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Multiplexed one-step RT-PCR VP7 and VP4 genotyping assays for rotaviruses using updated primers☆

Mathew D. Esona[a,*], **Rashi Gautam**[a], **Ka Ian Tam**[a], **Alice Williams**[b], **Slavica Mijatovic-Rustempasic**[a], and **Michael D. Bowen**[a]

[a]Gastroenteritis and Respiratory Viruses Laboratory Branch, Division of Viral Diseases, NCIRD, Centers for Disease Control and Prevention, Atlanta, GA 30329, USA

[b]Furman University, Greenville, SC, USA

Abstract

The current two-step VP7 and VP4 genotyping RT-PCR assays for rotaviruses have been linked consistently to genotyping failure in an estimated 30% of RVA positive samples worldwide. We have developed a VP7 and VP4 multiplexed one-step genotyping assays using updated primers generated from contemporary VP7 and VP4 sequences. To determine assay specificity and sensitivity, 17 reference virus strains, 6 non-target gastroenteritis viruses and 725 clinical samples carrying the most common VP7 (G1, G2, G3, G4, G9, and G12) and VP4 (P[4], P[6], P[8], P[9] and P[10]) genotypes were tested in this study. All reference RVA strain targets yielded amplicons of the expected sizes and non-target genotypes and gastroenteritis viruses were not detected by either assay. Out of the 725 clinical samples tested, the VP7 and VP4 assays were able to assigned specific genotypes to 711 (98.1%) and 714 (98.5%), respectively. The remaining unassigned samples were re-tested for RVA antigen using EIA and qRT-PCR assays and all were found to be negative. The overall specificity, sensitivity and limit of detection of the VP7 assay were in the ranges of 99.0–100%, 94.0–100% and 8.6×10^1 to 8.6×10^2 copies of RNA/reaction, respectively. For the VP4 assay, the overall specificity, sensitivity and limit of detection assay were in the ranges of 100%, 94.0–100% and ≤1 to 8.6×10^2 copies of RNA/reaction, respectively. Here we report two highly robust, accurate, efficient, affordable and documentable gel-based genotyping systems which are capable of genotyping 97.8% of the six common VP7 and 98.3% of the five common VP4 genotypes of RVA strains which are responsible for approximately 88.2% of all RVA infections worldwide.

Keywords

Rotavirus; VP7 and VP4 genes multiplexed gel-based genotyping; Updated primers

☆The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Names of specific vendors, manufacturers, or products are included for public health and informational purposes; inclusion does not imply endorsement of the vendors, manufacturers, or products by the Centers for Disease Control and Prevention or the US Department of Health and Human Services.

*Corresponding author at: 1600 Clifton Rd, NE, MSG-04, Atlanta, GA 30333, USA. mdi4@cdc.gov (M.D. Esona).

Conflicts of interest

No potential conflicts of interest were disclosed.

1. Introduction

Group A rotaviruses (RVA) are important pathogens that cause severe diarrheal disease in children less than five years of age, leading to severe dehydration and often death (Estes and Kapikian, 2007; Parashar et al., 2009). Recent reports revealed an estimated 453,000 preventable pediatric fatalities in low-income countries of Africa and Asia attributable to RVA (Tate et al., 2012). In industrialized nations, RVA infection rarely results in death, but remains the most common cause of hospitalizations for acute gastroenteritis in children and leads to major medical and societal costs. For example, in the United States alone, approximately 50,000 children are hospitalized and nearly one billion US dollars in societal costs can be attributed to RVA infections annually (Parashar et al., 1998; Payne et al., 2011).

RVA 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). The viral genome encodes for six structural proteins (VP1-VP4, VP6 and VP7) and five or six non-structural proteins (NSP1-NSP5/NSP6) (Estes and Kapikian, 2007). Encompassing the dsRNA are 3 protein layers, a central core (VP2), an inner protein layer (VP6), and an outer layer comprised of VP7 and VP4 proteins (Estes and Kapikian, 2007). The traditional binomial classification of RVA is based upon serotype/genotype specificities and the sequence diversity of the 2 outer proteins, VP7 (glycosylated, G-type) and VP4 (protease-sensitive, P-type) (Estes, 1996). These two outer capsid proteins, VP7 and VP4, contain multiple antigenic epitopes that can induce the production of neutralizing antibodies, which are a primary target for vaccine development (Estes, 1996; Estes and Kapikian, 2007). To date, at least 27 G- and 37 P-genotypes have been recognized and approximately 73 G/P genotype constellations of RVAs infecting humans have been reported (Matthijnssens et al., 2009, 2011; Trojnar et al., 2013). Of all possible combinations of RVA, 6 genotypes (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and to a lesser but increasing extent G12P[8], are currently the most important genotypes in humans worldwide and are associated with an estimated 80–90% of the RVA disease burden (Banyai et al., 2012; Iturriza-Gomara et al., 2011; Matthijnssens et al., 2009, 2010).

