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VIROLOGY

Monitoring Shedding of Five Genotypes of RotaTeq Vaccine Viruses by Genotype-Specific Real-Time Reverse Transcription-PCR Assays

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ABSTRACT RotaTeq (RV5) is a widely used live attenuated pentavalent rotavirus (RV) vaccine. Although fecal shedding of RV vaccine strains persists for long time periods, it is unclear how each vaccine strain replicates in intestinal tissue and is excreted in stool. To examine this issue, we established RV5 genotype-specific real-time reverse transcription-PCR (RT-PCR) assays. Five real-time RT-PCR assays were designed for the VP7 gene in genotypes G1, G2, G3, G4, and G6. All assays exhibited excellent linearity, and the detection limit was 1 infectious unit (IU)/reaction for G2, G4, and G6 and 10 IUs/reaction for G1 and G3. No cross-reactivity was observed among G genotypes. The inter- and intra-assay coefficients of variation were less than 3%. The assays were used to examine 129 stool samples collected from eight infants who received RV5. In cases 1 and 2, who received three rounds of vaccination, RV shedding decreased gradually with the number of vaccinations. G1 and G6 shedding appeared to be predominant in comparison to shedding of the other genotypes. Patterns of fecal shedding of the five genotypes of vaccine viruses differed between the eight vaccine recipients. RV5 genotype-specific real-time RT-PCR assays will be useful to study the molecular biology of RV5 replication in infants and experimental animals.

KEYWORDS RotaTeq, genotype, human rotavirus, real-time RT-PCR, rotavirus vaccine, shedding

Rotavirus (RV), which belongs to the family *Reoviridae*, consists of 11 segments of double-stranded RNA surrounded by a triple-layered capsid comprising a core, inner, and outer capsid. Based on the antigenic and genetic features of the inner capsid protein VP6, RV has been categorized into eight recognized groups (A to H) (1), and two species were identified recently (I and J) (2, 3). Group A RVs are a leading cause of gastroenteritis in children and causes substantial morbidity and mortality worldwide (4). For the majority of human group A RVs (RVAs), three genogroups have been established: two major genogroups represented by the reference strains Wa (genogroup 1 genes) and DS-1 (genogroup 2 genes) and one minor genogroup represented by reference strain AU-1 (genogroup 1 genes) (5, 6). The genotype of RV is determined based on the sequences of the VP7 and VP4 genes, which define the viral G and P genotypes, respectively (7). Four strains, G1P[8], G2P[4], G3P[8], and G4P[8], are predominant worldwide, including in Latin America. Thus, four common G types (G1, G2, G3, and G4) in conjunction with P[8] or P[4] represent over 88% of the strains analyzed worldwide. In addition, genotype G9 viruses associated with P[8] or P[6] were shown to

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have emerged as the fifth globally important G type, with a relative frequency of 4.1% (8, 9). Because several G-P combinations, such as G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], are commonly prevalent around the world (10, 11), it is thought that the host immune response against these five genotypes plays important roles in preventing severe RV gastroenteritis (RVGE).

Currently, there are two commercially available live attenuated RV vaccines, Rotarix (RV1; GlaxoSmithKline, Rixensart, Belgium) and RotaTeq (RV5; Merck and Co., Whitehouse Station, NJ, USA) (12). RV1 is a monovalent vaccine derived from the most common human RV genotype (G1P[8]) attenuated by serial passage in cell culture (13). It was anticipated that the host immune response to RV1 administration could confer cross-protection against other common genotypes of RV. On the other hand, RV5 is a pentavalent human-bovine RV reassortant vaccine that contains the five most prevalent genotypes (G1, G2, G3, G4, and P[8], which is carried as G6P[8]) and was designed to induce type-specific protective immunity against these common strains in children (14). Although the conceptual mechanism for preventing severe RVGE differs between the two RV vaccines, previous studies demonstrated that both vaccines are generally safe and equally efficacious in clinical trials (13, 15, 16). Both are live attenuated vaccines, and vaccine viruses are excreted into stool after vaccination (13, 16–24). Additionally, transmission of vaccine virus strains from vaccine recipients has been observed in healthy children (15). Although RV5 vaccine shedding was examined by Vesikari et al. (16), the kinetics of fecal shedding of each genotype remain unclear.

