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PCR-based approach to distinguish group A human rotavirus genotype 1 vs. genotype 2 genes

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Group A rotaviruses (RVs) are eleven-segmented, double-stranded RNA viruses and important causes of severe diarrhea in children. A full-genome classification system is readily used to describe the genetic makeup of individual RV strains. In this system, each viral gene is assigned a specific genotype based upon its nucleotide sequence and established percent identity cut-off values. However, a faster and more cost-effective approach to determine RV gene genotypes is to utilize specific oligonucleotide primer sets in RT-PCR/PCR. Such primer sets and PCR-based genotyping methods have already been developed for the VP7-, VP6-, VP4- and NSP4-coding gene segments. In this study, primers were developed for the remaining seven RV gene segments, which encode proteins VP1, VP2, VP3, NSP1, NSP2, NSP3, and NSP5/6. Specifically, primers were designed to distinguish the two most common human RV genotypes (1 vs. 2) for these genes and were validated on several cell culture-adapted human and animal RV strains, as well as on human RVs from clinical fecal specimens. As such, primer sets now exist for all eleven genes of common human RVs, allowing for the identification of reassortant strains with mixed constellations of both genotype 1 and 2 genes using a rapid and economical RT-PCR/PCR method.

1. Introduction

Group A rotaviruses (RVs) are important pathogens that cause acute gastroenteritis in children less than five years of age, leading to severe dehydration and even death (Estes and Kapikian, 2007; Parashar et al., 2009). Current reports indicate that each year over 450,000 preventable fatalities in developing countries are attributable to RVs, and the societal cost of these infections is estimated at upwards of $1 billion annually in the US alone (Payne et al., 2011; Tate et al., 2012). RVs are members of the *Reoviridae* family and exist as non-enveloped, triple-layered icosahedral capsids surrounding an eleven-segmented, double-stranded RNA (dsRNA) genome (Estes and Kapikian, 2007). Each genome (i.e., gene) segment codes for either a single viral structural protein (VP1, VP2, VP3, VP4, VP6, or VP7) or a single viral non-structural protein (NSP1, NSP2, NSP3, NSP4, or NSP5). In some RV strains, gene 11 codes for two non-structural proteins (NSP5 and NSP6) from overlapping open-reading frames. Historically, RVs have been classified based upon the antigenicity of the three major capsid proteins: VP7 (G-serotypes), VP4 (P-serotypes) and VP6 (subgroups) (Fischer and Gentsch, 2004). However, a more recent full-genome classification scheme is now readily used to describe the genetic makeup of individual RV strains (Matthijnssens et al., 2008). In this system, a specific genotype is assigned to each of the eleven viral gene segments based on their nucleotide sequences and established percent identity cut-off values. The nomenclature for the full-genome genotype of a RV strain is now written in the format Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where each letter corresponds to a single gene encoding a viral protein(s) (i.e., VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6) and *x* is a number that corresponds to the gene genotype.

The implementation of this full-genome classification system revealed that, while the G/P-genotypes of human RVs can vary, the non-G/P-genotype genes are usually either all genotype 1 or all genotype 2. For example, of the 333 human RV strains that had been fully sequenced as of December 2012, 312 of them have genotype constellations of either I1-R1-C1-M1-A1-N1-T1-E1-H1 or I2-R2-C2-M2-A2-N2-T2-E2-H2 (Matthijnssens and Van Ranst, 2012). Human RVs with mixed constellations of genotype 1 and 2 genes can be created in the laboratory and have occasionally been isolated from children with gastroenteritis (Matthijnssens and Van Ranst, 2012). However, it is currently hypothesized that such reassortant strains are less fit than those with pure genotype 1 or pure genotype 2 constellations (McDonald et al., 2009, 2011, 2012). To test this hypothesis, resource and time-efficient methods

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are needed to differentiate genotype 1 vs. 2 genes of human RVs as part of very large-scale (>1000 specimens) epidemiological surveillance studies. With such methods in-place, investigators can begin to answer important questions related to the diversity of circulating strains and the role of gene reassortment in RV evolution.

One approach that has been used to streamline studies of RV diversity relies on specific oligonucleotide primers to differentiate the common gene genotypes of strains using RT-PCR/multiplex PCR. In this approach, viral RNA is first converted into cDNA and amplified for 10 cycles using RT-PCR and universal primers designed against highly conserved gene regions. The product of this reaction is then re-amplified by PCR in the presence of one of the universal primers as well as two or more genotype-specific primers, which were designed against variable gene regions. The resulting nested genotype-specific amplicons are sufficiently different in size, allowing them to be easily discerned following agarose gel electrophoresis. Such universal and genotype-specific primers have been described for the VP7-coding gene (genotypes G1, G2, G3, G4, G9, and G12) and the VP4-coding gene (genotypes P[4], P[6], and P[8]) (Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994; Iturriza-Gómara et al., 2004). Similar approaches and primer sets have since been developed for the VP6-coding gene to differentiate genotype I1 vs. I2/I3 (Lin et al., 2008; Thongprachim et al., 2010). Moreover, for the NSP4-coding gene, primer sets exist to discriminate among genotypes E2, E1, and E3 (Rodrìguez-Dìaz et al., 2008). In the current study, primer sets were developed to distinguish between the two most common genotypes for the other seven non-G/P-type genes encoding: VP1 (R1 vs. R2), VP2 (C1 vs. C2), VP3 (M1 vs. M2), NSP1 (A1 vs. A2), NSP2 (N1 vs. N2), NSP3 (T1 vs. T2), and NSP5/6 (H2 vs. non-H2). The results show that these primers specifically and efficiently recognized the genotype 1 and/or 2 genes of cell-culture adapted and/or primary clinical RV strains by RT-PCR/PCR. Thus, the preliminary full-genome genotypes of most human RVs can now be deduced in a rapid, high-throughput, and cost-effective manner without the requirement for nucleotide sequencing.

