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Short Communication
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One-step Quantitative RT-PCR Assays for Detecting, Genotyping and Differentiating Wild-Type Group a Rotaviruses and Vaccine (Rotarix® and RotaTeq®) Strains in Stool Samples

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Text

Group A rotaviruses (RVA) are the leading cause of morbidity and mortality due to severe diarrhoea among children <5 years of age, with nearly 200,000 deaths per year attributable to RVA infection globally [1]. RVA belong to the Reoviridae family and have been classified as a separate genus Rotavirus. There are 8 wild-type rotavirus groups identified (designated groups A to H), 4 of which (groups A, B, C and H) cause disease in humans. Among these 4 groups, RVA are responsible for more than 90% of all infections in humans [2]. RVA are traditionally genotyped based on the sequence diversity of the two outer capsid proteins, VP7 (glycosylated, G-type) and VP4 (protease sensitive, P-type), that elicit host-neutralizing antibodies and reassort independently from one another *in vivo* [3]. Six G-genotypes (G1, G2, G3, G4, G9 and G12) and three P genotypes (P(4), P(6) and P(8)) reassort in different wild-type combinations (G1P(8), G2P(4), G3P(8), G4P(8), G9P(8), and G12P(8)) that account for approximately 80% - 90% of RVA infections worldwide. Two live-attenuated, oral RVA vaccines (RotaTeq®, Merck & Co. NJ, USA and Rotarix®, GSK Biologicals, Rixensart, Belgium) have been licensed in >100 countries worldwide since 2006 [4]. In 2009, the World Health Organization (WHO) issued a global recommendation for use of RVA immunization in all children <5 years, to provide protection against severe diarrhoea caused by the major RVA strains in circulation [1]. Rotarix® is a monovalent vaccine consisting of a human G1P(8) strain and RotaTeq® is a pentavalent vaccine consisting of 5 human-bovine reassortants expressing the 4 most common human RVA G-types, G1, G2, G3, and G4 and the most common human RVA P-type, P(8), on a parental bovine Wistar Calf 3 (WC3) strain backbone. RVA vaccines have significantly reduced RVA associated acute gastroenteritis (AGE) and mortality in countries that have implemented RVA vaccination [5]. However, Rotarix® and RotaTeq® are live vaccines that can replicate in vaccinated children and are shed in feces post vaccination [6,7]. RVA have a segmented genome (11 double stranded RNA gene segments) and thus are capable of reassortment. In reassortment, two RVA strains infecting the same cell swap genomic segments to produce progeny with a mosaic genome containing individual genes from each parent [8]. RotaTeq® component strains can reassort with each other to produce reassortant strains causing gastroenteritis [8]. Reassortant strains derived from vaccine strains Rotarix® [9] and RotaTeq® [10-12] have been associated with AGE in vaccinated [8,13-16] and unvaccinated children [17]. Rapid identification of causative agent associated with diarrhea is essential to ensure administration of the appropriate patient management and to control potential AGE outbreaks. Sensitive and specific high-throughput assays are required to monitor the prevalence of vaccine strains in AGE cases and the emergence of new vaccine-derived strains following RVA vaccine introduction. Diagnostic techniques to detect RVA in stool samples include electron microscopy, virus isolation in cell culture, polyacrylamide gel electrophoresis (PAGE) of viral segments, enzyme immunoassays (EIAs), passive particle agglutination tests, immunochromatographic tests, RT-PCR and real time or quantitative RT-PCR (qRT-PCR) [3,18]. Molecular techniques are most sensitive and specific to rapidly detect and genotype RVA strains. Several multipathogen detection assays are commercially available to simultaneously detect various enteropathogens (viruses including RVA, bacteria and parasites) causing gastroenteritis [3]. Molecular techniques used to genotype RVA strains include RT-PCR followed by gel based genotyping, qRT-PCR, Sanger sequencing, and next generation sequencing [3]. Using qRT-PCR assays to detect and genotype RVA offers several advantages over traditional RT-PCR assays including increased sensitivity, higher throughput, faster turnaround time, and quantification of viral loads.