Molecular methods for RV genotyping have been developed using microarray hybridization (25), restriction fragment length polymorphism (26), one-step or two-step conventional reverse-transcription PCR (RT-PCR) assay followed by gel-based genotyping of PCR amplifications (27–29), and nucleotide sequencing (30). Real-time RT-PCR assays have several advantages, including greater sensitivity than the conventional nested PCR (31), higher throughput, reduced turnaround time, and the ability to quantify viral RNA. In this study, we developed RV5 genotype-specific real-time RT-PCR assays to discriminate five different VP7 genotypes of RV5 and examined the kinetics of fecal shedding of these five genotypes in vaccinated infants.

MATERIALS AND METHODS

Viruses and RNA extraction. Commercially available RV5 was used as a vaccine strain. Human RVA strains KU G1P[8], DS-1 G2P[4], YO G3P[8], and ST3 G4P[6] and bovine RVA strain WC3 G6P[5] were used as representative wild-type viruses for the corresponding RV genotypes. These RVA strains were propagated as described previously (32). Briefly, RVA strains were pretreated with trypsin (type IX, from porcine pancreas) (10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C and propagated in MA104 cells in Eagle's minimum essential medium (Nissui, Tokyo, Japan) without fetal calf serum but containing trypsin (1 μg/ml).

The supernatant of viral cultures was used for RNA extraction. RNA was extracted from 140 μl of RV5 vaccine suspension, each wild-type strain, and stool samples using a QIAamp viral RNA minikit (Qiagen, Valencia, CA, USA). After extraction, RNA was stored at −80°C.

Primers, probes, and conditions for the genotype-specific real-time RT-PCR assays. Locations and sequences of primers and probes for genotype-specific real-time RT-PCR assays are shown in Table S1 in the supplemental material. The primers and probes were designed to discriminate between G1, G2, G3, G4, and G6 using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA) based on the reference sequences of vaccine strains (GenBank accession numbers GU565057.1 for G1, GU565068.1 for G2, GU565079.1 for G3, GU565090.1 for G4, and GU565046.1 for G6). To increase the sensitivity of the assays, ZEN double-quenched probes (Integrated DNA Technologies) were used.

Genotype-specific real-time RT-PCR assays were carried out using a TaqMan RNA-to-C_T 1-Step kit (Applied Biosystems, Foster City, CA, USA). Single-well denaturation, reverse transcription, and amplification were performed on a StepOne real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) in standard mode. PCR conditions were as follows: RT step for 15 min at 48°C, DNA polymerase activation and RT inactivation for 10 min at 95°C, and 45 cycles of denaturation for 15 s at 95°C, primer annealing for 30 s (G1, at 56°C; G2, no step; G3, at 51°C; G4, at 50°C; G6, at 62°C), and extension/emission of fluorescence for 60 s at 60°C. The real-time RT-PCR mixture (25-μl total volume) contained 12.5 μl of 2× RT-PCR mixture, 5 μl of template RNA, 40× TaqMan RT enzyme mixture, 200 nM probe, 400 nM primer, 100× 6-carboxy-X-rhodamine (ROX) reference dye, and nuclease-free water. All samples were subjected to heat denaturation at 95°C for 5 min.

Standard curves and positive control. RNA extracted from RV5 was used to prepare standard curves for each genotype-specific real-time RT-PCR assay.

