Journal of Virological Methods 213 (2015) 157–163

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Molecular genotyping and quantitation assay for rotavirus surveillance

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Article history:
Received 2 October 2014
Received in revised form 1 December 2014
Accepted 2 December 2014
Available online 17 December 2014

Keywords:
Rotavirus
Diarrhea
Genotyping
Multiplex real time RT-PCR

A B S T R A C T

Rotavirus genotyping is useful for surveillance purposes especially in areas where rotavirus vaccination has been or will be implemented. RT-PCR based molecular methods have been applied widely, but quantitative assays targeting a broad spectrum of genotypes have not been developed. Three real time RT-PCR panels were designed to identify G1, G2, G9, G12 (panel GI), G3, G4, G8, G10 (panel GII), and P[4], P[6], P[8], P[10], P[11] (panel P), respectively. An assay targeting NSP3 was included in both G panels as an internal control. The cognate assays were also formulated as one RT-PCR-Luminex panel for simultaneous detection of all the genotypes listed above plus P[9]. The assays were evaluated with various rotavirus isolates and 89 clinical samples from Virginia, Bangladesh and Tanzania, and exhibited 95% (81/85) sensitivity compared with the conventional RT-PCR-Gel-electrophoresis method, and 100% concordance with sequencing. Real time assays identified a significantly higher rate of mixed genotypes in Bangladeshi samples than the conventional gel-electrophoresis-based RT-PCR assay (32.5% versus 12.5%, $P < 0.05$). In these mixed infections, the relative abundance of the rotavirus types could be estimated by Cq values. These typing assays detect and discriminate a broad range of G/P types circulating in different geographic regions with high sensitivity and specificity and can be used for rotavirus surveillance.

1. Introduction

Rotavirus is one of the most common causes of diarrheal disease in young children globally and leads to two million hospitalizations and more than a half million deaths every year (Parashar et al., 2003, 2009). Rotavirus vaccine has been recommended by WHO for all national immunization programs (Babji and Kang, 2012; World Health Organization, 2013). Rotaviruses belong to the *Reoviridae* family and are classified into G- and P-types based on sequence or antibody reactivity to two outer viral proteins, VP7 and VP4, respectively. To date, >70 different G-type and P-type combinations have been identified (Matthijnssens et al., 2011). The G/P type of rotaviruses can fluctuate both temporally and geographically. Although cross-protection occurs with rotavirus vaccines, the extent and durability of this protection is unclear, thus uncommon strains may become prevalent or new strains may emerge under vaccine pressure (Assis et al., 2013; Gurgel et al., 2008; Hull et al., 2011; Kirkwood et al., 2009; Matthijnssens et al., 2009; Zeller et al., 2010). Pre-rotavirus vaccine surveillance reports from 1996 to 2007 (Banyai et al., 2012) provided a comprehensive landscape of rotavirus strain distribution worldwide. Prospective longitudinal surveillance post-rotavirus vaccine has been called for using robust genotyping technologies (Dennehy, 2013; Gentsch et al., 2009b).

Multiplex RT-PCR followed by gel-electrophoresis discrimination based on amplicon length has been the primary rotavirus genotyping method (Gentsch et al., 1992; Gouvea et al., 1990). Among 281 rotavirus typing studies within 12 years (Banyai et al., 2012), nearly all of the studies used RT-PCR, with 30% in combination with sequencing. Other methods used have included southern blot, northern blot, reverse line blot hybridization, PCR-ELISA, and

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; Cq, quantification cycle.

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http://dx.doi.org/10.1016/j.jviromet.2014.12.001
0166-0934/

RFLP. Probe-based real time PCR may offer more sensitive and specific detection and avoids post-amplicon manipulation and potential risk of contamination. Many one step singleplex real time RT-PCR assays have been designed for a variety of targets for rotavirus detection, such as VP6, NSP3, NSP4, VP2, but real time RT-PCR platform has not been adapted widely for rotavirus genotyping. Recently, Kottaridi et al. developed two panels of real time RT-PCR assays for detection of G1, G2, G3, G4, G9, P[4] and P[8] and showed good agreement with the conventional PCR assays but a two step was used and the selection of types was limited (Kottaridi et al., 2012).

In this work, three panels of 5-plex internally controlled one step real time PCR reactions were developed for identification and quantitation of G1–4, G8, G9, G10, G12 and P[4], P[6], P[8], P[10], P[11]. Alternately a 15-plex RT-PCR-Luminex assay was developed for identification of the same genotypes plus P[9]. These assays were evaluated with clinical specimens from three different regions of the world.