Currently, routine characterization of RVA into G- and P-genotypes in human stool and environmental samples is performed by molecular methods (Das et al., 1994; DiStefano et al., 2005; Gentsch et al., 1992; Gouvea et al., 1990; Iturriza-Gomara et al., 2004; Simmonds et al., 2008). Immunoassay characterization using monoclonal or polyclonal antibodies (Beards et al., 1984; Coulson et al., 1987; Taniguchi et al., 1987; Ward et al., 1991) is used rarely today. In the last two and a half decades, at least four conventional two-step multiplexed RT-PCR strategies for RVA G-typing (Das et al., 1994; Gouvea et al., 1990; Iturriza-Gomara et al., 2004; Taniguchi et al., 1992) and two for P-typing (Gentsch et al., 1992; Simmonds et al., 2008) have been developed and used widely. In addition, the use of assays such as restriction fragment length polymorphism analysis (Iturriza Gomara et al., 2002), sequence based analysis (Barman et al., 2004; DiStefano et al., 2005), multiplexed capture and type-specific primer extension (Lovmar et al., 2003), and hybridization to oligonucleotide probes (Santos et al., 2008; van Doorn et al., 2009) for RVA genotyping have been described. These genotyping assays have been invaluable in defining the importance of individual RVA G and P-types. They have been used to genotype

approximately 110,000 strains, and also have highlighted RVA genetic diversity (Bányai et al., 2012; Gentsch et al., 2005; Matthijnssens et al., 2009; Seheri et al., 2014). On a global level, however, recent issues with the effectiveness of these typing assays have been identified. First, as a result of genetic drift, genotype-specific PCR primers have increasingly failed to amplify the VP7 and VP4 genes of globally common strains, including those of the most common strain G1P[8] (Adah et al., 1997; Banyai et al., 2005; Cunliffe et al., 2001; Iturriza-Gomara et al., 2000; Parra and Espinola, 2006). Secondly, increasing numbers of studies have reported detection of novel human RVA G and P types, for example G12 and P[14], which are not typeable because genotype-specific primers are absent from the multiplexed primer mixtures (Banyai et al., 2003, 2004; Cooney et al., 2001; Iturriza Gomara et al., 2004; Rahman et al., 2005; Santos et al., 1998; Solberg et al., 2009). Finally, these RT-PCR assays occasionally mis-classify strains due to cross-priming of one genotype-specific primer to another genotype (Aladin et al., 2010; Esona et al., 2010b; Mitui et al., 2012).

Currently, two live-attenuated oral RVA vaccines, Rotarix (GlaxoSmithKline Biologicals, Belgium) and RotaTeq (Merck & Co., Inc., United States), have been licensed in more than 100 countries and are being introduced into routine immunization programs in the United States and other countries in Latin America, Europe, Africa and Asia (Armah et al., 2010; Benhafid et al., 2012; Cunliffe et al., 2012; Madhi et al., 2012; Ruiz-Palacios et al., 2006; Seheri et al., 2012; Vesikari et al., 2007a,b). Countries considering using these vaccines to reduce RVA disease have introduced strain surveillance programs to provide strain prevalence data in the pre-vaccine era and to judge the impact of the vaccine after introduction. When these new vaccines become widely used, surveillance will be important to determine if some strains escape immunity induced by the vaccines, whether rare strains emerge and if vaccine strains reassort in humans or circulate in children. This has raised the need for a robust, accurate, efficient, affordable and documentable typing system. The gel-based multiplexed one-step RT-PCR protocol developed here has all the above-mentioned characteristics. First, the gel-based RT-PCR is low cost or affordable and does not require specialized equipment and highly-trained laboratory personnel. Secondly, the one-step RT-PCR approach developed and validated in this study involves fewer manipulation steps to obtain RVA genotype data compared to previously-developed genotyping assays, thus reducing chances of cross-contamination. Previously described RT-PCR plus hemi-nested PCR assays are prone to cross-contamination because of the greater number of manipulation steps involved and innate proclivity of hemi-nested PCR to produce spurious bands. Thirdly, these assays use primers designed from contemporary VP4 and VP7 sequences. Therefore with the above-mentioned advantages, these one-step RT-PCR assays will be more beneficial in the developing countries, and especially in those countries with few trained personnel and limited laboratory space. Hence, the primary aim of the study is to develop and validate novel RVA VP7 and VP4 genotyping assays, using a multiplexed one-step Reverse transcriptase-PCR protocol with updated primers that specifically detects the most common RVA G-genotypes (G1, G2, G3, G4, G9 and G12) and P-genotypes (P[4], P[6], P[8], P[9] and P[10]) in human stool.

2. Materials and methods

2.1. Ethics statement

For domestic surveillance samples, institutional review board approvals were obtained from the CDC and from individual study sites. All clinical samples tested in this study were de-identified so they could not be linked back to cases.

2.2. Test samples

2.2.1. Reference strains—For reference RVA VP7 and VP4 genotypes, seventeen reference laboratory strains propagated in MA104 cells were used: Wa (G1P[8]), DS-1 (G2P[4]), P (G3P[8]), ST3 (G4P[6]), 116E (G9P[11]), US1205 (G9P[6]), WI61 (G9P[8]), L26 (G12P[4]), 1076 (G2P[6]), AU-1 (G3P[9]), 69M (G8P[10]), RRV (G3P[3]), SA11 (G3P[2]), CC425 (G3P[9]), RO1845 (G3P[9]), WC3 (G6P[5]) and OSU (G5P[7]).

2.2.2. Clinical samples—A total of 725 clinical stool samples, consisting of 658 rotavirus EIA positives with known VP7 and VP4 genotypes and 67 rotavirus EIA negatives were included in this study. The VP7 and VP4 genotypes were previously determined by using two-step G and P genotyping assays (Das et al., 1994; Gentsch et al., 1992) and 644 were confirmed by sequencing. The samples consisted of VP7 genotypes G1 (100); G2 (145); G3 (78); G4 (24); G9 (70); and G12 (228); and VP4 genotypes P[8] (460); P[4] (131); P[6] (50); P[9] (2); and P[10] (2). Samples with known RVA mixed G and P types ($n = 13$) were also included. All clinical stool samples were obtained from routine domestic and international RVA surveillance conducted by CDC. RotaTeq vaccine components and accession numbers (G1, GU565057; G2, GU565068; G3, GU565079; G4, GU565090; G6, GU565046 and P[5], GU565055; P[8], GU565044) and Rotarix vaccine components and accession number (G1, JX943614 and P[8], JX943612) were also analyzed.