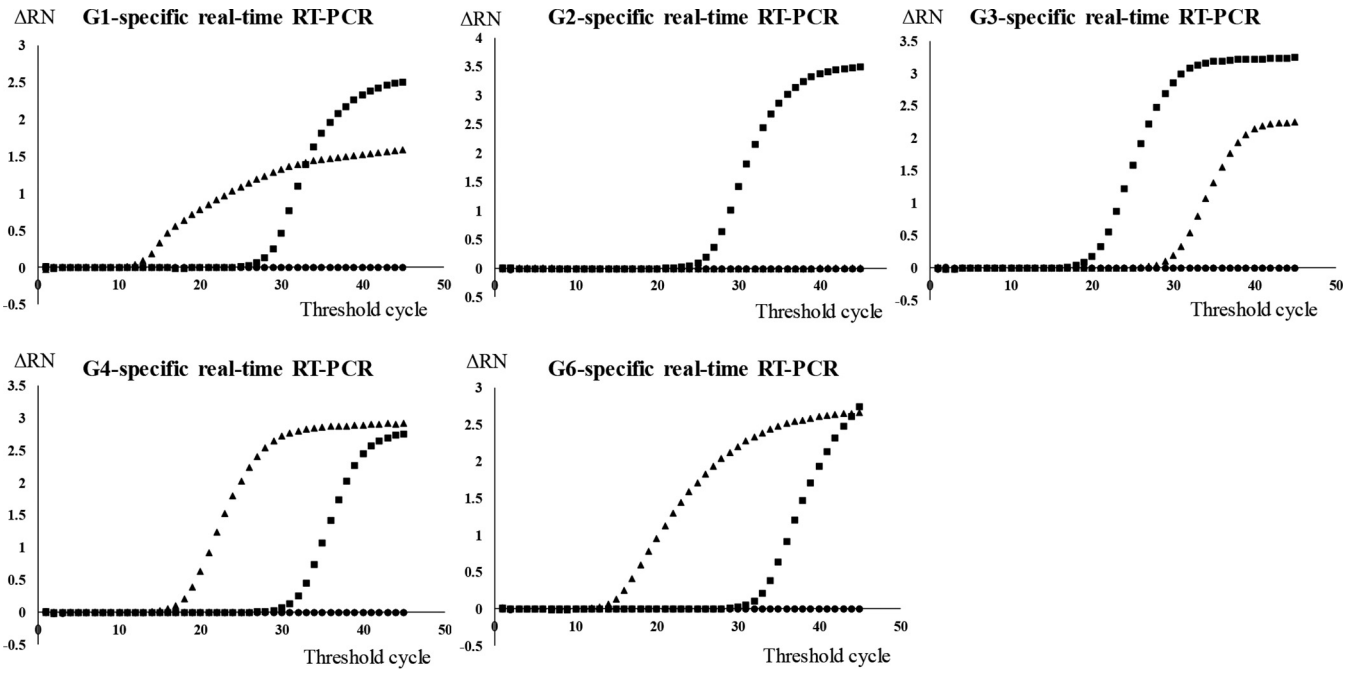

FIG 1 Specificity of one-step real-time RT-PCR assay for discrimination of five specific G genotypes of RotaTeq. ■, RNA extracted from RotaTeq; ▲, RNA extracted from a G genotype-specific rotavirus wild-type strain in the corresponding real-time RT-PCR; ●, RNA extracted from a rotavirus wild-type strain of another G genotype in the corresponding real-time RT-PCR.

According to the prescribing information, RV5 contains ≥2.2 × 10^6 infectious units (IU) of G1, ≥2.8 × 10^6 IU of G2, ≥2.2 × 10^6 IU of G3, ≥2 × 10^6 IU of G4, and ≥2.3 × 10^6 IU of P1A[8] in 2 ml of vaccine solution. Serial dilutions of each genotype were used to make each standard curve for real-time RT-PCR assays. RNA extracted from RV5 was used as a positive control for all genotype-specific real-time RT-PCR assays.

Reproducibility and repeatability of genotype-specific real-time RT-PCR assays. To assess the reproducibility (intra-assay variation) and repeatability (interassay variation) of genotype-specific real-time RT-PCR assays, duplicate experiments were performed with each wild-type strain. Reproducibility was determined by measuring the samples five times on separate days within the same experiment (to assess the intra-assay variation) and between four different assays (interassay variation). The coefficient of variation (CV) of the threshold cycle (C_T) value was defined as the ratio of the standard deviation to the mean.

Clinical samples. A total of 129 stored stool samples serially collected from eight infants (cases 1 to 8) who received RV5 in a previous clinical study approved by our institutional review board (approval no. 14-140) were used in experiments aimed at evaluating the clinical reliability of the assays. In cases 1 and 2, stool samples were collected for 14 days after the first and second vaccination and for 11 days (case 1) or 8 days (case 2) after the third vaccination in the foster home. Cases 3 to 8 were patients hospitalized in a neonatal intensive care unit (NICU) who received RV5 during hospitalization. The stool samples were collected for 9 days only after the first vaccination. Specimens were stored at −20°C until the analysis. Ten percent suspensions (1 ml) of each stool sample were prepared in physiological saline solution. Alternatively, swab samples were rinsed in 200 μl of physiological saline solution. Each suspension was clarified by centrifugation for 20 min at 4,000 × *g*, and 140 μl of the supernatant was used for RNA extraction.