2. Materials and methods

2.1. Specimens

Representative rotavirus isolates were selected for evaluating analytical performance, including Wa (G1P[8]), DS-1 (G2P[4]), AU-1 (G3P[9]), ST3 (G4P[6]), 69M (G8P[10]), 116E (G9P[11]), I-321 (G10P[11]), L26 (G12P[4]). Fecal samples tested previously positive for rotavirus by ELISA were provided from studies at the International Centre for Diarrhoeal Disease Research, Bangladesh, Kilimanjaro Christian Medical Centre, Tanzania, and Division of Consolidated Laboratory Services, Virginia. Bangladeshi samples were selected from a birth cohort study (2008–2009) in the Mirpur region of Dhaka (Mondal et al., 2012). Tanzanian samples were collected from inpatients with diarrhea from Kilimanjaro Christian Medical Centre and referral hospitals in Moshi from February 2008 to June 2009. More than 70% of the rotavirus positive samples were from children under age five. Virginia specimens were rotavirus positive diarrheal specimens collected during routine outbreak investigations (from February to April, 2011) by Division of Consolidated Laboratory Services, Virginia. All studies were approved by the University of Virginia, International Centre for Diarrhoeal Disease Research, Bangladesh and Kilimanjaro Christian Medical Centre institutional review boards.

2.2. RNA extraction

Nucleic acid was extracted from fecal samples using the QuickGene RNA tissue kit SII (Fujifilm, Tokyo, Japan) as described previously (Liu et al., 2011).

2.3. Multiplex one step real time RT-PCR

Genotype specific primers and probes were designed in the variable regions of VP7 and VP4 and adapted or modified from published assays wherever feasible (Aladin et al., 2010; Gentsch et al., 1992; Gouvea et al., 1990; Iturriza-Gomara et al., 2004) (Table 1). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and Biosearch Technologies (Novato, CA). Real time RT-PCR was performed with AgPath-ID RT-PCR kit (Life Technologies, Carlsbad, CA) with a CFX system (Bio-Rad, Hercules, CA). Three panels were formulated as panel GI, including G1, G2, G9, and G12; panel GII, including G3, G4, G8, G10; panel P, including P[4], P[6], P[8], P[10], P[11]. An internal control assay targeting NSP3 was incorporated in both G panels with final primer and probe concentration at 200 nM and 100 nM, respectively (Zeng et al., 2008). The samples were denatured by incubation at 95 °C for 5 min then on ice prior to mixing with the one step real time RT-PCR reagents. The cycling condition included reverse transcription at 45 °C for 20 min, denaturation at 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 55 °C for 1 min.

2.4. RT-PCR-Luminex assay

RT-PCR-Luminex assays were performed with QIAamp One Step RT-PCR kit (Qiagen, Valencia, CA) followed by luminex detection with a BioPlex system (Bio-Rad) (Liu et al., 2011). Final concentrations of biotinylated and non-biotinylated primers were 300 nM and 200 nM, respectively, for all the genotype specific primer assays, except 150 nM and 100 nM for NSP3. Carboxylate microspheres were labeled with oligonucleotide probes with amino modifier, and luminex detection was performed as described previously (Liu et al., 2011). The cutoff set for positivity was two-fold Median Fluorescence Intensity above background (nuclease free water).

2.5. Amplicon sequencing

PCR amplicon was generated with consensus primers for VP7 (Beg9 and End9) and VP4 (con2 and con3) described previously (Gentsch et al., 1992; Gouvea et al., 1990), and sequenced by GENEWIZ (South Plainfield, NJ). Con2 and con3 for VP4 were modified slightly, forward primer 5′-TGGCTTCRCTCATTTATAGACA-3′, reverse primer 5′-ATTTCNGACCATTTATAWCC-3′.

2.6. Generation of RNA transcripts

Consensus VP7 or VP4 amplicon from a positive sample for each genotype was cloned, amplified and in vitro transcribed according to the previous protocol (Liu et al., 2011), with T7 RNA polymerase and SP6 RNA polymerase, respectively. The two RNA transcript products were mixed in equal molar concentration measured with Nanodrop (Bio-Rad) in 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, then denatured at 90 °C for 3 min and hybridized by cooling down slowly to room temperature to generate double stranded templates. Non-denaturing 2% agarose gel electrophoresis was run to ensure the formation of double stranded RNA. For analytical performance, double stranded RNA transcripts were spiked into lysis buffer during extraction of fecal samples from healthy donors.

2.7. Statistics

Correlation was tested by regression analysis using the analysis of variance (ANOVA) tests. Mixed infection rates were compared with chi-square test. All P values were two-tailed and values of <0.05 were considered statically significant.

3. Results

3.1. Analytical performance of the real time RT-PCR assays

Linearity and limit of detection were determined using in vitro transcripts of VP7 and VP4 sequences corresponding to the interrogated G/P-types. As shown in Table 2, Pearson coefficient varied between 0.98 and 1.00 for linearity. Limit of detection, defined as the lowest concentration to achieve 100% detection in ten spiked samples, was 10^6 copies of in vitro transcripts per gram of stool (equivalent to 200 copies per RT-PCR reaction prior to extraction). The average quantification cycle (Cq) values at the Limit of detection were designated as the analytical cutoff used for further data analysis. Specificity of the assays was tested with 8 rotavirus isolates, each was positive for the expected G/P genotypes, and there were no false VP7 or VP4 detections. Furthermore, no cross-reaction