RT-PCR amplification of the RNAs with poly (A) tails were performed and then products were analyzed by PAGE. To demonstrate the possibility of this process, Mir48 polyadenylation was done as an internal control. The poly(A)-tagged dsRNAs were used for the amplification and detection of normal and rearranged segments of RF rotavirus using RT-PCR.

RT-PCR with polyadenylated normal and rearranged rotavirus RNAs

The RNA molecules with the poly (A) tails (following polyadenylation) were amplified by RT-PCR. To ensure rotavirus genome amplification and prevent non-specific *in vitro* reactions, one set anchor primers from the zebra fish genome sequence was nominated to replicate in all genome segments of rotavirus for increasing test accuracy. Double stranded cDNA synthesis was done using the Moloney murine leukemia virus (M-Mulv/ Thermo Scientific, Germany) and poly(T)-anchor primers R: 5'CACCTCCTTGTTGTTTTCTTCAGTTTTTTTTTTTTTTTTTTTT*G3'(44nt), F: 5'TCGTAGTAGTCGCTCTGATGGTTTTTTTTTTTTTTTTTT*G 3' (42nt), the *G is nucleotide triphosphate modifications for primer annealing to the C terminus of poly(A) tailed RNA. The presence of G nucleotide is due to the existence of a gtgacc conserved sequence at the end of rotavirus genome. Thus, in order for the oligo dt to connect to the rotavirus gene, guanine nucleotide in the primer was used, therefore, G-oligo-dt-anchor primer attached to the beginning of the gene, and also the presence of one or two nucleotides at the beginning of sequence causes primer specific binding to the end of the poly A sequence.

Amplification of cDNA was performed using an anchor primerR:5'CACCTCCTTGTTGTTCTTCAG3'(23nt), F:5'TCGTAGTAGTCGCTCTGATGG3' (21nt) (Eurofins, UK).The RT mixture (20 µL) contained 7 µL of RNA, 1 µL of M-Mulv, 1 µL of each oligodt-anchor primers (= 10 pmol), 2 µL reaction buffer (10x), 0.5 µL dNTP (10 mM), 7/5 µL DEPC water. And each 25 µL PCR reaction mixture contained 0.3 µL of *Taq* DNA polymerase (Sinaclone, Iran) (5 u/µL), 4µL of cDNA, 1 µL of each anchor primer (= 10 pmol), 2.5 µL reaction buffer (10x), 0.5 µL dNTP (10 mM), 15/7 µL DEPC water. Furthermore, each reaction contained DMSO (5%) or Betaine (1/8 mM) (Sigma, Aldrich, Germany) as PCR additives for increasing specificity and reliability of PCR reactions.

The RT reaction was incubated at 42°C for 45 min in a thermal cycler. The first step during cycling of PCR was 72°C for 10 min to fill incomplete (gap sequence) cDNA ends to produce intact DNA following an initial denaturation for 15 s at 95°C. This DNA was amplified by 35 cycles consisting of 94°C for 30 s, 52°C for 30 s and 72°C for 3 min, and a final extension step at 72°C for 7 min was performed.

RT-PCR with unmodified RNAs

According to the pattern of rearrangements of the genes for sensitivity comparison, in a similar situation, RT-PCR reactions were performed by purified normal rotaviral RNAs with specific primers (Eurofins, UK) for nonstructural gene segments (Table 1). Each 20 µL RT reaction mixture contained 1 µL of avian

Table 1. Specific primers for RT- PCR reaction of nonstructural gene segments of normal and rearranged rotavirus

Gene segment	Size (bp)	Forward primer	Reverse primer
NSP1	1611	5'GCTTTTTTTATGAAAAGTCTTGTGG3'	5'GTTCACATTTTAT GCTGCCTAG-3'
NSP2	1059	5'CGTCTCAGTCGCCGTTCG3'	5'GGTCACATAA GCGCTTTCAATT3'
NSP3	1104	5'5'ATTTAATGCTTTTCAGTGGTTGATGC3'	5'GGTCACATAACG CCCCTATAGCC3'