2. Materials and methods

2.1. Oligonucleotide primer design

Nucleotide sequence alignments of human RV genotype 1 and 2 genes (encoding VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5/6) were created using the ClustalX algorithm implemented in Geneious™ Pro (v.5.6.3). Universal forward and reverse primers were designed against highly conserved gene regions. Primers specific for genotype 1 or 2 genes (forward or reverse, depending upon the gene) were designed against regions that were variable between the two genotypes, but conserved within the genotype. GenBank accession numbers of human RV gene sequences used to create the alignments for primer design are shown in Table 1, and primer sequences developed in this study are shown in Table 2. Also shown in Table 2 are the primers developed by Lin et al., and Rodrìguez-Dìaz et al., for the VP6- and NSP4-coding genes, respectively (Lin et al., 2008; Rodrìguez-Dìaz et al., 2008). Primers for G/P-genotyping using RT-PCR/PCR can be found in several published manuscripts (Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994; Iturriza-Gómara et al., 2004). Lyophilized primers were generated by Life Technologies (Carlsbad, CA, USA), resuspended in DEPC-treated water to a working concentration of 5–10 pmol/μl, and stored at −20 °C.

2.2. Purification of viral RNA templates

Cell culture-adapted human RVs (strains Wa, DS-1, AU-1, ST3, A64, and L26), the porcine RV strain OSU, and the bovine RV strain UK were generously provided by Dr. John T. Patton (National Institutes of Health). To prepare viral RNA from these strains, approximately 1×10^7 monkey kidney (MA104) cells were infected with trypsin-activated RV as described previously (Arnold et al., 2009). Infections proceeded at 37 °C/5% CO_2 for 3–5 days until the cell monolayer was completely lysed. Total RNA was extracted from the lysate using Trizol® LS (Life Technologies; Carlsbad, CA, USA) as per the manufacturer's protocol. The extracted RNA was concentrated by isopropanol precipitation, resuspended in 25–50 μl of DEPC-treated water, and stored at −20 °C. Viral RNA from RV-positive clinical fecal specimens was a gift from Dr. Carl Kirkwood (Murdoch Childrens Research Institute, Victoria, Australia) and was extracted and G/P-genotyped as described previously (Kirkwood et al., 2011). The specimens were collected from children with RV gastroenteritis in West Australia and/or Victoria during the years of 2009–2012 as part of a routine surveillance program.

2.3. RT-PCR using universal primers for preparation of genotyping templates

To convert viral RNA into cDNA and amplify it for downstream genotyping, one-step RT-PCR was performed using the Super Script® One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Life Technologies; Carlsbad, CA, USA). RNA was denatured at 95 °C for 10 min in 50% dimethyl sulfoxide (DMSO) prior to being used as template. Each 25-μl reaction contained 12.5 μl of the Super Script® mix, 0.5 μl enzyme, 0.5 μl universal forward primer, 0.5 μl universal reverse primer, 1.0 μl of the RNA:DMSO mixture, and 10.0 μl DEPC-treated water. The reactions were incubated in a thermocycler at 50 °C for 30 min, 94 °C for 2 min, and then 10 cycles of [94 °C for 30 s; 48 °C for 30 s; 68 °C for 1 min]. A final extension step occurred at 68 °C for 10 min. In some experiments, the universal forward and reverse primers for the VP1-, VP2-, VP3-, NSP1-, NSP2-, NSP3- and NSP5/6-coding genes were combined into a single reaction. More specifically, each 25-μl reaction contained 12.5 μl of the Super Script® mix, 0.5 μl enzyme, 0.5 μl of each universal primer (7.0 μl total), 1.0 μl of the RNA:DMSO mixture, and 4.0 μl DEPC-treated water, with the same cycling parameters described above.

2.4. Genotyping templates using multiplex PCR and specific primers

To determine the genotype of the universal RT-PCR products, multiplex PCR was performed using a nested universal primer and both the genotype 1- and 2-specific primers. Each 25-μl reaction contained 21 μl of the AccuPrime™ *Pfx* Supermix (Life Technologies; Carlsbad, CA), 1.0 μl of the nested universal primer and 1.0 μl each of both genotype-specific primers and 1.0 μl of the RT-PCR (from above). Control reactions containing both universal forward and reverse primers, but lacking the genotype-specific primers were supplemented with 1.0 μl of DEPC-treated water to maintain a consistent reaction volume. The reactions were heated to 94 °C for 2 min in the thermocycler and then underwent 35 cycles of [94 °C for 30 s; 48 °C for 30 s; 68 °C for 1 min], followed by a 10 min final extension step at 68 °C. Reaction products were electrophoresed in 2% agarose-TAE (Tris-acetate-EDTA) gels and visualized by ethidium bromide staining and exposure to ultraviolet light. Gel images were captured using a BioRad ChemiDoc Imaging System. Figures were prepared using Adobe Photoshop (v12.0) and Illustrator (v15.0). To verify the specificity of the amplification, PCR products were excised from the gels, purified using the Qiagen Gel Extraction kit (Qiagen; Gaithersburg, MD, USA), and directly sequenced as described previously (Rippinger et al., 2010).

Table 1
Genotypes and accession numbers for human RV gene sequences used for primer design.

