DETECTION OF VACCINE-DERIVED ROTAVIRUS STRAINS IN NON-IMMUNOCOMPROMISED CHILDREN UP TO 3-6 MONTHS AFTER VACCINATION

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JUKKA MARKKULA: DETECTION OF VACCINE-DERIVED ROTAVIRUS STRAINS IN NON-IMMUNOCOMPROMISED CHILDREN UP TO 3-6 MONTHS AFTER VACCINATION

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RotaTeq® on elävä, oraalisesti kolmesti (2, 3 ja 5 kk) annosteltava rotavirusrokote, joka koostuu ihmisen ja naudan rotavirusten yhdistelmistä. Villityypin rotavirusten tapaan myös rokotetyypin viruksien on havaittu erittyvän ja muodostavan uusia kombinaatioita rokotteen sisältämien kantojen välillä.

Tutkimuksen tarkoituksena oli tutkia rokotetyypin rotaviruksen erityksen yleisyyttä lapsilla. Aineistona käytettiin Tampereen yliopistollisessa sairaalassa syyskuun 2009 ja elokuun 2011 välisenä aikana hoidettuja ripulitautia (AGE) tai hengitystieinfektiota (RTI) sairastavia lapsia, joilta kerättiin ulostenäyte. Tutkimukseemme osallistui 333 lasta, jotka olivat saaneet vähintään yhden annoksen RotaTeq® rokotetta. Lapset jaettiin tutkimusryhmiin taudin diagnoosin perusteella: 182 RTI, 56 AGE, ja 62 lapsella oli molempien oireita. Ulostenäytteistä tutkittiin PCR-menetelmällä rotaviruksen pintaproteiinit VP7, VP4 ja VP6 mahdollisen rokoteviruksen tunnistamiseksi. Rokoteperäisiä yhdistelmäviruksia yritettiin kasvattaa soluviljelmissä.

Tutkimuksessamme RotaTeq® rokoteviruksen erittyminen oli yleistä, 17 %:lla (49) lapsista havaittiin RotaTeq® VP7 ulosteesta. Näistä 30 lapsella oli RTI, 12 AGE ja 7 molempia oireita. RotaTeq® G1 oli yleisin genotyyppi kaikissa ryhmissä. Erityksen kestoa tutkittiin RTI lapsilla, joita pidimme terveiden verrokkeina. Eritystä havaittiin 14/28 (50 %) näytteistä, jotka oli otettu 1. ja 2. rokoteannoksen välissä, 10/38 (26 %) 2. ja 3. annoksen välissä, ja 6/117 (5 %) 3. rokoteannoksen saaneiden näytteistä. On kuitenkin mahdollista, että erittyminen on alkanut jo ensimmäisen rokoteannoksen jälkeen, joten todellisuudessa erityksen kesto voi olla havaittua pidempi.

Tutkimuksessa havaitsimme, että RotaTeq® rokotevirusten erittyminen on yleistä myös lapsilla, joilla ei ole gastroenteriitin oireita. Pitkään jatkuva virusten erittyminen ja täten myöskin lisääntyminen suolistossa viittaa pitkittyneeseen, jopa krooniseen infektioon. Löydöksen kliininen merkitys on kuitenkin epäselvä ja vaatii lisätutkimuksia.

Introduction

Rotavirus (RV) is globally the most common cause of acute gastroenteritis (AGE) in young children with an estimated death toll of 453.000 annually [1]. In developed countries RV is an important cause of hospitalizations. Two live oral RV vaccines were licensed in 2006 [2, 3], and many countries have introduced universal RV vaccination programs.

RotaTeq® (Merck & Co., Whitehouse Station, NJ, USA) is a live oral pentavalent human-bovine reassortant RV vaccine, consisting of five vaccine viruses, each with bovine RV strain WC-3 (G6P[5]) backbone reassorted with either VP7 surface proteins of human RVs G1, G2, G3 and G4, or with human VP4 outer surface protein P[8]. In Finland, RotaTeq® vaccine was taken into the national immunization program in September 2009, and is administered on a three dose schedule at the ages of 2, 3 and 5 months. The coverage is over 95 %, and the impact of RotaTeq® vaccinations on RV gastroenteritis (RVGE) seen in hospital in Finland has been impressive with 91 % reduction in vaccine eligible age groups [4, 5].