2.2.3. Non-RVA target—A total of six non-RVA targets including rotavirus group B (RVB), rotavirus group C (RVC), adenovirus (types 40 and 41) and norovirus (genotypes GI. 3b and GII.4) were also analyzed.

2.3. RNA extraction of test samples

RNA from 17 reference virus strains, 725 clinical samples and non-RVA targets were extracted using the MagMax Viral RNA Isolation kit (Life Technologies, New York, NY) on the automated KingFisher extraction platform (Thermo Electron Corporation, Vantaa, Finland), the MagNA Pure Compact RNA Isolation Kit on the automated MagNA Pure Compact Instrument (Roche Applied Science, Indianapolis, IN, USA) and the Viral NA Large Volume Kit II on the automated MagNA Pure 96 instruments (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. All extracted nucleic acids were stored at −80 °C until analyzed.

2.4. Primer design

To generate a consensus sequences for the most common RVA VP7 genotypes (G1, G2, G3, G4, G9 and G12) and VP4 genotypes (P[4], P[6], P[8], P[9] and P[10]), a total of 1800 gene sequences with known lineages and sub-lineages and years of isolation/detection/submission

from 1983 to 2012 were obtained from the GenBank database. Four hundred of these sequences were representatives of known lineages and sub-lineages of the five common VP4 genotypes (Donato et al., 2012; Magagula et al., 2014; Nakagomi et al., 2012; Nyaga et al., 2014), while 1400 were representatives of known lineages and sub-lineages of the six common VP7 genotypes (Donato et al., 2012; Esona et al., 2013; Magagula et al., 2014; Nakagomi et al., 2012; Nyaga et al., 2014; Stupka et al., 2009, 2012). The consensus sequences obtained from multiple alignments of the six most common RVA VP7 genotypes (G1, G2, G3, G4, G9 and G12) and five most common VP4 genotypes (P[4], P[6], P[8], P[9] and P[10]) were used to design oligonucleotide primers for specificVP7 and VP4 genotypes, respectively. Because the sequences of strains with VP4 P[9] and P[10] genotypes from GenBank were very conserved, the P[9] and P[10] antisense primers previously described (Gentsch et al., 1992) were incorporated into the VP4 genotyping assay. Candidate VP7 and VP4 universal forward primers and multiple primer sets of VP7 and VP4 genotype-specific reverse primers were designed manually. Degenerate bases were introduced into the primer sequences to account for sequence variation observed in sequence alignments of the above-mentioned VP7 and VP4 genotypes. The primer sequences were checked for specificity using NCBI-Nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were checked for self-annealing sites, hairpin loop formation and 3′ complementarity using the IDT oligonucleotide calculator (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). For primers with melting temperatures (Tm) in the range of 30–40 °C, the Tm's were increased to 45–50 °C, using AP-dC (G-clamp) base analogs as required (Glen Research, VA, USA). All primers were synthesized at the Biotechnology Core Facility, Centers for Disease Control and Prevention and purified by high-performance liquid chromatography (HPLC).

2.5. Screening, optimization, and validation of VP7 and VP4 genotyping assays

Multiple forward and reverse primer sets designed for VP7 genotypes (G1, G2, G3, G4, G9 and G12) and VP4 genotypes (P[8], P[4], P[6], P[9] and P[10]) were screened using RVA cultured strains bearing the common VP7 and VP4 genotypes, as well as stool samples including G1P[8], G1P[6], G1P[4], G2P[4], G2P[6], G2P[8], G3P[8], G3P[6], G4P[8], G4P[4], G4P[6], G9P[8], G9P[4], G9P[6], G12P[8], G12P[6], G12P[4] and other genotypes in circulation. For both the VP7 and VP4 multiplexed conventional one-step RT-PCR assays, the primer sets with the best sensitivity and specificity for detecting the genotypes of each of the RVA cultured strains were selected for optimization. Primers for each genotype were also selected based on the predicted amplicon size generated, which in tend could be used to identify individual RVA genotypes. Selected VP7 and VP4 primer sets were optimized by performing each assay at several primer concentrations in the range of 0.5–6 μM. The primer concentrations showing amplification of its respective template without non-specific amplification or cross reactivity were selected for subsequent development. The optimized VP7 and VP4 multiplexed one-step RT-PCR genotyping assays were then validated using reference laboratory strains and clinical samples. The sequences, nucleotide positions and expected amplicon sizes of the selected forward and reverse primers for both VP7 and VP4 assays are shown in Table 1.