RV strain name*	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP5/6
RVA/human-tc/USA/**Wa**/1974/G1P[8]	R1-FJ423113	C1-FJ423114	M1-FJ423115	A1-FJ423117	N1-FJ423120	T1-JX406753	H1-FJ423123
RVA/human-tc/USA/**D**/1974/G1P[8]	R1-EF583021	C1-EF583022	M1-EF583023	A1-EF672571	N1-EF672573	T1-EF672572	H1-EF672576
RVA/human-wt/IND/**61060**/2006/G1P[8]	R1-HQ609554	C1-HQ609557	M1-HQ609560	A1-HQ609566	N1-HQ609569	T1-HQ609572	H1-HQ609578
RVA/human-wt/BEL/**BE00036**/2008/G1P[8]	R1-HQ392311	C1-HQ392318	M1-HQ392320	A1-HQ392313	N1-HQ392312	T1-HQ392319	H1-HQ392314
RVA/human-wt/BEL/**BE00029**/2008/G1P[8]	R1-HQ392239	C1-HQ392242	M1-HQ392241	A1-HQ392243	N1-HQ392234	T1-HQ392240	H1-HQ392235
RVA/human-wt/USA/**VU05-06-13**/2006/G1P[8]	R1-JF490578	C1-JF490588	M1-JF490587	A1-JF490586	N1-JF490579	T1-JF490583	H1-JF490580
RVA/human-wt/USA/**VU06-07-1**/2007/G1P[8]	R1-JF490875	C1-JF490885	M1-JF490884	A1-JF490877	N1-JF490876	T1-JF490880	H1-JF490878
RVA/human-tc/USA/**DS-1**/1976/G2P[4]	R2-EF583025	C2-EF583026	M2-EF583027	A2-EF672578	N2-EF672580	T2-EF672579	H2-EF672583
RVA/human-wt/CHN/**TB-Chen**/???/G2P[4]	R2-AY787653	C2-AY787652	M2-AY787654	A2-AY787647	N2-AY787648	T2-AY787649	H2-AY787651
RVA/human-tc/USA/**P**/1974/G3P[8]	R1-EF583037	C1-EF583038	M1-EF583039	A1-EF672599	N1-EF672601	T1-EF672600	H1-EF672604
RVA/human-wt/USA/**DC1563**/1974/G3P[8]	R1-FJ947175	C1-FJ947176	M1-FJ947177	A1-FJ947179	N1-FJ947182	T1-FJ947181	H1-FJ947185
RVA/human-wt/USA/**2008747332**/2008/G3P[8]	R1-HM773689	C1-HM773690	M1-HM773691	A1-HM773693	N1-HM773696	T1-HM773695	H1-HM773699
RVA/human-wt/USA/**2008747336**/2008/G3P[8]	R1-HM773678	C1-HM773679	M1-HM773680	A1-HM773682	N1-HM773685	T1-HM773684	H1-HM773688
RVA/human-tc/GBR/**ST3**/1975/G4P[6]	R1-EF583045	C1-EF583046	M1-EF583047	A1-EF672613	N1-EF672615	T1-EF672614	H1-EF672618
RVA/human-tc/BRA/**IAL28**/1992/G5P[8]	R1-EF583029	C1-EF583030	M1-EF583031	A1-EF672585	N1-EF672587	T1-EF672586	H1-EF672590
RVA/human-tc/USA/**Se584**/1998/G6P[9]	R2-EF583041	C2-EF583042	M2-EF583043	A3-not incl.	N2-EF672608	T1-EF672607	H3-not incl.
RVA/human-wt/COD/**DRC86**/2003/G8P[8]	R2-DQ005125	C2-DQ005124	M2-DQ005123	A2-DQ005119	N2-DQ005118	T2-DQ005117	H2-DQ005115
RVA/human-tc/GBR/**69M**/1980/G8P[10]	R2-EF576937	C2-EF583014	M2-EF576915	A2-EF672557	N2-EF672559	T2-EF672558	H2-EF672562
RVA/human-tc/USA/**Wi61**/1983/G9P[8]	R1-EF583049	C1-EF583050	M1-EF583051	A1-EF672620	N1-EF672622	T1-EF672621	H1-EF672625
RVA/human-tc/UK/**A64**/1987/G10P[14]	R2-EF583017	C2-EF583018	M2-EF503819	A3-not incl.	N2-EF672566	T6-not incl.	H3-not incl.
RVA/human-tc/PHL/**L26**/1987/G12P[4]	R2-EF583033	C2-EF583034	M2-EF583035	A2-EF672592	N1-EF672594	T2-EF672593	H1-EF672597
RVA/human-wt/GER/**GER172-08**/2008/G12P[6]	R1-FJ747625	C1-FJ747626	M1-FJ747627	A1-FJ747631	N1-FJ747632	T1-FJ747633	H1-FJ747635

* Common strain name is highlighted in bold print.

3. Results

3.1. Oligonucleotide primer design

Nucleotide sequence alignments were performed using human RV genotype 1 and 2 genes (encoding VP1, VP2, VP3, NSP1, NSP2, NSP3, and NSP5/6) to identify conserved and variable regions for primer design. The RV gene sequences used in these alignments were (i) from cell-culture adapted human strains as well as from strains found in clinical fecal specimens, (ii) from human strains isolated on different dates from various geographical locations, and (iii) from human strains that exhibit diverse G/P-genotypes (Table 1). The rationale for choosing diverse human RV strains for primer design was to increase the chances that the primers will accurately recognize and discriminate between a broad array of genotype 1 and/or 2 genes in epidemiological studies.

For each gene, two highly conserved regions (≤1 kb apart) were chosen for the design of universal forward and universal reverse primers, which would be expected to amplify both genotype 1 and 2 genes of human RVs in RT-PCRs (Fig. 1 and Table 2). In contrast, gene regions that varied between genotypes 1 and 2, yet that remained highly conserved within each genotype, were identified for the design of specific primers (Fig. 1 and Table 2). The genotype 1- and 2-specific primers were located within the boundaries of the universal primers and were >90 bps apart, allowing the resulting PCR amplicons to be discerned following gel electrophoresis (Fig. 1). For the VP1-, VP2-, VP3- and NSP1-coding genes, the genotype-specific primers were designed as forward primers, to be paired with universal reverse primers in multiplex PCR (Fig. 1 and Table 2). For the NSP2- and NSP3-coding genes, the genotype-specific primers were designed as reverse primers, to be paired with universal forward primers in multiplex PCR (Fig. 1 and Table 2). For the NSP5/6-coding gene, no appropriate variable regions were identified in the alignments for genotype-specific primer design. However, due to an insertion in the 3′ untranslated region (UTR) of the NSP5/6 gene, human RV genotype 2 genes are ~150 bp longer than non-genotype 2 genes (Fig. 1). Therefore, a universal reverse primer was designed against the extreme 3′ terminus, and when paired with an upstream universal forward primer, it would create different sized RT-PCR amplicons from genotype 1 vs. 2 genes (Fig. 1). Specifically, a 537-bp product would be expected for genotype 2 genes, while other human RV genes (including those that are genotype 1) would yield only the smaller, 385-bp amplicon (Fig. 1).