In wild-type RVGE, shedding of RV in stools usually lasts for 7-10 days [6]. Prolonged shedding of wild-type RV has been described up to 57 days after hospital admission for RVGE in previously healthy children, and up to 25 days in asymptomatic children [7, 8]. Shedding of RotaTeq® vaccine strain viruses was reported to be low in pre-licensure studies[3], because the testing method, the plaque assay in cell culture, was insensitive. In the REST study, using the plaque assay, the rate of shedding of RotaTeq® vaccine virus was 32/360 (8.9 %) after the first dose, 0/249 after the second dose and 1/385 (0.3 %) after the third dose, and the duration of shedding was 1-15 days with peak from 4 to 6 days.[9] Considerably higher rates of vaccine virus shedding were recently detected when the REST study samples were re-examined with enzyme-linked immunosorbent assay (ELISA) and VP6 reverse transcription polymerase chain reaction (RT-PCR) [10].

Other recent studies using ELISA to detect RV antigen have shown shedding rates of RotaTeq® vaccine viruses up to 21.4 % in vaccinated children, who have had AGE symptoms after vaccination [11, 12]. A study by Hsieh et al. used real-time polymerase chain reaction (qPCR) for detection of RV RNA, and found very high rates of shedding (80-90 %) after the first dose, while the corresponding rate of shedding using ELISA antigen detection was 20-30 %. The maximum duration of shedding was 28 days, after the first dose.[13] Prolonged shedding and transmission of RotaTeq® vaccine viruses have been reported in premature infants and children with severe combined immunodeficiency (SCID) and, occasionally, in immunocompetent children [14-17].

Several studies on children have detected presence of vaccine-derived double-reassortant RV G1P[8] (vdG1P[8]) in the stools in association with AGE symptoms [11, 16-19], suggesting that vdG1P[8] may be of greater virulence than the original vaccine strains. Shedding of vdG1P[8] has also been associated with sibling transmission [16].

We examined the occasional presence of RotaTeq® vaccine viruses in young children seen in hospital mainly for respiratory tract infection (RTI) and correlated these findings with history of RV vaccination. This approach yielded new information on the extent, duration, and type of RotaTeq® vaccine virus shedding.

Materials and methods

Clinical methods

A prospective study on the etiology of AGE and RTI in children was conducted at Tampere University Hospital from September 1, 2009 to August 31, 2011. The study was approved by the Ethics Committee of Pirkanmaa Hospital District. A written consent of a parent or legal guardian was obtained before enrollment of the child into the study.[20] Children, who were seen in the pediatric outpatient clinic or admitted into the pediatric ward with AGE or who were admitted into the pediatric ward with RTI, were eligible for the study. Many children actually had symptoms of both AGE and RTI. Patients with nosocomial AGE were also included.[5, 20]

Information about possible RV vaccination was enquired from the parents and confirmed from the records of the respective well baby clinic by a study nurse. A stool sample was collected during the hospitalization or, if not successful, the parents were provided with a sample kit to send a stool specimen within two weeks from home.

Laboratory methods

Viral RNA was extracted from stool samples using Qiagen QIAamp Viral RNA Mini Kit (Hilden, Germany) according to the manufacturer's instructions. The RNA extracts were stored in a freezer at -70°C until tested by RT-PCR for RV. RV VP7 and VP4 sequences were detected using previously described primers and methods [21-22]. VP6 sequences were detected as previously described [19, 23].

Positive RT-PCR detections were further analyzed by sequencing using the same primers as in RT-PCR. Positive amplicons (VP7, VP4 and VP6) were purified using Qiagen QIAquick Gel Extraction Kit (Hilden, Germany) and sequenced using BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Applied Biosystems, Foster City, CA) on an ABI PRISM™ 310 Genetic Analyzer. Sequences were analyzed with Sequencher™ 4.9 and compared with published reference strains from GeneBank (http://www.ncbi.nlm.nih.gov/BLAST/, Nucleotide blast).

RV VP6 antigen was determined from RotaTeq® VP7 positive stool samples by ELISA, using the IDEIA Rotavirus Kit (Oxoid Ltd, Basingstoke, Hampshire, United Kingdom). Stools absorbed in a

diaper were not tested by ELISA, as the test could not be performed reliably. A sample with an optical density read >0.15 by spectrophotometry at a wavelength of 450 nm was considered as positive. Positive stool samples were propagated in MA104 cells as described previously with the modification of using minimum essential medium (MEM) with 0.5 µg/mL of trypsin instead of MEM containing 100 U/mL penicillin, 100 U/mL L-glutamine and 100 µg/mL streptomycin [24].

