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Detection of Fecal Shedding of Rotavirus Vaccine in Infants Following Their First Dose of Pentavalent Rotavirus Vaccine

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Abstract

Studies on rotavirus vaccine shedding and its potential transmission within households including immunocompromised individuals are needed to better define the potential risks and benefits of vaccination. We examined fecal shedding of pentavalent rotavirus vaccine (RV5) for 9 days following the first dose of vaccine in infants between 6 and 12 weeks of age. Rotavirus antigen was detected by enzyme immunoassay (EIA), and vaccine-type rotavirus was identified by nucleotide sequencing based on genetic relatedness to the RV5 VP6 gene. Stool from 22 (21.4%) of 103 children contained rotavirus antigen-positive specimens on ≥1 post-vaccination days. Rotavirus antigen was detected as early as post-vaccination day 3 and as late as day 9, with peak numbers of shedding on post-vaccination days 6 through 8. Vaccine-type rotavirus was detected in all 50 antigen-positive specimens and 8 of 8 antigen-negative specimens. Nine (75%) of 12 EIA-positive and 1 EIA-negative samples tested culture-positive for vaccine-type rotavirus. Fecal shedding of rotavirus vaccine virus after the first dose of RV5 occurred over a wide range of post-vaccination days not previously studied. These findings will help better define the potential for horizontal transmission of vaccine virus among immunocompromised household contacts of vaccinated infants for future studies.

Keywords

gastroenteritis; rotavirus; vaccine

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1. Introduction

In 2006, the pentavalent human-bovine reassortant rotavirus vaccine, RotaTeq (RV5; Merck & Co., Whitehouse Station, NJ), was licensed in the United States after major clinical trials demonstrated its efficacy in preventing rotavirus gastroenteritis. [1, 2] In a large phase III efficacy trial, fecal shedding of vaccine virus was evaluated in a subset of participants on days 4 to 6 after each of the 3 administered doses, usually given at ages 2, 4, and 6 months. [2] Shedding was seen in 17 (12.7%) of 134 participants after the first dose, 0 of 109 after the second dose, and 0 of 99 after the third dose. In the Rotavirus Efficacy and Safety Trial (REST), shedding was evaluated among a subset of subjects during the 4 to 6 day period after each dose and among all subjects who submitted a stool sample positive for rotavirus antigen at any time. [3] Shedding was seen in 32 (8.9%) of 360 subjects after the first dose, 0 of 249 after the second dose, and 1 (0.3%) of 385 after the third dose. The potential for horizontal transmission of vaccine virus was not evaluated in either trial.

Exposure to a dose as low as 10 wild-type rotavirus particles may result in infection in a susceptible individual [4–6], and the attack rate of wild-type rotavirus in close contacts of infected children may range anywhere from 15 to 50%. [7–9] Thus, it is biologically plausible that fecal shedding of rotavirus vaccine virus can potentially lead to horizontal transmission to close contacts of vaccinated infants and subsequent clinical disease. In fact, a case of pentavalent rotavirus vaccine virus transmission from one healthy, immunocompetent sibling to another healthy, immunocompetent sibling resulting clinical disease requiring treatment in an emergency department has been reported. [10] This potential for transmission is significant for immunocompromised individuals who are at risk for developing more severe and/or chronic infection, which may include extraintestinal infection and severe, protracted diarrhea as has been seen in individuals with primary immunodeficiencies and bone marrow or solid organ transplants infected with wild-type rotaviruses, and more recently, in infants with severe combined immunodeficiency and vaccine-type rotavirus. [11–19] According to the recommendations for rotavirus vaccination by the Advisory Committee on Immunization Practices (ACIP), infants living in households with individuals who have impaired immune status can be vaccinated, as the benefit afforded from protection against wild-type rotavirus disease for these individuals outweighs the theoretical low risk for transmission of vaccine virus and subsequent vaccineassociated disease. [1] Nevertheless, studies on rotavirus vaccine virus shedding and its potential transmission in these households should be carried out to better define the potential risks and benefits of vaccination. This may be important in areas where rotavirus-associated mortality is low and providers are more wary of the potential risks of vaccination. [7]

Since the low rates of RV5 fecal shedding observed in the pre-licensure trials were observed during a limited time period of 4 to 6 days following vaccination, assessment of RV5 shedding over a greater time period will help better define the potential for horizontal transmission of rotavirus vaccine virus among household contacts of vaccinated infants. Therefore, we conducted the following study to further characterize fecal shedding of RV5 in infants following their first dose of vaccine, the dose after which the greatest proportion of shedding was observed during the prelicensure trials.