Whenever possible, as allowed by the target sequences, primers were designed to contain ≥29% GC content, to end in either a 3′ G or C, and to avoid self-complementarity. Of the 26 primers designed, 10 of them end in a 3′ G or C, and all are expected to avoid self-complementarity (Table 2). Moreover, of the 14 universal primers designed, 11 of them exhibit GC contents within the optimal range (Table 2). However, universal primers VP1-Ur, VP3-Uf, and NSP1-Ur each have lower than optimal GC contents (17%, 25%, and 28%, respectively) due to fact that the conserved

Table 2
Universal and genotype 1- or 2-specific primers for all nine of the non-G/P-type genes of human RVs.

Protein-coding gene	Primer (name/sequence 5′–3′)	%GC
VP1	**VP1-Uf**/TCA GGA ATA GCT GAT GAA ATT GC	39
	VP1-Ur/AAT AAT TGA TAA CAT CCA TAA TTA	17
	VP1-GT1f/TAC TAT TAA TGA CAT TTT GCG TGA	29
	VP1-GT2f/GCA TAC CAA AAA TAG ATG CTG ATA	33
VP2	**VP2-Uf**/GCT ATT AAA GGC TCA ATG GCG TAC	46
	VP2-Ur/GGA TGT AGA ATT GAT GGA TAA TTG	33
	VP2-GT1f/GAC TTA CCA CAA CAA AAT GAA CG	39
	VP2-GT2f/AGA AGT TGT AAC GGA CAG TCA TGA	42
VP3	**VP3-Uf**/CTA ATC TCA CTA CAC ATA ATA TAT	25
	VP3-Ur/GTA TCC AAT GGA TCC CAC GTC TCA	50
	VP3-GT1f/ATG ATT ATG AGA ATA ACA TAG TTT	21
	VP3-GT2f/AAA TAT AGA AGA TTA TTT ATT ACC A	16
NSP1	**NSP1-Uf**/GTT TGT CAA TGG TGT AGT CAA TAT	33
	NSP1-Ur/TAT GAT TTG ATG TCA CAT AAT TTG G	28
	NSP1-GT1f/CAC GTA ATT GTA GTG AAT TAT CTT C	32
	NSP1-GT2f/TAA TAA GTT TGC AAA CAC AAT TAA A	20
NSP2	**NSP2-Uf**/ATG GCT GAG CTA GCT TGC TTT TGT	46
	NSP2-Ur/CTT CAT CCA TCT TTC TAT CAG TTG	38
	NSP2-GT1r/ACA AGT TCT TTA ACA CAT ACA TC	30
	NSP2-GT2r/CAG CAT ATT TCC ATT TTT ATT ATT AC	23
NSP3	**NSP3-Uf**/CAA GAT GGA GTC TAC TCA GCA GAT	46
	NSP3-Ur/GGT TTT TGA CAG TGT TAG CTT TTA	33
	NSP3-GT1r/TAA AAA TGT TCT GTC ATA ATC TTG A	24
	NSP3-GT2r/TTA TAT ATT TGT AAT TCA TTG ATA	13
NSP5/6	**NSP5/6-Uf**/GCT GGC GTG TCT ATG GAT TCA TCA	50
	NSP5/6-Ur/GGT CAC AAA ACG GGA GTG GGG AGC	63
NSP4*	**NSP4sense**/GGC TTT TAA AAG TTC TGT TCC GAG	42
	NSP4anti/GGT CAC ATC AAG ACC ATT CC	50
	NSP4FW/GGA ATG GCG TAT TTT CC	47
	NSP4-A/TGT TCT TTG TAA CGT GTC	39
	NSP4-B/CTT GCG GTG AAG AGT TCG G	58
VP6**	**VP6-R**/GTC CAA TTC ATN CCT GGT GG	>50
	6EBG.303/AAY GTR TGT ATG GAT GAR ATG	>29
	3END.682c/GTM GTT AAM ACY CTD CGG	>39
	6END.1082c/ATA YTC TTG ACG YAC TGC G	>42

* Rodrìguez-Dìaz et al. (2008).
** Lin et al. (2008).

