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Designing and validation of new primers for efficient genotyping of animal G3 rotaviruses

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ABSTRACT

The present study describes the problem of genotyping failures of animal rotaviruses with existing and in-use G3 genotyping primers. To overcome the problem, published and in-use G3 typing primers with sequences of VP7 genotyping regions from human and animal G3 rotavirus isolates were evaluated. The sequence alignment analysis showed that existing in-use G3 primers exhibit higher complementarities with rotavirus isolates of G6, G8 and G10 genotype specificities. The existing G3 primers showed up to 9 nucleotide mismatches with the animal origin rotavirus isolates of G3 genotype specificity. The modified G3 genotyping primers yielded positive amplification in all the G3 isolates of animal origin, with no incorrect amplification with any other group A rotavirus genotypes viz. G6 and G10. We advise the use of the proposed primers in molecular surveillance studies to discover the truly dominant genotypes of rotaviruses in animals.

Key words: group A rotavirus, animals, G3, G6, G10 genotype, VP7 gene, genetic diversity, multiplex-RT-PCR genotyping, G3 primers

Introduction

Rotavirus is the leading cause of neonatal diarrhea and is associated with heavy mortality and morbidity in humans, as well as in animals, throughout the world

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(FAGIOLO et al., 2005; PARASHAR et al., 2006; DHAMA et al., 2009; MALIK et al., 2012; MALIK et al., 2013a, 2013b; NITURE et al., 2011). This situation is much more grave in developing countries, where socio economic conditions further favor the survival and spread of the virus in nature (RAO et al., 2003; GHOSH et al., 2007; KUSUMAKAR et al., 2008; SAVITA et al., 2008; BASERA et al., 2010; KUSUMAKAR et al., 2010). Group A rotaviruses are most predominant and highly divergent. To accommodate all the variants, group A rotaviruses are further classified into serotypes and genotypes. The dual G and P genotyping classification system, based on the sequence variations in the genes encoding VP4 and VP7 proteins, has been adopted for molecular epidemiological studies (ESTES and KAPIKIAN, 2007; FALCONE et al., 1999; GOUVEA et al., 1990; GULATI et al., 1999; MINAKSHI et al., 2001 and 2005; ITURRIZA-GOMARA et al., 2004). For the sequence based genotyping strategy, specific primers are selected from the consensus regions of the virus genes, not matching with other genotypes, resulting in distinct amplicon sizes, which are easily discernable on gel. So far, 27G (G1-27) types and 35P (P[1-35]) rotavirus genotypes have been described (COLLINS et al., 2010; MAES et al., 2009; MATTHIJNSSENS et al., 2010; MALIK et al., 2012). The genomic configuration of the rotavirus makes it more prone to point mutations and genomic reassortment, leading to the emergence of newer genotypes. Sometimes accumulation of point mutations in the genotyping regions of VP7/VP4 genes leads to failure in typing of the rotavirus isolates (ITURRIZA-GOMARA et al., 2004; MANUJA et al., 2008; SHARMA et al., 2009). To overcome the problem of primer mismatch, modified or degenerate primers have also been suggested (ITURRIZA-GOMARA et al., 2004). A few studies had also reported detection of a different G type in RT-PCR, which was later identified as a different genotype on sequencing (ITURRIZA-GOMARA et al., 2004; ALADIN et al., 2010). Although, sequencing and sequence based automated genotyping through RotaC software are much more reliable ways for confirming the circulating genotype, they are not practical for large scale testing of samples, particularly in developing countries, due to economics and limited infrastructure facilities.

G3 rotaviruses, initially recognized to be affecting humans, have been detected in canine, caprine, porcine, equine and lapine forms, with an increasing trend in bovines, particularly in India, where incidences of G3 have been on the increase since its first reporting in 2002 in central India (VARSHNEY et al., 2002; GHOSH et al., 2007). While working on bovine rotaviruses in central, northern and foothills of western temperate Himalaya of India during 2008-2010, we found a number of G3 rotaviruses in bovines, which were confirmed through automated genotyping tool for rotaviruses (RotaC v1.5) and sequence blast (data not shown). Initially, the multiplex RT-PCR genotyping strategies of GOUVEA et al. (1990) and ITURRIZA-GOMARA et al. (2004) which are commonly employed for typing of animal G3 rotaviruses, were used to genotype the bovine samples. The aET3 (GOUVEA et al., 1990) primer showed dual infection with either G3G6 or G3G10 genotypes, while all these samples failed to give positive amplification with

Ituriza-Gomara primers (ITURRIZA-GOMARA et al., 2004). The samples which showed dual infection were later confirmed through sequencing as either G6 or G10 (data not shown). We extended the analyses with the aim to (i) check the complementarities of the in-use G3 primers (GOUVEA et al., 1990; ITURRIZA-GOMARA et al., 2004) with animal rotaviruses from India and other parts of the world, (ii) check variability in the genotyping regions of the VP7 gene within G3 rotavirus isolates of animal and human origin, and (iii) modify or propose new genotype specific primers for G3 rotavirus isolates of animals.