Results

During the two-year study period a total of 1610 children with symptoms of AGE, RTI, or both, were recruited into the study and 1002 (62.2 %) children provided stool samples. Of these children, 333 (33.2 %) had been vaccinated with RotaTeq® at any time after September 1, 2009 and formed the patient material of our study. Of the 333 children, 56 (16.8 %) were seen or admitted for AGE, 182 (54.7 %) were admitted for RTI, and 95 (28.5 %) had both AGE and RTI symptoms during the clinic visit or hospital stay. The mean age of the study population was 258 days, ranging from 56 to 643 days; 70.6 % were males.

RV detection and types

Out of the 333 stool samples from children who had ever been vaccinated with RotaTeq® vaccine, 53 (15.9 %) were RV positive by RT-PCR specific for VP7. Four (7.5 %) were identified as wild-type RVs and 49 (92.5 %) as RotaTeq® vaccine type RVs by sequence analysis of VP7. All 4 wild-type associated cases occurred in children who were hospitalized with AGE or a combination of AGE and RTI symptoms, and the wild-type RV genotypes were G4P[8] (3 cases) or G9P[8] (1 case). Of those children, 3 had received the full vaccine regimen and one had received one dose of RotaTeq® vaccine. It is assumed that these cases were breakthrough RV infections in vaccinated children. No vaccine-type virus was detected concomitantly with the wild-type RV in these cases.

Of the 49 vaccine strain positive children 12 (24.5 %) had AGE, 30 (61.2 %) had RTI, and 7 (14.3 %) had both AGE and RTI symptoms. RV antigen by ELISA was tested from 30 (61.2 %) of the 49 VP7 positive cases. ELISA could not be performed from the remaining 19 samples due to an insufficient amount or type of the sample (diaper). Out of those 30 samples, only 5 (16.7 %) were ELISA positive; of these 1 was from a child with AGE and 4 from children with RTI. The ELISA positive child with AGE was detected with RotaTeq® G1P[8] as did all the 4 children with RTI, one child was also detected with additional P[5] VP4.

VP6 RT-PCR was positive in 47 (95.9 %) and negative in 2 (4.1 %) of the 49 vaccine VP7 positive cases. The negative results were from one child with RTI and another child with AGE. Bovine vaccine type VP6 was detected in 46 cases and human type P[8] VP6 in 1 child with RTI,

described later. VP6 RT-PCR was negative in 2 cases. VP4 RT-PCR was positive in 35 and negative in 14 cases.

RotaTeq® G1 was by far the most common G-type as it was detected in 46 of the 49 vaccine type VP7 positive cases. Of these, 30 children had RTI. The combination G1P[8] was detected in 11 children (36.7 %) (once concomitantly with RotaTeq® G6). G1P[5] was detected in 4 children (13.3 %), and one child had RotaTeq® G1 with both P[5] and P[8]. VP4 reassortant G6P[8] was detected in two children with RTI symptoms, and one child, mentioned above and described later, was detected with human VP6 reassorted with RotaTeq® G1 and human P[8]. (Fig 1)

Genotypic distribution of vaccine origin RVs in children with AGE (N=12) was even more diverse than in children with RTI. Double-reassortant G1P[8] was detected in 4 children (33.3 %). RotaTeq® G1 with P[5] and P[8] was found in the stools of two children (16.7 %). Other genotypes were detected only one each and are shown in Figure 1.

In children with both AGE and RTI symptoms (N=7) RotaTeq® G1 alone, RotaTeq® G1P[8] and RotaTeq® G1 with P[5] and P[8] were detected in two children each. One child was detected with RotaTeq® G1P[5]. (Fig. 1)

