Potentially Pathogenic Organisms in Stools and Their Association With Acute Diarrheal Illness in Children Aged <2 Years

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Key points in 40 words or less:

We investigated the association between a range of commonly tested organisms in stool and acute diarrheal disease in healthy Australian children aged <2 years, identified the leading organisms, and highlighted the high rates of asymptomatic shedding of potentially pathogenic organisms.

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ABSTRACT

Background: Acute diarrheal illness (ADI) causes a substantial disease burden in high-income countries. We investigated associations between potentially pathogenic organisms in stools and ADI by polymerase chain reaction (PCR) in Australian children aged <2 years.

Methods: Children in a community-based birth cohort had gastrointestinal symptoms recorded daily and stool samples collected weekly until their second birthday. Diarrhea was defined as ≥3 liquid or looser than normal stools within a 24-hour period. PCR assays tested for 11 viruses, 5 bacteria, and 4 protozoa. Detections of a new organism or of the same following at least two negative tests were linked to ADIs, and incidence rates and estimates of association with ADI were calculated.

Results: 154 children provided 11,111 stool samples during 240 child-years of observation, and 228 ADIs were linked to samples. Overall, 6,105 (55%) samples tested positive for a target organism. The incidence rate of 2,967 new detections was 11.9 (95% confidence interval 11.4–12.3) per child-year, with 2,561 (92%) new detections unrelated to an ADI. The relative risk of an ADI was 1.5–6.4 times greater for new detections of adenovirus, enterovirus, norovirus GII, parechovirus A, wild-type rotavirus, sapovirus GI/II/IV/V, *Salmonella*, *Blastocystis*, and *Cryptosporidium*, compared to when these were absent.

Conclusions: Wild-type rotavirus, norovirus GII, sapovirus GI/II/IV/V, adenovirus 40/41, and *Salmonella* were associated with ADI in this age group and setting. However, high levels of asymptomatic shedding of potential pathogens in stools from children may contribute to diagnostic confusion when children present with an episode of ADI.

INTRODUCTION

Acute diarrheal illness (ADI) is an important cause of global childhood mortality and morbidity [1]. Although rotavirus vaccines have been successful in high-income countries at reducing hospitalization from ADI in young children, the burden associated with ADI remains substantial [2-5]. In 2013 there were 70,553 ADI-related hospitalizations among children aged <5 years in the United States resulting in USD $226.5 million in direct medical costs, while in Australia there were 7,668 hospital admissions in 2016 in this age group costing USD $33.3 million [2, 6]. While molecular detection by multiplex polymerase chain reaction (PCR) assays are now used widely for diagnosing gastrointestinal illnesses, the clinical significance of many organisms included in diagnostic panels is unknown, especially if they cause infections beyond the gastrointestinal tract [7, 8].

Studies of organisms shed in the stools of young children and their association with ADI episodes have been limited by their cross-sectional design [9], where even with matched controls it is not possible to accurately differentiate between organisms responsible for an ADI and incidental subclinical infection or prolonged shedding from an earlier infection [10]. Consequently, there are limited data describing organism-specific risks. Ideally, longitudinal birth cohort studies with regular and frequent stool sampling irrespective of symptoms are required. To date such studies are few and have been undertaken in low/middle-income countries [11]. Thus, to help inform the role of PCR assays in identifying the cause of ADI in young children, we used birth cohort data to provide measures of association between 19 potentially pathogenic organisms and vaccine-type rotavirus shed in stools and ADI in healthy children aged <2 years residing in an Australian subtropical city during the rotavirus vaccine era.

METHODS

Data sources

The Observational Research in Childhood Infectious Diseases (ORChID) study (clinicaltrials.gov: NCT01304914) was a longitudinal, community-based, birth cohort of children in the first 2 years of life, describing acute diarrheal episodes and their associated microbiology in stool swabs [11]. Between September 2010 and October 2012, mothers were progressively recruited from antenatal clinics in Brisbane, a subtropical Australian city. Full inclusion and exclusion criteria, and data collection details are described in the Supplementary Methods. Briefly, healthy, term-born children were followed until their second birthday. Parents provided socio-demographic, health, pregnancy, and birth details at enrolment, and recorded the daily number of loose stools in a standardized diary, which was mailed monthly to the researchers. Telephone interviews were conducted quarterly to update feeding and childcare attendance details. Formal childcare was defined as regulated care outside the child's home. The duration of exclusive breastfeeding was defined as the period from birth to the time of introducing milk formula or solids, whichever was sooner. The Children's Health Queensland, the Royal Brisbane and Women's Hospital, and The University of Queensland Human Research Ethics Committees approved the study. Parents provided informed consent.

