Journal of Virological Methods 266 (2019) 65–71

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Evaluation of different genomic regions of Rotavirus A for development of real time PCR

Madhuri S Joshi*, Shital G. Deore, Atul M. Walimbe, Sujata S. Ranshing, Shobha D. Chitambar*

Enteric Viruses Group, ICMR-National Institute of Virology, Pune, India

ARTICLE INFO

Keywords:
Acute gastroenteritis
Rotavirus A
Real time polymerase chain reaction
VP6 gene
Sensitivity

ABSTRACT

The nucleotide alignment of all 11 genes of human Rotavirus A (RVA) strains revealed suitability of NSP2, NSP3 and VP6 genes for the development of real time PCR (qRT-PCR). Evaluation of qRT-PCR assays using known rotavirus ELISA positive and negative fecal specimens showed non-overlapping ranges of Mean ± 3SD cycle threshold (Ct) values for NSP3 and VP6 based assays. Using serial dilutions of purified RVA, high sensitivity of VP6 qRT-PCR assay (1.95×10^{-5} pg/μL of RNA) was recorded as compared to NSP2 and NSP3 qRT-PCR assays (1.95×10^{-4} pg/μL of RNA). Further, evaluation of the VP6 qRT-PCR assay involving 266 fecal specimens and frequency polygon analysis of the data indicated cut-off value of 35 for Ct with high sensitivity (126/131, 96%) and specificity (12/12, 100%). This VP6 qRT-PCR assay will be a useful diagnostic tool to evaluate clinical presentations in rotaviral gastroenteritis under different conditions such as breast feeding and administration of rotavirus vaccines.

1. Introduction

Acute gastroenteritis (AGE) is the major cause of 1.5–2.5 million deaths and 3–5 billion morbidity cases yearly amongst children < 5 years of age, globally. The majority of the episodes of viral gastroenteritis are due to rotavirus (RV), norovirus (NoV), astrovirus (AstV) and adenovirus (AdV) infections. Among them, rotavirus infections are predominant.

Rotavirus, a member of the family *Reoviridae*, is classified into nine serogroups, A—I, based on the antigenicity of the inner capsid protein VP6. Globally, infections with serogroups A (RVA), B (RVB), C (RVC) and H (RVH) have been detected in humans with predominance of RVA in the pediatric population (Alam et al., 2007). Thirty seven percent of the estimated deaths attributable to diarrhea in children < 5 years of age are caused by rotavirus (Tate et al., 2016).

The Rotavirus Classification Working Group (RCWG) proposed the classification system based on the 11 RNA genomic segments. To date 36 G genotypes and 51 P genotypes have been identified in VP7 and VP4 genes respectively (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg). Amongst these, G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] genotype combinations are frequently detected globally in RVA strains which cause infections in humans. The occurrence of unusual rotavirus G-genotypes (G5-G6, G8, G10, G11 and G20) and P-genotypes (P[1]-P[3], P[5], P[7], P[9]-P[11], P[14], P[19] and P[25]) is believed to be introduced into the human population through interspecies transmission and/or reassortment events (Cook et al., 2004).

A variety of assays that have been developed and used to detect RVA in fecal specimens include electron microscopy (EM), isolation of virus in cell culture, enzyme immunoassays (ELISAs), reverse-transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (qRT-PCR) (Kapikian et al., 1974; Wyatt et al., 1983; Knisley et al., 1986; Wilde et al., 1992; Iturriza-Gómara et al., 2004; Mijatovic-Rustempasic et al., 2016).

Being speedy, sensitive and specific, molecular assays extend several advantages over other conventional methods. Among these, several qRT-PCR assays have been developed focusing VP2, VP4, VP6, VP7, NSP3, and NSP4 genes for detection of RVA specific RNA (Kang et al., 2004; Pang et al., 2004; Logan et al., 2006; Min et al., 2006; Freeman et al., 2008; Gutiérrez-Aguirre et al., 2008; Jothikumar et al., 2009; Adlhoch et al., 2011; Pang et al., 2011; Kottaridi et al., 2012). However, due to the continuous accumulation of point mutations in rotavirus genome, reassortment of related genomic segments in rotavirus strains and zoonotic transmission of animal rotavirus strains that may introduce novel genotypes and/or lineages in humans (Matthijnssens et al., 2009; Martella et al., 2010), it is important to explore different genes of RVAs for their comparative utility in qRT-PCR assays from time to time. The present study was conducted to evaluate all of the 11

---

* Corresponding authors at: Enteric Viruses Group, ICMR- National Institute of Virology, 20-A, Dr. Ambedkar Road, P. O. Box No. 11, Pune 411 001,India.
E-mail addresses: jmadhuri10121968@gmail.com, joshi.ms@niv.co.in (M.S. Joshi), drshobha.niv@gmail.com (S.D. Chitambar).

https://doi.org/10.1016/j.jviromet.2019.01.017
Received 30 November 2018; Received in revised form 28 January 2019; Accepted 28 January 2019

genes of RVAs for optimal design of primers and probes for qRT-PCR assays. The data obtained was utilized to select suitable genes and their sequences for the development of sensitive and specific qRT-PCR assay to detect RVA RNA in fecal specimens from children who were hospitalized for acute gastroenteritis.

2. Materials and methods

2.1. Designing primers and probes for qRT-PCR

The partial or complete nucleotide sequences of RVA structural and non-structural genes representing all of the genotypes detected in human infections (http://rega.kuleuven.be/cev/viralmetagenomics/viralclassification/rcwg) were retrieved from GenBank data base. Multiple nucleotide sequences were aligned with Clustal W program (Tamura et al., 2013) utilizing 57, 68, 77, 125, 178, 213, 59, 355, 26 and 37 sequences respectively of the VP1, VP2, VP3, VP4, VP6, VP7, NSP1, NSP2, NSP3, NSP4 and NSP5 genes. The sequence alignments of all genes were examined for conserved stretches and multiple consensus sets of primers and probes were designed manually. The specificity of the stretches of respective genes selected finally was further checked using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sets of primers and probes were examined for self-annealing sites, hairpin loop formation, 3′ complementarities and melting temperatures (Tm) using IDT oligonucleotiodes calculator (http;//www.idtdna.com/analyzer/Applications/OligoAnalyzer). Nucleotide variations in the selected sequence stretches of different genes were curated by introducing degenerate bases. All primers and probes that were selected were synthesized by Applied Biosystems, USA. The GenBank accession numbers of the strains referred to indicate the locations of the primers and probes and size of PCR products are described in Table 1. The TaqMan MGB probes for the NSP2, NSP3 and VP6 genes were labeled with NED, VIC and 6-FAM reporter dyes at the 5′ end respectively.