Materials and methods

Sampling, processing and viral RNA isolation. The viral RNA polyacrylamide gel electrophoresis (RNA-PAGE) positive 37 faecal samples for the rotavirus, collected from bovine calves up to 3 months of age and exhibiting signs of diarrhea from dairy farms of central, northern India and foothills of western temperate Himalaya of India were subjected for G (VP7) typing. The viral RNA was extracted from 500 µl of rotavirus positive (10% w/v) faecal suspension using equal volume of TriReagent-LS (Sigma, St. Louis, USA). The precipitated RNA was finally dissolved in 25 µL of Nuclease Free Water (NFW) and assessed qualitatively and quantitatively using Nanodrop Spectrophotometer (ND-1000, ThermoScientific, USA). Selected isolates were cloned and confirmed through sequencing and automated genotyping RotaC software (http://rotac.regatools.be) (MAES et al., 2009). The sequence variability and primer complementarities with common genotypes of animals were evaluated using a number of sequences retrieved from NCBI data base, with an aim to update and monitor the suitability of the in-use primers.

RT-PCR and genotyping. RT-PCR was performed as described by GOUVEA et al. (1990) with slight modifications to detect rotavirus. Reverse--transcription for cDNA synthesis from viral RNA was performed using Random Hexamer primers (0.2 µg/µL, Fermentas). Initially, 50 ng of viral RNA, 0.5 µL Random Hexamer primer, 2 µL dimethyl sulphoxide (DMSO) were added in a thin walled 0.5 mL PCR tube and the reaction mixture was incubated at 95 °C for 5 min for denaturing the dsRNA strands and immediately snap chilled on ice. Then 4 µL of 5X RT buffer (Promega, Madison USA), 2 µL of 10 mM dNTPs (Fermentas, Lithuania), 40 U RNase Inhibitor (Ambion, USA) and 200 U MMLV RT (Promega, Madison USA) were added and kept for incubation at 37 °C in a thermocycler for 1 hr. (HR-PCR-96G; Haier, Shanghai, China). The RT was denatured at 80 °C for 5 min. A negative control (NFW) and a positive control RNA from a rotavirus cell-culture isolate were also included in each RT-PCR reaction run.

The full length VP7 gene PCR was performed by using 3 µL cDNA, 5 µL 10X PCR buffers, 1.5 µL 25 mM $MgCl_2$, 2 µL DMSO, 1µL dNTP, 1.5 µL each of 30 pmol of forward and reverse primers, 1.5 µL 1.25U of Taq DNA Polymerase (Fermentas, Lithuania) and the volume constituted to 50 µL with NFW. The first round of amplification of full-length

VP7 (1062bp) consisted of initial denaturation at 94°C for 4 min followed by 35 cycles of 45 sec at 94 °C, 90 sec at 46 °C, and 90 sec at 72 °C, followed by a final incubation at 72 °C for 10 min. Amplified products were resolved by conventional agarose gel electrophoresis (1% w/v) at 100V for 1 hr in 1X TAE buffer with 0.5 µg/mL ethidium bromide and viewed under UV transilluminator and documented.

Multiplex nested PCR for determination of G type was performed as per the method and primers of GOUVEA et al. (1990) for G3 (aET3) and ITURRIZA-GOMARA et al. (2004). Primers used for the full length amplification and subsequently nested PCR for determination of genotypes for the VP7 gene are given in Table 1. Genotyping was performed using 3µL of 1:100 diluted PCR product of first round and the typing primers (G3) with the reverse primer (used for the generation of first round product of VP7 gene). The cycling conditions for the second round were same as that of the first round. The amplicons were analysed as described above.

Table. 1. Primers used for first round VP7 gene amplification and subsequent RT-PCR multiplexing of group A rotaviruses with genotype specific primers

	Primer	Sequence (5'…..3')	Position	Product size	Reference
VP7 Full length	VP7-F	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1-28	1062bp	TANIGUCHI et al. (1992)
	VP7-R	GGTCACATCATACAATTCTAATCTAAG	1062-1036		
G typing	G3 F	CGTTTGAAGAAGTTGCAACAG	689-709	373bp	GOUVEA et al. (1990)
	G3-F	ACGAACTCAACACGAGAGG	250-268	813bp	ITURRIZA-GOMARA et al. (2004)
	G6-F	GATTCTACACAGGAACTAG	481-499	581bp	GOUVEA et al. (1990)
	G10-F	ATGTCAGACTACARATACTGG	666-686	396bp	ITURRIZA-GOMARA et al. (2004)
New primers	G3-F	CTAATTCNACACAAGAAG	251-268	812bp	This paper

Cloning, sequencing and analysis. The full length amplicons of the VP7 gene (1062 bp) of selected bovine rotavirus isolates, showing G3G6 and G3G10 dual types, were excised from the gel and purified by High Pure PCR Product Purification kit (Roche Diagnostics, Hannover, Germany) with final elution in 20µL elution buffer. The purified products (50 ng) were ligated into pJET1.2/blunt vector (Fermentas, Lithuania) following the manufacturer's instructions, and transformed into *E. coli* Top10 cells. The transformed products were plated in LB-agar plate containing Ampicillin (50µg/mL) and kept at 37°C for 12-16 hrs. for the colonies to develop. Colony PCR was performed to check

the presence of the insert, using 2X Master Mix (Fermentas) (0.05 units/µl *Taq* DNA Polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, and 0.4 mM dGTP and 0.4 mM dTTP) and vector specific primers (pJET-F and pJET-R). The plasmid was isolated from positive clones using GeneJet Plasmid Isolation kit (Fermentas, Lithuania) and quantified in a Nanodrop Spectrophotometer. The positive plasmids were selected and sequenced using Automated Sequencer (ABI 3130, USA). Sequence data from these samples were submitted in the NCBI GenBank database under the following accession numbers: B-46 (HM235510), B-48 (HM235511), B-68 (HM235512), and PTN-970 (HQ199897).