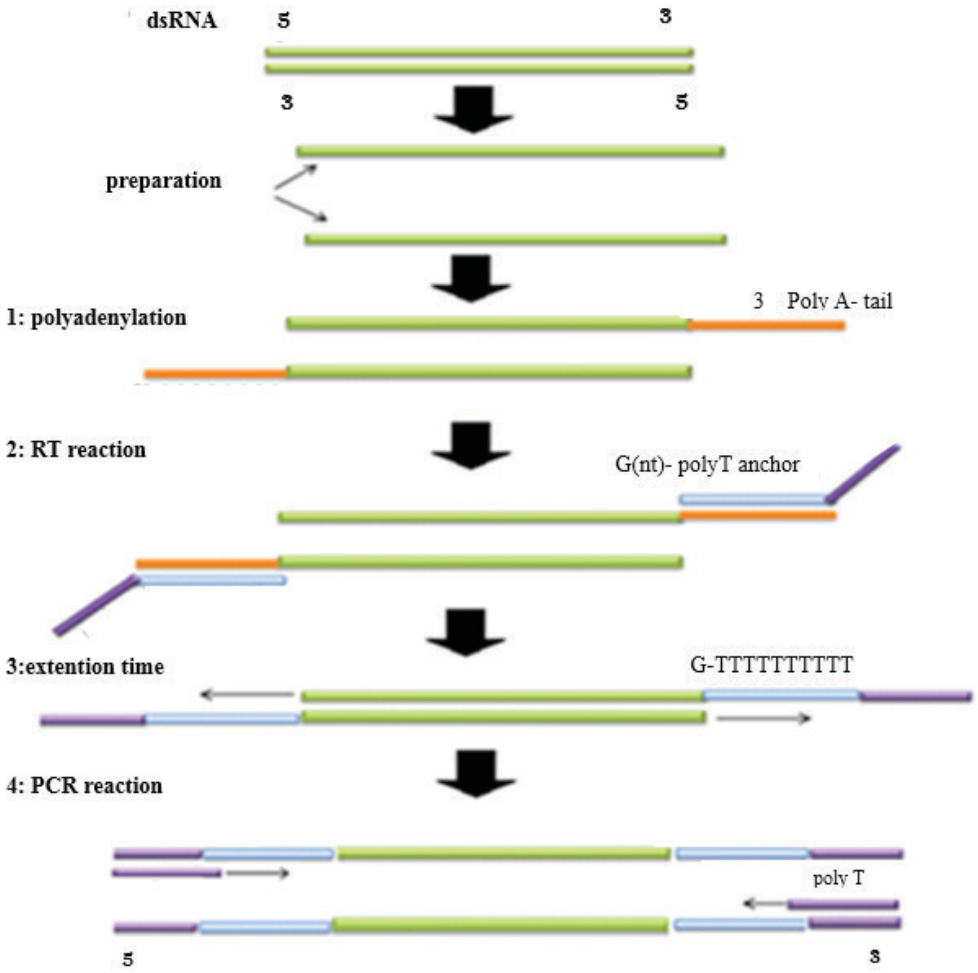

Figure 1. Schematic picture showing the four main steps of Poly (A) -Tailed Universal Reverse Transcription PCR method.

myeloblastosis virus (M-MuLV) RT enzyme (Thermo Scientific, Germany), 7 µL of total RNA, 1 µL of each specific primers, 2 µL reaction buffer, 0.5 µL dNTP and 7/5 µL DEPC water. Each 25 µL PCR reaction mixture contained 0.3 µL of *Taq* DNA polymerase (Sinaclone, Iran), 4 µL of cDNA, 1 µL of each specific primer, 2.5 µL reaction buffer, 0.5 µL dNTP, 15/7 µL DEPC water. The RT reaction was incubated at 42°C for 45 min in a thermal cycler. PCR amplification was performed by an initial denaturation step for 5 min at 95°C followed by 35 cycles consisting of 94°C for 30 s, 65°C for 30 s and 72°C for 2/5 min, and a final extension step at 72°C for 7 min. It is necessary to note that in the two mentioned methods, the absence of DNA contamination was verified by performing the same reactions without M-MuLV RT enzyme (negative control). All RT-PCRs of this study were performed with a PCR thermal cycler Applied Biosystems (Veriti® 96-Well-ABI-USA). The amplified product was confirmed using polyacrylamide gel electrophoresis (10%) and silver staining.

