

Detection of Viruses in Weekly Stool Specimens Collected During the First 2 Years of Life: A Pilot Study of Five Healthy Australian Infants in the Rotavirus Vaccine Era

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Several viruses are associated with gastroenteritis in infants. This pilot study, nested within a larger community-based project of early childhood infections, collected daily symptom data and 511 weekly stool samples from five healthy, fully vaccinated, term infants from birth until their second birthday. Real-time PCR assays were used to detect six enteric viruses. Frequent, silent shedding of one or more of the six viruses was observed, particularly involving adenovirus where shedding could be for up to 3 months without gastrointestinal symptoms. These pilot data demonstrate that a positive PCR result for enteric viruses may not always indicate the cause of childhood gastroenteritis. *J. Med. Virol.* 89:917–921, 2017.

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KEY WORDS:

INTRODUCTION

Acute gastroenteritis (AGE) associated with viruses, such as rotavirus, norovirus, and adenovirus, is a major contributor globally to childhood mortality and morbidity [Liu et al., 2012]. Of these viruses, rotavirus is the main cause of severe diarrhea in young children accounting in 2013 for more than 210,000 childhood deaths worldwide [Tate et al., 2016]. The recent addition of oral rotavirus vaccines onto the national immunization schedules of developed countries, such as Australia, has been followed by an overall decrease in childhood diarrheal illness [Buttery et al., 2011; Dey et al., 2012]. In these

countries other enteric viral pathogens, especially noroviruses, are now the leading cause of severe childhood AGE [Koo et al., 2013].

Recent advances in molecular detection methods have helped reduce the diagnostic gap in children with AGE [Amar et al., 2007]. These highly sensitive techniques have also aided the discovery of several new enteric viruses, including human bocavirus and anellovirus, although whether these novel agents are genuine pathogens remains controversial [Kapusinszky et al., 2012]. Successful national rotavirus vaccine programs and recent discoveries of novel viruses have raised important questions over the relative contributions of existing and emerging enteric viruses to childhood diarrhea. So far, most studies have been cross-sectional, lacking suitable controls, and based in healthcare settings, increasing the likelihood of selection bias [Scallan et al., 2005; Lambert et al., 2012]. The aim of this community-based pilot study was to assess the prevalence of enteric virus infections and their association with gastrointestinal symptoms in the first 2 years of life in five otherwise healthy infants recruited prospectively from birth.

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MATERIALS AND METHODS

The Observational Research in Childhood Infectious Diseases (ORChID) project is a community-based, prospective birth cohort study examining the nature and frequency of acute respiratory and gastrointestinal infections in infants up until age 2 years in the subtropical city of Brisbane, Australia [Lambert et al., 2012]. Parents recorded in a daily diary any fever, respiratory, or gastrointestinal symptoms in their infant and collected nose and stool swab samples weekly from birth until the infant's second birthday. The nose and stool swab samples were submitted each week to the research laboratory by regular surface mail. In order to inform the logistics for planning, a larger study involving all 158 infants in the birth cohort, a convenience sample of the first five ORChID participants to complete the study with a full set of stool samples were included in this pilot. At 2, 4, and 6 months of age, each had received three doses of oral human-bovine reassortant rotavirus vaccine (RotaTeq; Merck & Inc., Co., NJ) as part of Queensland's publically funded immunization program (actual immunization dates confirmed by the Australian Childhood Immunisation Register). The Human Research Ethics Committees of the Children's Health Queensland Hospital and Health Service, the Royal Brisbane and Women's Hospital and The University of Queensland approved the study.

Six enteric viruses were tested using polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (Rt-PCR) assays. The choice of viruses was determined primarily by availability of PCR templates within the laboratory and included established enteric virus pathogens (rotavirus, norovirus), recognized and emerging respiratory viruses that can be associated with gastrointestinal symptoms (adenovirus—subgroup F, human coronavirus, human bocavirus-1), and systemic viruses shed from the gastrointestinal tract (enterovirus).