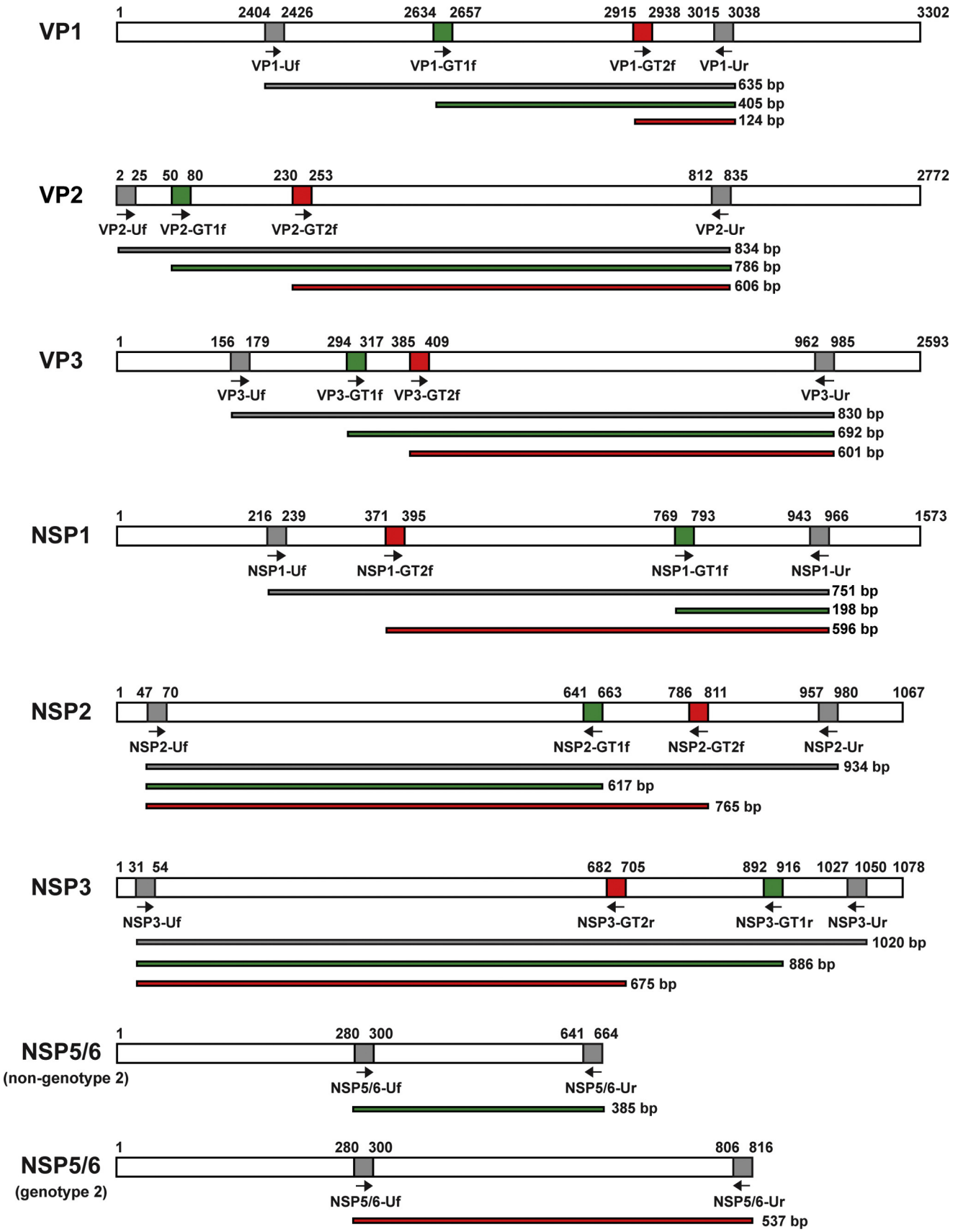

Fig. 1. Cartoon schematics of RV genes encoding VP1, VP2, VP3, NSP1, NSP2, NSP3, and NSP5/6. The figure shows the relative locations of the universal (gray boxes), genotype 1-(green box), and genotype 2-(red box) specific primers for each gene and is not drawn to scale. The nucleotide numbers corresponding to each primer are shown at the top of the schematics and primer orientation (forward or reverse) is indicated with an arrow. All nucleotide numbers are based on that of strain 61060, except for the VP2-coding gene (based upon VU06-07-1) and the genotype 2 NSP5/6-coding gene (based upon TB-Chen). Beneath each schematic are the expected products for PCRs using the different primers. Universal amplicons (gray lines), genotype 1 amplicons (green lines) and genotype 2 amplicons (red lines) are shown with sizes (in bps) listed to the right.

Table 3
Summary of RT-PCR/PCR results with the cell culture-adapted RVs.

RV gene type	# Genes amplified/# genes tested for each primer set[a]		
	Universal primers (RT-PCR)	Genotype 1-specific primers (multiplex PCR)[b,c]	Genotype 2-specific primers (multiplex PCR)[b,c]
Human genotype 1	**14/16**	**11/11**	0/11
Porcine genotype 1	**6/7**	**5/5**	0/5
Human genotype 2	**16/16**	0/15	**15/15**
Bovine genotype 2	**4/4**	0/4	**4/4**
Genotype 3	6/11	1/3	0/3
Genotype 6	0/1	not applicable	not applicable
Genotype 7	**1/1**	**1/1**	0/1

[a] Successful primer-template combinations are shown in bold.
[b] Excludes genes that failed to amplify with universal primers in RT-PCRs.
[c] Excludes the NPS5/6-coding gene, as the strategy for this gene relies on universal primers only.

regions of these genes are very AT-rich (Table 2, Fig. S1, and data not shown). Likewise, 8 of 12 genotype-specific primers exhibit GC contents within the optimal range, but the genotype-specific primers for the VP3- and NSP3-coding genes each have lower than optimal GC contents (16–24%) because the regions with the best inter-genotypic variability are also very AT-rich (Table 2, Fig. S1, and data not shown).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2013.08.025.

3.2. Testing the functionality and specificity of universal and genotype-specific primers

To validate the functionality of the designed primers, RT-PCRs were first performed using RNA templates derived from well-characterized, cell culture-adapted human strains for which full-genome sequences are known. These RVs have purely genotype 1 genes (strains Wa and ST3), purely genotype 2 genes (strain DS-1), or mixed genotype 1 and 2 genes (strain L26) (Fig. 2A) (Heiman et al., 2008; Matthijnssens et al., 2008). To test the extent of primer specificity, RT-PCRs were also performed using RNAs derived from human RVs that contain genotype 3 genes (strains AU-1 and A64), as well as RNA from animal RVs with genotype 1, 2, 3, 6, and/or 7 genes (porcine strain OSU and bovine strain UK) (Fig. 2A) (Heiman et al., 2008; Matthijnssens et al., 2008). In this experiment, RT-PCRs proceeded for 10 cycles, and the amplicons were not detectable following gel electrophoresis (data not shown). A very small amount (1/25 vol) of each universal RT-PCR was used as template in subsequent PCRs containing (i) only the universal primers or (ii) a nested universal primer and both genotype 1- and 2-specific primers. For the NSP5/6-coding gene, just the PCRs with universal primers were performed, since no genotype-specific primers were developed for this segment. The PCR products were electrophoresed in 2% agarose-TAE gels and visualized using ethidium bromide stain (Fig. 2B–H). The sizes of the amplicons, and therefore the putative genotypes of the genes, were determined using molecular weight standards. The bands were also excised from the gels and directly sequenced to confirm their identities (data not shown).

The universal primers efficiently amplified 14 of the 16 tested human RV genotype 1 genes and 16 of the 16 tested human RV genotype 2 genes by RT-PCR (Fig. 2 and Table 3). In fact, of the 32 human RV genotype 1 and 2 genes tested, the only two failures occurred with the universal primers: (i) the VP3-coding gene of strain ST3 and (ii) the NSP1-coding gene of strain Wa (Fig. 2D and E). The universal primers also amplified some of the more divergent RV genes, including: (i) 6 of the 7 tested genotype 1 genes from the porcine RV OSU, (ii) 5 of the five tested of the genotype 2 genes and 1 genotype 7 gene from the bovine strain UK, and (iii) 6 of the 11 genotype 3 genes from human or animal RVs (Fig. 2 and Table 3). As expected, for the NSP5/6-coding gene the larger 537-bp product was seen for the genotype 2 gene of strain DS-1 following universal primer amplification, while all other strains (with non-genotype 2 genes) only showed the smaller 385-bp product (Fig. 2H). A faint 385-bp product was also detected for the NSP5/6-coding gene of DS-1, suggesting that a cryptic or non-specific primer binding site may be present upstream of the 3′ UTR. Testing of additional laboratory strains suggest that this non-specificity is related only to the DS-1 virus, as no other tested genotype 2 gene produced lower molecular weight product (data not shown). Together, these results suggest that the universal primers recognize and amplify many human RV genotype 1 and 2 genes, as well as some non-genotype 1/2 genes and non-human RV genes, by RT-PCR.