One-step, singleplex, qRT-PCR assays have been developed for detection of Rotarix® and RotaTeq® vaccine strains in stool samples [19]. Primary qRT-PCR assays were designed for vaccine-specific targets in the Rotarix® NSP2 gene, which shows the highest degree of genetic dissimilarity between the Rotarix® vaccine strain and other G1P(8) strains and the RotaTeq® VP6 gene which detects the presence of the bovine-RVA WC3 backbone in all five RotaTeq® component strains. Secondary qRT-PCR assays have been developed for the Rotarix® VP4 gene targeting two nucleotide mismatches between Rotarix® vaccine strain and wild-type G1P(8) strains. Three RotaTeq® component strains contain a VP3-WC3 gene and two component strains contain a VP3-human gene, thus two secondary qRT-PCR assays were developed flanking the nucleotides specific to RotaTeq® VP3-WC3 and RotaTeq® VP3-human genes. Designed qRT-PCR assays were validated on sequence confirmed stool samples containing vaccine strains, wild-type RVA strains of different genotypes and RVA negative stools obtained from routine domestic and international RVA surveillance conducted by the CDC [19]. For quantification, standard curves were generated using dsRNA transcripts derived from RVA gene segments. The qRT-PCR singleplex assays exhibited 92-100% sensitivity, 94-100% specificity and a limit of detection of 1-140 copies per reaction [19]. These singleplex qRT-PCR assays are used routinely to detect and distinguish Rotarix® and RotaTeq® vaccine strains from wild-type RVA strains in surveillance studies. The singleplex qRT-PCR

assays developed to detect Rotarix® and RotaTeq® vaccine strains [19] are used to detect Rotarix® and RotaTeq® vaccine strains using Rotarix NSP2 and RotaTeq VP6 primary assays, respectively, in AGE stool samples that tested positive for RVA by EIA or NSP3 gene qRT-PCR (Figure 1) [18]. The positive vaccine samples can be confirmed by secondary singleplex qRT-PCRs, the Rotarix VP4 assay for Rotarix® vaccine and the RotaTeq VP3-WC3 and the RotaTeq VP3-human assays for RotaTeq® vaccine.

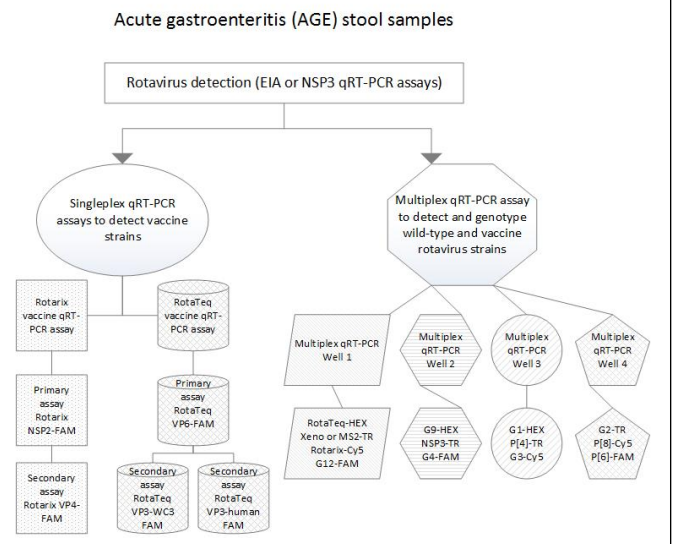

Figure 1: Flow chart showing qRT-PCR assays developed for RVA detection (NSP3 assay), vaccine strain detection in singleplex format (Rotarix® NSP2-primary assay, Rotarix® VP4-secondary assay; RotaTeq® VP6- primary assay, RotaTeq® VP3-WC3 and VP3-human-secondary assays) and multiplex qRT-PCR (13 components) assay to detect and genotype RVA wild-type and vaccine strains in AGE stool samples.