2. Materials and Methods

2.1 Study Participant Enrollment

Study enrollment began in May 2008 and ended in February 2009. Recruitment of study participants occurred at the Broadway Practice of the Ambulatory Care Network of New York-Presbyterian Hospital, located in the Inwood neighborhood of northern Manhattan in New York City. This practice serves over 4000 pediatric patients each year. In 2008, approximately 19% of these patients were under 2 months of age. The majority of the population (66%) is Latino, with 76% of patients receiving Medicaid.

This study enrolled infants between 6 and 12 weeks of chronological age who had already been deemed eligible to receive their first dose of RV5 by their primary care providers. Primary care providers or study team members obtained verbal consent for participation from primary caretakers under an approved protocol by the Institutional Board Review Office of Columbia University. Study participant recruiters excluded infants if their caretakers did not speak English or Spanish, since information sheets and stool collection kit instructions were available only in these two languages. Administration of other licensed childhood vaccines was not restricted.

2.2 Stool Sample Collection

Once a provider or study recruiter obtained verbal consent, the primary caretaker was given a stool collection kit and instructed to obtain swabbed samples of the infant's stool once daily during the 9 days following vaccination with the first dose of RV5. All samples were de-identified and assigned a unique study number separate from the medical record. No patient information was collected.

Each stool collection kit included the following items: 9 double-ended swab culturettes (BBL™ CultureSwab™ Liquid Stuart Medium, Becton, Dickinson, and Company, Franklin Lakes, NJ),9 labels, one for each post-vaccination day to identify the day the swab was obtained, 9 plastic bags with post-vaccination day labels, 1 specimen retention box to hold all bagged specimens, 1 information pamphlet with photographic instructions, and hand sanitizer. Caretakers were instructed to dip the culture swabs into stool present in the infant's diaper to obtain a sample. The swabs were then labeled with the appropriate post-vaccination day and placed into a plastic bag also labeled with the corresponding post-vaccination day. The bags were stored inside the retention box and kept refrigerated for the duration of the collection period. No swabs were obtained on days that the infant did not stool. Study team members provided follow up calls to the caretakers at the beginning and near the end of the collection period to answer any questions. At the end of the 9-day collection period, participants were instructed to return the retention box with all the swabs to the clinic, when they were given a $15 gift card to reimburse them for their time and travel expenses. The study team was notified daily if samples were returned, and once notified, study team members transported the returned kits to the New York-Presbyterian/ Columbia Clinical Virology Laboratory for rotavirus antigen testing.

2.3 Detection of RV5

To screen for rotavirus vaccine virus, all stool samples were first tested for the presence of rotavirus antigen. Antigen positive samples then underwent reverse transcription-polymerase chain reaction (RT-PCR) amplification and nucleotide sequencing of specific gene segments of the bovine rotavirus strain WC3, the parent strain of the pentavalent RV5 vaccine. Each of the 5 vaccine strains (vaccine-type rotavirus), contains 9 or 10 genes derived from WC3 and 1 or 2 genes from human rotaviruses. [20] A subset of antigen positive samples underwent rotavirus cultivation in MA104 cells to confirm the presence of live virus. Methods for each of these steps are outlined below.

2.3.1 Rotavirus Antigen Detection—Rotavirus antigen was detected by enzyme immunoassay (EIA) (Premier Rotaclone kit, Meridian Diagnostics, Cincinnati, Ohio) according to the manufacturer's protocol. All samples were stored at −80°C after EIA testing. All EIA-positive samples and a subset of 8 randomly chosen, EIA-negative samples that were sent as control specimens for quality assurance subsequently were sent for rotavirus genotyping by RT-PCR and nucleotide sequencing and virus culture at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA.