2.6. VP7 and VP4 multiplexed one-step RT-PCR genotyping assays

A conventional multiplexed one-step RT-PCR was performed on purified RNA using the one-step RT-PCR kit (QIAGEN, Inc., Valencia, CA) as per the manufacturer's recommendations with slight modification. In brief, the final reaction volume was changed from 50 μl to 30 μl while keeping the concentrations of the other reagent within the recommended range. For the VP7 multiplexed reaction, a single universal forward primer whose sequence is conserved among the VP7 genes of genotypes G1, G2, G3, G4, G9 and G12 and a cocktail of six reverse amplification primers specific and complementary to variable regions of the VP7 genes of the same genotypes were used. The forward primer was used at a final molarity of 6 μM, while the genotype-specific primers were used at a final molarity of 1 μM each. For the VP4 assay, a single forward primer at final molarity of 5 μM and a cocktail of five reverse amplification primers specific and corresponding to variable regions of genotypes P[4], P[6], P[8], P[9], and P[10] at final molarity of 1 μM each was used. After denaturation of the RNA at 97 °C for 5 min on a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Inc., Foster City, CA), the reactions were incubated on ice until reverse transcription. RT and amplification was performed using the following parameters: 50 °C for 30 min; 95 °C for 15 min then 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min, repeated for a total of 25–35 cycles (depending on the EIA OD value of the sample); 72 °C for 7 min; and then 4 °C hold. RT-PCR genotyping product was electrophoresed on 3% agarose gels containing GelRed (Biotium, Heyward, CA, USA) for 2 h at 100 V and products were detected under UV transillumination or were analyzed on the LabChip® GX instrument (Caliper Life Sciences, MA, USA) using a HT DNA 1K reagent kit (Dual protocol DNA Analysis and Quantitation) with the HT DNA Extended Range LabChip (Caliper Life Sciences, MA, USA).

2.7. Determination of limit of detection (LOD) of the VP7 and VP4 conventional multiplexed one-step RT-PCR genotyping assays

The LODs for the VP7 and VP4 genotyping assays were determined using stool samples with known G and P genotypes (G1P[8], G2P[4], G3P[8], G4P[6], G9P[8] and G12P[8]) and high viral antigen concentration (OD values ranging from 2.5 to 3.0). For VP4 genotypes P[9] and P[10], laboratory culture strains AU-1 (G3P[9]) and 69M (G8P[10]) were used. These samples were serially diluted 10^{-1} to 10^{-9} in a RVA-negative stool suspension and then re-tested by Premier™ Rotaclone® EIA (Meridian Bioscience, Inc., Cincinnati, OH, USA). RNA was extracted and tested by NSP3 qRT-PCR (Mijatovic-Rustempasic et al., 2013) and the new VP7 and VP4 multiplexed one-step RT-PCR genotyping assays.

2.8. qRT-PCR assay

In order to establish the LODs, 10-fold dilution of the NSP3 dsRNA transcript positive control (10^{-4} to 10^{-12}) were prepared in DEPC-treated water containing 100 ng/μl yeast carrier RNA (Ambion, Austin, TX) and tested by NSP3 qRT-PCR assay as described previously (Mijatovic-Rustempasic et al., 2013). A standard curve was generated by plotting the log of copy numbers against cycle threshold (C_t) value and copy numbers calculated as described previously (Mijatovic-Rustempasic et al., 2013). It has been reported that each

RVA virion contains equimolar amounts of each of the 11 segments (McDonald and Patton, 2011; Patton, 1990), hence the copy numbers of NSP3 gene per reaction obtained were assumed to be equivalent to that of the VP7 and VP4 genes. To determine the LODs for the diluted stool samples, a dsRNA transcript dilution served as a standard in each assay. Each template and non-template control (NTC) was tested in duplicate. The average C_t value for each reaction was determined and copy numbers were calculated as previously described (Mijatovic-Rustempasic et al., 2013).

2.9. Evaluation of alternative RT-PCR kits

The RNA extracts from each diluted sample were tested by the new G and P one-step RT-PCR assays using three commercially available one-step RT-PCR kits according to manufacturer's instruction with slight modification. In brief, the final reaction volume was changed from 50 μl to 30 μl while keeping the concentrations of the other reagent within the recommended range. These kits include Qiagen one-step RT-PCR kit (Qiagen, Germantown, MD, USA), MyTaq™ one-step RT-PCR kit (Bioline, Taunton, MA, USA) and SuperScript® III one-step RT-PCR System with Platinum® *Taq* High Fidelity DNA Polymerase (Life Technologies, Grand Island, NY, USA). All diluted stool samples were tested using the new VP7 and VP4 one-step RT-PCR assays as described above. The primer concentrations and the cycling conditions were the same for all three commercial kits. The VP7 (G) and VP4 (P) RT-PCR amplicons were analyzed by electrophoresis of the amplified products in 3% agarose gels containing GelRed (Biotium, Heyward, CA, USA) and products were detected under UV transillumination. The LODs were determined as the lowest level of stool dilution that yielded an amplification product and a correct genotype determination.

2.10. Assay performance calculations

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each assay were calculated using standard procedures.

3. Results

3.1. Limit of detection (LOD)

Prior to extraction of these diluted samples, EIA results show that all the OD values were below the cut-off OD value of 0.150 and thus below the sensitivity limits of 5.0×10^5 particles/ml as reported in the kit instructions.

Extracts from serially diluted stool samples were analyzed for the presence of the template using the new G and P conventional multiplexed one-step RT-PCR genotyping assays and the NSP3 qRT-PCR assay (Mijatovic-Rustempasic et al., 2013). For the conventional RT-PCR assays, analysis by gel electrophoresis revealed a single, genotype-specific amplicon of the expected size for all RVA samples (data not shown). For the VP7 genotyping assay, the first 6 out of 9 dilutions had amplifiable template for genotypes G2, G3, G4, and G12 and the LODs were 8.6×10^2, 8.6×10^2, 5×10^2 and 8.6×10^1 copies of NSP3 RNA per reaction, respectively (Table 2). Genotypes G1 and G9 had amplifiable template in the first 5 out of 9 dilutions corresponding to a LODs of 3.8×10^2 and 2.2×10^2 copies of NSP3 RNA per reaction, respectively (Table 2). Interestingly, the VP7 assay was able to detect a

genotype G1 (LOD = 5.2×10^1 copies of NSP3 RNA per reaction) in a single sample previously reported as having a genotype G9 only. For the VP4 assay, the first 6 out of 9 dilutions had amplifiable template for genotypes P[4], P[6] and P[10] and with LOD of 8.6×10^2, 8.6×10^2 and ≤1 copies of NSP3 RNA per reaction, while genotypes P[8] and P[9] had amplicon in 5 (LOD = 3.8×10^2 copies of NSP3 RNA per reaction) and 4 (LOD = 8.2×10^2 copies of NSP3 RNA per reaction) out of 9 dilutions, respectively (Table 2). The three commercial one-step RT-PCR kits, Qiagen one-step RT-PCR kit (Qiagen), MyTaq™ one-step RT-PCR kit (Bioline) and SuperScript® III one-step RT-PCR System with Platinum® *Taq* High Fidelity DNA Polymerase (Life Technologies) produced equivalent results.