RESULTS

Linearities and sensitivities of RV5 genotype-specific real-time RT-PCR assays. The linearity and sensitivity of RV5 genotype-specific real-time RT-PCR assays were evaluated using serial dilutions of RNA extracted from RV5. Excellent linearity was obtained (date not shown), and a high correlation was obtained between the concentration of the diluted RNA and the C_T value of each genotype in all genotype-specific real-time RT-PCR assays. Detection limits were 1 IU/reaction for G2, G4, and G6 and 10 IU/reaction for G1 and G3.

Specificity of RV5 genotype-specific real-time RT-PCR assays. Although only the vaccine strain was amplified by the G2-specific real-time RT-PCR assays, both vaccine and wild-type genotypes were coamplified by the other four genotype-specific real-time RT-PCR assays (Fig. 1). In each G-specific real-time RT-PCR assay, only the matched

TABLE 1 Intra-assay variability of real-time RT-PCR assays for discrimination of five specific G genotypes of RotaTeq

Amt of RNA (IU/reaction)	Intra-assay variability by genotype[a]														
	G1			G2			G3			G4			G6		
	C_T	SD	CV (%)	C_T	SD	CV (%)	C_T	SD	CV (%)	C_T	SD	CV (%)	C_T	SD	CV (%)
1	ND	ND	ND	39.11	0.47	1.2	ND	ND	ND	41.20	0.72	1.7	37.31	0.57	1.5
10	33.32	0.61	1.8	35.66	0.53	1.5	34.34	0.64	1.9	36.76	0.46	1.3	34.08	0.73	2.1
1×10^2	29.46	0.40	1.4	31.82	0.41	1.3	30.51	0.67	2.2	32.65	0.44	1.4	30.45	0.73	2.4
1×10^3	25.90	0.72	2.8	28.18	0.73	2.6	26.49	0.46	1.7	28.45	0.70	2.5	26.53	0.48	1.8
1×10^4	21.93	0.22	1.0	24.47	0.47	1.9	22.70	0.38	1.7	24.35	0.33	1.3	22.43	0.26	1.2
1×10^5	18.29	0.23	1.3	20.79	0.47	2.3	19.09	0.28	1.5	20.18	0.52	2.6	18.89	0.36	1.9

[a]To determine intra-assay variability, mean cycle threshold (C_T) values and coefficients of variation (CVs) were calculated on five consecutive days. Standard samples serially diluted from RotaTeq were used. The CV of the C_T value is expressed as the ratio of the standard deviation (SD) and the mean. IU, infectious units; ND, not detected.

wild-type strain was specifically amplified. So, no cross-reaction between the other G genotypes was observed in any of the genotype-specific real-time RT-PCR assays.

Intra- and interassay variability of RV5 genotype-specific real-time RT-PCR assays. To evaluate the reproducibility of RV5 genotype-specific real-time RT-PCR assays, we evaluated inter- and intra-assay variability. To determine the intra-assay variability, the C_T values of the standard curves were measured in duplicate, and the mean C_T values and coefficients of variation were calculated on five consecutive days (Table 1). To evaluate the reproducibility of the methods, the coefficient of variation was defined as the ratio of the standard deviation to the mean. As shown in Table 1, the coefficients of variation of intra-assay experiments were less than 2.8% for five G genotypes. To evaluate reproducibility between the assays, C_T values were measured in quadruplicate (Table 2). As shown in Table 2, the coefficients of variation of the interassay examination were less than 2.9% for five G genotypes.

Reliability of assays for analyzing clinical samples. To examine replication of the five different genotypes after vaccination, we analyzed stool samples collected from vaccine recipients using the RV5 genotype-specific assays. In case 1, fecal shedding of G6 predominated after the first vaccination, whereas shedding of G1 predominated after the second and third vaccinations (Fig. 2A). In case 2, both G1 and G4 shedding predominated after the first vaccination, and G1 shedding predominated after the second vaccination (Fig. 2B). In both cases 1 and 2, fecal shedding of vaccine viruses decreased as the number of vaccinations increased. As shown in Fig. 3, the predominantly shed genotypes were G3 in case 3, G6 in case 4, G1, G2, and G6 in case 5, G1 and G4 in case 6, and G1 and G6 in case 8. Fecal shedding of all five genotypes was low in case 7.

