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Differentiation of RotaTeq® vaccine strains from wild-type strains using *NSP3* gene in reverse transcription polymerase chain reaction assay

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ABSTRACT

RotaTeq® is a live attenuated human-bovine reassortant vaccine against rotaviruses that is used worldwide. However, shedding of the virus used in RotaTeq® has been detected in the feces of children following vaccination by the oral route, possibly affecting community immunity. Therefore, a simple and efficient method to discriminate between virulent and RotaTeq® vaccine strains is required. In this study, a novel one-step multiplex reverse-transcription polymerase chain reaction (RT-PCR) assay targeting the *NSP3* gene was developed to detect RotaTeq® vaccine strains in fecal samples. RotaTeq® vaccine viruses were successfully distinguished from known wild-type rotavirus genotypes. In addition, the developed assay was able to detect rotaviruses in clinical stool samples obtained from South Korea during the 2011–2013 rotavirus seasons. Of the 1106 stool specimens from children with acute gastroenteritis that were screened, 286 rotaviruses were genotyped. RotaTeq® vaccine strains were identified in 39 samples (13.6%). The novel RT-PCR assay that was developed could be used to detect and discriminate between RotaTeq® vaccine strains that are shed in fecal matter, and to estimate the quantification of virus that has been shed after vaccination.

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1. Introduction

Rotaviruses are the most common etiological agents of acute diarrhea in children worldwide. These viruses are transmitted among individuals *via* the fecal-oral route (Cortese and Parashar, 2009). Infection with a rotavirus can result in a spectrum of illnesses that range from subclinical infection to mild diarrhea, to severe and occasionally fatal dehydration (Sergio and Leon, 2009). Rotaviruses cause approximately 453,000 deaths annually in children younger than 5 years, with a mortality rate of 86 deaths per 100,000 people worldwide. The majority of rotavirus-related deaths occur in developing and low-income countries with poor healthcare standards (Tate et al., 2012; WHO, 2013).

Rotavirus is a genus of the *Reoviridae* family, which contains eight species (A–H) (Matthijnssens et al., 2012b). The viral genome is composed of 11 double-stranded ribonucleic acid (dsRNA) segments, with each segment encoding a gene. These 11 genes encode six structural proteins (*VP1–4*, *VP6*, *VP7*) and five/six nonstructural proteins (*NSP1–NSP5/6*) (Estes et al., 2007). The outer capsid of the rotavirus particle comprises the *VP7* glycoprotein and the protease sensitive *VP4*. These proteins define the G and P genotypes, and confer host specificity, virulence, and protective immunity (Estes and Cohen, 1989). To date, at least 27 G and 37 P genotypes have been reported in humans and animal species (Matthijnssens et al., 2012a; Trojnar et al., 2013). The global distribution of G and P genotypes indicates that five G genotypes (G1–G4, G9) and three P genotypes (P[4], P[6], P[8]) are most commonly associated with human rotavirus infections (Matthijnssens et al., 2009; Santos and Hoshino, 2005). In South Korea, rotavirus infection is the most common cause of acute diarrhea in young children, particularly during the winter months. Surveillance studies have shown that genotypes G1–4 are the most common rotaviruses, and their distribution is likely dependent on seasonal and geographic influences (Than and Kim, 2013).

The administration of rotavirus vaccines in developing and developed countries is recommended by the World Health Organization (WHO) (WHO, 2007). In 2006, two vaccines were approved and made available worldwide. The RV1 vaccine (Rotarix™; GlaxoSmithKline Biologicals, Rixensart, Belgium) uses an attenuated G1P[8] rotavirus strain, and is administered in a 2-dose series

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(O'Ryan, 2007). The RV5 vaccine (RotaTeq®; Merck and Co Inc, West Point, PA, USA) is a live bovine-human reassortant vaccine containing five reassortant strains, representing human G1, G2, G3, G4, and P[8] rotavirus genotypes. This vaccine is administered as a 3-dose series (Heaton and Ciarlet, 2007).

The first pre-licensure trials of RV1 and RV5 were conducted in Europe and the Americas. The trials demonstrated that these vaccines were highly effective (85–98%) against severe rotavirus disease, with no risk of intussusception, and conferred protection against a range of rotavirus strains (WHO, 2007). In 2009, following additional proof of their efficacy in Africa and Asia, the WHO recommended these vaccines for routine immunization of infants throughout the world (Burki, 2013). RV1 and RV5 were introduced in South Korea in September 2007 and July 2008, respectively. Although these vaccines have not yet been integrated into routine immunization schedules in South Korea, the economic benefits of vaccination will help build a strong case for their use in preventing rotavirus-related illnesses.

Rotavirus shedding in the feces of vaccinated children has been detected following vaccination (Anderson, 2008). In addition, new reassortant strains have recently been identified in stool samples (Hemming and Vesikari, 2012). Transmission among siblings has also been detected, along with co-infection by rotaviral and non-rotaviral agents of gastroenteritis (Donato et al., 2012; Payne et al., 2009; Smith et al., 2011). These factors might complicate the evaluation of rotavirus transmission following vaccination, and the spread of these strains within populations.