2.3.2 Viral RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Rotavirus double-stranded RNA (dsRNA) was extracted using the guanidine and silica method modified from that of Boom et al. 1990. [21] Using the RT-PCR assay, the extracted dsRNA was used as template for amplification and detection by RT-PCR using primers designed to be specific for the VP6 gene segment of strain WC3. Samples with sequences 100% identical to the VP6 gene of WC3 strain of bovine rotavirus were designated as vaccine strains. In order to confirm the presence of other WC3 vaccine strain segments, fragments of the NSP1 and VP7 genes of a subset of EIA-positive samples were also amplified and sequenced. The following steps were performed: Viral RNA was denatured at 97°C for 5 minutes. Then, reverse transcription, followed by RT-PCR, were carried out using the Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA, USA). The RT-PCR reaction was carried out with an initial reverse transcription step at 42°C for 30 min, followed by PCR activation at 95°C for 15 min, and 30 cycles of amplification with the following cycling conditions using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems Group, Foster City, CA): 30 seconds at 94°C, 30 seconds at 42°C, 45 seconds at 72°C, a final extension of 7 minutes at 72°C, and a 4°C cooling step. Amplicons were run in a 1% agarose gel. The desired bands were then excised and purified with the QIAquick Gel extraction kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's protocol and saved for sequencing (see below). The forward and reverse primers used for the amplification of the partial VP6, NSP1, and VP7 gene segments of the WC3 strain were as outlined below. *VP6*, WC3-VP6F (forward primer), nt 240–261 (5'CTA GAT GCT AAC TAC GTC GAA 3'); WC3-VP6R (reverse primer), nt 630–650 (5'TCA AAT TGT TGT GTA TTG GC 3'); *NSP1*, WC3-NSP1F (forward primer), nt 131–150 (5'CCA GTG ACA AAA TAT AAA GG 3'); WC3-NSP1R (reverse primer), nt 464–484 (5'CTC AAG ATT TAT AGT CAA TGC 3'); *VP7*, WC3-VP7F (forward primer), nt 688–708 (5'ACG TTT GAA ACA GTT GCG ACA 3'); WC3-VP7R (reverse primer), nt 823–841 (5'CGC CTA CCT GTA TGA CTG C 3'). These oligonucleotides were designed based on the sequences

of the WC3 NSP1 (Accession number; EF990699), WC3 VP6 (Accession number; AF411322), and WC3 VP7 (Accession number; AY050272) genes. [22]

2.3.3 Real-time RT-PCR—The dsRNA extracts were amplified in duplicate by a rotavirus NSP3 gene real-time RT-PCR (rt RT-PCR) assay modified for use on ABI 7500 Fast Real-Time PCR System (Applied Biosystems).[23] For standard curve development, cells infected with reference strain Wa, grown in roller tubes and purified on $CsCl_2$ gradients as previously described, was used. [24] The concentration of nucleic acid was determined by NANODROP 1000 version 3.6 (Thermo Scientific, DE, USA) to be 4.3 ng/μl. To generate a standard curve, a 10-fold dilution series (10^0 to 10^7) was made with elution buffer and aliquots were stored at −80°C. Each dilution was amplified in duplicate using the same NSP3 assay and copy number calculated as described previously. [23]

2.3.4 Nucleotide Sequencing—Purified WC3-VP6 (410 bp) and, if applicable, WC3-NSP1 (353 bp) and WC3-VP7 (153 bp) amplicons were subjected to cycle sequencing using the same consensus primers as for RT-PCR and a Big Dye Terminator cycle sequencing ready kit (Applied Biosystems, Inc., Foster City, California). Cycle sequencing products were purified using Centri-sep spin columns (Princeton Separations, Inc, Adelphia, NJ, USA), dried in a DNA speed Vac^R (Savant Instruments Inc. Holbrook, NY, USA), and reconstituted in 15 μl of Hi-Di formamide. Automated separation and base-calling of the cycle sequencing products were performed using an ABI 3130 XL sequencer (Applied Biosystems, Foster City, CA, USA). Overlapping sequence fragments were assembled using the Sequencher program (Gene Codes Corporation, Inc., Ann Arbor, Michigan). Nucleotide sequence similarity searches of the GenBank database (release 143.0) were performed using the National Center for Biotechnology Information (NCBI, National Institutes of Health, Bethesda, MD, USA) Basic Local Alignment Search Tool (BLAST). [25]