DISCUSSION

Fecal shedding of vaccine viruses can cause transmission of vaccine virus(es) from vaccinated children to unvaccinated contacts, potentially inducing herd immunity. On the other hand, such transmission poses a risk of vaccine-derived disease in immuno-

TABLE 2 Interassay variability of real-time RT-PCR assay for discrimination of five specific G genotypes of RotaTeq

Amt of RNA (IU/reaction)	Interassay variability by genotype[a]														
	G1			G2			G3			G4			G6		
	C_T	SD	CV (%)	C_T	SD	CV (%)	C_T	SD	CV (%)	C_T	SD	CV (%)	C_T	SD	CV (%)
1	ND	ND	ND	38.96	0.74	1.9	ND	ND	ND	39.25	0.16	0.4	39.25	0.67	1.7
10	31.14	0.22	0.7	35.33	0.49	1.4	34.82	0.46	1.3	35.24	0.38	1.1	34.67	0.31	0.9
1×10^2	28.15	0.13	0.5	31.75	0.57	1.8	31.24	0.22	0.7	31.87	0.18	0.6	31.23	0.12	0.4
1×10^3	24.56	0.06	0.3	28.30	0.23	0.8	26.97	0.16	0.6	27.45	0.73	2.7	27.57	0.14	0.5
1×10^4	20.54	0.02	0.1	24.25	0.38	1.6	23.29	0.16	0.7	23.40	0.50	2.1	23.65	0.06	0.3
1×10^5	16.79	0.03	0.2	20.90	0.39	1.9	19.02	0.03	0.2	18.90	0.54	2.9	20.09	0.01	0.1

[a]To determine interassay variability, standard samples serially diluted from RotaTeq were used. Cycle threshold (C_T) values were measured in quadruplicate. The coefficient of variation (CV) of the C_T value is expressed as the ratio of the standard deviation (SD) and the mean. IU, infectious units; ND, not detected.

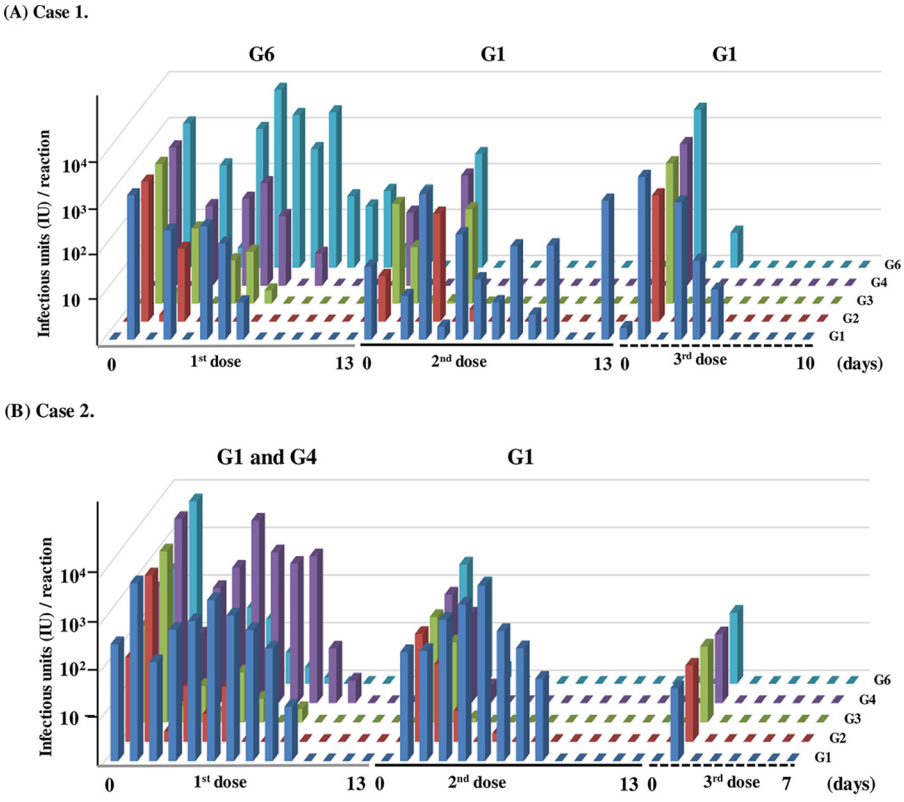

FIG 2 Excretion of five different rotavirus strains after RotaTeq vaccination in cases 1 and 2, which completed three rounds of vaccination. Predominant shedding of genotypes is shown as follows: blue, G1; brick red, G2; green, G3; purple, G4; turquoise, G6.

compromised contacts (22, 33). Reassortant strains derived from RV5 or RV1 are associated with acute gastroenteritis in vaccinated (34, 35) and unvaccinated children (36). Moreover, previous studies suggested that widespread use of the RotaTeq vaccine has led to the introduction of vaccine genes into circulating human RVs (37, 38). Therefore, characterization of the RV genotype of circulating RVA strains is important also in order to distinguish between wild-type and vaccine strains or vaccine-reassortant strains (37, 38). In this study, we developed RV5 genotype-specific real-time RT-PCR assays that can discriminate and quantify each genotype and evaluated their reliability for analysis of clinical specimens.