2.2. RNA extraction and qRT-PCR assay

The specificity and limit of detection (LOD) in qRT-PCR assays utilizing different primers and probes of NSP2, NSP3 and VP6 genes were determined using MA-104 cell line adapted and sucrose gradient purified human rotavirus 3 (HRV-3) YO strain at the RNA concentration of 19.5 ng/μL (Nano Photometer,Implen, Germany). Serial ten-fold dilutions of purified HRV-3 were prepared in a supernatant of RVA ELISA (GA Generic assays GmbH, Dahlewitz, Germany) and RT-PCR negative (30%) fecal specimen. RNA was extracted from fecal supernatants spiked with HRV-3 using automated MagNA™ 96 viral RNA isolation kit (USA) according to the manufacturer's instructions and tested by the NSP2, NSP3 and VP6 gene based qRT-PCR assays. Briefly, RNA was subjected to denaturation at 95 °C for 5 min followed by flash chilling in ice for 2 min. The qRT-PCR was carried out using a TaqMan One step RT-PCR master mix (20 μL) (Applied Biosystems, Foster City, CA, USA) containing 0.33 μL of each of the forward and reverse primers as well as TaqMan probe (10 μM concentration each) and 5 μL of denatured RNA under the following conditions: 48 °C for 30 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 55 °C for 1 min using a ABI 7500 Real time PCR System (Fast Dx, Applied Biosystems, Foster City, CA, USA).

To compare LOD obtained by the use of VP6 gene sequence derived primers of the present study with that of the conventional VP6 gene primers published earlier (Iturriza Gómara et al., 2002), conventional RT-PCR assays were performed utilizing One Step RT-PCR kit (QIAGEN, Germany). PCR products were analyzed by agarose gel-electrophoresis and visualized under UV transilluminator.

2.3. Preparation of reference RNA standard from RVA VP6 gene

Complete VP6 gene (1.3 kb) of HRV-3 strain, was amplified by conventional RT-PCR using the primers NIVF1-VP6 [GGATCC-5′GGCTTTTAAACGAAGTCTT (1–19)] and NIVR1-VP6 [GCGGCCGC-5′GGTCACATCCTCTCACT (1338–1356)] with T7 promoter sequences at 5′ end of both primers and cloned into pGEM-T easy vector with BamH1 and Not1 restriction enzyme sites (Promega, USA). The presence of insert in the plasmid was confirmed by restriction digestion and PCR. Transcription was carried out by RiboMax™ Large scale RNA production system with T7 polymerase (Promega, Madison, USA). RNA was purified using TRIzol LS reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was estimated by Nano Photometer (Implen, Germany) and serial 10-fold dilutions of quantified RNA (1.2×10^{12} molecules/ μL) were used as reference standard RNA. A standard curve was generated by plotting the log dilution of HRV3 RNA transcript against Ct value to determine the sensitivity of the assays. The copy number (RNA concentration) was calculated by using the equation, Copy number (molecules/μL) = [concentration (ng/μL) X 6.022×10^{23} (molecules/mol)]/ [length of amplicon X 340 (g/mol) X10^9 (ng/g)] as described earlier (Mijatovic-Rustempasic et al., 2013).

2.4. Study specimens

During surveillance studies conducted between 2009 and 2012 in Western India for detection of rotavirus strains and genotypes, fecal specimens were collected from children < 5 years, hospitalized for acute gastroenteritis. Rotavirus positivity was detected in 35–40% of the specimens by Rotavirus antigen detection ELISA (GA Generic assays GmbH, Dahlewitz, Germany) (Chitambar et al., 2014). Two hundred and sixty-six specimens comprising known rotavirus negative (n = 132)

Table 1
Primers and probes used for amplification and detection of rotavirus A.

Name	Nucleotide sequence (5'-3')	Accession number of the referred strain and location of the primer/probe	Amplicon size
NSP2 gene			
SM 10 (F)	GGCTTTTAAAGCGTCTCAGT (Tm 55)	AB022770 (1-20)	101bp
SM 11 (R)	AATYTATAGCTATCRTTCTCYARATG (Tm 51)	AB022770 (102-77)	
SM 12 (P)	CCATGGCTGAGCTAGCTTGCTT (Tm 64)	AB022770 (45-67)	
NSP3 gene			
SM 7 (F)	ACCCTCTATGAGCACAATAGTTA (Tm 55)	AB22771 (1002-1025)	70bp
SM 8 (R)	TCACATAACGCCCCTATAGCC (Tm 59)	AB22771 (1072-1052)	
SM 9 (P)	AGCTAACACTGTCAAAAACCTAA (Tm 54)	AB22771 (1027-1049)	
VP6 gene			
SM4 (F)	GTNTTTCCACCAGGYATGA (Tm 57)	AB022768 (1101-1119)	237bp
SM5 (R)	GGTCACATCCTCTCACTA (Tm 46.35)	AB022768 (1356-1339)	
SM6 (P)	CACCATCHAGRGARGATAA (Tm 50)	AB022768 (1147-1165)	

F: Forward primer; R: Reverse primer; P: Probe; N: A/G/C/T; Y:C/T; H: A/C/T; R: A/G.