Results

Cell culture

The CPE of rotavirus in the MA-104 cells consisted of cytoplasmic degeneration and cell detachment from the monolayer was markedly produced. There were no significantly different signs of cell damage in both normal and rearranged viruses.

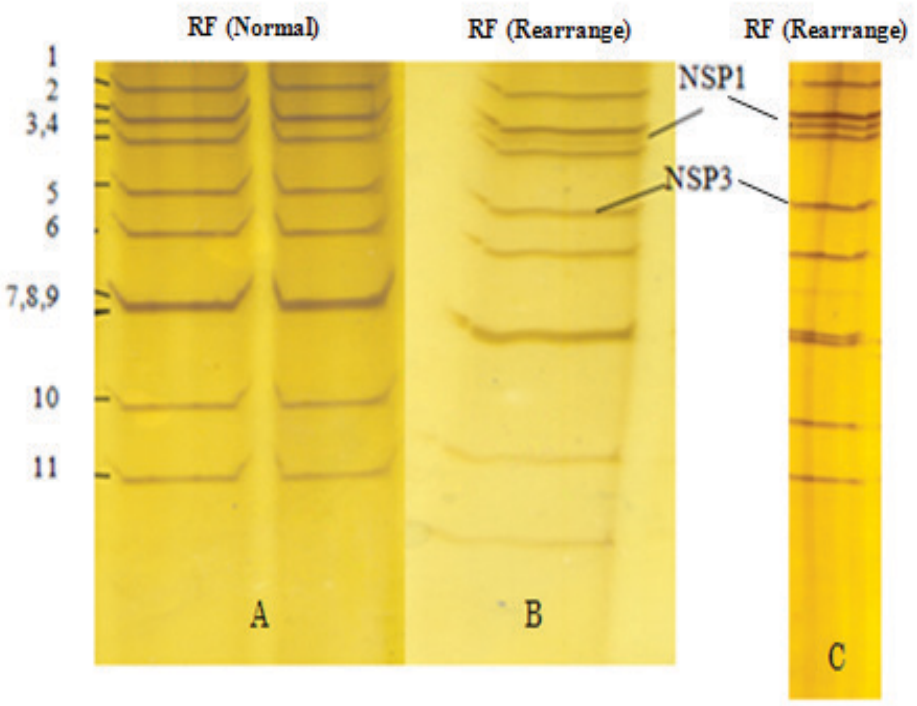

Figure 2. Normal **(A)** and rearranged **(B, C)** electropherotype patterns of rotavirus dsRNA in 10% polyacrylamide gels and silver staining. Rearranged RF rotavirus *NSP1* gene is observed between segment 3 and 4 and *NSP3* is placed on location of segment 5 .

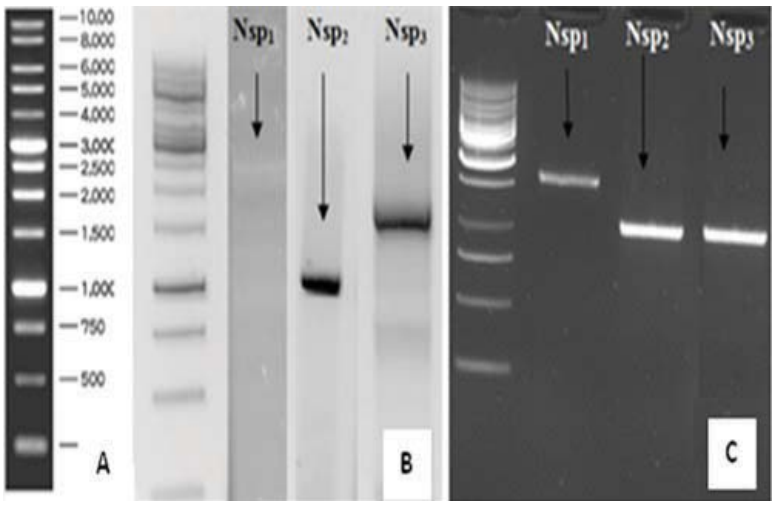

Figure 3. RT-PCR amplification of rotavirus RF strain in agarose gels (1%); **A)** 1 kb molecular weight marker. **B)** rearranged viral product. **C)** Normal viral product.