The sequence chromatogram was visualized in ABI sequencing analysis software. Mega BLAST was performed with the deduced sequence within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/Blast) to confirm the presence of gene specific to rotaviruses. The automated genotyping tool for rotavirus (RotaC, http://rotac.regatools.be) was used for the confirmation of the sequences obtained (ITURRIZA-GOMARA et al., 2004). Animal and human group A rotavirus sequences for genotype G3, from different geographical locations within India and rest of the world, were retrieved from the NCBI nucleotide database. Sequence analysis was performed using the LaserGene software package (Dnastar Inc., Madison, WI).

New G3 genotype specific primers for animals. To overcome the problem of genotyping failure, mispriming and accommodating animal rotavirus strains, new primers were designed by modifying the earlier primers of ITURRIZA GOMARA et al. (2004) and tested practically with G3 bovine and equine rotavirus isolates and other reference genotypes (G6 (UK and NCDV) and G10 isolates. These primers were also tested *in silico* for complementarities with G3 and other common genotypes of animals (G3, G5, G6, G8 and G10).

Results and discussion

The availability of correct information on circulating rotavirus genotypes is of prime importance to formulate an effective vaccine for either humans or animals. With the use of multiplex RT-PCR strategy, informative epidemiological data has been generated on G and P types circulating throughout the world. However, failure in rotavirus genotyping, due to the accumulation of point mutations in the VP7 gene, has also been extensively reported, particularly in G1, G2, G7, G8, G9 and G10 genotypes (ADAH et al., 1997; DIWAKARLA and PALOMBO, 1999; ITURRIZA-GOMARA et al., 2001; SANTOS et al., 2003; MARTELLA et al., 2004). To overcome primer binding failure, degenerate primers were suggested by ITURRIZA-GOMARA et al. (2004).

G3 genotype is an emerging rotavirus type, and it has been detected in many parts of the world over the last two decades. Conflicting reports on G3 typing of rotaviruses with

	aET3(Gouvea et al., 1990)	G3 (Gomara et al., 2004)
Genotype/Species/Yr•/	690 700 710	250 260 270
Country/Acc. No.	---+----------+----------+-	--+----------+----------+
	--CGTTTGAAGAAGTTGCAACAG--	--ACGAACTCAACRCGAGAGG--
G3 Bo 2008 India HQ199897	G A . A . . . . . G . . T . . . . A T	A T G . T . . T . . C . . . . A . . . A . A G
G3 Bo 2002 India AF386914	C A . A . . . . . G . . T . . . . C T	A T G . T . . T . . T . . A . A . . . A . A G
G3 Bo 2008 India HM235512	G A . A . . . . . G . . T . . . . A T	A T G . T . . T . . C . . A . A . . . A . A G
G3 Bo 2008 India HM235510	G A . A . . . . . G . . T . . . . A T	A T G . T . . T . . C C . C . A . . . A . A G
G3 Bo 2001 UK AF448852	C A . . . . . C T	A T . . A . . . . . G . . A . A . . . A . A A
G3 Bo 2001 UK AF427124	C A . . . . . C T	A T . . A . . . . . G . . A . A . . . A . A A
G3 Ca 1979 USA EU708928	G A . A . . . . . G . . . . . A C C	A C G . T . . T . . T . . A . A G . . A . A G
G3 Ca 1980 USA EU708917	G A . A . . . . . G . . . . . A C C	A C G . T . . T . . T . . G . A G . . . A G
G3 Eq 2007 India DQ981477	G A . A . . . . . G . . T . . . . C T	A T G . T . . T . . C . . A . A . . . A . A G
G3 Eq 2007 India DQ981479	G A . A . . C . . . . . T . . G . C T	A T G . T . . T . . T . . G . A . . . A . A G
G3 Eq 2005 Germany AY750921	G A . A . . C . . . . . A . . . . G . . . C T	A T . . T . . T . . T . . A . A . . . A . A A
G3 Eq 2005 Germany AY750922	A A . A . . C . . . . . G A . . . . G . . . A C T	A T . . T . . T . . T . . A . A . . . A . A A