Table 2
: Statistical analysis of Ct values of known RVA ELISA positive and negative specimens using NSP2, NSP3 and VP6 qRT-PCR assays.

qRT-PCR assay	Mean Ct ± SD and 99% range (mean Ct -3SD, mean Ct + 3SD)	
	qRT PCR reactive/ Known ELISA positive (n = 30/30)	qRT PCR reactive/ Known ELISA negative (n = 5/24)
NSP2	21.5 ± 5.1 (6.3, 36.8)	37.2 ± 1.5 (32.7, 41.8)
NSP3	21.4 ± 2.9 (12.6, 30.1)	40.2 ± 1.3 (30.3, 44.1)
VP6	18.04 ± 3.75 (6.8, 29.3)	38.2 ± 1.4 (34.04, 42.4)

and positive (n = 134) were included in the present study. Rotavirus strains detected in ELISA positive fecal specimens were found to contain common (G1P[8], G2P[4], G9P[8], G12P[6]), unusual (G9P[4], G2P[6], G12P[8], G19P[8]P[4] and non-typeable (G1P[NT], G2P[NT], GNTP[8], G9P[NT], G12P[NT]), GNTP[6]), GNTP[NT], genotype combinations by multiplex PCR (Iturriza-Gómara et al., 2004). Fecal specimens from 49 neonates suffered due to gastroenteritis outbreak and 26 fecal specimens obtained from a group of asymptomatic children, < 5 years, hospitalized for diseases other than diarrhea were also tested. These specimens were tested negative for NoV, enteric AdV, human AstV, Aichivirus (AiV), Enterovirus (EV) and human Bocavirus (BoV) by the methods as described earlier (Chitambar et al., 2012; Lasure and Gopalkrishna, 2017).

2.5. qRT-PCR, quality control and estimation of viral load in fecal specimens

RNA reference standard of VP6 gene of HRV-3 in serial ten-fold dilutions and RNA of each of the 30% suspensions of fecal specimens extracted using an automated MagNA™ 96 viral RNA isolation kit, were subjected to qRT- PCR assay. Negative control (autoclaved distilled water) was incorporated in each of the assays. To avoid cross contamination of RNA, extraction of RNA from standard preparation and fecal specimens and qRT-PCR amplifications were performed in separate laboratory rooms. Specificity of the designed primers and probes for RVA was checked in the qRT-PCR assay by testing fecal specimens detected positive for other enteric viruses [NoV (n = 11), Human AdV (n = 10), Human AstV (n = 8), AiV (n = 3), EV (n = 10), human BoV (n = 5)] in previous studies conducted in the laboratory (Chitambar et al., 2012; Lasure and Gopalkrishna, 2017). Using SDS software (Applied Biosystems), the runs were analyzed to obtain quantitative data for estimation of the target gene concentration.

2.6. Statistical analysis

All statistical analysis was performed in PASW Statistics 18. Using ELISA as the gold standard, the Receiver Operating Characteristic (ROC) analysis was performed for determination of optimal cut-off for Cycle threshold (Ct) value to classify specimen as RVA positive or negative. The ROC was produced by plotting Sensitivity against (1-Specificity) for different candidate values of Ct which were obtained by varying Ct value over its possible range according to pre-defined rule as implemented in PASW.

3. Results

3.1. qRT-PCR primers and probes design

According to the criteria described for designing of primers and probes (http://www.idtdna.com/analyzer/Applications/Oligoanalyzer/), suitable consensus nucleotide stretches were identified in three (NSP2, NSP3 and VP6) of the eleven genes of RVA. The remaining 8 genes showed lack of conserved stretches or their suitability for primer designing in multiple alignments obtained by retrieving respective GenBank sequences. The suitability of the stretches of selected genes was further confirmed in multiple alignments obtained by retrieving the sequences of NSP2 (n = 1874), NSP3 (n = 683) and VP6 (n = 1218) genes from human RVA strains available in GenBank using the key words “Group A rotavirus” [organism] AND “VP6/NSP3/NSP2” [gene name]. The site was considered as degenerate when consensus was lower than 95%.

3.2. NSP2, NSP3 and VP6 qRT-PCR assays

The primers and probes based on NSP2, NSP3 and VP6 genes were employed in qRT-PCR assays involving known ELISA positive (n = 30) and negative (n = 24) specimens. Known ELISA positive specimens containing common (G1P[8], G2P[4], G9[8], G12P[6]), unusual (G9P[4],G1P[4]) and non-typeable (GNTP[6], GNTP[NT]) rotavirus strains showed a range of Ct values between 14.5–33.8, 16.7–28.2 and 12.6–26 in NSP2, NSP3 and VP6 qRT-PCR assays, respectively. Of the 24 ELISA negative specimens, 19 were nonreactive and remaining 5 showed Ct values > 35 in all of the qRT-PCR assays. Using the theory of Gaussian distribution, the range of central 99% observations was estimated by Mean-3*SD, Mean + 3*SD formula (Table 2). The Mean ± 3SD values in the NSP3 and VP6 qRT-PCR assays showed non-overlapping ranges of Ct values for the ELISA positive and negative specimens, however similar observation was not recorded for the NSP2 qRT-PCR assay.

The serial ten-fold dilutions (neat to 10^{-10}) of HRV3 spiked in rotavirus ELISA negative fecal specimen showed reactivity in ELISA up to the dilution of 10^{-2}. The viral RNA extracted from these serial dilutions was used for the detection of LOD of the newly developed NSP2, NSP3 and VP6 based qRT-PCR assays. The 10^{-8} dilution containing 1.95×10^{-4} pg/μL of RNA was the highest dilution that showed presence of HRV3 RNA by qRT-PCR targeting NSP2 and NSP3 gene while the VP6 gene based qRT-PCR detected spiked HRV3 up to the dilution of 10^{-9} and estimated to contain 1.95×10^{-5} pg/μL of RNA.

3.3. RT-PCR

The performance of conventional diagnostic VP6 gene based RT-PCR assay described earlier (Iturriza Gómara et al., 2002) was compared with that of the RT-PCR carried out with the primers (SM4 and SM5) of the VP6 qRT-PCR of the present study. In the conventional RT-PCR, amplicons of 379 bp size were detected up to the dilution of 10^{-7} of HRV3 (Fig. 1a) while RT-PCR of the present study showed PCR products of 255 bp size up to the dilution of 10^{-9} (Fig. 1b).