The detection limits of RV5 genotype-specific real-time RT-PCR assays were 1 IU/reaction for G2, G4, and G6, and 10 IU/reaction for G1 and G3, equivalent to that of a previously reported nonspecific real-time RT-PCR method for detecting RV (39) that was considered sufficiently sensitive for monitoring fecal shedding of vaccine viruses using clinical specimens.

Except for the G2-specific real-time RT-PCR assay, the assays amplified both the vaccine and wild-type strains of each G genotype (Fig. 1). However, no cross-reactivity among the genotypes tested was detected. Therefore, these methods can reliably discriminate between the five different genotypes contained in the RV5 vaccine, despite the cross-reaction between wild-type and vaccine strains in the G1-, G3-, G4-, and G6-specific real-time RT-PCR assays. Additionally, based on the intra- and interassay variability tests in the initial validation analysis, these assays are highly reproducible and suitable for the examination of clinical specimens.

As shown in Fig. 2, all G genotype RV loads decreased gradually with the number of vaccinations. RV IgA antibodies in intestinal tissue play an important role in protective immunity against RV disease. Moreover, we previously reported that fecal RV RNA load decreases gradually as the number of vaccinations increases (15). Naturally acquired RV

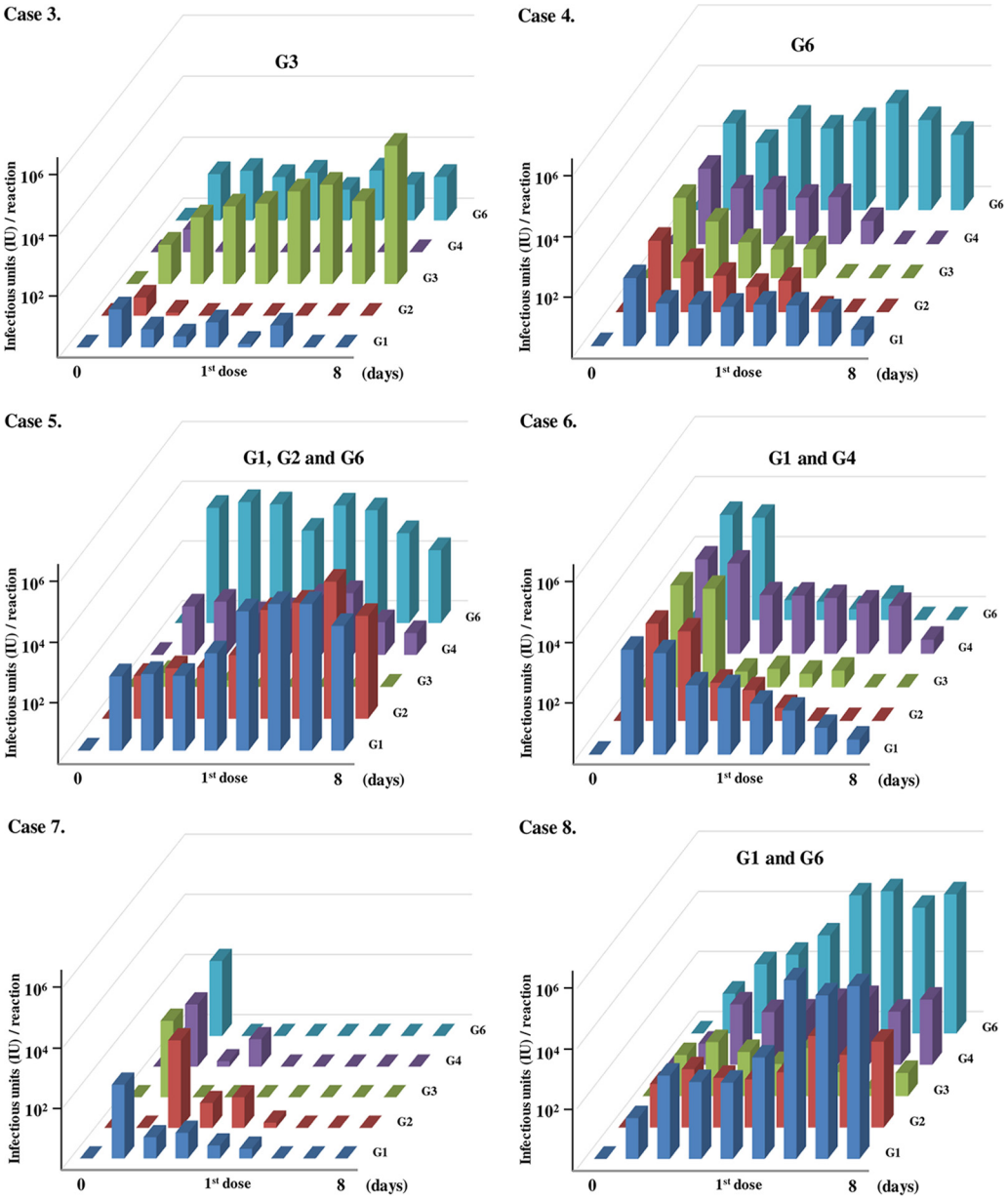

FIG 3 Excretion of five different rotavirus strains after RotaTeq vaccination in cases 3 to 8. Samples from these six cases were collected only after the first vaccination, and predominant shedding of genotypes is shown as follows: blue, G1; brick red, G2; green, G3; purple, G4; turquoise, G6.

infection induces immunity against RV and decreases the severity of the subsequent RV infection (40). A large cohort study of the natural course of RV infection, performed in Mexico, revealed that the subsequently identified genotypes tend to be different from the previously infecting genotype (40). Interestingly, the RV genotypes predominantly excreted in cases 1 (G6) and 2 (G4) at the time of the first vaccination were barely detected in fecal excretion after the second vaccination. Thus, our data support the idea of genotype-specific protective immunity, as proposed based on a previous clinical study (40). To confirm this hypothesis, future studies should test the association between fecal shedding of an RV genotype and the genotype-specific neutralizing antibody response.

In this study, among 12 separate administrations of RV5, G1 genotype shedding was predominant in seven vaccinations, and G6 genotype shedding was predominant in