Genome analysis

The results showed the generation of gene rearrangement through 8 serial passages of normal rotavirus RF strain using MOI ≥ 1. The rearranged segments of *NSP1* and *NSP3* genes with different migration pattern in PAGE were observed in the background of normal RF counterpart. In the period of viral replication rearranged forms of rotavirus RF strain were generated in cell culture. As seen in Figure 2, rearranged RF rotavirus *NSP1* gene is observed between segment 3 and 4 and *NSP3* is placed on location of segment 5 (Normal (A) and rearranged (B, C).[10]

Poly (A) -RT-PCR

After confirming the electrophoretic pattern of genomic rearrangements in non-structural parts, amplification of these components by specific RT-PCR and Poly (A) RT-PCR using nonstructural gene primers was shown. Amplification of normal and rearranged genome reaction was performed by RT-PCR and the normal genome was amplified readily (Figure 3, C), whereas weak amplification of the rearranged genes was observed (Figure 3, B). The full length products of the genes, encoding *NSP1*, 2,3 of normal and rearranged rotavirus, were amplified by Poly (A) RT-PCR and DNA fragments were sequenced. Rearranged segments were evaluated by Poly (A) RT-PCR method and silver staining (Figures 4 and 5). The column A in Figure 4 indicate whole genome amplification of rotavirus and column B belong to the specific amplification of gel purified normal *NSP4* gene segment as a control.

As shown in Figure 5, lane4 mir48 were checked for careful

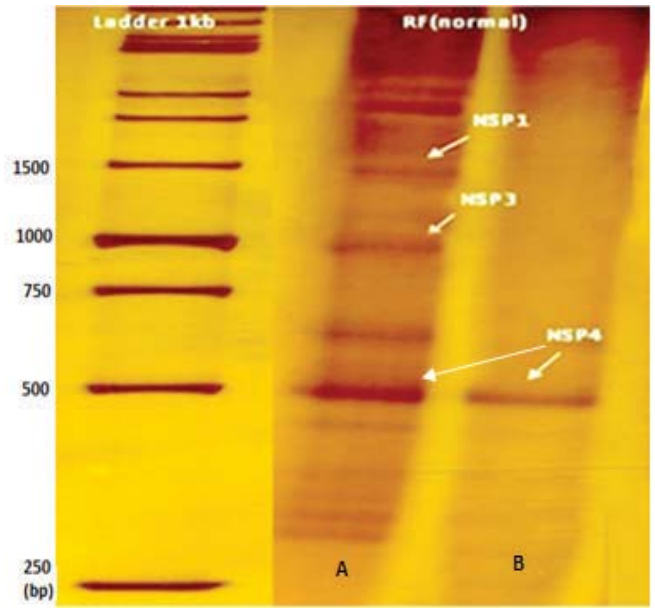

Figure 4. Demonstration of Poly (A) RT-PCR reaction. Normal rotavirus profile in 10% polyacrylamide gels and silver staining.

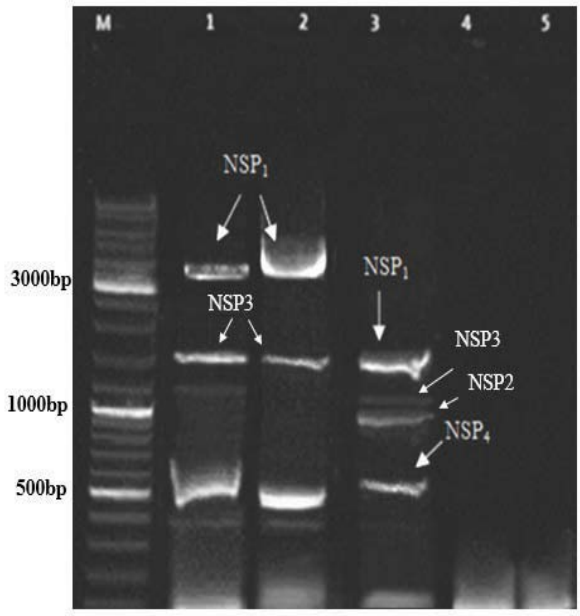

Figure 5. Demonstration of Poly (A) RT-PCR amplification of normal and rearranged rotavirus in 1% agarose gel. **Lane 1 & 2)** amplification of rearranged rotavirus; **Lane 3)** normal rotavirus; **Lane 4)** mir48 as positive control. **Lane 5)** negative control. M: Mix molecular weight marker.