It is difficult to distinguish between wild-type rotavirus strains and those used in vaccines by either regular EIA or RT-PCR methods. Standard diagnosis of rotavirus-related gastroenteritis is based on an enzyme immunoassay that recognizes the group A rotavirus VP6 protein in stool specimens (Donato et al., 2012; Payne et al., 2009; Smith et al., 2011). In contrast, reverse transcription polymerase chain reaction (RT-PCR) assays can detect and identify all species and genotypes of human rotavirus (Hemming and Vesikari, 2012); however, sequencing analysis is required to distinguish vaccine-derived viruses from their wild-type counterparts (Than et al., 2015). There are few reports regarding the detection of rotavirus vaccine strains in clinical samples (Gautam et al., 2014; Rose et al., 2010). The majority of rotavirus surveillance reports have focused on the detection of RotaTeq®-derived strains, and have been conducted in the USA, Belgium, Australia, and South Korea (Donato et al., 2012; Hemming and Vesikari, 2012; Payne et al., 2009; Than et al., 2015).

In this study, a novel one-step multiplex RT-PCR assay targeting the bovine NSP3 gene was developed to differentiate RotaTeq® vaccine viruses from wild-type rotaviruses. The developed assay was used to screen clinical stool specimens from Korean children hospitalized with acute gastroenteritis between March 2011 and February 2013. The novel method that was developed could be widely used to distinguish RotaTeq® virus strains that are shed in clinical samples because of its specificity, sensitivity, speed, and low cost.

2. Materials and methods

2.1. Ethics and vaccination

Stool specimens were collected from infants hospitalized for severe gastroenteritis between March 2011 and February 2013 at Chung-Ang University Hospital (Seoul, South Korea). This study was approved by the Human Subjects Institutional Review Board (IRB) of Chung-Ang University College of Medicine (Seoul, Korea; protocol number #2011-10-06). Written informed permission was obtained from all participants; for the children enrolled in this study, written informed consent was obtained from their parents. The provision of informed consent also included permission to use the data for future research purposes under conditions of anonymity. RotaTeq® vaccine records were obtained from hospital records, or by interviewing the parents. In general, infants were 8–10-weeks old when they were vaccinated with the first dose of RotaTeq® (mean time 6 ± 3 days). Almost all infants were hospitalized for 3 days with episodes of watery diarrhea (three times per day), pain in the right upper quadrant of the abdomen, and vomiting (non-projectile and non-bilious, containing consumed food).

2.2. Viruses and clinical samples

Known RotaTeq® viruses were obtained from a commercial RotaTeq® vaccine stock (Chung-Ang University Hospital), along with strains adapted to cell culture- ($n = 3$), and non-cell culture-adapted ($n = 9$) strains, as detected by Than et al. (2015). Known wild-type rotaviruses of various genotypes were identified in our laboratory during the 2008–2010 rotavirus seasons. These included cell culture-adapted (G1, $n = 10$; G2, $n = 2$; G3, $n = 4$, G4, $n = 3$, G9, $n = 3$; G11, $n = 1$; G12, $n = 2$) and non cell-culture adapted (G1, $n = 10$; G2, $n = 10$; G3, $n = 10$, G4, $n = 10$; G9, $n = 10$) strains. For clinical samples, a total of 1106 stool samples were collected from Korean infants hospitalized for acute gastroenteritis at Chung-Ang University Hospital from March 2011 to February 2013. All stool samples were diluted 10-fold with phosphate-buffered saline (PBS; pH 7.4) and clarified by centrifugation ($10,000 \times g$, 10 min).

2.3. Virus RNA isolation

Viral dsRNA was extracted from diluted stool samples using Trizol reagent (Gibco BRL, Grand Island, NY, USA). In brief, 0.3 mL of the diluted sample was mixed with 0.7 mL of Trizol reagent and 0.2 mL of chloroform:isoamyl alcohol (24:1). After centrifugation ($12,000 \times g$, 10 min), RNA was precipitated with isopropanol and centrifuged again ($12,000 \times g$, 10 min). The RNA pellet was washed with 70% ethanol, dissolved in 20 μL of RNase-free water, and stored at −80 °C until required.

2.4. Oligonucleotide primer design and synthesis

To detect RotaTeq® viruses, primers NSP3F-B (5′-TGG CCG ATA CTA GAA CTA CAG A-3′, nt 288–308) and NSP3R-B (5′-TGA TTA CAT CAA TGG AAT TTA GC-3′, nt 820–842) were designed based on highly conserved regions of bovine *NSP3* from the parent vaccine strains WI79-9 (GenBank accession number GU565060), SC2-9 (GU565071), WI78-8 (GU565082), BrB-9 (GU565093), and WI79-4 (GU565049) (Matthijnssens et al., 2010), using CLUSTAL-X 2.1 (Table 1). Primers were synthesized by Life Technologies (Seoul, South Korea). The primer sequences used displayed some variation with common circulating human rotavirus sequences that are in GenBank (Supplementary Table 1).

2.5. RT-PCR specificity using reference oligonucleotide primers

Purified rotavirus RNA (5 μL) was denatured at 97 °C for 5 min in the presence of dimethyl sulfoxide. One-step multiplex RT-PCR assays were carried out using a Qiagen OneStep RT-PCR kit (Qiagen, Westburg, Germany). Each assay was conducted in a 25 μL volume with optimal concentrations of primers NSP3F-B and NSP3R-B, or a mix of primers NSP3F-B, NSP3R-B, Beg9, and End9 used. Synthesis of cDNAs was conducted at 42 °C for 45 min, followed by 30 amplification cycles (94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min), and a final extension step (72 °C for 10 min). Amplicons were separated by electrophoresis on 1.2% (w/v) SeaKem LE agarose gels (FMC Bioproducts, Rockland, ME, USA). Gels were stained with ethidium

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