For The NSP5/6-gene, the universal primer set was sufficient to genotype the human RV genes by RT-PCR. However, for the other six genes of this study, genotype-type specific primers were needed. Of the 11 human RV genotype 1 templates that were synthesized by the universal primers in RT-PCRs for these genes, all 11 were also correctly amplified by the genotype 1-specific primers in multiplex PCRs (Fig. 2 and Table 3). Likewise, all 15 of the human RV genotype 2 templates that were synthesized by the universal primers in RT-PCRs for these genes, all 15 were also correctly PCR-amplified by the genotype 2-specific primers (Fig. 2 and Table 3). Importantly, in the multiplex PCRs, there was no detectable cross-amplification of human RV genotype 1 genes with the genotype 2-specific primers and vice versa. However, as expected, there were some strain-to-strain differences in robustness of the PCRs. For example, due to intra-genotypic variation, strain L26 showed faint bands corresponding to genotype 2 amplicons for the VP3- and NSP1-coding genes, while strain DS-1 showed brighter bands (Fig. 2D–E, and S1). The genotype 1-specific primers, which were designed to recognize human RV genes, also amplified the porcine RV genotype 1 VP1-, VP2-, NSP1-, NSP2-, and NSP3-coding cDNAs of strain OSU (Fig. 2B, E–G, and Table 3). In a similar manner, the genotype 2-specific primers also recognized the bovine RV genotype 2 VP1-, VP2-, VP3-, and NSP2-coding templates from strain UK (Fig. 2B–D, F, and Table 3). The only non-genotype 1/2 genes that were amplified by the specific primers were the genotype 3 VP1- and VP2-coding genes of AU-1 and the genotype 7 NSP3-coding gene of strain UK (Fig. 2G and Table 3). This result suggests that, although the genotype-specific primers correctly differentiate genotype 1 vs. genotype 2 genes in multiplex PCR, they may not discriminate between human vs. animal genes of the same genotype nor would they differentiate genotype T1 vs. genotype T7.

3.3. Validation of approach with RVs in human fecal specimens

To further validate this PCR-based genotyping approach, viral RNA from 5 G1P[8]-positive and 5 G2P[4]-positive human fecal specimens were tested. In this experiment, the universal primers for the VP1-, VP2-, VP3-, NSP1-, NSP2-, NSP3-, and NSP5/6 genes