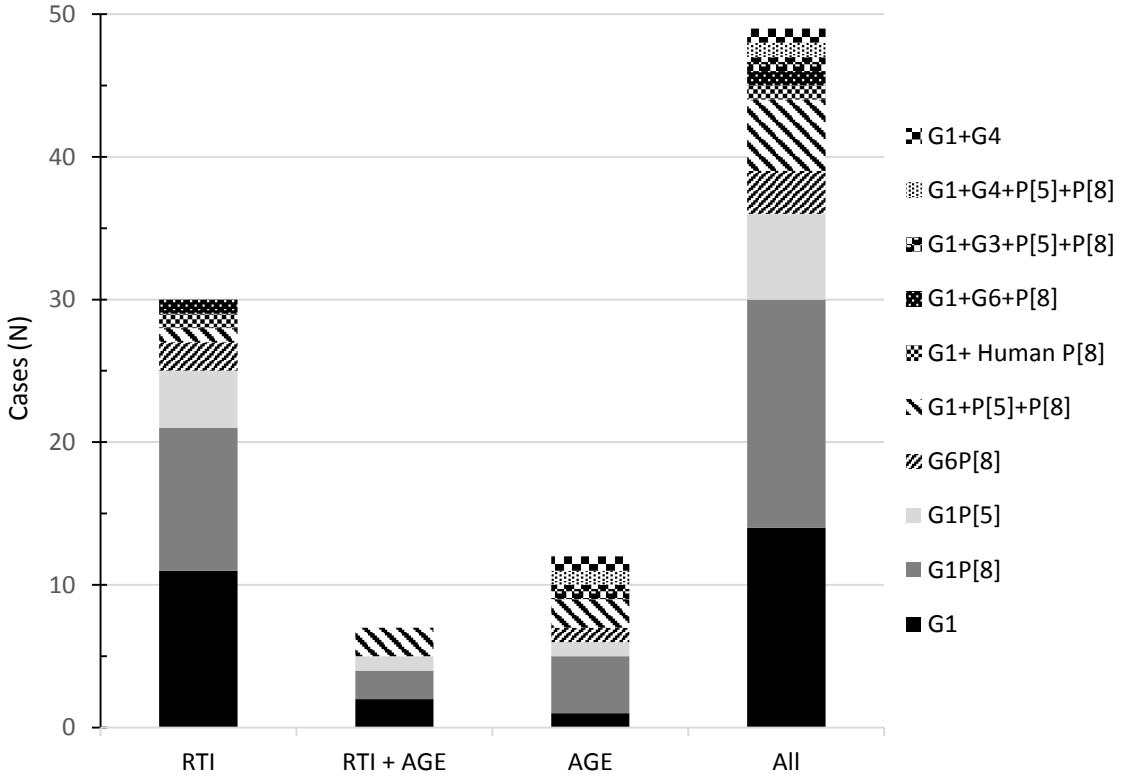

Figure 1. Genotypic distribution of children detected with RotaTeq® vaccine strains detected in stool samples of children with respiratory tract infection (RTI), acute gastroenteritis (AGE), or both.

One child hospitalized for RTI was detected with RotaTeq® vaccine origin VP7, together with human wild-type VP4 and VP6. The stool sample of this child was obtained 10 days after the

second dose of RotaTeq® vaccine. The sample was extracted for several times, and RT-PCR and sequencing were done twice for each protein from each extraction, but the RT-PCR and sequencing results remained identical. The sample could not be propagated in MA104 cells.

Vaccine-derived G1P[8] double-reassortants

Sixteen (32.7 %) children out of 49 were detected concomitantly with vaccine-derived VP7 G1 and VP4 P[8] and bovine VP6 of vaccine origin. In 4 cases the virus had already been verified as true double-reassortant rather than only concomitant shedding of vaccine originated proteins. These 4 children with AGE symptoms and RotaTeq® G1P[8] double-reassortant virus have been previously reported [19]. In the remaining 12 cases, the same vaccine-derived G1P[8] (vdG1P[8]) rotavirus was now detected in 10 children with RTI and in 2 children with AGE and RTI symptoms.

Stool samples of three children detected with RotaTeq® vdG1P[8], who had recently received the first dose of RotaTeq® vaccine and were ELISA positive, were cultured in MA104 cells. Stool samples were obtained 6 days (1 case) and 14 days (2 cases) after the vaccination. Two of the samples showed no sign of growth in cell culture, but in one case (sample obtained 6 days after vaccination), the virus was successfully propagated and remained stable in three passages of cell culture.

Shedding of RotaTeq® vaccine viruses in children with RTI

Of the 182 children with RTI, 28 had received the 1st dose, 38 had received two doses and 116 had received all three doses of RotaTeq® vaccine at the time they were hospitalized for RTI. In these children, shedding of RotaTeq® vaccine virus was detected in 14/28 (50.0 %) after the first and before the second dose. After the second and before the third dose vaccine virus shedding was detected in 10/38 cases (26.3 %), and after the third dose 6 out of 116 cases (5.2 %). The oldest child with RotaTeq® vaccine virus detection was aged 8 months. The age distribution of the children is shown in Figure 2.