Nucleic acid extractions were performed as described previously [Ye et al., 2015]. Each stool sample was homogenized in approximately 2.5 ml S.T.A.R buffer (Roche Diagnostics, Castle Hill, Australia) to make up a 10% stool suspension. The suspension was centrifuged to remove large particles before the supernatant (200 μ l) underwent nucleic acid extraction. Extraction efficiency and inhibition were monitored using an equine herpes virus spike [Ye et al., 2013] and PCR. Real-time PCR and Rt-PCR testing of specimens for DNA and RNA viruses, respectively, was performed using primers and probes for adenovirus [Alsaleh et al., 2014], enterovirus [Maunula et al., 2008], norovirus group II [Kageyama et al., 2003], rotavirus [Pang et al., 2011], human bocavirus-1 [Tozer et al., 2009], and human coronavirus OC43, 229E, NL63 [Gunson et al., 2005] and human coronavirus HKU [Dare et al., 2007].

Adenovirus positive samples were further characterized using an established adenovirus genotyping

method [Sarantis et al., 2004]. Each adenovirus-positive sample was re-amplified by PCR using primers spanning the hypervariable regions (HVRs-7) of the hexon gene. PCR products were sent for automated bidirectional sequencing at the Australian Genome Research Facility sequencing laboratory at The University of Queensland.

Rotavirus-positive samples were screened for vaccine strains by a RotaTeq VP6 gene-specific quantitative RT-PCR assay [Gautam et al., 2014]. Rotavirus-positive samples negative for the RotaTeq VP6 gene were further genotyped using an established method based on the rotavirus G (VP7) and P (VP4) sequences [Kirkwood, 2010].

The symptom diary captured gastrointestinal symptoms daily. A symptomatic episode (AGE), was defined as >3 loose stools and/or vomiting within a 24-hr period. A symptomatic episode started on the first day of AGE symptoms and concluded with the last day of symptoms; a new episode required at least 3 asymptomatic days between it and the previous episode to commence.

To examine whether symptoms were associated with higher viral loads, linear regression models were constructed with episode type (symptomatic/asymptomatic) entered as the main effect and semi-quantitative cycle threshold (Ct) values from the real-time PCR assays as the outcome. All analyses were performed with Stata v11.0 (StataCorp, College Station, TX).

RESULTS

Overall, 511 stool samples (98.1% of maximum anticipated specimens) were collected from the five infants during the 2-year study period. The median (interquartile range) time for these specimens to reach the laboratory from the day of collection was 3 (2–4) days. At least one virus was detected in 208 (40.7%) samples. Adenovirus was detected most frequently ($n=131$; 25.6%), followed by enterovirus (63; 12.3%), norovirus (26; 5.1%), human bocavirus-1 (21; 4.1%), rotavirus (7; 1.4%), and human coronavirus (3 OC43, 1 NL63, 1 229E; 1.0%). A single virus was detected in 166 positive samples (79.8%), while two were co-detected in 39 samples (18.8%), and three in three samples (1.4%). The most frequent co-detections were for adenovirus and enterovirus ($n=23$, 54.7% of co-detections), followed by norovirus with either adenovirus ($n=6$, 14.2%) or enterovirus ($n=7$, 16.7%).

Of the 131 adenovirus positive samples, 62 (47.3%) were genotyped successfully. The observed genotypes were adenovirus serotypes 31 ($n=9$) and 12 ($n=2$) of subgenus A, serotypes 1 ($n=8$), 2 ($n=37$), and 5 ($n=2$) of subgenus C, and serotype 41 of subgenus F ($n=4$). The failure to genotype the remaining 69 adenovirus-positive samples was attributed to viral load being below the detection limit of the genotyping protocol. The mean Ct for successfully genotyped

detections was 32.3 cycles (SD 7.0) and 39.3 cycles (SD 3.2) for the non-genotyped detections; with a mean difference of 7.0 cycles (95%CI 5.1–8.8).