3.2. Testing of reference virus strains using the VP7 and VP4 conventional multiplexed one-step RT-PCR genotyping assays

To evaluate these 2 genotyping assays, we assembled a panel of 17 characterized reference RVA strains carrying the most common VP7 and VP4 genotypes (see strain list in Section 2). Analysis of these reference laboratory strains was carried out by multiplexed one-step RT-PCR amplifications. For the VP7 assay, use of a single universal forward (designated as VP7uF) and genotype-specific reverse primers for G1, G2, G3, G4, G9 and G12 (designated as G1-R4, G2-R4, G3-R1, G4-R2, G9-R2 and G12-R2) yielded visible dsDNA products of the predicted sizes for all 14 of the target strains tested and did not detect any of the non-target (G5, G6, G8) strains (Fig. 1A). Also, for VP4, use of a single universal forward (designated as VP4uF) and genotype-specific reverse primers for P[8], P[4], P[6], P[9] and P[10] (designated as P[8]-R2, P[4]-R5, P[6]-R2, P[9]-4T-1 and P[10]-5T-1) yielded visible dsDNA products of the predicted sizes for 11 of the target strains tested and did not detect any of the non-target (P[2], P[3], P[5], P[11]) strains (Fig. 1B). For the VP7 assay, the absence of a product band for strains OSU, WC3 and 69M, (Fig. 1A, lanes 16, 17 and 18) was due to the fact that G5, G6 and G8 specific primers were not included in this assay and for the VP4 assay, the absence of product bands in lanes 12–17 of Fig. 1B was because primers specific for these VP4 genotypes were not included in this assay.

3.3. Clinical samples

A summary of the results obtained from the 725 clinical samples with both the VP7 and VP4 conventional multiplexed one-step RT-PCR genotyping assays is presented in Table 3. For the VP7 assay, a total of 711 (98.1%) of the clinical samples could be genotyped and in 14 (1.9%) of the samples a genotype could not be assigned. Further investigation revealed that 13 of these 14 samples initially genotyped as G1 ($n = 1$), G2 ($n = 5$), G3 ($n = 5$), and G9 ($n = 1$) and not sequence confirmed, had negative EIA OD values in the range of 0.044–0.046. One of the samples was EIA positive but RNA was completely degraded as determined by polyacrylamide gel electrophoretic (PAGE) RNA analysis. The G4 ($n = 24$) and mixed ($n = 13$) samples used in this assay were all identified. For the VP4 assay, a total of 714 (98.5%) samples were genotyped, while 11 (1.5%) samples that were previously genotyped as P[4] ($n = 5$), P[8] ($n = 3$) and P[6] ($n = 3$) could not be genotyped. These 11 samples had negative EIA values in the range of 0.044–0.046 and were in the same group of negative EIA samples mentioned above. Also, the P[9] ($n = 2$) and P[10] ($n = 2$) samples, as well as the mixed P types ($n = 10$) subjected to this assay were all correctly identified. For VP7, the mixed genotypes were mostly G2/G12, G4/G12, G1/G9, G2/G3, G1/G3/G4 (RotaTeq vaccinee

stool), G2/G3, and G1/G3/G4 (RotaTeq vaccine components), while P[4]/P[8] and P[6]/P[8] were the most common mixed VP4 genotypes seen (Fig. 1C). When RNAs extracted from the RotaTeq and Rotarix vaccines and from RotaTeq vaccinees were tested using the VP7 and VP4 assays, mixed genotypes G1/G3/G4 were detected from both RotaTeq extracts and a G1 was detected in Rotarix extract. The VP4 assay was able to detected the P[8] genotype from all three extracts. However, irrespective of targeted G and P genotypes, the VP7 and VP4 assays exhibited 94–100% sensitivity and 99–100% specificity with a PPV of 100% and NPV of 99.1–100% (Table 3).