2.3.4 Cultivation and Plaque Purification of Rotavirus in MA104 Cells—In order to determine if viable vaccine strain virus was present in stool samples of PCR-positive samples, a subset of these samples was culture-adapted using a modified version of previously described procedures. [26, 27] Briefly, 1 ml of a 10% PBS suspension for each stool sample was filtered by passage through a 0.45μM sterile filter (Krackeler Scientific, Inc., Albany, NY) and activated in the presence of 15μg of trypsin (Gibco Laboratories, Grand Island, N.Y, USA) for 1 hour at 37°C. Confluent monolayers of MA104 cells in roller tubes were washed with Dulbecco Modified Eagle Medium (DMEM) to remove serum-containing media and then inoculated with the trypsin-treated stool suspensions. After virus adsorption, the cells were washed gently once to remove unabsorbed virus, and 2 ml of DMEM containing neomycin (Gibco Laboratories) and 20μg of trypsin per ml were added. The tubes were incubated at 37°C on a roller drum and checked daily for cytopathic effect (CPE). Tubes were harvested when CPE was complete or at day 4 and frozen at −80°C. After freezing and thawing twice, infected cell lysates were treated with trypsin. Then, 0.1 ml of each lysate was used to inoculate roller tubes of MA104 cells again as described above. After 3 passages, lysates were tested for rotavirus by EIA (Premier Rotaclone). Lysates that were EIA-positive were plaque purified in monolayers of MA104 cells [24]. After 3 rounds of plaque purification, individual plaques were used as inoculum for MA104

cell cultures to prepare a virus stock. These samples also underwent RT-PCR amplification and nucleotide sequencing.

2.4 Data Analysis

Cumulative and daily proportions of fecal rotavirus vaccine virus shedding during the 9 days following vaccination were calculated. Duration of shedding and peak shedding days within the 9-day collection period were determined.

3. Results

A total of 198 infants were enrolled. One hundred-three (52%) collection kits were returned to the clinic. Fifty-nine (57%) of these 103 kits were returned with a complete set of 9 swabs; the 103 kits had a total of 830 swab specimens. Forty-three (42%) kits were returned within 4 days after the expected date of completion (Table 1). Twenty-one (20%) were returned within 5–14 days. Six (6%) were returned after a period of time greater than 14 days. The range of days for date of return after completion of stool collection included 0 days (i.e., stool samples returned on the final day of stool collection) to 54 days. The expected completion dates for stool collection were unknown for 33 (32%) kits, as the study team had not been notified when the kits were given to caretakers.

3.1 Rotavirus Antigen Testing

Twenty-two (21.4%) of 103 collection kits contained rotavirus EIA-positive swabs on 1 or more post-vaccination days. Of the 59 kits with complete sets of swabs, 12 (20.3%) had 1 or more positive swabs. Shedding occurred as early as post-vaccination day 3 and as late as post-vaccination day 9 (Table 2). Peak proportions of shedding occurred on post-vaccination days 6 through 8. Looking specifically at the shedding patterns of those infants who had EIA-positive specimens, the mean duration of shedding within the 9 days post-vaccination was 2 days; 5 (23%) infants had positive EIA-positive specimens on 1 day only, 9 (41%) infants on 2 days, 5 (23%) infants on 3 days, and 3 (13%) infants on 4 days (Table 3). The first EIA-positive specimen was detected on day 6 or later of sample collection in a majority (77%) of the infants. There was no discernible pattern in timing or length of shedding among those with known kit distribution and return dates, nor was there a discernible pattern to indicate that longer return times were associated with later shedding (Table 3).