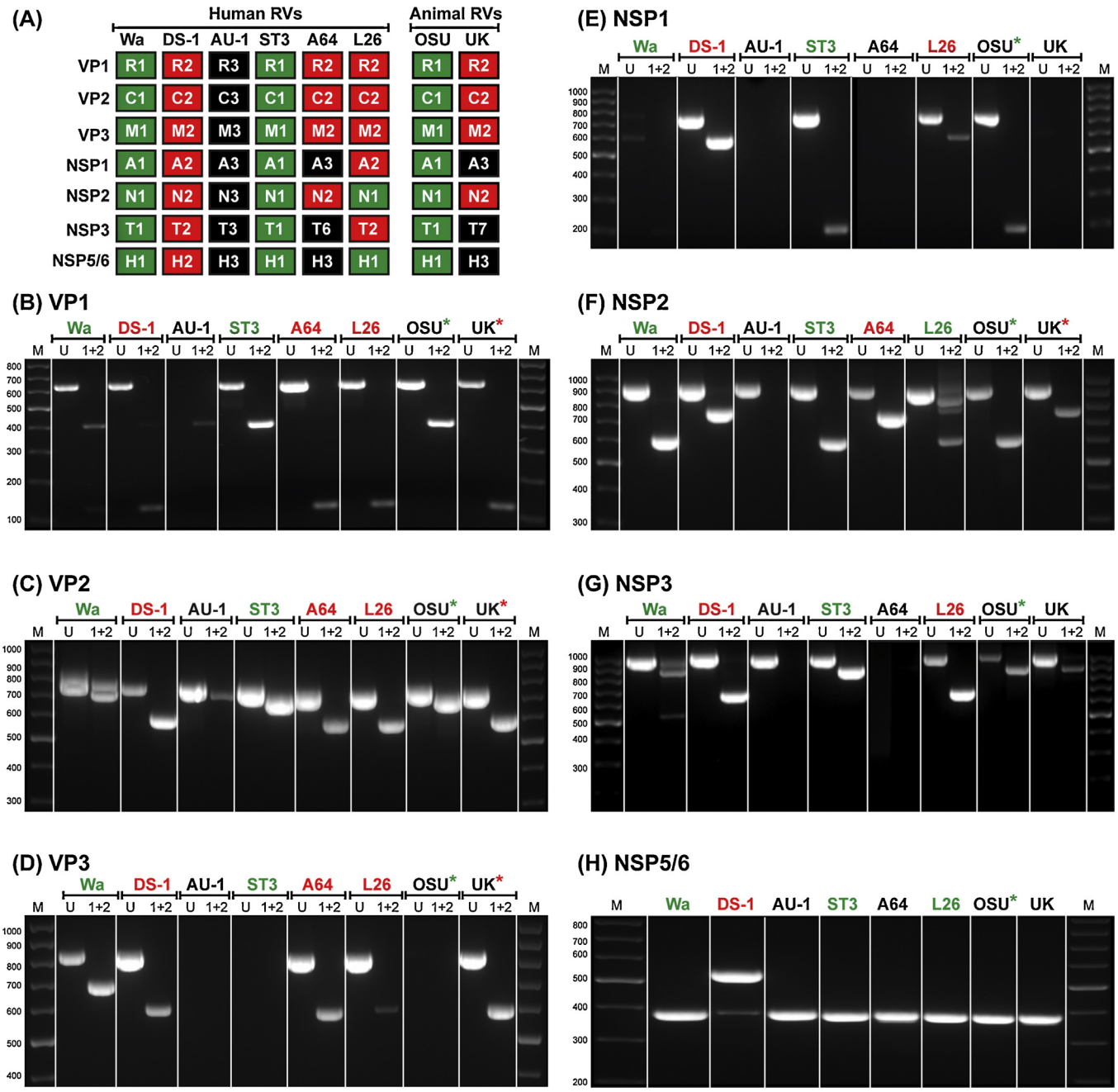

Fig. 2. Functionality and specificity of primers in RT-PCR/PCR using RNA from cell culture-adapted strains. (A) Genotype constellations of cell culture-adapted human or animal RVs. Genotype 1 genes are shown in green, genotype 2 genes are shown in red, and non-genotype 1/2 genes are shown in black. (B)–(H) Results of RT-PCR/PCR. Viral RNAs of various strains were converted to cDNAs and further amplified using universal primers and RT-PCR. These templates were then individually programmed into PCRs containing either the universal forward and reverse primers as a control (U) or a nested universal primer plus both the genotype 1- and 2-specific primers (1+2). Approximately 10 μl of the PCRs were electrophoresed in 2% agarose-TAE gels. A DNA ladder was used as a molecular weight marker and sizes (in bps) are listed to the left of each gel image. Strain names are listed at the top of each gel image. Green print represents human strains with genotype 1 genes, red print represents human strains with genotype 2 genes, and black print represents all other genes. The genotype 1 genes of the porcine RV strain OSU are noted by green asterisks. The genotype 2 genes of the bovine RV strain UK are noted by red asterisks.

were used simultaneously at the RT-PCR step, so as to improve the cost-effectiveness of the genotyping approach. Then, 1.0 μl (1/25 vol) of the RT-PCR was used as template for separate gene-specific PCRs containing (i) only the universal primers (data not shown) or (ii) a nested universal primer and both genotype 1- and 2-specific primers (Fig. 3). Again, for the NSP5/6-coding gene, the universal forward and reverse primers were used. The PCR products were electrophoresed in 2% agarose-TAE gels and visualized using ethidium bromide stain. The sizes of the amplicons, and therefore the putative genotypes of the genes, were determined using molecular weight standards. The bands were also excised from the gels and directly sequenced to confirm their identities (data not shown).

Following multiplex PCR, the G1P[8] RVs showed genotype 1-sized amplicons for the VP1-, VP2- VP3-, NSP1-, NSP2, NSP3-coding genes, and they showed a non-genotype 2-sized amplicon (385 bp) for the NSP5/6-coding gene (Fig. 3 and data not shown). In contrast, the G2P[4] RVs showed genotype 2-sized amplicons for genes encoding VP1, VP2, VP3, NSP1, NSP3 and NSP5/6 genes (Fig. 3 and data not shown). In contrast to what

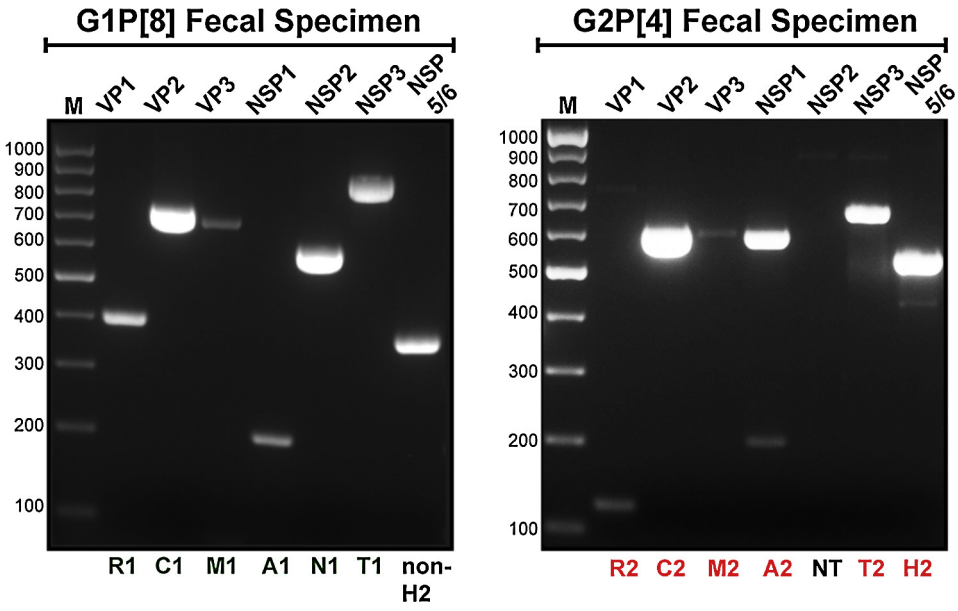

Fig. 3. Optimizing the cost-effectiveness of PCR genotyping and validation of the method with RVs from human fecal specimens. Viral RNA was converted into cDNAs and further amplified using all seven sets of universal primers in a single RT-PCR. These templates were then individually programmed into PCRs containing both the genotype 1- and 2-specific primers. PCRs were electrophoresed in 2% agarose-TAE gels. A DNA ladder was used as a molecular weight marker and sizes (in bps) are listed to the left of each gel image. The deduced genotype of each gene, based on the size of the PCR amplicon, is listed on the bottom of each gel image. Putative genotype 1 genes listed in green and putative genotype 2 genes are listed in red. The genotype 2 NSP2-coding gene was non-typeable (NT) using this combined approach.

was seen for the NSP5/6-coding gene of the cell culture-adapted G2P[4] strain DS-1, those of the clinical specimens only produced a larger 537-bp product following RT-PCR with the universal primers. Unfortunately, the two genotype 2-specific primers for VP3 and NSP2 did not work well when using the combined RT-PCR approach (Fig. 3 and data not shown). This result suggests that single-gene reactions may need to be done or that these particular primers may need further optimization following future field testing. Nonetheless, the cumulative results suggest that the genotype-specific primers recognize and appropriately amplify the genes of RVs in clinical fecal specimens and that combining several primers at the RT-PCR step may be used to reduce the cost of genotyping.