A one-step, multiplex qRT-PCR assay has been developed to detect and genotype wild-type RVA and vaccine strains (Rotarix® and RotaTeq®) in stool samples [20]. A one-step multiplex qRT-PCR containing 13 components assays was developed to: 1) detect RVA using the NSP3 assay ([18]); 2) detect Rotarix® and RotaTeq® vaccine strains ([19]); 3) genotype both wild-type and vaccine RVA strains for VP7 (G1, G2, G3, G4, G9 and G12 genotypes) and VP4 (P(4), P(6), and P(8) genotypes) genes; and 4) detect an internal process control, either Xeno armored RNA (Life Technologies Corp., Grand Island, NY, USA) or MS2 (ZeptoMetrix, Buffalo, NY, USA) [20]. The multiplex qRT-PCR assay containing 13 components is performed in 4 wells; each well contains master mix with primers and probes specific for 3-4 target genes with different reporter dyes (FAM, HEX, Texas Red (TR), and Cy5). The Well 1 reaction mixture contains primers and probes for RotaTeq-VP6-HEX, Xeno or MS2-TR, Rotarix-NSP2-Cy5 and G12-FAM. The Well 2 reaction mixture contains primers and probes for G4-FAM, NSP3-TR and G9-HEX. The Well 3 reaction mixture contains primers and probes for G1-HEX, P(4)-TR and G3-Cy5. The Well 4 reaction mixture contains primers and probes for G2-TR, P(8)-Cy5 and P(6)-FAM. The multiplex qRT-PCR assay was validated on large number of sequence confirmed stool samples (containing wild-type or vaccine/vaccine derived RVA strains), vaccine strains, RVA negative samples and lab-cultured strains of different genotypes. The multiplex qRT-PCR assay was validated by generating standard curves using dsRNA transcripts that were generated artificially from RVA gene segments. All the qRT-PCR assays were performed in 96-well ABI Fast plates using the GeneAmp EZ rTth RNA PCR kit (Applied Biosystems Inc.-ABI, Foster City, USA) which is now discontinued by ABI. In a multiplex format, NSP3, Rotarix® and RotaTeq®, VP7 and VP4 qRT-PCRs, displayed 81-93% efficiency with a limit of detection of 10-4000 copies per reaction [20]. All the qRT-PCR assays have been tested using the EMD Millipore-One step RT-PCR Master Mix kit (EMD Chemicals Inc., Billerica, MA, USA) and the limit of detection is comparable to that of the GeneAmp EZ rTth kit (unpublished data).

The vaccine detection singleplex assays have been incorporated into the one step multiplex qRT-PCR assay to detect and genotype wild-type RVA and vaccine strains [20]. The multiplex qRT-PCR assay can be used on AGE samples directly or on selected RVA positive samples tested by EIA or NSP3 gene qRT-PCR. This multiplex qRT-PCR (4 wells, 13 components) assay will detect RVA by NSP3 gene qRT-PCR, will detect vaccine strains (Rotarix® and RotaTeq®) by Rotarix NSP2 and RotaTeq VP6 qRT-PCRs and will genotype the RVA strains for six VP7 (G1, G2, G3, G4, G9, and G12) and three VP4 (P(4), P(6), and P(8)) genotypes along with a Xeno or MS2 internal process control. The samples testing positive for vaccine strains by multiplex qRT-PCR assay are confirmed for vaccine strains by using secondary assays, Rotarix-VP4 for Rotarix® positive samples and RotaTeq VP3-WC3 and RotaTeq VP3-human assays for RotaTeq® positive samples (Figure 1). We recommend that a representative subset of wild-type RVA samples and all the samples found to contain vaccine strains should be sequenced by Sanger sequencing or Next Gen sequencing to confirm the genotypes obtained by the multiplex qRT-PCR assay.

The limitations of singleplex and multiplex qRT-PCR assays evaluated in this study are that the samples with G and P combinations not covered in this assay won't be genotyped. Also, these assays won't detect reassortants between wild-type RVA rotavirus and vaccine strains involving gene segments other than VP4, VP7 and NSP2 genes for Rotarix® vaccine strain and VP4, VP7, VP6 and VP3 genes for RotaTeq® vaccine strains.

These qRT-PCR assays (both in singleplex and multiplex formats) facilitate high-throughput detection and genotype characterization of wild-type RVA, vaccine strains and RotaTeq® vaccine derived G1P(8) reassortant strains in stools samples from AGE patients. These assays will also determine the frequency of vaccine or RotaTeq® vaccine derived G1P(8) strains in stool samples during routine RVA surveillance.

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Disclaimer

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