Six of the seven rotavirus, positive samples were observed between the ages of 8 and 15 weeks, corresponding to the timing of the first dose of the RotaTeq vaccine. The remaining rotavirus positive sample was collected at 50 weeks of age. Genotyping showed that five rotavirus positive samples were the RotaTeq vaccine strain, while the sample at 50 weeks was a wild-type G1P[8] strain (subject 4). The remaining rotavirus positive sample (subject 1, week-9) could not be genotyped due to low viral load (Ct value = 39.1 cycles).

The distribution of virus detections among the five infants is summarized in the Table I and Figure 1 (with AGE episodes marked). Only 22 of the 208 (10.6%) virus-positive samples were associated with gastrointestinal symptoms and >1 virus was detected in seven (31.8%) of these episodes. Detection of >1 virus in asymptomatic virus detection episodes was 18.8% (35/186). Among individually detected viruses, just 14/131 (10.7%) adenovirus detections (serotype 2, (2/37); serotype 5 (1/2); serotype 31 (1/9); serotype 41 (2/4); and non-typable (8/69)) were associated with symptoms. Similarly, only 9/63 (14.3%) enterovirus, 7/26 (27.0%) norovirus, and 1/21 (4.8%) human bocavirus-1 detections were accompanied by symptoms. However, no gastrointestinal symptoms were associated with the seven rotavirus positive or five human coronavirus positive samples. Symptomatic enterovirus detections had lower viral load than asymptomatic detections (mean Ct value difference = 2.34 cycles; 95%CI = 0.1, 4.7) (Table I). No similar association was observed for other viruses. In contrast, and unlike the other four participants, subject 2 had 15 symptomatic AGE episodes

ranging from 1 to 5 days duration and where in 11 none of the six viruses were detected.

At various times, adenovirus, enterovirus, and norovirus each had periods of continuous virus shedding (Fig. 1), lasting as long as 11 weeks for adenovirus, 6 weeks for enterovirus, and 4 weeks for norovirus. Based on the adenovirus genotyping data, adenovirus serotype 2 was shed continuously for 11 weeks in subject 1 (weeks 37–47) and for 9-weeks in subject 3 (weeks 30–38). Sequential detections of different virus genotypes were also observed during periods of continuous adenovirus shedding. For example, subject 4 (the only infant not to have any recorded AGE episodes during their first 2 years of life) had serial detection of adenovirus types 2, 31, and 1, occurring between 80 and 90 weeks of age (Fig. 1).

DISCUSSION

In this pilot study, weekly stool samples from five children from a community-based cohort during the first 2 years of life were tested. Frequent shedding of one or more of the targeted six viruses was identified in weekly swabs, but often in the absence of gastrointestinal symptoms. Of these six viruses, norovirus genogroup II, was found to have the highest proportion of positive detections associated with gastrointestinal symptoms and is consistent with its relative importance as a cause of AGE in young children already immunized against rotavirus infections [Koo et al., 2013]. These findings confirm and extend those of previous observations, including from two infants in the first year of life where there was almost constant shedding of various enteric viruses—adenovirus, anellovirus, bocavirus, enterovirus, parvovirus, and picobirnavirus—in weekly stool

TABLE I. Number of Enteric Virus Detections and Their Association With Acute Gastroenteritis Symptoms in Weekly Stool Samples Collected From Five Healthy Infants During the First 2 Years of Life