G3 Eq 2002 Japan AB046466	G A . A . . C . . . . . G . . . . . G . . G . C T	A T G . T . . T . . T . . A . A . . . A . A A
G3 Eq 2006 Japan AB046465	A A . A . . C . . . . . G . . . . . G . . G . C T	A T G . T . . T . . T . . A . A . . . A . A A
G3 Eq 2006 Greece GQ266671	G A . . . . C . . . . . G A . . . . G . . . . C T	A T . . T . . T . . T . . A . A . . . A . A A
G3 Eq 2007 Greece GQ266668	G A . . . . C . . . . . G A . . . . G . . . . C T	A T . . T . . T . . T . . A . A . . . A . A A
G3 Po 1994 Argentina L35054	T T . A . . . . . A T . . . G . . C . A T . C A	A T . T . . . T . . . . . T A C . A G T . A A
G3 Po 1994 Argentina L35056	T T . . . . . G A C . . . G . . T . G C . C A	A T . . . . . T . . . . . A A T G A G T . A A
G3 Po 2006 Thailand DQ256503	T A . . . . C . . . . C T	A T G . A . . . . G . . G . A . . . A A A G
G3 Po 2006 Thailand DQ256502	T A . . . . C . . . . C T	A T G . A . . . . . G . . G . A . . . A A A G
G3 La 2003 Italy AF528204	A A . . . . . C T	A C G . T . . T . . C . . A . . . . . A . A G
G3 La 2003 Italy AF528201	G A . . . . . C T	A C G . T . . T . . C . . A . A . . . A . A G
G3 Hu 1996 Italy GU296430	T A . . . . C . . . . . . A . . . . C T	A T . . A . . . . A . A . . . A . A A
G3 Hu 1996 Italy GU296431	T A . . . . . A . . . . C C	A T . . A . . T . . . . . G . A T . . A . A G
G3 Hu 1998 Japan AB011971	T A . T . . . . G . A C . . . . . T G A T A G C	A T G . T . . . . . A . C . A . C A A T
G3 Hu 1998 Japan AB011972	T A . T . . . . . A C . . . . . T G A T A G C	A T G . T . . . . . A . A . A . C A A T
G3 Hu 1998 Japan AB011973	T A . T . . . . . A C . . . . . T G A T A G C	A T G . T . . . . . A . C . A . C A A T
G3 Hu 2001-03Japan EF088831	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2000 Belgium AY456382	G A . A . . . . . C T	A C G . T . . T . . C . . A . A . . . A . A G
G3 Hu 2005 Slovenia EU383000	T A . . . . . C C	A T G . A . . . . . G . . A . A . . . A . A G
G3 Hu 2001 USA AJ311738	T A . A . . . . . G . . . . . C T	A T . . A . . . . . G . A . . . A . A A
G3 Hu 2001 USA AJ311739	T A . A . . . . . G . . . . . C T	A T . . A . . . . . G . A . . . A . A A
G3 Hu 1993 USA U04350	C A . . . . . C T	A T . T . . . . . A . . . . . A A
G3 Hu 2005 Russia GQ117001	C T . . . . . A T G . . . . . T G A . A A T	A C G . T . . . . . T . . T . A . . . A . G A
G3 Hu 2008 Russia GU377159	C A . . . . . C T	A T G . A . . . . . G . A . . . A . G G
G3 Hu 2007 Russia GQ452925	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2004-06 Spain EU159190	C T . . . . . A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2004-06 Spain EU159191	C T . . . . . A A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 1997 China AF260957	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 1998 China AF260958	C A . . . . . C T	A T . T . . . . . G . . . . . A A
G3 Hu 2005 China EU708590	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2006 China EU708583	T A . . . . . C T	A T . . . . . T . . . . . G . . . . . A A
G3 Hu 2007 China GU985263	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2004 Malaysia AY870661	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2004 Malaysia AY900173	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2004 Thailand AY707793	C A . . . . . C T	A T . . . . . G . . G . . . . A A
G3 Hu 2009 Thailand GQ996896	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2001 Vietnam EF495121	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2008 Vietnam GQ129459	C A . . . . . C T	A T . . . . . G . . . . . A A
G3 Hu 2002 India AB081594	C A . . . . . C T	A T . T . . . . . A . . . . . A A
G3 Hu 2008 Germany FJ747619	C A . . . . . C T	A T . . . . . G . . . . . A A