3.4. VP6 qRT-PCR assay for fecal specimens

Among rotavirus ELISA positive fecal specimens (n = 134), 131 were reactive in VP6 qRT-PCR assay with Ct values ranging from 6.75 to 41.58 (median Ct value: 19). The remaining three specimens were non-reactive. Nearly 95% (126 /131) of the reactive specimens showed Ct values < 35. Of the 132 rotavirus ELISA negative specimens, 120 were non-reactive and 12 showed reactivity with Ct values > 35 (Table 3). Of the 49 fecal specimens collected from neonates, 7 specimens positive in rotavirus ELISA were also reactive in qRT-PCR assay with Ct values detectable between 15.35 and 27.4 (median Ct value 23.01). Ten of the 42 ELISA negative neonatal specimens, showed Ct values in the range of 21.56–38.84 with median Ct value 33.14. Among non-diarrheal fecal specimens negative in rotavirus ELISA (n = 26), 5 showed Ct values > 35. NoV, Human AdV, Human AstV, AiV, EV, human BoV RT-PCR positive fecal specimens (n = 47) were non-reactive in VP6 qRT-PCR.

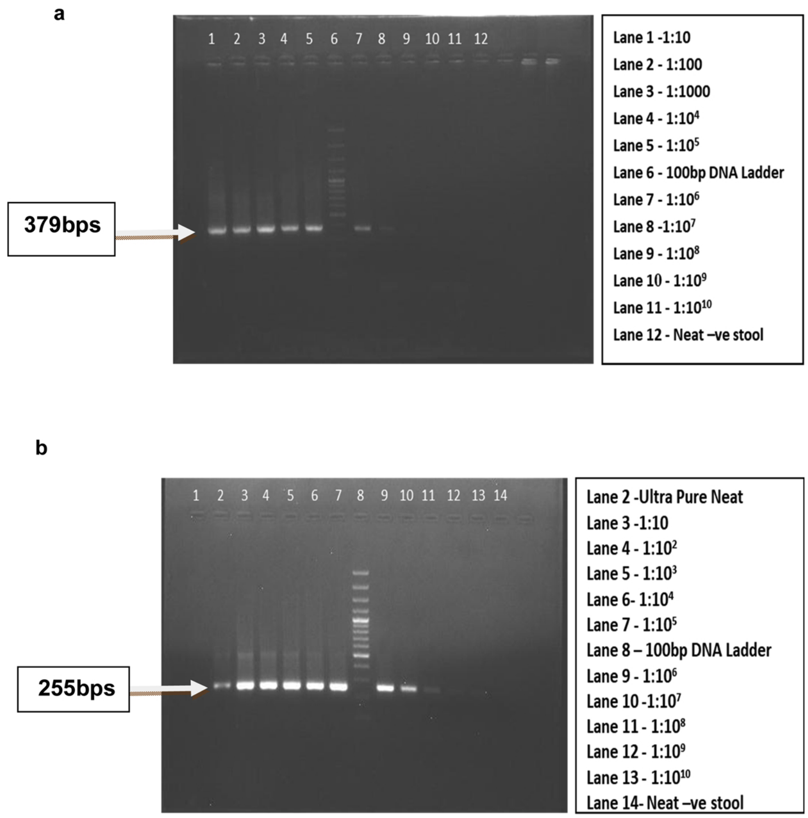

Fig. 1. Electrophoretic migration patterns of ethidium bromide stained PCR products obtained using primers of (a) conventional RT-PCR (product size: 379bps) and (b) VP6 qRT-PCR of the present study (product size: 255bps).

Table 3
Analysis of the Ct values of the known ELISA positive and negative specimens using VP6 qRT-PCR assay.

		VP6 qRT PCR assay		Total
		Ct Value < 35	Ct Value > 35	
ELISA	Positive	126	5	131
	Negative	0	12	12
Total		126	17	143

3.5. Determination of Cut-off for Ct value of VP6 qRT-PCR assay

In order to determine the cut off for Ct value, the data obtained from 134 ELISA positive and 132 ELISA negative specimens tested by VP6 qRT-PCR assay was used. Ct values of 143 VP6 qRT-PCR reactive specimens (131 ELISA positive and 12 ELISA negative) ranged from 6.75 to 44.7 while Ct value remained undetected for three ELISA positive and 120 ELISA negative specimens. A frequency polygon analysis of detectable Ct values showed a bimodal distribution with a clear distinction at Ct value of 33 (Fig. 2). The Ct values of 12 ELISA negative but qRT-PCR reactive specimens ranged from 35.2 to 44.7 with 38.2 ± 2.5 as Mean ± SD and 37.7 as median value. Among 131 ELISA positive specimens, Ct values ranged from 6.75 to 41.6 and showed 20 ± 7.05 as Mean ± SD while 19.2 as median value. The Mean Ct value +2 SD for ELISA positive specimens was found to be 34.1. Based on these observations, the Ct value of 35 was considered as a cut-off value to differentiate RVA positive from negative specimens. Using ELISA results as a standard, the ROC curve also supported the cut-off of 35 with 96.2% (126/131) sensitivity and 100% (12/12) specificity.

3.6. Efficiency of VP6q RT-PCR assay and estimation of viral load

To determine the efficiency of the VP6 qRT-PCR assay, serial 10 fold dilutions of VP6 RNA transcript were prepared and used as a standard in assay runs. Each template and non-template control was tested in duplicate. For accurate and precise measurements, a broad range of standard dilutions (10^{-4} to 10^{-11}) was used to construct the standard curves for RVA ($Y = -3.928x + 39.445$; R2 = 0.988; Efficiency – 79.7%) (Fig. 3 a and b). Analysis of the calibration data indicated that the range of the quantitative analysis was from 4.748×10^{7} to 4.7 copies per reaction, corresponding to Ct values of 8.46 to 36.97 respectively The plot of Ct values versus log transcript copy numbers indicated a linear correlation with a R^2 value of 0.988 and efficiency of the assay was calculated to be 79.7% (Fig. 3a and b).