4. Discussion

RT-PCR has been considered the method of choice for genotyping of RVA and thereby, regarded as the gold standard (Fischer and Gentsch, 2004). For this purpose, in 1990, the multiplexed hemi-nested RT-PCR based G genotyping assay was developed by Gouvea et al. (1990) and later other sets of G genotyping primers were published by other researchers (Das et al., 1994; Gouvea et al., 1990; Iturriza-Gomara et al., 2004). In 1992, the complimentary P genotyping multiplexed hemi-nested RT-PCR based assay was developed by Gentsch et al. (1992), and in 2009 Simmonds et al. established an alternative set of VP4 consensus primers (VP4F/VP4R) (Simmonds et al., 2008) for typing the P genotypes missed with the previously described Con3/Con2 consensus primers (Gentsch et al., 1992). These genotyping assays, some of which are more than 20 years old, have generated enormous amounts of useful epidemiological data which has highlighted RVA genetic diversity on a global level (Bányai et al., 2012; Gentsch et al., 2005; Matthijnssens et al., 2009; Seheri et al., 2014). Consequently, expanding genetic diversity, and genetic drift with the accumulation of point mutations at primer binding sites, have all been observed and linked to mistyping or failure of genotype-specific primers to correctly identify strains (Banyai et al., 2005; Cunliffe et al., 2001; Esona et al., 2010b; Mitui et al., 2012; Rahman et al., 2005; Solberg et al., 2009). Ongoing failures of these decade-old assays to correctly characterize RVA strains have contributed to an estimated 10–30% of strains being classified as non-typeable, a definite hindrance to RVA research, worldwide (Esona et al., 2010a; Gentsch et al., 2005). With rapid changes in the epidemiology of RVA, and the emergence of genotypes G9 and G12 in many parts of the world (Banyai et al., 2012; Esona et al., 2013; Iturriza-Gomara et al., 2011; Seheri et al., 2014), it is imperative to be able to efficiently determine the VP7 and VP4 genotypes of RVA strains in clinical samples collected in the pre- and post-vaccine introduction eras. Vaccine effectiveness must be accessed against common VP7 and VP4 genotypes components of the vaccines, as well as those not included, and this is dependent on the accuracy and sensitivity of typing methods for RVA strains. To maintain the sensitivity, specificity and accuracy, primers used in these RT-PCR-based typing methods must be regularly revised and updated (Fischer and Gentsch, 2004; Iturriza-Gomara et al., 2004; Masendycz et al., 1997; Simmonds et al., 2008). To accomplish this, we have developed and extensively validated RVA, VP7 and VP4 multiplexed genotyping one-step RT-PCR assays to identify the common six G- (G1–G4, G9 and G12) and five P- (P[4], P[6], P[8], P[9] and P[10]) genotypes. A noted advantage of these newly developed methods is that a G12 specific primer has been incorporated in the VP7 assay. Primer specific for G12 genotypes have been described by various investigators for confirmation of the G12

genotypes, however, the reaction has been done in separate reaction tube (Banerjee et al., 2007; Samajdar et al., 2006).

Validation performed in this study has shown that these newly designed VP7 and VP4 multiplexed one-step genotyping RT-PCR assays will consistently detect and correctly characterize the common six VP7 genotypes (G1–G4, G9 and G12) and five P genotypes (P[4], P[6], P[8], P[9], and P[10]), whether in culture supernatants, clinical stool samples or in vaccine stocks (with the exception of the G2 component of the RotaTeq vaccine stock).

We have demonstrated that the sensitivity of these novel VP7 and VP4 one-step genotyping RT-PCR assays depends on the VP7 and VP4 genotypes. Any significant sequence variation of the RVA genome of each genotype overtime will result in decreased sensitivity of these assays. The LOD varied for each VP7 G- and VP4 P-genotypes but irrespective of the assay or genotype, the LOD was found to be below the LOD of the EIA assay detection. This indicates that both VP7 and VP4 one-step genotyping RT-PCR assays are more sensitive than EIA assay and will improve the diagnosis of RVA in clinical samples if used simultaneously with EIA for screening and genotyping of rotavirus. Though the number of each segment of RVA per virion is not known, previous studies have shown that each RVA virion contains probable equimolar ratio of each of the 11 segments (McDonald and Patton, 2011; Patton, 1990). Therefore, the improved VP7 and VP4 genotyping assays developed in this study has an LOD in the range of 8.6×10^1 to 8.6×10^2 copies of NSP3 or VP7 and ≤1 to 8.2×10^2 copies of NSP3 orVP4 RNA per reaction, respectively.

The described VP7 and VP4 typing assays have been shown to consistently and correctly genotype RVA strains belonging in published lineages and sub-lineages of the six common G- and five common P-genotypes (Esona et al., 2013; Esteban et al., 2010; Le et al., 2011; Magagula et al., 2014; Martella et al., 2011; Mascarenhas et al., 2010; Nyaga et al., 2014; Stupka et al., 2009, 2012). The advantages of these two gel-based multiplexed one-step genotyping RT-PCR protocols are: (1) they do not require specialized equipment; (2) laboratory personnel need only basic skills to follow the protocols; (3) improved differentiation of samples with mixed genotypes; (4) this approach involves less handling of samples, is less labor-intensive, and less prone to sample cross-contamination; and (5) other commercially available one-step RT-PCR kits such as the MyTaq™ one-step RT-PCR kit (Bioline) and the SuperScript® III one-step RT-PCR System with Platinum® *Taq* High Fidelity DNA Polymerase (Life Technologies) can be used in place of the Qiagen one-step RT-PCR kit (Qiagen).

Though these two type-specific one-step genotyping RT-PCR primers were designed to generate highly specific amplification of the common six G- and five P-genotypes, they have a few limitations. First, the assays does not test for some rarer genotypes like G6, G8, G10, P[5], P[14]. Second, they do not contain a 3′ consensus primer which can be used to generate amplicons for sequencing in cases when the genotyping assay fails. In case of failure, previously published 5′ and 3′ consensus primer sets (Das et al., 1994; Gentsch et al., 1992; Gouvea et al., 1990) can be used to generate amplicon for sequencing.

Here we report two highly robust, accurate, efficient, and documentable gel-based multiplex genotyping systems. These assays are capable of genotyping 97.8% of the six common VP7 and 98.3% of the five common VP4 genotypes of RVA strains which are responsible for approximately 88.2% of all RVA infections (Banyai et al., 2012). Additionally they will replace the age-old two step hemi-nested VP7 (Das et al., 1994; Gouvea et al., 1990) and VP4 (Gentsch et al., 1992) genotyping RT-PCR assays which have been linked to genotyping failure in ≥30% of RVA positive samples worldwide (Gentsch et al., 2005) and are more prone to sample cross-contamination.