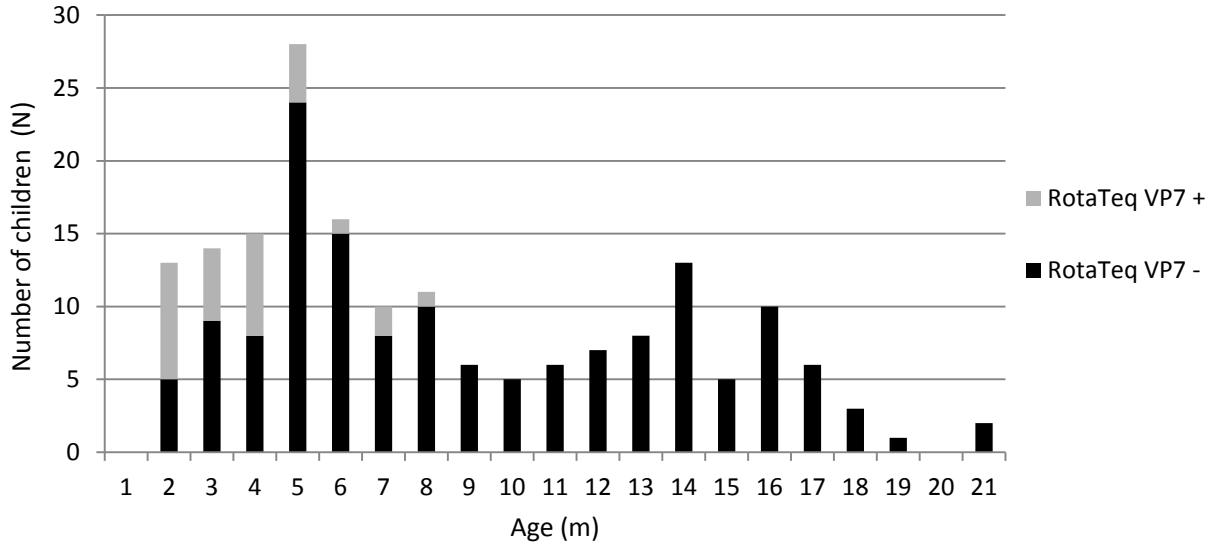

Figure 2. Age distribution of children hospitalized with respiratory tract infections and studied for RotaTeq® vaccine strains in stool samples.

Overall, RotaTeq® vdG1P[8] was commonly shed after each dose of the vaccine. After the first dose 7 children (50.0 %) shed RotaTeq® vdG1P[8] and 3 children G1 alone. After the second and the third dose of vdG1P[8] was less commonly found but RotaTeq® G1 was the most common genotype as it was detected in various combinations in 5 children (50.0 %) and in 3 children (50.0 %) after these doses, respectively. No other vaccine-derived human G-type but G1 was found in the samples collected from children with RTI. The vaccine strains and combinations in relation to the latest vaccine dose are shown in Table 1.

Genotype	I dose (N=14)	II dose (N=10)	III dose (N=6)	Total (N=30)
G1	3 (21.4)	5 (50.0)	3 (50.0)	11 (36.7)
vdG1P[8]	7 (50.0)	2 (20.0)	1 (16.7)	10 (33.3)
G1P[5]	1 (7.1)	2 (20.0)	1 (16.7)	4 (13.3)
G1+P[5]+P[8]	1 (7.1)	0	0	1 (3.3)
G1+Human P[8]	0	1 (10.0)	0	1 (3.3)
G6P[8]	1 (7.1)	0	1 (16.7)	2 (6.7)
G1+G6+P[8]	1 (7.1)	0	0	1 (3.3)

Table 1. Frequency, N (%), of rotavirus genotypes and their combinations detected after each dose of RotaTeq® vaccine in children hospitalized with respiratory infection.

The duration of shedding was counted from the date of the most recent vaccine dose and was over 14 days in 16 cases (53.3 %) and over 30 days in 9 cases (30.0 %). The median duration of shedding of RotaTeq® G1 containing vaccine strains, excluding the vdG1P[8] and concomitant shedding of G6P[8], was 22 days. The median duration of shedding of RotaTeq® vdG1P[8] was 14 days. The longest duration of shedding was 84 days counted from the third immunization in a child

detected with RotaTeq® G1 VP7, however VP4 and VP6 RT-PCRs were negative. The proportion of long-time (over 14 days) shedders became larger after each dose of vaccine received; after the first dose prolonged shedding was detected in 4/14 (28.6 %) children, respectively after the second 7/10 (70.0 %) and after the third dose 5/6 (83.3 %).