experimental design and optimization. Actually, to demonstrate the possibility of this approach, additional RT-PCRs were performed with specific primers on unmodified RNA. Surprisingly, although the result demonstrated the presence of rotaviral RNAs as expected, a weak amplification of the rearranged segments over their normal counterparts were observed. This result confirmed that RT-PCR favors the amplification of the shortest template rather than rearranged ones and cannot be used to efficiently detect rearranged sequences in a background of homologous normal sequences. Perhaps novel applicable and more sensitive tests are needed for detection of abnormal sequence through immunodeficiency (e.g: SCID child) instead of RT-PCR. Based on the electrophoretic pattern of the rearranged segments, a large addition of ~1500 and 400 bases in the fragments 5 and 7 was generated that encoded the *NSP1* and *NSP3* proteins, respectively. They show nucleotides increment through the PAGE analysis (Table 2).

Discussion

Rotavirus production through cultivation of virus by high MOI progressively generates rearranged forms of segmented RNA viruses. Several studies have shown the importance of genetic variation, and offer precious information about amplification, diversity, biology and evolution of segmented RNA viruses. Although PCR is popular and performed routinely for amplification of genes, it has some limitations, such as primer designing for unknown sequences. Such studies may provide the basis for RNA viruses evolution in rearrangement. Occasionally in these cases, the PCR primers (universal or specific) fail to detect and amplify target genes. This study is the first report of the sequence-independent amplification method illustrating the occurrence of rearrangement at a specific site. This novel protocol composed of two established techniques (RT-PCR and Polyadenylation) and is applicable for production of full-length cDNA product from non-polyadenylated RNAs of rotavirus normal and rearranged genome. This way, the probability of direct access to RNA in biological samples and in other words RNA-based studies can be provided that is particularly important and constructive. There are many advantages to this method, for example, Poly A tailing step as modification step of RNA molecules with sequences are known/unknown oligonucleotides that enable cDNA amplification without either random or specific primers. In this procedure, the DNA was produced without use of known primers that target nucleic acid-specific RNA or DNA (cDNA), and the procedure did not involve random priming or target-specific PCR primer. The primers were designed for the outer regions of templates.[11–13] Purified rotavirus RNA was first modified by the addition of a poly (A) tail, and oligonucleotides of adenosine are synthesized on each end of purified rotaviral dsRNA molecules which become priming sites for RT- PCR step.

Since the sequence information of different members of rotavirus

Table 2. Length of nonstructural gene segments after amplification

Gene segment	Normal (bp)	Rearranged (bp)
NSP1	1611	~ 3100
NSP2	1059	not detected
NSP3	1104	~ 1500
NSP4	486	not detected

group A is needed for phylogenetic classification, the Poly (A) -Tailed Universal Reverse Transcription PCR is a specific and applicable method, which can be used as a biological tool in phylogenetic studies. In the present study, a home-brewed assay based on novel sequence-independent amplification protocol (Poly(A) -Tailed Universal Reverse Transcription PCR) has been developed. Also Potgieter, et al. indicated that oligo-ligation with T4 RNA ligase could be improved by inclusion of PEG and hexamine cobalt chloride (HCC) in reactions.[7] Both of these reports are significantly sensitive and specific for comprehensive investigations of the genetic diversity in dsRNA virus populations. In near future, these novel improved methods are applied and optimized for other candidate viruses (various types of dsRNA viruses) as well as other groups of rotaviruses.

Conflict of interest

The authors of this article declare that they have no conflict of interest related to the material in the manuscript.

Acknowledgments

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