Although there are more sophisticated molecular techniques such as Surface Enhanced Raman Spectroscopy (Driskell et al., 2010) and various types of high-throughput next generation sequencing (Jere et al., 2011) for detection and characterization of rotavirus from stool samples, the cost associated with these methods is high and demands the expertise of specialized personnel. In conclusion, these novel G and P assays are simpler, less complex, faster and require less experienced personnel to perform. We believe that they will be useful for RVA characterization in studies worldwide for the foreseeable future.

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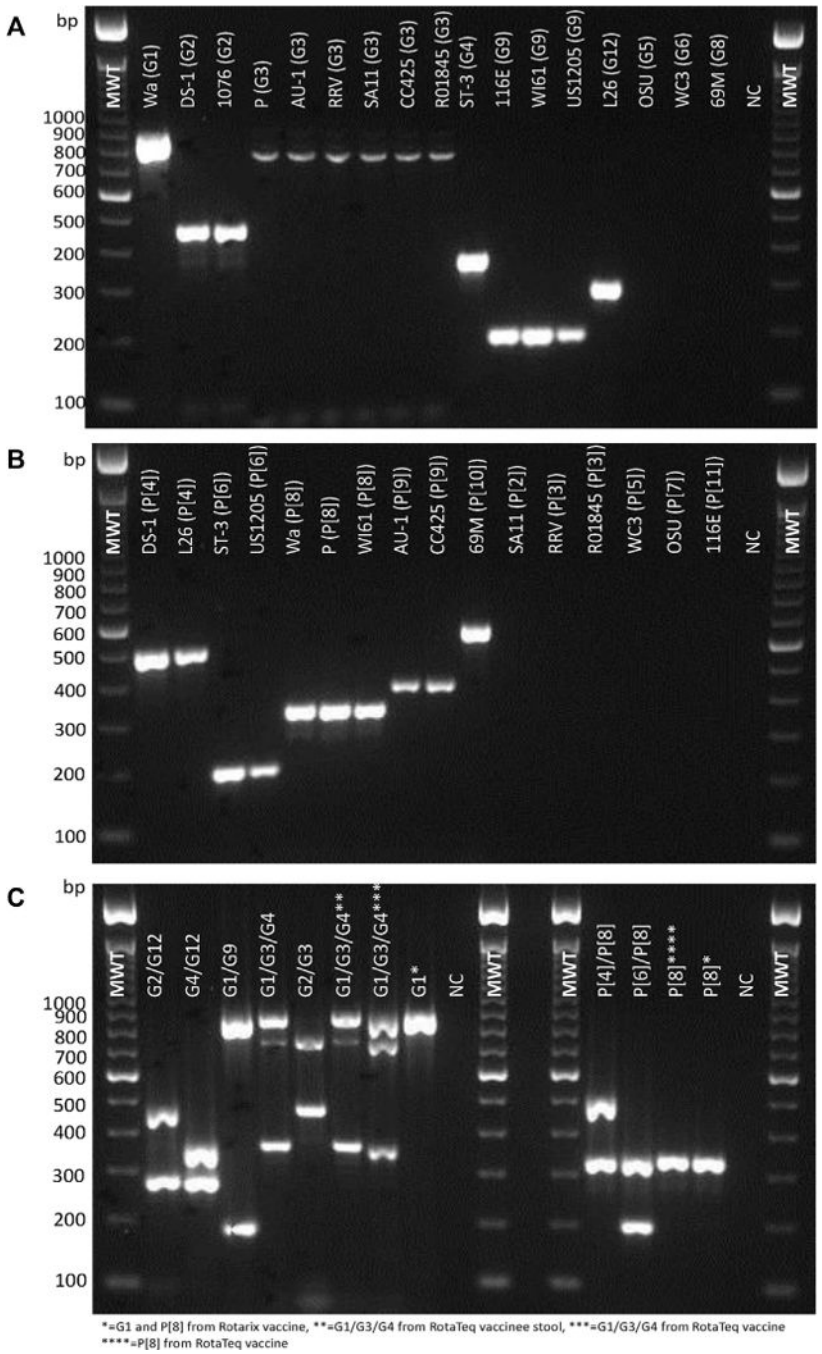

Fig. 1.

(A and B) Multiplexed one-step RT-PCR genotyping of RVA strains using the VP7 and VP4 gene segments. RVA dsRNA was extracted from cell lysates of reference virus strains and 4 μl of the eluate was analyzed. (A) G genotyping. Lanes: MWT, molecular weight markers (Tracklt™ 100 bp DNA ladder; Invitrogen, NY, USA), lanes 1 and 20; marker molecular sizes are indicated on the left in base pairs); 2–18, products amplified from dsRNA of human and animal RVA strains possessing genotypes G1 (lane 2, strain Wa), G2 (lane 3, strain DS-1), G2 (lane 4, strain 1076), G3 (lane 5, strain P), G3 (lane 6, strain AU-1), G3