3.2 Rotavirus RT-PCR

Vaccine-type (WC3-like) rotavirus alone was identified in all but 2 EIA-positive samples by RT-PCR with primers specific for the WC3-VP6 gene segment followed by nucleotide sequencing of the PCR amplicons. For the 2 samples that came from one infant, the WC3-VP7 gene segment was detected along with a wild-type VP6 gene segment, suggesting that the infant was shedding a human rotavirus in addition to vaccine virus. WC3-NSP1 and VP7 sequences were detected in all 12 samples tested for these gene segments. In addition, 8 EIA-negative specimens selected from seven infants with EIA-positive specimens on one or more post-vaccination days were also positive for vaccine-type rotavirus by PCR. In each case, the gene fragment tested was 100% identical to the corresponding gene fragment of WC3, the parent strain of the pentavalent rotavirus vaccine. Of these EIA-negative, PCR-

positive specimens, one was collected on post-vaccination day 2, two on post-vaccination day 5, two on post-vaccination day 6, one on post-vaccination day 7, and two on post-vaccination day 8.

Real-time RT-PCR (rtRT-PCR) was also conducted on EIA positive samples to assess viral load. Genome copy number per/gm of stool varied from about 4.5×10^{7} to 7.0×10^{12}, similar to the range described previously for EIA positive stool samples (Table 3). [23] In both cases the commercial Rotaclone assay was used for detecting rotavirus in stool samples. One sample that was positive by standard RT-PCR was negative by rtRT-PCR. We could find no discernable pattern between copy number of vaccine virus genome and the number of days post-vaccination when the sample was collected (Table 3).

3.3 Rotavirus cultivation

Twelve EIA-positive samples and 1 EIA-negative sample from five infants underwent rotavirus cultivation by three passages in cell culture. Nine (75%) of the positive samples and the 1 negative sample were culture positive for vaccine-type rotavirus. For those infants who had EIA-positive samples that were also culture positive, shedding began on post-vaccination day 5 post-vaccination or later (range: post-vaccination day 5 to post-vaccination day 9), and rotavirus titers ranged from 10 to 10^{6} PFU/ml. For the one infant with an EIA-negative, culture positive sample, this sample was obtained on post-vaccination day 2 (rotavirus titer: 107 PFU/ml), and the infant had subsequent EIA-positive samples on post-vaccination days 3, 4 and 7 that did not undergo rotavirus cultivation. There was no discernible pattern that could allow for correlation between titer and time post vaccination. The remaining samples were EIA-negative after 3 passages in MA104 cells and were not analyzed further. These 3 negative samples had been collected on post-vaccination days 5 and 6 from one infant and on post-vaccination day 8 from another infant.

4. Discussion

This study examined the characteristics of fecal shedding of pentavalent rotavirus vaccine in infants during the first 9 days following the first dose. We found that 21.4% of infants had evidence of vaccine virus shedding on at least one day during the 3 to 9 days following vaccination. Two previous studies demonstrated shedding in 8.9% and 12.7% of infants during the 4 to 6 days following vaccination with the first dose. Our results demonstrate that the duration of vaccine virus shedding can extend beyond 6 days following vaccination, thus extending the time period for potential transmission of vaccine virus. In addition, these results likely underestimate the potential for shedding and transmission, given our finding of EIA-negative/PCR-positive and EIA-negative/culture positive samples and our use of swabbed stool samples vs. bulk stool, which may yield smaller amounts of vaccine virus. Further, while the amount of infectious virus in stool samples was not directly estimated, the finding that we could efficiently cultivate vaccine virus from stool samples collected between days 6 to 9 post-vaccination suggests that sufficient infectious virus may be present for potential horizontal transmission of vaccine virus that may result in clinical disease. Our finding of vaccine virus genome copies in stool samples collected through post-vaccination day 9 at levels that are in the same range as wild type rotavirus infections is also consistent

with the potential for horizontal transmission of vaccine virus. This is supported by the reported case of vaccine virus transmission between 2 immunocompetent siblings. [10]