Rotaviral load was estimated in the 65/131 ELISA positive and qRT-PCR reactive specimens with Ct values ranging from 11.77 to 41.0. Sixty two specimens with Ct values < 35 were found to contain RNA copies between 1.9×10^{4} and $4.1 \times 10^{7\cdot}$ Remaining 3 specimens with Ct values > 35 showed RNA copies between 1.1×10^{3} and 8.8×10^{3} per reaction. Among ELISA negative diarrheal (n = 5) and non-diarrheal (n = 5) specimens with Ct values > 35, the viral load was found to be below 185 RNA copies per reaction.

Seven ELISA positive (with Ct values 15.3–27.4, RNA copies 1.82×10^{3} - 1.73×10^{8} per reaction) and ten ELISA negative (with Ct values 21.5–38.8, RNA copies 5.0×10^{6} - 270.0 per reaction) neonatal specimens were reactive in VP6qRT-PCR assay. The Mean Ct value (27.4± 7.3) of these 17 neonatal specimens was significantly higher than that of the 143 specimens from older children (21.1± 8.4) (t test, p = 0.0066). All 17 specimens were positive for VP6, NSP2 and VP7 RT-PCR and 14 of them were also positive for VP4 multiplex PCR (data not shown).

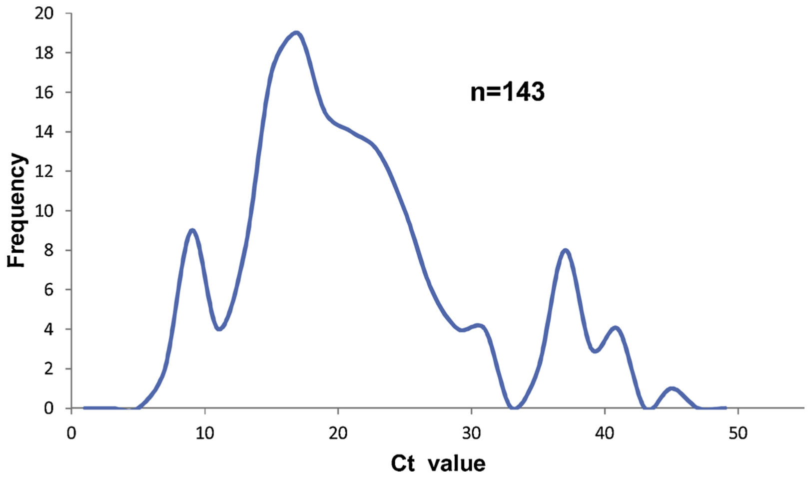

Fig. 2. Frequency polygon analysis of Ct values.

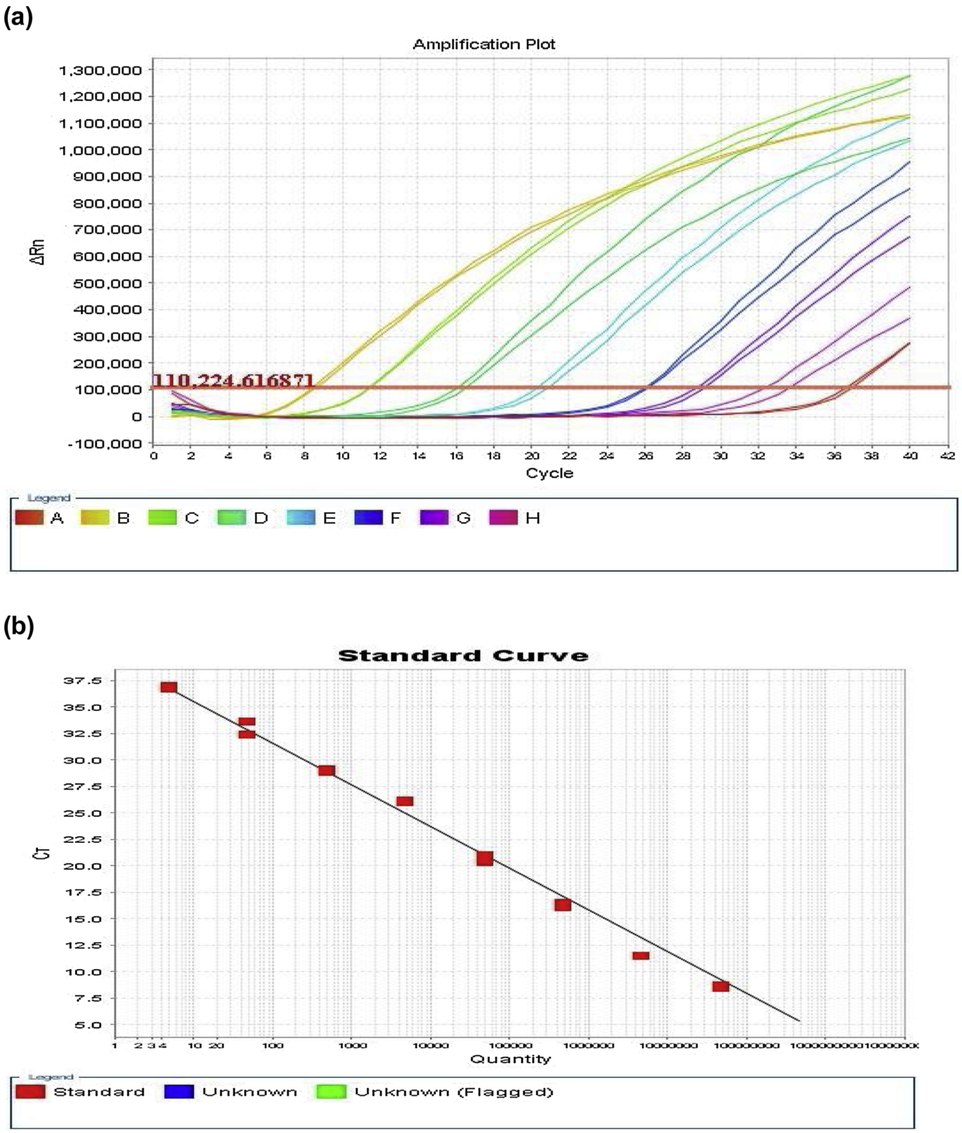

Fig. 3. (a) Amplification curve of HRV-3 YO strain in qRT-PCR using its respective 10 fold serial dilutions of RNA transcripts and (b) the linear relationship between log transcript copy number and quantitation cycle. Delta run (ΔRn) on Y axis refers to the fluorescence emission of a reaction minus the background fluorescence measured from early cycles of the real time PCR run before a logarithmic increase in fluorescence.