Discussion

In this study, we used children hospitalized for respiratory infection without AGE symptoms as proxies for healthy children to follow the shedding of RotaTeq® vaccine viruses. Among this pediatruc population, we detected the presence of RotaTeq® vaccine viruses in 16.5 %. This is higher than detected in the pre-licensure studies using the plaque assay method on samples collected within a week after each dose [3, 9, 25], but largely in line with more recent studies, which have shown shedding rates of around 20 % using RT-PCR or EIA as detection methods [11, 12, 26]. However, these studies were originally planned to detect RV soon after vaccination, whereas our study was an occasional survey of children seen in hospital and included many who had received the vaccine a long time before hospitalization.

The previous studies have not detected prolonged shedding in such scale; although Hsieh et al. detected RotaTeq® strains 28 days after inoculation [13]. Very long shedding, like our finding, up to the age of 8 months has not been described previously, although Patel et al. detected shedding over 200 days in immunocompromised children [15]. We detected that the proportion of prolonged shedding became higher after each dose of RotaTeq® vaccine. However, it is not certain after which dose the shedding started in such cases. It is possible that our occasional sampling detected children who became long time shedders already after the first dose.

Dominance of RotaTeq® G1 genotype in shedding was a new finding, as G1 alone, G1P[5] or vdG1P[8] were detected in the stool samples of 85.7 % of all children and 90.0 % of children with RTI symptoms. In pre-licensure studies of RotaTeq®, G1 and P[8] were also the most common genotypes to be shed after vaccination, also shedding of vdG1P[8] was detected already in these studies [27, 28]. In the composition of RotaTeq® vaccine, the amount of G1 and P[8] is nearly the same as the amount of G3 and G4, and lower than G2; therefore the higher shedding rate of RotaTeq® G1 cannot be explained by a higher inoculum [29]. Our study showed that the only unifying factor with prolonged shedding was RotaTeq® G1 VP7, and detected long duration of shedding of G1 whether the VP4 was P[5] or P[8]. The properties that make G1 such a predominant genotype in shedding remain unknown and need further study.

We also detected a high number, 16 in total, of children, who were shedding either vaccine-derived double-reassortant G1P[8] RVs or at least G1 and P[8] proteins of the vaccine viruses concomitantly. Of these, 4 cases of vdG1P[8] have been previously confirmed by cell culture as

stable double-reassortant RVs with the ability to cause AGE symptoms in children either after the first or the second dose of RotaTeq® vaccine within 14 days[19]. In this study we confirmed one ELISA positive vdG1P[8] double-reassortant RV case by cell culture in a child, who had received the first dose of RotaTeq® vaccine 6 days before hospital admission caused by RTI with no diarrhea. We cultured two other ELISA positive RTI cases in MA104 cells but no growth was detected. The other 9 cases were ELISA negative and were not cultured. Therefore we cannot confirm whether these detections are true vdG1P[8] double-reassortants or concomitant shedding of G1 and P[8] vaccine virus proteins but believe the former is more likely. Still, and if so, our finding of vdG1P[8] double-reassortants in asymptomatic children suggests that the double-reassortant is not always associated with diarrheal symptoms. However, we could not verify if these children had had AGE symptoms soon after the vaccination and before hospital admission, because we could not obtain a full medical history of these children. The potential of the vdG1P[8] to cause symptoms may still be higher than that of other vaccine-derived viruses, and there is a possibility that the double-reassortant is more infectious than other reassortants.

The major limitation of our study was the design of the original study, which was not planned to determine the rate or duration of shedding of RotaTeq® vaccine, but only provided stool samples at random time points. However, even with this less than optimal study design we could determine that long term shedding of vaccine viruses is not uncommon. While we could not determine after which dose the long-term shedding started, it is reasonable to speculate that already the first dose may select those individuals who eventually become long-term shedders.

Shedding of RV vaccine strains in asymptomatic children is usually not regarded as clinically significant, with the possible exception of transmission to susceptible or immunocompromised contacts. The use of sensitive RT-PCR in detection of vaccine viruses has been criticized, and the plaque assay in cell culture defended, on the grounds that RT-PCR may not detect live infectious viruses but only parts (RNA) of the virus [10]. However, a prolonged presence and multiplication of the vaccine viruses in the intestines even as detected by RT-PCR in this study is an indication of chronic infection in the intestines and may also be associated with clinical consequences, although these are unknown as yet.

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