Fig. 1. Sequence alignment of G3 primer region of GOUVEA et al. (1990) and ITURRIZA-GOMARA et al. (2004) with rotavirus isolates of G3 genotype specificity of bovine, canine, equine, porcine, lapine and human origin. The year indicates either the sample collection or sequence submission data as per records in NCBI GenBank, database.

	3ETa Gouvea 1990			G3 (Gomara et al., 2004)			Modified G3 primers		
Genotype/Species/Yr* /Country/Acc.no.	690	700	710	250	260	270	250	260	270
	---+---------+---------+-			--+---------+---------+			-+---------+---------+		
	--CGTTTGAAGAAGTTGCAACAG--			--ACGAACTCAACACGAGAGG--			--CTAATTCNACACAAGAAG--		
G3 Bo 2008 India HQ199897	CA.A.....G..T....CT			ATG.T..T..C....A...A.AG			TG.....C.....AG		
G3 Bo 2001 India AF386914	CA.A.....G..T....CT			ATG.T..T..T....A...A.AG			TG.....T.....AG		
G3 Bo 2008 India HM235512	GA.A.....G..T....A-			ATG.T..T..C....A...A.AG			TG.....C.....AG		
G3 Bo 2008 India HM235510	GA.A.....G..T....AT			ATG.T..T..CC.C.A...A.AG			TG.....CC.C.....AG		
G3 Bo 2002 UK AF448852	CA.....CT			AT..A.....G....A...A.AA			TA.A..C..G.....AA		
G3 Bov 2001UK AF427124	CA.....CT			AT..A.....G....A...A.AA			TA.A..C..G.....AA		
G3 Ca 1979 USA EU708939	GA.A.....G.....ACC			ACG.T..T..T..G.AG..A.AG			CG.....T..G..G....AG		
G3 Ca 2000 Italy AF271090	AA.A.....C..T....CT			ATG.T..T..C....AG..A.AG			TG.....C.....G....AG		
G3 Eq 2007 India DQ981479	GA.A..C.....T..G.CT			ATG.T..T..T..G.A...A.AG			TG.....T..G.....AG		
G3 Eq 2006 Japan AB197846	GA.A..C....N....G..G.CT			ATG.T..T..T....A...A.AA			TG.....T.....AA		
G3 Eq 2005 Germany AY750924	AA.A.....GA....G....CT			AT.TT..T..T....A...A.AA			TAT.....T.....AA		
G3 Eq 2006 Greece GQ266672	GA....C.....GA....G....CT			AT..T..T..T....A...A.AA			TA.....T.....AA		
G3 Po 1994 Argentina L35060	TA.A.....G...A.....AAT			ATG.A..T..G....A...A.AA			TG.A.....G.....AA		
G3 Po 2007 Slovenia EU348715	TA.....CT			ATG.....G....A...A.AG			TG.G..C..G.....AG		
G3 La 2003 AF528204	AA.....CT			ACG.T..T..C.....A.AG			CG.....C....G....AG		
G3 La 2003 AF528201	GA.....CT			ACG.T..T..C....A...A.AG			CG.....C.....AG		
G3 Hu 2002 India AB081594	AA.....CT			AT.T.....AA			TATG..C..A....G...G.AA		
G3 Hu 1993 USA U04350	CA.....CT			AT.T.....AA			TATG..C..A....G...G.AA		
G3 Hu 1998 China AF260958	CA.....CT			AT.T.....G.....AA			TATG..C..A..G.G...G.AA		
G3 Hu 2001-03 Russia EF495125	CA.....CT			AT.....G.....AA			TA.G..C..A..G.G...G.AA		
G3 Hu 2008 Germany FJ747619	CA.....CT			AT.....G.....AA			TA.G..C..A..G.G...G.AA		
G3 Hu 2008 Vietnam GQ129459	CA.....CT			AT.....G.....AA			TA.G..C..A..G.G...G.AA		
G3 Hu 2009 Thailand GQ996896	CA.....CT			AT.....G.....AA			TA.G..C..A..G.G...G.AA		
G5 Bo 2008 South Korea EU873013	TT.A.....AC...G..C.AT.CA			AT.T...T.....TAC.AGT.AA			TATG.....A..TAC.AGT.AA		
G5 Po 2006 Thailand DQ683523	CT.A.....AC...A..T.GT.CA			ATG.A..T..T...GCGAGT.AA			TG.A.....T...GCGAGT.AA		
G5 Po 2010 Ireland FJ617257	TT.A.....AC...A..T.AT.CA			ATG.....G..GATGAGT.AA			TG.G..C..G..GATGAGT.AA		
G5 Hu 1995 Brazil GAU41005	TT.A.....AC...A..T.GTACA			TTG....T..T..GGC.AGT.AA			TG.G.....T..GGC.AGT.AA		
G5 Hu 2000 China EF159576	CT.A.....AT...A..T.AT.CG			ATGT.....GATGAGT.AA			TGTG..C..A..GATGAGT.AA		
G6 Bo NCDV VP7 M12394	ACA.....AC.....G...AT			ATG.AG....T....A.AGT.AG			TG.AG.C..T.....AGT.AG		
G6 Bo UK VP7 X00896	ACA.....ACG.....G...AC			ACG.A.....T..G.A.AGT.AG			CG.A..C..T..G...AGT.AG		
G6 Bo 2010 India HM567170	TA.....AC.....G...ACT			ACG.A..T..T....AGAGT.AA			CG.A.....T.....GAGT.AA		
G6 Bo-Hu RotaTeq 1992 GU565046	CA.....AC.....G...ACG			ATG.A.....T....A.AGT.AG			TG.A..C..T.....AGT.AG		
G6 Bo 2002 India AF443298	CA.....AC.....G...ACG			ACG.A..T..T..G.A.AGT.AG			CG.A.....T..G...AGT.AG		
G6 Bo 2005 Australia GQ352364	CA.A.....AC.....G...ACG			ATG.....T..G.A.AGT.AG			TG.G..C..T..G...AGT.AG		
G8 Bo 2001 Nigeria AF361439	GA.T.....CT			ATCA...TGT.T..ACTTCT.AG			TCAG...GTAT..ACTTCT.AG		
G8 Bo 2009 Japan AB044294	AA.T.....G.....CT			ATCAA..GTGT.TAATTCT.AA			TCAA..CGTGT.TA.TTCT.AA		
G8 Hu 1999 UK AF143689	AA.T.....CT			ATCA...TGT.T..ACTTTCAGA			TCAG...GTAT..ACTTTCAGA		
G8 Hu 2000 Malawi FJ386443	GA.T.....A.....CT			ATCA...TGT.T..ACTTCC.AG			TCAG...GTAT..ACTTCC.AG		
G8 Bo 2002 Australia GQ398012	AA.T.....G.....CT			ATCAA..GTGT.TAATCCT.AG			TCAA..CGTGT.TA.TCCT.AG		
G8 Hu 2002 Brazil HM123844	GA.T.....CT			ATCA....GT.T..ACTTCA.AA			TCAG..CGTAT..ACTTC..AA		
G10 Bo 2006 Mexico FJ217204	AA.....C.....CT			ATGT...TG.T..TAA...T.AG			TGTG...G.T..TA....T.AG		
G10 Bo 2006 Turkey FJ598311	GA.A.....G..A.....CT			ATG.A..TA.T...AAG..T.AA			TG.A...A.T...A.G..T.AA		
G10 Hu 2002 Nigeria EF218662	GA.A.....G..G.....CT			ATG.A..TA.T...AAG..T.AA			TG.A...A.T...A.G..T.AA		
G10 Hu 2004 Co.Ivoire AY816181	GA.A.....G..G.....CT			ATG.A..TA.T...AAG..T.AA			TG.A...A.T...A.G..T.AA		
G10 Hu 2007 India GQ240619	AA.A.....G.....CT			ACGT....G.C.T.GAG..TAAA			CGTG..CG.C.T.G.G..TAAA		
G10 Hu 2007 USA EF672567	GA.A.....G..A.....CT			ATGT....G.T...AAG..C.AA			TGTG..CG.T...A.G..C.AA		