4. Discussion

The present study reports evaluation of multiple alignments of complete or partial nucleotide sequences of all eleven genomic RNA sequences of human RVA strains and eligibility of the NSP2, NSP3 and VP6 genes for designing primers and probes in the qRT-PCR assays. The nucleotide alignments of respective genes obtained in the study were also utilized to examine the primer and probe sequences of corresponding genes published earlier (Iturriza Gómara et al., 2002; Kang et al., 2004; Pang et al., 2004; Logan et al., 2006; Freeman et al., 2008; Jothikumar et al., 2009; Yasmon et al., 2010; Pang et al., 2011; Liu et al., 2011; Gautam et al., 2016). The designing of NSP2 gene derived primers and probe described in a single study (Gautam et al., 2016) was based on the Rotarix vaccine strain, and hence, was useful only for determining the frequency of Rotarix vaccine strain and vaccine derived reassortant strains associated with acute gastroenteritis. On the other hand, the NSP3 gene based TaqMan assay has been reported widely for detection of RVA (Pang et al., 2004; Freeman et al., 2008; Jothikumar et al., 2009; Pang et al., 2011; Mijatovic-Rustempasic et al., 2016). The primer and probe sequences of this gene initially designed by Pang et al., (2004) were utilized in other studies after modifications (Freeman et al., 2008; Pang et al., 2011; Liu et al., 2011). The modified primer and probe sequences when examined along with nucleotide alignment of 683 GenBank strains in the present study, showed their suitability for detection of majority of the human RVA strains except mcs 65, mcs 72 and mcs 90 (GenBank accession numbers: JX307624 – JX 307,626) strains of G8P[4] genotype combination (Mukherjee et al., 2013) due to the substitution (C→T) at extreme 3′ end in the forward primer (NVP3-F Deg). The primers and probe designed from 5′ end sequence of NSP3 gene have also been described by Jothikumar et al., (2009). Although the primers from this study did not show any mismatches in the alignment of GenBank strains included in the present study, probe (JVKP-FAM) showed 18.8% of nucleotide frequency of “A” at position 73 in place of “G” which is in close proximity to the 3′ end of the probe indicating the need to modify this position as Y (G/A) to avoid false negative results.

The use of the VP6 gene sub-grouping region derived primers and probes in RT-PCR and/or qRT-PCR have been reported in several studies (Iturriza Gómara et al., 2002; Kang et al., 2004; Iturriza-Gómara et al., 2009; Yasmon et al., 2010). Analysis of forward primer, (VP6F) described in these studies using nucleotide alignment of GenBank strains (n = 1218) indicated mismatched nucleotide at the extreme of 3′ end in three GenBank strains (Fig. 4). Besides this, multiple substitutions were noted in the middle of the primer in multiple strains (Fig. 4), thus suggesting the necessity of appropriate modifications (V→N, C→Y, R→D) in the sequence as well as in the length of the primer (Fig. 4).

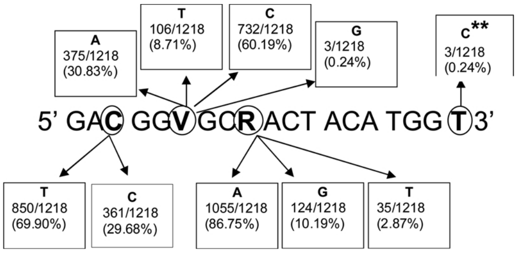

Fig. 4. Determination of frequency of nucleotide substitutions in VP6 F primer published earlier* using GenBank nucleotide sequences from human RVA strains (n = 1218).
* Iturriza Gomara et.al (2002).
** Three GenBank strains EU372734- G9P[8], HM066153- G2P[4], JQ069587-G1[8] with
extreme 3′ end mismatch.

Logan et al. (2006) described qRT-PCR assay consisting of 2 forward primers (Rota A fwd1 and Rota A fwd2), 2 reverse primers (Rota A rev1 and Rota A rev2) and two probes (Rota A probe 1VIC and Rota A probe 2VIC) designed from 5′ end of VP6 gene sequence. This assay ensured maximum sensitivity; however use of two probes was uneconomical making it unpractical for the purpose of routine diagnosis. In addition to this, rotavirus strain [KF035111.1, G4P[14]] showed substitution (T→C) at extreme 3′ end in both probes.

The limitations of the NSP2, NSP3 and VP6 derived primers and probes published in earlier studies have been overcome in the newly developed qRT-PCR assays of the present study by selecting conserved stretches of the genes and/or incorporating degenerate primers in the assays. The performance of the NSP2, NSP3 and VP6 gene based qRT-PCR assays of the present study was assessed by using known rotavirus ELISA positive and negative specimens. The NSP2 qRT-PCR assay displayed slightly higher Ct ± SD values as compared NSP3 and VP6 gene based qRT-PCR assays (Table 2) during testing of the clinical specimens with RNA detection sensitivity similar to that of the NSP3 qRT-PCR assay. However, Mean ± 3SD values estimated using Gaussian theorem showed overlapping pattern in the known ELISA positive and negative samples, thus making NSP2 qRT-PCR assay unfit for diagnostic purpose.

Among the remaining assays developed in the present study, non overlapping patterns of Mean ± 3SD values estimated in a similar manner were shown by the NSP3 qRT-PCR and VP6 qRT-PCR assays for known ELISA positive and negative samples. However, lowest Ct values for the known RVA positive clinical specimens and 10 fold more sensitivity with RNA detectable limit of 1.95×10^{-5} pg/μL encouraged us to select VP6 qRT-PCR assay for further study. One hundred fold more sensitivity of the VP6 qRT-PCR primers as compared to those of conventional VP6 RT-PCR diagnostic assay (Iturriza Gómara et al., 2002) was also confirmed by the RT-PCR assay in the present study (Fig. 3 a and b).