Stool samples were collected weekly and mailed to the laboratory (median time in transfer: 3 days, inter-quartile range: 2–4) where they were stored at -80°C and batch-tested at a later date. Real-time PCR assays were employed to detect the viruses, bacteria, and protozoa (Supplementary Methods, Supplementary Table 1). Positive detection was defined at cycle threshold values ≤40. A positive generic adenovirus PCR result, which was negative for adenovirus 40/41 was categorized as indicating non-group F adenovirus.

Definitions

Diarrhea was defined as ≥3 loose (liquid or looser than normal) stools within a 24-hour period. ADI episodes consisted of ≥1 day of diarrhea, separated by ≥3 days without loose stools [12]. New detection episodes were either the detection of a new organism or, if the same organism, this required ≥2 negative preceding PCR test results for that organism or a period of ≥28 days from when the organism was last detected. If the organism tested positive for the first time ever in a child, it was labelled a first detection. A new single detection occurred when the organism was newly detected, and no other organisms were identified in stools taken within 7 days before or 7 days after this event. Co-detections identified more than one organism present in the same stool sample. When a new detection occurred within 7 days of ADI onset it was categorized as symptomatic. Duration of detection episodes was counted from the day of the first positive test until the day of the last positive test.

Analysis

Participant and family characteristics were presented as descriptive statistics, and time-varying characteristics (study participation, exclusive breastfeeding, formal childcare enrolment) are displayed over age categories using frequencies and percentages. Prevalence of positive PCR test results and the count, shedding duration, and incidence rate (IR) of new detection episodes were calculated for each organism. Poisson regressions employing robust variance estimates were used to calculate the IRs. The at-risk period was entered as the exposure-time variable, which included the day of the new detection, but excluded the remainder of the detection episode and the subsequent refractory period. The association between organism-specific IRs and age was presented in graphical form. To calculate the measures of pathogenicity detailed below, the baseline and symptom diary data were merged with the PCR test results. The frequencies of negative/positive stool samples associated with symptomatic/asymptomatic

periods were calculated using an algorithm (Supplementary Figure 1). The association between new detection episodes and ADI was presented as risk ratios (RRs) and risk differences (RDs), calculated using generalized linear models (binomial family, log link for RR and identity link for RD) with robust variance estimates. Univariable analyses were followed by multivariable models adjusting for age, older child in household, exclusive breastfeeding, and formal childcare attendance. To aid comparison with other studies, odds ratios, attributable risk, and attributable fraction in the exposed were calculated (Supplementary Methods). Sensitivity analyses removed the potential effects of co-detections and protection from previous infections by investigating the association between new single detections and first detections with ADI, respectively. Two-tailed *p*-values <0.05 were considered statistically significant. Stata 16 (College Station, TX: StataCorp LLC) was used.

RESULTS

The ORChID study collected 87,641 child-days (240 child-years) of symptom data from 154 children, and 11,111 stool samples (Supplementary Figure 2). Most (55%) children were born into families above the 75th percentile of household incomes, 65% had no older children in the household, 53% were exclusively breastfed for the first 3 months of life, and by 18 months 63% attended formal childcare. In all, 94% of children had received 3 doses of the government-funded rotavirus vaccine by 32 weeks of age (Table 1).

Prevalences, incidence rates, and duration of detections

Of the 11,111 stool samples, 6,105 (55%) tested positive for any organism (excluding vaccine-type rotavirus). Amongst the 6,105 positive samples there were 3,447 (56%) single detections, and 1,739 (28%) and 919 (15%) co-detections with 2 and 3 or more organisms, respectively. The most prevalent viruses were non-group F adenovirus (*n*=1,993/11,111; 18%), parechovirus

A (*n*=1,423; 13%), and enterovirus (*n*=1,339; 12%). *Clostridioides difficile* (*n*=1,602; 14%) was the most common bacterial species detected, with all other bacteria present in <1% of samples; *Shigella* and *Yersinia* were not detected. The most common protozoan was *D. fragilis* (*n*=904; 8%), with *Giardia* (*n*=166; 1%) the only other protozoan with a prevalence >1%. Vaccine-type rotavirus was detected in 8% (*n*=909) of samples, while the wild-type rotavirus was identified in 0.4% (*n*=41) (Table 2).