	Adenovirus	Enterovirus	Norovirus group II	Rotavirus	Human bocavirus-1	Human coronavirus
Asymptomatic detection						
No. of swabs	117	54	19	7	20	5
Ct count; mean(SD)	36.01 (6.26)	35.60 (3.35)	32.49 (3.13)	38.85 (3.45)	34.51 (7.76)	41.75 (1.09)
Symptomatic detection						
No. of swabs	14	9	7	0	1	0
Ct count; mean(SD)	35.95 (7.49)	33.26 (2.37)	30.54 (2.83)	–	25.64 (–)	–
Mean difference between asymptomatic and symptomatic detections; (95%CI)	0.06 (–3.52, 3.64)	2.34 (0.01–4.67)	1.95 (–0.84 to 4.73)	–	8.87 (–7.78 to 25.52)	–
Subject number and PCR positive swabs						
Infant 1 (n = 102)	25	0	0	2	0	1 (229E)
Infant 2 (n = 106)	12	12	5	0	4	0
Infant 3 (n = 102)	32	19	5	0	5	1 (OC43)
Infant 4 (n = 95)	32	14	6	4	3	2 (NL63; OC43)
Infant 5 (n = 106)	30	18	10	1	9	1 (OC43)
Total (n = 511)	131	63	26	7	21	5

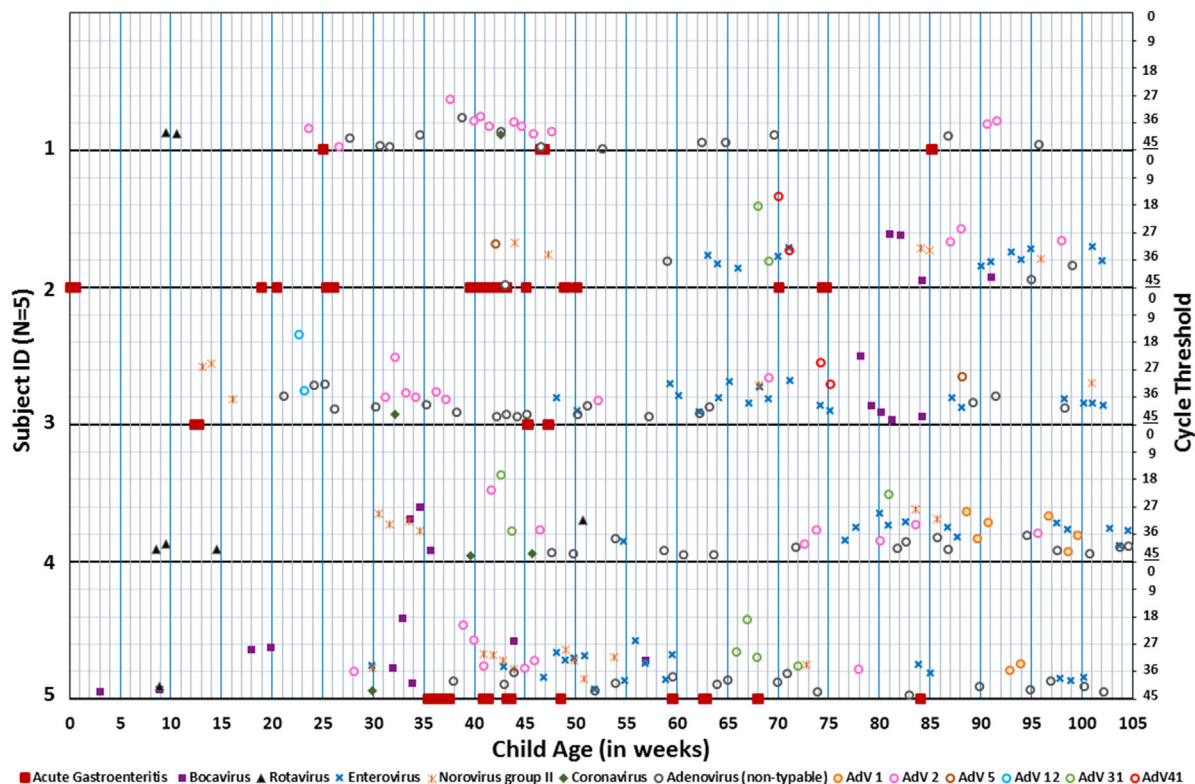


Fig. 1. Weekly virus detections with corresponding cycle threshold (Ct) values and symptoms of acute gastroenteritis in five healthy infants during the first 2 years of life. (Please note that for subject 2 there are distinct AGE episodes at weeks 25 and 26, and also at weeks 39 and 44, but that these cannot be distinguished in the figure due to the small scale).

samples with only occasional minor symptoms being present [Kapusinszky et al., 2012].