Fig. 2. Sequence alignment of G3 primer region of GOUVEA et al. (1990) and ITURRIZA-GOMARA et al. (2004) and proposed G3 primers with rotavirus isolates of G6, G8, G10 and G3 genotypes from animal and human origin. The year indicates either the sample collection or sequence submission data as per records in NCBI GenBank, database.

either cross-priming with G8 strains (GOUVEA et al., 1990) or G10 strains (ITURRIZA-GOMARA et al., 2004) have been observed. To evaluate the in-use RT-PCR strategies (GOUVEA et al., 1990; ITURRIZA-GOMARA et al., 2004) for G3 typing of the animal rotaviruses, primers that target regions within the rotavirus capsid gene (VP7) were analyzed.

The VP7 gene sequence alignment of animals and human rotaviruses from different parts of the world, when aligned with the in-use G3 genotyping oligonucleotide primers of GOUVEA et al. (1990) and ITURIZZA-GOMARA et al. (2004), revealed a series of point mutations, which might result in either cross priming or primer binding failures at the 3' end of the G3 specific primer binding site (Fig. 1 and 2). Multiple sequence alignment of G3 (aET3) primer region of GOUVEA et al. (1990) with animal G3 isolates showed 3 common mutations in Indian bovine strains, while UK bovine strains showed no variability. Canine strains showed 3 variations in this region and equine strains from different parts of the world showed up to 6 mismatches. Porcine strains from Argentina showed maximum variability at 7 positions, while porcine strains from Thailand showed only one mismatch. The lapine G3 strains from Italy showed maximum complementarity in the region selected by GOUVEA et al. (1990). This primer region (689'-709) also showed maximum complementarities with human G3 isolates from China, Malaysia, Thailand, Vietnam, India, Slovenia, and Germany (Fig. 1) while G3 isolates from Spain, Belgium, Italy, and USA showed 1 to 2 variations. A few of the human G3 isolates from Japan and Russia showed numbers of point mutations, some even completely blocking the 3 prime portion of this primer region (Fig. 1). Like these, GOUVEA et al. (1990), ITURRIZA-GOMARA primers (250-269 position) also showed complementarities with human G3 isolates from Spain, China, Malaysia, Thailand, Vietnam, India and Germany. However, they showed 4-5 mismatches with human G3 isolates from Italy, Japan, Belgium, Slovenia, USA and Russia (Fig. 1). The same primer region in VP7 gene sequences from G3 rotavirus isolates of animal origin (bovine, equine, canine, lapine and porcine) showed more mismatches (up to 9) (Fig. 1).

To check the cross priming/false priming with other common genotypes (G3, G5, G6, G8 and G10) of animals (also reported in humans), sequence alignment was performed with the sequences retrieved from animals and humans from different parts of the world. The sequence analysis showed that though Gouvea primers (GOUVEA et al., 1990) show more complementarities with G3 isolates of humans and animals, they are also cross priming at more than 8 nucleotides at the 3 prime end of G8 and up to 6 nucleotides with G10 isolates (Fig. 2). However, apart from 2 substitutions towards the 3 prime end, Gouvea primers (GOUVEA et al., 1990) also show priming at more than 10 nucleotides with G6 isolates (Fig. 2). These primers however did not show any cross priming with G5

isolates of bovine, porcine or humans (Fig. 2). Whereas, the G3 primers of ITURIZZA-GOMARA et al. (2004) showed no cross-matching with any of the other genotypes (G5, G6, G8 and G10) of animal or human origin (Fig. 2), confirming their suitability for specific G3 genotyping of rotavirus isolates.

To overcome the genotyping failure using in-use G3 primers, we modified the Iturriza-Gomara primers to cover a majority of the G3 isolates of animal origin, including bovine, canine, equine, porcine and lapine from different parts of the world (Fig. 2). *In silico* analysis showed that these primers exhibit complementarity with the majority of animal G3 isolates and show no cross priming with either G5, G6, G8 or G10 genotypes from animals or humans. The modified primers are recommended for genotyping of animal G3 rotaviruses, while Iturriza-Gomara primers should be preferred for genotyping of human G3 rotavirus isolates. The suitability of these modified primers was also tested on field isolates of bovine and equine G3 genotype isolates obtained from different geographical areas. The bovine and equine G3 isolates were successfully amplified in optimized PCR conditions at 50°C annealing. The cross priming was also evaluated in lab on reference UK and NCDV strains (G6) and G10 isolates available in our laboratory. The bovine G3 isolates which gave dual genotype results with Gouvea primers yielded no such results with modified primers. The VP7 sequence analysis from G3 and G10 isolates confirms the availability of a very low number of variable regions differing in these two genotypes, thus the region selected by Iturriza-Gomara et al. (14) was used with degenerate primers at 5 positions (252 (T for G), 255 (T for C), 258 (N for A/G/C/T), 263 and 267 (A for G)) to amplify the bovine (UK and Indian strain), equine, canine, porcine and lapine G3 strains from different parts of the world (Fig. 2). The modification was also undertaken with deletion of one nucleotide at the 5 prime end, which has mutated in all animal rotavirus G3 strains (Fig. 2).

Conclusions

In conclusion, analyses of in-use G3 primers showed that substitutions are different in human than in animal rotavirus strains. The results confirm that ITURIZZA-GOMARA et al. (2004) primers could be used for genotyping of human group A rotavirus isolates and the proposed new primers for genotyping of animal G3 isolates.