The VP6 qRT-PCR assay carried out on the known RVA ELISA positive specimens (n = 134) indicated presence of RVA genome in 131 specimens. Three specimens non-reactive in VP6qRT-PCR assay were non-typable by multiplex PCR applied for genotyping and negative in VP6 RT-PCR indicating absence of viral genome in capsids or false positivity in ELISA test as reported earlier (Kang et al., 2013). Among the RVA ELISA negative specimens of diarrheal and non-diarrheal individuals majority were non-reactive with exception of 17 specimens with Ct values > 35 suggesting absence of acute phase of the disease due to rotavirus infection. Among the neonates, the Mean Ct value qRT-PCR reactive specimens was markedly higher than that of older children (n = 17, 27.4 ± 7.3 vs n = 143, 21.1 ± 8.4; $p = 0.0066$) thereby indicating low content of viral load and/or limited replication of rotavirus in the neonatal gut.

RVA VP6 qRT-PCR assay did not show any cross reactions with other enteric viral pathogens namely NoV, AdV, AstV, AiV, EV, BoV displaying specificity of the assay only for RVA. Further, the utility of the VP6 qRT-PCR assay to detect different vaccine strains (Rotarix- Acc. No.KX954619.1; Rotavac - Acc. No.FJ361206.1; RotaTeq-BrB-9/1996/G4P7[5]- Acc. No.GU565089.1; RotaTeq-WI78-8/1992/G3P7[5]- Acc. No.GU565078.1; RotaTeq-SC2-9/1992/G2P7[5]) - Acc. No.GU565067.1; RotaTeq-WI79-9/1992/G1P7[5]- Acc. No.GU565056.1; RotaTeq-WI79-4/1992/G6P1A[8]- Acc. No.GU565045.1) was confirmed by locating the primers and probe in the corresponding GenBank nucleotide sequences (data not shown). The frequency polygon analysis and ROC analysis also indicated cut-off value of 35 for Ct with high sensitivity (126/131, 96%) and specificity (12/12, 100%) of VP6 qRT-PCR assay.

To summarize, the present study reports development of a sensitive and specific TaqMan based qRT-PCR assay for detection of RVA. The use of this assay could be extended for better understanding of spectrum of gastroenteritis under different conditions.

Acknowledgements

We are grateful to Dr. AC Mishra, former Director Indian Council of

Medical Research - National Institute of Virology (ICMR-NIV) and Dr. DT Mourya, Director ICMR-NIV for their constant support. The thanks are due to Mr. Prabhakar Jadhav for collection of specimens and Mr. Manohar Shinde for technical assistance. The funds for the project were provided by the ICMR- NIV, Ministry of Health and Family Welfare, Government of India.

References

Adlhoch, C., Kaiser, M., Hoehne, M., MasMarques, A., Stefas, I., Veas, F., et al., 2011. Highly sensitive detection of the group A rotavirus using apolipoprotein H-coated ELISA plates compared to quantitative real-time PCR. Virol. J. 8, 63. https://doi.org/10.1186/1743-422X-8-63.

Alam, M.M., Kobayashi, N., Ishino, M., Ahmed, M.S., Ahmed, M.U., Paul, S.K., et al., 2007. Genetic analysis of an ADRV-N-like novel rotavirus strain B219 detected in a sporadic case of adult diarrhea in Bangladesh. Arch. Virol. 152 (1), 199–208.

Chitambar, S.D., Gopalkrishna, V., Chhabra, P., Patil, P., Verma, H., Lahon, A., et al., 2012. Diversity in the enteric viruses detected in outbreaks of gastroenteritis from Mumbai, western India. Int. J. Environ. Res. Public Health 9, 895–915.

Chitambar, S.D., Ranshing, S.S., Pradhan, G.N., Kalrao, V.R., Dhongde, R.K., Bavdekar, A.R., 2014. Changing trends in circulating rotavirus strains in Pune, western India in 2009-2012: emergence of a rare G9P[4] rotavirus strain. Vaccine 32, A29–32. https://doi.org/10.1016/j.vaccine.2014.03.027.

Cook, N., Bridger, J., Kendall, K., Iturriza-Go´mara, M., El-Attar, L., Gray, J., 2004. The zoonotic potential of rotavirus. J. Infect. 48, 289–302.

Freeman, M.M., Kerin, T., Hull, J., McCaustland, K., Gentsch, J., 2008. Enhancement of detection and quantification of rotavirus in stool using a modified real-time RT-PCR assay. J. Med. Virol. 80, 1489–1496.

Gautam, R., Mijatovic-Rustempasic, S., Esona, M.D., Tam, K.I., Quaye, O., Bowen, M.D., 2016. One-step multiplex real-time RT-PCR assay for detecting and genotyping wild-type group A rotavirus strains and vaccine strains (Rotarix® and RotaTeq®) in stool samples. Peer J 4, e1560. https://doi.org/10.7717/peerj.1560.

Gutiérrez-Aguirre, I., Steyer, A., Boben, J., Gruden, K., Poljsak-Prijatelj, M., Ravnikar, M., 2008. Sensitive detection of multiple rotavirus genotypes with a single reverse transcription-real-time quantitative PCR assay. J. Clin. Microbiol. 46, 2547–2554.

Iturriza Gómara, M., Wong, C., Blome, S., Desselberger, U., Gray, J., 2002. Molecular characterization of VP6 genes of human rotavirus isolates: correlation of genogroups with subgroups and evidence of independent segregation. J. Virol. 76, 6596–6601.

Iturriza-Gómara, M., Kang, G., Gray, J., 2004. Rotavirus genotyping: keeping up with an evolving population of human rotaviruses. J. Clin. Virol. 31, 259–265.

Iturriza-Gómara, M., Elliot, A.J., Dockery, C., Fleming, D.M., Gray, J.J., 2009. Structured surveillance of infectious intestinal disease in pre-school children in the community:' the Nappy Study'. Epidemiol. Infect. 37 (7), 922–931.