The PCR results of the 11,111 stool samples included 9,929 positive organism-specific results (excluding vaccine-type rotavirus), of which 2,967 (30%) (Table 2) were new detections of any organism with an overall IR of 11.9 (95% confidence interval [CI] 11.4–12.3) per child-year. The IRs of individual organism-specific new detections ranged from 0 to 2.8 per child-year (Table 2) and was 1.0 (95% CI 0.9–1.2) for vaccine-type rotavirus. IRs generally increased after 6 months of age, peaking in the second year of life (Figure, Supplementary Figures 3a–b). The median duration of new detection episodes ranged from 1 (astrovirus, norovirus GI, wild-type rotavirus, *D. fragilis*) to 5 weeks (*Blastocystis*) with wide variation in maximum shedding times ranging up to >60 weeks for *C. difficile* and *Giardia* (Supplementary Figure 4). However, caution should be exercised when examining the maximum shedding times for non-group F adenovirus and enterovirus as prolonged shedding may represent sequential infections involving different genotypes [13].

Merging of datasets

PCR and symptom data were successfully merged for 96% of tests (Supplementary Table 2) and 90% (*n*=228/253) of observed ADIs. Most new detection episodes with merged symptom data (92% [2,561/2,780]; Supplementary Table 2) were not associated with the beginning of

an ADI. In contrast, many ADIs (64% [147/228]) were associated with at least 1 new detection. Most (54–92%) new detections were single detections (Supplementary Table 3).

Measures of pathogenicity

Ten potentially pathogenic species/groups were significantly associated with an ADI episode in univariable analysis (non-group F adenovirus, adenovirus 40/41, enterovirus, norovirus GII, parechovirus A, wild-type rotavirus, sapovirus GI/II/IV/V, *Salmonella*, *Blastocystis*, *Cryptosporidium*). Risk was greatest for wild-type rotavirus (RR=6.4 [95% CI 3.0–13.3]; RD=23.4% [95% CI 2.8–44.0]), followed by *Salmonella* (RR=5.3 [95% CI 2.2–12.6]; RD=18.7% [95% CI -1.0–38.4]) and norovirus GII (RR=4.1 [95% CI 2.8–6.0]; RD=12.8% [95% CI 6.9–18.7]). Results from multivariable (i.e., adjusted) models were similar, although non-group F adenovirus, enterovirus, and *Blastocystis* were no longer significant (Table 3). There was no significant association between vaccine-type rotavirus and ADI (RR=0.6 [95% CI 0.3–1.2]; RD=-1.8% (95% CI -3.8–0.1]). Other measures of association between pathogens and ADI are displayed in Supplementary Table 4. In sensitivity analyses the magnitude of effects were largely unchanged, except parechovirus, Blastocystis, and Cryptosporidium were no longer significant when the outcome was new single detections, enterovirus was no longer significant when the outcome was first detections, and astrovirus first detections became significant.

DISCUSSION

During the ORChID study, 55% of stool samples tested positive for at least one potentially pathogenic organism. The IR of organism-specific new detections ranged up to 2.8 new detections per child-year, increased after 6 months of age for both symptomatic and asymptomatic infections, and peaked between ages 12–24 months. Overall, viruses were

detected more often than bacterial or protozoal species. Although 92% of new detections were from children without ADI symptoms, new detections of adenovirus, enterovirus, norovirus GII, parechovirus A, wild-type rotavirus, sapovirus GI/II/IV/V, *Salmonella*, *Blastocystis*, and *Cryptosporidium* were associated with 1.5–6.4 times higher risk of ADI. For the remaining organisms and for vaccine-type rotaviruses, the risks of ADI were not significant, either from a possible lack of association (astrovirus, human bocavirus, *C. difficile*, *D. fragilis*, *Giardia*) or due to low prevalence (norovirus GI, *Campylobacter*, *Shigella*, *Yersinia*).