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Conflict of Interest/Competing Interests

As such there is no conflict of interest.

References

ADAH, M. I., A. ROHWEDDER, O. D. OLALEYLE, H. WERCHAU (1997): Nigerian rotavirus serotype G8 could not be typed by PCR due to nucleotide mutation at the 3' end of the primer binding site. Arch. Virol. 142, 1881-1887.

ALADIN, F., S. NAWAZ, M. ITURRIZA-GÓMARA, J. GRAY (2010): Identification of G8 rotavirus strains determined as G12 by rotavirus genotyping PCR: updating the current genotyping methods. J. Clin. Virol. 47, 340-344.

BASERA, S. S., R. SINGH, N. VAID, K. SHARMA, S. CHAKRAVARTI, Y. P. S. MALIK (2010): Detection of rotavirus infection in bovine calves by RNA-PAGE and RT-PCR. Ind. J. Virol. 21, 144-147.

COLLINS, P. J., V. MARTELLA, C. BUONAVOGLIA, H. O'SHEA (2010): Identification of a G2-like porcine rotavirus bearing a novel VP4 type, P [32]. Vet. Res. 41, 73-75.

DHAMA, K., R. S. CHAUHAN, M. MAHENDRAN, S. V. S. MALIK (2009): Rotavirus diarrhea in bovines and other domestic animals. Vet. Res. Commun. 33, 1-23.

DIWAKARLA, C. S., E. A. PALOMBO (1999): Genetic and antigenic variation of capsid protein VP7 of serotype G1 human rotavirus isolates. J. Gen. Virol. 80, 341-344.

ESTES, M. K., A. Z. KAPIKIAN (2007): Rotaviruses. In: Fields Virology (Knipe, D. M., P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, S. E. Straus, Eds.). 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA, pp. 1917-1974.

FAGIOLO, A., R. CRISTINA, L. OGLA, B. ANTONIO (2005): Buffalo Pathologies. In: Buffalo Production and Research, (Antonio, B., Ed.), FAO, Rome, pp. 249-296.

FALCONE, E., M. TARANTINO, L. Di. TRANI, P. CORDIOLI, A. LAVAZZA, M. TOLLIS (1999): Determination of bovine rotavirus G and P serotypes in Italy by PCR. J. Clin. Microbiol. 37, 3879-3882.

GHOSH, S., V. VARGHESE, S. SAMAJDAR, M. SINHA, N. KOBAYASHI, T. N. NAIK (2007): Molecular characterization of bovine group A rotavirus G3P[3] strains. Arch. Virol. 152, 1935-1940.

GOUVEA, V., N. SANTOS, M. D. C. TIMENETSKY (1994): Identification of bovine and porcine rotavirus G types by PCR. J. Clin. Microbiol. 32, 1338-1340.

GOUVEA, V., R. I. GLASS, P. WOODS, K. TANIGUCHI, H. F. CLARK, B. FORRESTER, Z. Y. FANG (1990): Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens. J. Clin. Microbiol. 28, 276-282.

GULATI, B. R., N. OSAMU, K. YUMI, N. TOYOKO, R. PANDEY (1999): Relative frequencies of G and P types among rotaviruses from Indian diarrheic cow and buffalo calves. J. Clin. Microbiol. 6, 2074-2076.

ITURRIZA-GOMARA, M., D. CUBITT, U. DESSELBERGE, J. GRAY (2001): Amino acid substitution within the VP7 protein of G2 rotavirus strains associated with failure serotype. J. Clin. Microbiol. 39, 3796-3798.

ITURRIZA-GOMARA, M., G. KANG, J. GRAY (2004): Rotavirus genotyping: Keeping up with an evolving population of human rotaviruses. J. Clin. Virol. 31, 259-265.

KUSUMAKAR, A. L., SAVITA, Y. P. S. MALIK, MINAKSHI, G. PRASAD (2008): Occurrence of mammalian group A rotavirus in swine population of central India. Ind. J. Anim. Sci. 78, 475-477.

KUSUMAKAR, A. L., SAVITA, Y. P. S. MALIK, MINAKSHI, G. PRASAD (2010): Genomic diversity among group A rotaviruses from diarrhoeic children, piglets, buffalo and cow calves of Madhya Pradesh. Ind. J. Microbiol. 50, 83-88.

MAES, P., J. MATTHIJNSSENS, M. RAHMAN, M. VAN RANST (2009): Rota C: a web-based tool for the complete genome classification of group A rotaviruses. BMC Microbiol. 9, 238.

MALIK, Y. P. S., K. SHARMA, N. VAID, S. CHAKRAVARTI, K. M. CHANDRASHEKAR, S. S. BASERA, R. SINGH, MINAKSHI, G. PRASAD, B. R. GULATI, K. N. BHILEGAONKAR, A. B. PANDEY (2012): Occurrence of group A rotavirus mixed G and P genotypes in bovines: Predominance of G3 genotype and its emergence in combination with G8/G10 types. J. Vet. Sci. 13, 271-278.

MALIK, Y. P. S., N. KUMAR, K. SHARMA, A. A. HAQ, A. KUMAR, M. PRASAD (2013a): Sequence and phylogenetic analysis of bovine rotavirus isolates (G6 genotypes) from India. Adv. Anim. Vet. Sci. 1, 41-43.