Jothikumar, N., Kang, G., Hill, V.R., 2009. Broadly reactive TaqMan assay for real-time RT-PCR detection of rotavirus in clinical and environmental specimen. J. Virol. Methods 155, 126–131.

Kang, G., Iturriza-Gomara, Wheeler J.G., Crystal, P., Monica, B., Ramani, S., et al., 2004. Quantitation of group A rotavirus by real-time reverse-transcription-polymerase chain reaction: correlation with clinical severity in children in South India. J. Med. Virol. 73, 118–122.

Kang, G., Desai, R., Arora, R., Chitamabar, S.D., Naik, T.N., Krishnan, T., et al., 2013. Diversity of circulating rotavirus strains in children hospitalized with diarrhea in India, 2005–2009. Vaccine 31, 2879–2883. https://doi.org/10.1016/j.vaccine.2013.04.030.

Kapikian, A.Z., Kim, H.W., Wyatt, R.G., Rodriguez, W.J., Ross, S., Cline, W.L., et al., 1974. Reovirus like agent in stools: association with infantile diarrhea and development of serologic tests. Science 185, 1049–1053.

Knisley, C.V., Bednarz-Prashad, A.J., Pickering, L.K., 1986. Detection of rotavirus in fecal specimens with monoclonal and polyclonal antibody-based assay systems. J. Clin. Microbiol. 23, 897–900.

Kottaridi, C., Spathis, A.T., Ntova, C.K., Papaevangelou, V., Karakitsos, P., 2012. Evaluation of a multiplex real time reverse transcription PCR assay for the detection and quantitation of the most common human rotavirus genotypes. J. Virol. Methods 180, 49–53.

Lasure, N., Gopalkrishna, V., 2017. Molecular epidemiology and clinical severity of Human Bocavirus (HBoV) 1-4 in children with acute gastroenteritis from Pune, Western India. J. Med. Virol. 89, 17–23. https://doi.org/10.1002/jmv.24593.

Liu, J., Kibiki, G., Maro, V., Maro, A., Kumburu, H., Swai, N., et al., 2011. Multiplex reverse transcription PCR Luminex assay for detection and quantitation of viral agents of gastroenteritis. J. Clin. Virol. 50, 308–313. https://doi.org/10.1016/j.jcv.2010.12.009.

Logan, C., O'Leary, J.J., O'Sullivan, N., 2006. Real-time reverse transcription-PCR for detection of rotavirus and adenovirus as causative agents of acute viral gastroenteritis in children. J. Clin. Microbiol. 44, 3189–3195.

Martella, V., Bányai, K., Matthijnssens, J., Buonavoglia, C., Ciarlet, M., 2010. Zoonotic aspects of rotaviruses. Vet. Microbiol. 140, 246–255. https://doi.org/10.1016/j.vetmic.2009.08.028.

Matthijnssens, J., Bilcke, J., Ciarlet, M., Martella, V., Bányai, K., Rahman, M., et al., 2009. Rotavirus disease and vaccination: impact on genotype diversity. Future Microbiol. 4, 1303–1316.

Mijatovic-Rustempasic, S., Tam, K.I., Kerin, T.K., Lewis, J.M., Gautam, R., Quaye, O., et al., 2013. Sensitive and specific quantitative detection of rotavirus A by one-step real-time reverse transcription-PCR assay without antecedent double-stranded-RNA denaturation. J. Clin. Microbiol. 51, 3047–3054. https://doi.org/10.1128/JCM.01192-13.

Mijatovic-Rustempasic, S., Esona, M.D., Williams, A.L., Bowen, M.D., 2016. Sensitive and specific nested PCR assay for detection of rotavirus A in samples with a low viral load. J. Virol. Methods 236, 41–46. https://doi.org/10.1016/j.jviromet.2016.07.007.

Min, B.S., Noh, Y.J., Shin, J.H., Baek, S.Y., Min, K.I., Ryu, S.R., et al., 2006. Assessment of the quantitative real-time polymerase chain reaction using a cDNA standard for human group A rotavirus. J. Virol. Methods 137, 280–286.

Mukherjee, A., Mullick, S., Deb, A.K., Panda, S., Chawla-Sarkar, M., 2013. First report of human rotavirus G8P[4] gastroenteritis in India: evidence of ruminants-to-human zoonotic transmission. J. Med. Virol. 85, 537–545.

Pang, X.L., Lee, B., Boroumand, N., Leblanc, B., Preiksaitis, J.K., Yu Ip, C.C., 2004. Increased detection of rotavirus using a real time reverse transcription-polymerase chain reaction (RT-PCR) assay in stool specimens from children with diarrhea. J. Med. Virol. 72, 496–501.

Pang, X.L., Cao, M., Zhang, M., Lee, B., 2011. Increased sensitivity for various rotavirus genotypes in stool specimens by amending three mismatched nucleotides in the forward primer of a real-time RT-PCR assay. J. Virol. Methods 172, 85–87.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197.

Tate, J.E., Burton, A.H., Boschi-Pinto, C., Parashar, U.D., 2016. Global, regional, and national estimates of rotavirus mortality in children < 5 years of age, 2000-2013. Clin. Infect. Dis. (Suppl. 2), S96–105. https://doi.org/10.1093/cid/civ1013.

Wilde, J., Van, R., Pickering, L., Eiden, J., Yolken, R., 1992. Detection of rotaviruses in the day care environment by reverse transcriptase polymerase chain reaction. J. Infect. Dis. 166, 507–511.

Wyatt, R.G., James, H.D., Pittman Jr., A.L., Hoshino, Y., Greenberg, H.B., Kalica, A.R., et al., 1983. Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes. J. Clin. Microbiol. 18, 310–317.

Yasmon, A., Harahap, E.D., Dwipoerwantoro, P.G., Dewi, B.E., 2010. Detection of human group A and C rotaviruses in pediatric patients with acute gastroenteritis by real time RT-PCR assay: a preliminary study. MAKARA KESHATAN 14 (2), 65–69. https://doi.org/10.7454/msk.v14i2.685.