ADIs and new organism detections

Merging datasets revealed symptoms did not always have a matching new organism detection. Overall, 36% of ADIs could not be attributed to any new detections, which might be due to non-infectious causes of ADI [4, 7, 14]. This result is also consistent with other studies using molecular diagnostic platforms. A pediatric hospital-based study from the United States utilizing a multiplex PCR assay capable of detecting 23 gastrointestinal pathogens failed to detect an organism in 48% of 1,089 diarrheal stool samples [15]. Similarly, the multinational MAL-ED birth cohort study from low-income countries could not detect pathogens in 35% of 6,625 ADI stools from children aged <2 years, despite employing a PCR assay able to detect 29 different enteric pathogens [16]. Furthermore, 92% of positive stool samples were from children without an ADI. This asymptomatic shedding contributes to diagnostic confusion when ADI is attributed to an organism that may be shedding from an incidental subclinical infection, a recent previous, infection, or from infection outside of the gastrointestinal tract [13]. It has been suggested by some experts that measuring illness severity scores (e.g., Vesikari score) and viral loads (e.g., Ct values as a semi-quantitative estimate) might help improve the diagnostic utility of PCR testing, including interpreting the results [17, 18].

Recognized pathogens

Our results confirmed the importance of wild-type rotavirus, norovirus GII, sapovirus GI/II/IV/V, adenovirus 40/41, *Salmonella*, and *Cryptosporidium*. As expected in a highly vaccinated cohort, wild-type rotavirus detections were uncommon at 0.4% and an IR of 0.08 episodes per child-year. However, rotaviruses were associated with the greatest risk of an ADI episode. The prevalence of the two norovirus genogroups that are included in bivalent candidate vaccine trials was markedly lower at 0.6% (GI) and 3.6% (GII) of samples than found in European children (aged ≤4 years; GI: range 4.2–4.3%, GII: 6.5–18%) [19]. Moreover, in our cohort only GII was positively associated with ADI. The association between sapovirus and ADI was slightly stronger than reported previously [18], while other studies in high-income countries reported non-significant RRs ranging 0.9–1.4, possibly due to partial protection from previous infections in their comparatively older cohorts [9, 20, 21]. The prevalence of adenovirus 40/41 at 1.6% was lower than reported in European children aged ≤4 years (8.3%), but these viruses remained significantly associated with ADI [22]. For *Salmonella*, the observed IR of symptomatic new detections during the ORChID study was considerably greater than the notification rate observed by national surveillance data for the same age group in Queensland between 2010–2013 (1,215 new detections per 100,000 child-years during ORChID versus about 375 notifications per 100,000 children aged <2 years per year) [23], highlighting the difference between active community-level research and passive healthcare-based surveillance. *Cryptosporidium* is considered an important cause of ADI in high-income countries [24], as it was during our study, although the association with ADI was not consistent across sensitivity analyses and it may have been influenced by co-detections.

Ninety-four percent of participants were vaccinated against rotaviruses, and vaccine-type rotavirus strains were detected more frequently than wild-type rotaviruses. Consistent with our

earlier studies, vaccine-strain detections clustered around the times of vaccination [25, 26]. Evidence for an association of vaccine-strains with ADI as a possible adverse effect of rotavirus vaccination was not found. The inclusion of vaccine-strains served as a negative control to provide reassurance that measures of association with ADI are likely to be reliable estimates in this cohort.

However, our results were inconclusive for *Campylobacter*, which was likely from a lack of symptomatic new detections, and for *Shigella* and *Yersinia* because of absent detections. While *Campylobacter* are an important cause of ADI in Australia, unlike in low and middle-income countries, they are proportionately more common in older children [27].

Two other recognized pathogens, astrovirus and *Giardia* were not found to be significantly associated with ADI. Indeed, astrovirus is considered worthy of vaccine development [28], but the results from our study could not confirm their association with ADI other than for first detections. The results for *Giardia* appear to have been affected by low numbers resulting in a RR with a wide confidence interval.

Organisms not considered gastrointestinal pathogens in this age group

Some organisms tested are not generally accepted as causing ADI. Yet, we report significant associations for non-group F adenovirus and enterovirus. While diarrhea may have been part of a systemic response to infections by these viruses, it is also possible specific virus-types, such as adenovirus 31/52, or one of several enterovirus species may be etiologic agents of ADI [29-31]. However, although the association with ADI for individual types would be stronger

than our estimates for a larger group, given dilution by non-pathogenic types, after adjustment for potential confounding factors the RR for both viruses became non-significant.