Of note, in the current study was the duration of shedding observed for certain adenovirus types. While not all adenovirus-positive samples could be assigned a genotype (due to low viral load), adenovirus, especially adenovirus type 2 could be shed for almost 3 months without gastrointestinal symptoms being present. Unlike co-detection of different adenovirus genotypes, sequential detection of various adenovirus types as observed in subject 4 over a 10-week period has received little attention in the literature. Two of the three genotypes (types 2 [subgenus C] and 31 [subgenus A]) had been detected intermittently beforehand in this subject. It is plausible that the serial detections reported in this infant represent independent reactivations of these two genotypes, whose genogroups infect lymphoid tissue at different sites of the gastrointestinal tract [Kosulin et al., 2015], followed by a new adenovirus type 1 [subgenus C] infection.

Only one of these five fully immunized children had a wild type rotavirus strain detected; a subclinical G1P[8] infection identified at 50 weeks of age. Of the remaining six rotavirus-positive samples, five were confirmed as a vaccine strain. The sixth sample was detected 10-days after the first dose of RotaTeq vaccine, could not be genotyped, but the next sample

from this subject collected 1-week later was confirmed RotaTeq vaccine strain positive. These data support findings from a recent Australian study where RotaTeq vaccine viruses accounted for 72% of rotavirus PCR positives samples in children aged <32-weeks submitting stool samples for diagnostic testing [Schepetiuk et al., 2015].

This pilot study also identified issues with the predictive value of molecular diagnostic assays in stool samples from infants. While PCR is very sensitive, most detections in these stool samples were not associated with gastrointestinal symptoms. Other studies have suggested that this limitation could be overcome in children with AGE by measuring viral loads using real-time PCR Ct values and developing cut-off values for detections that were meaningful clinically [Corcoran et al., 2014]. However, with this small sample population, only the Ct values for enterovirus detections were associated significantly with symptoms. Given enterovirus is recognized as only a minor contributor to AGE (any gastrointestinal symptoms are likely part of a more systemic illness, including fever and/or rash), and that the observed difference in Ct values between symptomatic and asymptomatic virus detections was relatively small (2.34 cycles or <1-log difference in viral load), drawing further conclusions from these data must be done with caution. Moreover, unlike blood, where a defined volume/cell number can be quantified,

it is difficult to accurately assess a viral load from stool samples that may vary widely in their fluid content. Although real-time PCR Ct values from stool samples were used as a surrogate, semi-quantitative estimate of viral load, this is still a limitation where Ct values in liquid stool may underestimate viral load.

The current pilot study only involved five participants and some recognized viral agents associated with AGE were not tested, including astrovirus and norovirus group I viruses. Nevertheless, this study assisted in refining methods and workflow for the much larger task of stool testing for the entire ORChID cohort [Lambert et al., 2012]. It also found that it is possible to detect multiple viruses in parent-collected stool specimens returned to the laboratory by surface mail. Additional questions for the ORChID cohort include further details on the nature and duration of virus shedding in the stools of healthy infants, if important biological differences exist between the major adenovirus and enterovirus genotypes shed in stools, including their associations with gastrointestinal (and other) symptoms, and whether a Ct value cut-off can be used to improve diagnostic accuracy of viral AGE in young children.

In summary, these data highlight the complexity of viral shedding from the gastrointestinal tract and determining disease-pathogen associations in healthy infants and young children. The results affirm and extend previous observations of frequent and subclinical shedding of multiple and diverse viruses in the stools of two siblings during their first year of life.

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