four. Thus, shedding of G1 and G6P[8] genotypes appeared to predominate over shedding of other genotypes (Fig. 2 and 3). According to previous preclinical trials aimed at evaluating the safety and efficacy of RV5, neutralizing antibody responses against G1 and G6 were superior to those against G2, G3, and G4, and vaccine efficacies were almost equivalent among the five genotypes (16). The present findings, together with data from previous preclinical trials (41, 42), suggest that the G1 and G6P[8] genotypes can proliferate predominantly *in vivo* and may induce a strong immune response against G1, G6, and P[8] genotypes in vaccinated infants. Future studies should measure genotype-specific neutralizing antibody titers and other immunological factors to determine whether the level of fecal shedding of RV is correlated with the immune response (43–46).

Although the number of cases in this study was limited, patterns of fecal shedding of the five different genotypes of vaccine viruses appeared to differ among the vaccine recipients. For example, in case 7, shedding of all five RV vaccine strains was limited. Various factors such as breast feeding and maternal antibodies may interfere with the host immune response. Breast milk contains RV-specific antibodies and other neutralizing factors (47–50), including maternal antibody (51, 52), which may result in suppression of RV vaccine proliferation in intestinal tissue. Vaccine efficacies are lower in developing countries (53, 54) than in developed countries (55, 56). Therefore, in the future, it will be important to elucidate the precise mechanisms controlling vaccine virus replication in intestinal tissue and the host immune response against RV.

In summary, we developed RV5 genotype-specific real-time RT-PCR assays capable of measuring genotype-specific viral shedding in RV5-vaccinated infants. According to our analysis of clinical samples, it is likely that shedding of G1 and G6 is predominant in vaccinated infants. Additionally, because patterns of fecal shedding of the five different genotypes of RV5 appeared to differ among the vaccine recipients, some individual factors may contribute to control of vaccine virus replication in intestinal tissue. The novel RV5 genotype-specific real-time RT-PCR assays should be useful for high-throughput molecular screening of stool samples. Furthermore, the assays could be a valuable tool for the study of the molecular biology/replication of RV5 components in vaccinated infants and also in suitable animal models.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00035-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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