For other organisms not considered gastrointestinal pathogens in healthy children, the positive result for parechovirus A appears to have been caused by co-detections with other pathogens, in-line with previous analyses [32]. Also, *Blastocystis* is a suspected intestinal commensal rather than a genuine pathogen in immunocompetent hosts [33], but had a significant unadjusted association of a considerable magnitude with ADI. This significant finding, however, was imprecise from low numbers with wide confidence intervals, was limited to first detections, appears to have been influenced by co-detections, and disappeared in the adjusted analysis.

While *C. difficile* is a recognized gastrointestinal pathogen in older children and adults, especially following exposure to antibiotics, in neonates and young infants it is only associated with high rates of asymptomatic colonization [34]. During the ORChID study the infection rate was consistently low in the first 6 months of life (cumulative incidence: 20%) and then peaked between 7–15 months of age (with an average of approximately 2.0 new detections per child-year). This observation is consistent with a recent systematic review of 95 studies where *C. difficile* detection rates were highest in those aged 6–12 months and coincided with cessation of exclusive breastfeeding between 4–6 months of age [35, 36].

Finally, despite *D. fragilis* being the most prevalent protozoa in our study, it had no association with ADI, which supports it being generally considered an intestinal commensal [33, 37].

Strengths and limitations

The study strengths are its frequent and regular collection of stool samples, regardless of symptoms, during the period of greatest intestinal adaptation following birth. Sensitivity analyses confirmed our main findings. However, the ORChID study does have limitations, such as reduced generalizability for older children and lower income settings. The selective PCR testing strategy prevented us from exploring the incidence and risk of other agents that might have been present had an unbiased meta-genomic testing approach been adopted. However, this was not possible with the large number of samples involved. The PCR assays targeted species and did not differentiate between different genogroups/types within that species. In this regard, the lack of association between human bocavirus and ADI may have been from human bocavirus-1 being included in the pan-bocavirus assay, as this subtype is no longer implicated in ADI [38]. Our analyses were not adjusted for the potential effect of partial or cross-protective immunity from prior exposure to the same pathogen or different genogroups within the same species [39]. Co-detections were not explored further other than by descriptive analysis. Symptoms of acute respiratory illness were not linked to new detections in stool, which may have affected our conclusions for non-group F adenovirus. Because of its frequent and non-specific nature in very young children, we did not include vomiting in our definition of an ADI. The sample size was insufficient to draw meaningful conclusions for some organisms. Our analyses focused on the initial positive test result marking the beginning of new detections, and thus subsequent positive detections from ongoing shedding were excluded.

Conclusions

Although the development of multiplex molecular diagnostic platforms has made it possible to detect potentially pathogenic organisms in stools with greater sensitivity, the clinical utility of multiplex PCR platforms remains controversial [40]. Here we reaffirmed that wild-type

rotavirus, norovirus GII, sapovirus GI/II/IV/V, adenovirus 40/41, and *Salmonella* are associated with ADI in young children. However, human bocavirus, *C. difficile*, and *D. fragilis* may not be genuine pathogens in this age group and setting. The reporting of positive stool PCR results for these three organisms in young otherwise healthy children with ADI is consequently difficult to justify and clinicians should exercise caution when presented with such results. High asymptomatic shedding rates of potential pathogens in stools also complicates the interpretation of results from highly sensitive, molecular-based tests when used for routine testing of ADI, especially if an infectious cause is not considered likely. Despite these misgivings, multiplexed real-time PCR assays are replacing conventional methods for identifying gastrointestinal pathogens. To help define the role of these assays in routine testing of diarrheal stools, future studies should include samples from asymptomatic and ill subjects at the community level [41]. Cost-effectiveness analyses are also needed, but these have proven challenging without an adequate reference standard and uncertainties with interpreting positive test results [42]. Such studies, however, will provide evidence to further advise public health policymakers on surveillance, sanitation, food/water quality, and vaccination.

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CONFLICT OF INTEREST

KG participated in a rotavirus strain outbreak advisory board for GlaxoSmithKline (GSK), Wavre, Belgium. DMW reports research funding from SpeeDx Pty Ltd unrelated to the study. MDN reports stock options as a past employee of GSK (2014-2021).