MALIK, Y. P. S., N. KUMAR, K. SHARMA, R. SHARMA, H. KUMAR, A. KUSUMAKAR, SAVITA, S. SHUKLA, K. M. CHANDRASHEKAR (2013b): Epidemiology and genetic diversity of rotavirus strains associated with acute gastroenteritis in bovine, porcine, poultry and human population of central India, 2004-2008. Adv. Anim. Vet. Sci. 1, 111-115.

MANUJA, B. K., MINAKSHI, A. MANUJA, B. R. GULATI, G. PRASAD (2008): A novel genomic constellation (G10P[3]) of group A rotavirus detected from buffalo calves in northern India. Virus Res. 138, 36-42.

MARTELLA, V., V. TERIO, S. ARISTA, G. ELIA, M. CORRENTE, A. MADIO, A. PRATELLI, M. TEMPESTA, A. CIRANI, C. BUONAVOGLIA (2004): Nucleotide variation in the VP7 gene affects PCR genotyping of G9 rotavirus identified in Italy. J. Med. Virol. 72, 143-148.

MATTHIJNSSENS, J., M. RAHMAN, M. CIARLET, M. ZELLER, E. HEYLEN, T. NAKAGOMI, R. UCHIDA, Z. HASSAN, T. AZIM, O. NAKAGOMI, M. VAN RANST (2010): Reassortment of human rotavirus gene segments into G11 rotavirus strains. Emerg. Infect. Dis. 16, 625-630.

MINAKSHI, G. PRASAD, Y. P. S. MALIK, R. PANDEY (2005): G and P genotyping of bovine group A rotaviruses in faecal samples of diarrheic calves by DIG-labeled probes. Ind. J. Biotechnol. 4, 93-99.

MINAKSHI, R. PANDEY, G. PRASAD, Y. P. S. MALIK (2001): VP4 gene specific RT- PCR for detection of bovine group A rotaviruses. Ind. J. Anim. Sci. 71, 611-613.

NITURE, G. S., A. G. KARPE, M. PRASAD (2011): Characterization of buffalo, poultry and human rotaviruses in Western India. Vet. arhiv 81, 307-319.

PARASHAR, U. D., C. J. GIBSON, J. S. BRESSE, R. I. GLASS (2006): Rotavirus and severe childhood diarrhea. Emerg. Infect. Dis. 12, 304-306.

RAO, C. D., M. R. JAGANNATH, B. C. VARSHNEY, M. DAS, B. S. Y. REDDY (2003): Genomic diversity through gene reassortment and antigenic drift and molecular epidemiology of rotaviruses in India. In: Genomic Diversity and Molecular Epidemiology of Rotaviruses. (N. Kobayashi, Ed.) Research Signpost, Trivandrum, India, pp. 55-74

SANTOS, N., E. M. VOLOTAO, C. C. SOARES, M. C. ALBUQUERQUE, F. M. DA SILVA, V. CHIZHIKOV, Y. HOSHINO (2003): Vp7 gene polymorphism of serotype G9 rotavirus strain and its impact on G genotype determination by PCR. Virus Res. 93, 127-138.

SAVITA, A. L. KUSUMAKAR, Y. P. S. MALIK, MINAKSHI, G. PRASAD (2008): Detection and characterization of Group A and D avian rotaviruses in India. Ind. J. Biotechnol. 7, 554-556.

SHARMA, G., A. TAKU, R. CHHABRA, M. A. BHAT (2009): Prevalence of bovine diarrhoea in Jammu. Ind. J. Virol. 20, 7-12.

TANIGUCHI, K., F. WAKASUGI, Y. PONGSUWANNA, T. URASAWA, S. UKAE, S. CHIBA, S. URASAWA (1992): Identification of human and bovine rotavirus serotypes by polymerase chain reaction. Epidemiol. Infect. 109, 303-312.

VARSHNEY, B., M. R. JAGANNATH, R. R. VETHANAYAGAM, S. KODHANDHARAMAN, H. V. JAGANNATH, K. GOWDA, D. K. SINGH, C. D. RAO (2002): Prevalence of, and antigenic variation in, serotype G10 rotaviruses and detection of serotype G3 strains in diarrheic calves: implications for the origin of G10P11 or P11 type reassortant asymptomatic strains in newborn children in India. Arch. Virol. 147, 143-165.

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SAŽETAK

U radu je opisan problem neuspjeha genotipizacije životinjskih rotavirusa s dosadašnjim početnicama za genotipizaciju G3. Za rješavanje tog problema vrednovane su objavljene i rabljene početnice za G3 sa sekvencijama za VP7 područja genotipizacije izolata rotavirusa podrijetlom od ljudi i životinja. Analiza poravnanja sekvencije pokazala je da rabljene početnice G3 pokazuju veću komplementarnost s izolatima rotavirusa genotipa G6, G8 i G10. Postojeće početnice G3 bile su u devet nukleotida nepodudarne s rotavirusima G3 životinjskog podrijetla. Preinačene početnice za genotipizaciju G3 pokazale su se uspješnima za umnožavanje sva tri izolata G3 podrijetlom od životinja bez pogrješnog umnožavanja bilo kojeg genotipa skupine A rotavirusa odnosno G6 i G10. Preporučuje se upotreba predloženih početnica u molekularnim istraživanjima za dokaz uistinu dominantnih genotipova rotavirusa u životinja.

Ključne riječi: skupina A rotavirusa, životinje, genotip G3**,** G6, G10, gen VP7, genetska raznolikost, multipla TR-PCR genotipizacija, početnice G3