This study has several limitations. First, the study population was restricted to one pediatric clinic with a relatively homogeneous population, thereby limiting the ability to generalize the results. Second, we had to rely on the caretakers to properly collect samples. While the majority seemed to do well with the instruction pamphlet and follow up calls, some voiced confusion over the collection process, more specifically the exact start date. We attempted to ameliorate this issue with follow up phone calls on the day of vaccination or the collection start date, but encountered many unanswered calls or disconnected numbers. Given the de-identification of study samples, it is difficult to ascertain how many of those who returned complete sample collection kits were individuals who initially had been confused and whether these samples were collected properly. In addition, storage and transport of the collection kits may have affected stability of the vaccine virus, which currently is unknown. This may account for why shedding was not seen on the first 2 days following vaccination. However, this would result in an underestimation of the rate of vaccine virus shedding, leading to an overestimated sample size for a transmission study. On the other hand, shedding of vaccine virus may be similar to shedding of wild-type rotavirus, which is mostly detectable by EIA 3 to 7 days after infection. Third, due to resource constraints, we were unable to test all negative samples for vaccine virus by RT-PCR and/or culture. Testing of these specimens may have led to a higher estimate of vaccine virus shedding since one EIA-negative specimen was actually positive for vaccine type virus by RT-PCR and culture. Finally, we cannot be sure for how long shedding continued as shedding continued up to post-vaccination day 9 in some cases.

5. Conclusion

In summary, the findings from this study demonstrate that fecal shedding of rotavirus vaccine virus after the first dose of RV5 can occur over a wider range of post-vaccination days than previously studied. Given these findings and the finding that live vaccine-virus was isolated from most samples analyzed by cultivation in cell culture, additional clarification of the potential risks and benefits of vaccination within households with immunocompromised individuals in future transmission studies is needed.

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Table 1

Time interval between completion of stool collection and kit return

Days Post-Completion	No. of Kits (%)
0–4 days	43 (42%)
5–14 days	21 (20%)
>14 days	6 (6%)
Unknown return interval	33 (32%)

Table 2

Fecal Shedding of RV5 by post-vaccination day

Fecal Shedding of RV5									
Day post-vaccination	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**	**Day 9**
# Samples returned	90	92	92	90	92	93	95	92	94
# EIA positive for rotavirus antigen	0	0	2	3	4	10	13	11	7
# PCR positive for vaccine virus	0	0	2	3	4	10	13	11	7
% positive per day	0.0%	0.0%	2.0%	3.0%	4.0%	10.7%	13.6%	12.0%	7.4%

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Table 3

RV5 shedding patterns and viral loads (copies per gram of stool) of infants with rotavirus positive stool specimens*

Infant	Return interval (days)†	Post-vaccination Day								
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
1	4		3.6×10^{11}	4.4×10^{10}	7.9×10^{10}	NS‡	NS	§		
2	2	NS				3.6×10^{11}	4.5×10^{9}			
3	0						6.3×10^{9}	3.5×10^{11}		
4	0	NS						3.6×10^{12}	1.4×10^{12}	
5	0							4.4×10^{11}	4.8×10^{12}	3.2×10^{10}
6	Unknown						1.1×10^{12}	7.9×10^{11}	1.6×10^{12}	7.9×10^{11}
7	Unknown			3.2×10^{12}	7.0×10^{12}	6.3×10^{9}	5.6×10^{10}			
8	1								6.3×10^{11}	3.2×10^{12}
9	1				NS	NS	1.0×10^{9}	NS	7.8×10^{9}	
10	Unknown						9.6×10^{9}			
11	Unknown							5.4×10^{8}	1.5×10^{9}	
12	8								6.6×10^{7}	7.8×10^{9}
13	3					3.2×10^{9}				
14	13			NS			4.0×10^{9}	3.2×10^{10}	NS	
15	12				NS		7.9×10^{10}	2.0×10^{10}		
16	Unknown			NS	NS			3.5×10^{11}		
17	10					1.3×10^{12}	2.0×10^{11}	5.4×10^{8}	7.8×10^{9}	
18	3			NS	5.6×10^{11}	7.0×10^{11}	2.5×10^{8}	1.6×10^{10}	5.6×10^{10}	
19	Unknown				NS		NS	2.9×10^{10}	3.0×10^{12}	2.8×10^{12}
20	Unknown						2.7×10^{12}	2.8×10^{11}	3.5×10^{10}	
21	2								4.5×10^{7}	1.4×10^{8}
22	21	NS			NS	3.5×10^{10}	2.0×10^{10}	4.4×10^{10}	5.4×10^{10}	neg

* Shaded boxes represent post-vaccination days with rotavirus EIA-positive specimens

† Return interval = the number of days from post-vaccination day 9 to specimen return date

‡ NS=no specimen

§ Specimen not tested