(lane 7, strain RRV), G3 (lane 8, strain SA11), G3 (lane 9, strain CC425), G3 (lane 10, strain RO1845), G4 (lane 11, strain ST-3), G9 (lane 12, strain 116E), G9 (lane 13, strain WI61), G9 (lane 14, strain US1205), G12 (lane 15, strain L26), G5 (lane 16, strain OSU), G6 (lane 17, strain WC3), G8 (lane 18, strain 69M), and (lane 19, negative control). (B) P genotyping. Lanes: MWT, molecular weight markers (TrackIt™ 100 bp DNA ladder, lanes 1 and 19), 2–16, products amplified from dsRNA of human and animal RVA strains possessing genotypes P[4] (lane 2, strain DS-1), P[4] (lane 3, strain L26), P[6] (lane 4, strain ST-3), P[6] (lane 5, strain US1205), P[8] (lane 6, strain Wa), P[8] (lane 7, strain P), P[8] (lane 8, strain WI61), P[9] (lane 9, strain AU-1), P[9] (lane 10, strain CC425), P[10] (lane 11, strain 69M), P[2] (lane 12, strain SA11), P[3] (lane 13, strain RRV), P[3] (lane 14, strain RO1845), P[5] (lane 15, strain WC3), P[7] (lane 16, strain OSU), P[11] (lane 16, strain 116E), and (lane 17, negative control). (C) Mixed G and P genotyping. Lanes: MWT, molecular weight markers (TrackIt™ 100 bp DNA ladder, lanes 1, 11, 13 and 19), 2–8, products amplified from RVA strains in stool possessing mixed G genotypes G2/G12 (lane 2), G4/G12 (lane 3), G1/G9 (lane 4), G1/G3/G4 (lane 5), G2/G3 (lane 6), G1/G3/G4** (lane 7, mixed genotypes from RotaTeq vaccinee stool), G1/G3/G4*** (lane 8, mixed genotypes from RotaTeq vaccine), G1* (lane 9, strain from Rotarix vaccine), negative control (lane 10) and for mixed P genotypes, P[4]/P[8] (lane 14), P[6]/P[8] (lane 14), P[8]**** (lane 15, strain from RotaTeq vaccine), P[8]* (lane 16, strain from Rotarix vaccine), and negative control (lane 17).

Table 1

Primers used for G and P genotyping assays.

Primer	Sequence (5′-3′)	Polarity	Gene	Genotype	Nucleotide position strand	Approximate amplicon size, bp	Reference
VP7uF	GGC TTT AAA ARM GAG AAT TTC CG	+	VP7	Generic	1–23		This study
G1-R4	ACA TTA GAR CCA CCA ACT TGT AT	−	VP7	G1	850–828	849	This study
G2-R4	CAT TAT AAT CAC AAT ACA GTT G	−	VP7	G2	463–442	462	This study
G3-R1	CGT CAG TAA TYA CTA RTT TYT CAG CTG	−	VP7	G3	733–707	732	This study
G4-R2	ATA GWG TAT {C} TT TCC ATT CAK TGT C	−	VP7	G4	358–337	357	This study
G9-R2	GGT GAT ACA ATR ACA ATA AGT AAA AG	−	VP7	G9	185–160	184	This study
G12-R2	GAA GTC ATA AAA YTY TCT TGT TG	−	VP7	G12	284–262	283	This study
VP4uF	TGG YTT CVC TCA TTT ATA GAC A	+	VP4	Generic	11–32		This study
P[4]-R5	GCA TYC CTA CAA GTC TAT TAY TAG	−	VP4	P[4]	508–485	497	This study
P[6]-R2	ACC ATC GAG TAC TGG YTC TAT YGT TG	−	VP4	P[6]	210–185	199	This study
P[8]-R2	GYG GTT CAA YAG CAA CKA CT	−	VP4P[4]	P[8]	350–330	339	This study
P[9]-4T-1	TGA GAC ATG CAA TTG GAC	−	VP4	P[9]	402–385	391	Gentsch et al. (1992)
P[10]-5T-1	ATC ATA GTT AGT AGT CGG	−	VP4	P[10]	594–575	583	Gentsch et al. (1992)

F, forward; R, reverse; VP, structural protein; bp, base pair; R, A or G; Y, C or T; V, A, C or G; N, A, C, G or T; {C}, AP-dC (G-clamp).

Table 2

LODs of the G and P genotyping assays.

Gene segment	LOD EIA value	LOD in copies of NSP3 RNA/reaction
VP7 genotypes		
G1	0.104*	3.8×10^2
G2	0.126*	8.6×10^2
G3	0.082*	8.6×10^2
G4	0.067*	5×10^2
G9	0.067*	2.2×10^2
G12	0.054*	8.6×10^1
VP4 genotypes		
P[4]	0.126*	8.6×10^2
P[6]	0.082*	8.6×10^2
P[8]	0.104*	3.8×10^2
P[9]	0.122*	8.2×10^2
P[10]	0.081*	≤1

* Below limit of EIA assay detection (0.15).

Table 3

Performance of the VP7 (G) and VP4 (P) conventional multiplex one-step RT-PCR genotyping assays.

One-step RT-PCRgenotyping assay	Total samples tested	Total samples genotyped	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
VP7 (G genotype)						
G1	100	99	99.0	100	100	100
G2	145	100	97.0	100	100	99.1
G3	78	73	94.0	100	100	99.2
G4	24	24	100	100	100	100
G9	70	69	99.0	99.0	100	99.2
G12	228	226	99.1	100	100	100
Mixed**	13	13	ND	ND	ND	ND
Negative***	67	0	ND	ND	ND	ND
VP4 (P genotype)						
P[4]	131	126	99.0	100	100	100
P[6]	50	47	94.0	100	100	99.6
P[8]	460	457	99.3	100	100	100
P[9]	2	2	100	100	100	100
P[10]	2	2	100	100	100	100
Mixed**	13	13	ND	ND	ND	ND
Negative***	67	0	ND	ND	ND	ND

** Mixed G were G2/G12, G4/G12, G1/G9, G1/G3/G4, and G2/G3, while mixed P genotypes were P[4]/P[8] and P[6]/P[8].

*** Negative by NSP3 qRT-PCR and OD values were below limit of EIA assay detection.