4. Discussion

Ongoing molecular epidemiological studies seek to gain insight into the diversity and evolutionary dynamics of RVs circulating at various geographical locations. RT-PCR/multiplex PCR is regarded as an appropriate high-throughput and cost-effective method for typing human strains in these studies (Fischer and Gentsch, 2004). Usually, the VP7- and VP4-coding genes (and occasionally the VP6- and NSP4-coding genes) of RVs in fecal specimens are assigned genotypes according to their PCR-amplification by specific primer sets (Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994; Iturriza-Gómara et al., 2004; Fischer and Gentsch, 2004; Lin et al., 2008; Rodrìguez-Dìaz et al., 2008; Thongprachim et al., 2010). In the current study, the RT-PCR/PCR-based genotyping approach was extended to the remaining seven RV genes (encoding VP1, VP2, VP3, NSP1, NSP2, NSP3, and NSP5/6), allowing for rapid differentiation of genotype 1 vs. genotype 2 genes. The impetus for developing these primers was to gain a more comprehensive understanding of the frequency of human RVs showing mixed genotype constellations (i.e., with both genotype 1 and genotype 2 genes).

Universal forward and reverse primers designed against conserved gene regions were effective at amplifying the vast majority of tested human RV genotype 1 and 2 genes by RT-PCR. The universal primers were also able to amplify some other genes, in addition to human RV genotype 1 or 2 genes. In particular, the universal primers worked well on the genotype 1 and 2 genes of the animal RVs, strains OSU and UK, respectively. The universal primers also amplified C3, N3, T3, H3, and T7 genes from culture-adapted RV strains. Because genotype 3-specific primers have not yet been developed for the VP2-, NSP2-, NSP3- and NSP5/6 genes, the fact that the universal primers recognize them in RT-PCRs may be beneficial to investigators. Indeed, the control RT-PCR reactions for these rare genes could be directly sequenced to determine their genotype. Of the cell culture-adapted human RV strains, only two failures were found: (i) the VP3-coding gene of from strain ST3 (genotype M1) and (ii) the NSP1-coding gene of strain Wa (genotype A1). The universal reverse primers for these genes each show 4 nucleotide mismatches when compared to the sequences of ST3 or Wa, which may explain the lack of RT-PCR products (Fig. S1). Nonetheless, the universal primers for the VP3- and NSP1-coding genes closely match those of other M1 or A1 sequences, and they proved effective on RVs from primary fecal specimens. As such, it is expected that the universal primers for the VP3- and NSP1-coding genes will be useful in epidemiological surveillance studies.

Genotype 1- and 2-specific primers, which were designed against gene regions that varied inter-genotypically but not intra-genotypically, were found to discriminate between human RV genotype 1 vs. 2 templates in multiplex PCRs. Importantly, in the multiplex PCRs, there was no detectable cross-amplification of human RV genotype 1 templates with the genotype 2-specific primers and vice versa. The genotype 1- and 2- specific primers designed here also recognized many of the cognate genotype genes of the animal RV strains OSU and UK. Thus, while human and animal genes sharing the same genotype can often be discerned phylogenetically based upon nucleotide sequences, they would not be differentiated by multiplex PCR with the primers designed in this study (McDonald et al., 2011). Likewise, the genotype 7 NSP3-coding gene of strain UK was recognized by the genotype 1-specific primer; this result is not surprising given that there are only 3 nucleotide mismatches between their sequences (data not shown). Therefore, for the NSP3-coding gene, the current primer sets would not distinguish among human RV genotype 1, porcine RV genotype 1 and bovine RV genotype 7. A similar result was found

for the genotype 3 VP2-coding gene of strain AU-1, which was recognized by the genotype 1 specific primers. However, the primer sets easily discriminate genotype 1-like genes from genotype 2 genes and, thus, will be useful in studies aimed at understanding the prevalence of RVs with mixed genotype 1 and 2 constellations.

Some strain-to-strain differences in robustness of the PCRs were noticed for a few genotype-specific primers. For example, strain L26 showed faint bands corresponding to genotype 2 amplicons for the VP3- and NSP1-coding genes, while other strains (e.g., DS-1) showed brighter bands. Sequence analysis suggests that the weak amplification for L26 gene may reflect very modest intra-genotypic variation at the primer design site (Fig. S1). Indeed, the genotype-specific primers for the VP3-coding gene consistently produced faint bands with the RVs from clinical fecal specimens; conversely, the amplicons for all other genes, including those for the NSP1-coding gene were fairly robust (Fig. 3 and data not shown). It is expected that the primers designed in this study will be functional in discriminating genotype 1 vs. genotype 2 genes of human RVs, but that some may require further optimization following rigorous field testing.

When combined with the previously described primers sets for the VP7-, VP4-, VP6- and NSP4-coding genes, the new primers developed in this study will enable the full-genome genotyping of the most common human RVs using RT-PCR/multiplex PCR (Gouvea et al., 1990; Gentsch et al., 1992; Das et al., 1994; Iturriza-Gómara et al., 2004; Fischer and Gentsch, 2004; Lin et al., 2008; Rodrìguez-Dìaz et al., 2008; Thongprachim et al., 2010).

It is estimated that, using this approach, the genotype constellations of human RVs could be deduced by RT-PCR/PCR for less than $10 USD/specimen, whereas nucleotide sequencing would cost upwards of $150 USD/specimen. Thus, this RT-PCR/multiplex PCR genotyping approach could reasonably be employed to screen fecal specimens for the presence of RVs containing mixed constellations of genotype 1 and 2 genes on a very-large scale (>1000 specimens). Nonetheless, it is important to note that this approach is meant to complement, but not replace, the genetic analysis of human RVs using nucleotide sequencing. PCR-based genotyping should simply be used as a facile and inexpensive way of screening RVs to identify representative strains for downstream nucleotide sequencing. It is recommended that nucleotide sequencing be used to confirm the genotypes of human RVs in cases of non-typeable genes, or whenever evidence of cross-amplification by genotype-specific primers is seen, as this might indicate a co-infected specimen.

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