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TABLES

Table 1. Characteristics of the 154 participants at birth and over time in the ORChID study

	***n* (%)**
Delivery type: vaginal	105 (68%)
Sex: female	80 (52%)
Season of birth:	
spring	43 (28%)
summer	42 (27%)
fall	26 (17%)
winter	43 (28%)
Household income[a] (*N*=151):	
<$67,500 (<50th percentile)	17 (11%)
$67,500 to <$115,000 (50–74th percentile)	51 (34%)
≥$115,000 (≥75th percentile)	83 (55%)
Older siblings in household at birth:	
none	100 (65%)
one	38 (25%)
two or more	16 (10%)
Number of children participating[b,c]:	
at 1 month of age (*N*=154)	150 (97%)
at 6 months of age (*N*=154)	135 (88%)
at 12 months of age (*N*=154)	117 (76%)
at 18 months of age (*N*=154)	109 (71%)
at 23 months of age (*N*=154)	101 (66%)

Number of children exclusively breastfeed[c]:	
at 1 month of age	97 (64%)
at 3 months of age	81 (53%)
at 4 months of age	67 (44%)
at 5 months of age	44 (29%)
at 6 months of age	14 (9%)
Number of children in formal childcare[c]:	
at 1 month of age	0 (0%)
at 6 months of age	17 (12%)
at 12 months of age	60 (45%)
at 18 months of age	80 (63%)
at 23 months of age	88 (70%)
Rotavirus vaccination[d] (*N*=131):	123 (94%)
Season of stool sampling (*N*=11,111):	
spring	2,807 (25%)
summer	2,763 (25%)
fall	2,789 (25%)
winter	2,752 (25%)

N = number of observations; [a] gross, per year, in Australian Dollars, 2010–12; [b] defined as the end of daily symptom observations; [c] results from life tables which consider children who have not reached the outcome by the end of their participation; [d] completed 3 vaccine doses by 32 weeks of age, 23 participants left the study before reaching this time limit. Queensland began vaccinating children with RotaTeq in a 3-dose schedule administered orally at 2, 4, and 6 months of age, with the age limit for the 3rd dose at 32 weeks.

Table 2. Incidence rates of potentially pathogenic organism detection episodes in stools during the ORChID study (*N*=11,111)

	Organism	Prev.	New detection episodes		
			Number	Exposure (child-years)[a]	Incidence rate per child-year (95% CI)
Viruses	Adenovirus (non-group F)	17.9%	544	192	2.84 (2.61, 3.09)
	Adenovirus (40/41)	1.6%	81	243	0.33 (0.27, 0.42)
	Classic human astrovirus	2.0%	106	241	0.44 (0.36, 0.53)
	Human bocavirus	7.8%	338	223	1.52 (1.36, 1.69)
	Enterovirus	12.1%	491	210	2.36 (2.16, 2.57)
	Norovirus GI	0.6%	40	246	0.16 (0.12, 0.22)
	Norovirus GII	3.6%	186	236	0.79 (0.68, 0.91)
	Parechovirus A	12.8%	276	213	1.31 (1.16, 1.47)
	Rotavirus (wild-type)[b]	0.4%	19	247	0.08 (0.05, 0.12)
	Sapovirus GI/II/IV/V	4.1%	196	235	0.84 (0.73, 0.96)
Bacteria	*Campylobacter*	0.3%	13	247	0.05 (0.03, 0.09)
	Clostridioides difficile A/B	14.4%	256	208	1.23 (1.09, 1.39)
	Salmonella	0.4%	14	247	0.06 (0.03, 0.10)
	Shigella	1.0%	0	248	0.00
	Yersinia	0.8%	0	248	0.00
Protozoa	*Blastocystis*	1.0%	14	245	0.06 (0.03, 0.10)
	Cryptosporidium	0.8%	32	246	0.13 (0.09, 0.18)
	Dientamoeba fragilis	8.1%	350	221	1.59 (1.43, 1.76)
	Giardia intestinalis	1.5%	11	244	0.05 (0.02, 0.08)

[a] *at-risk period but excluding shedding of that organism and the subsequent refractory period;*

[b] *N=11,077 as 34 stool samples were unable to be genotyped; prev. = prevalence; G = genogroup.*

Table 3. Associations (95% confidence intervals) between new potentially pathogenic organism detection episodes and acute diarrheal illness, as observed during the ORChID study

		New detections				**Sensitivity analyses**	
		Unadjusted		**Adjusted**		**New single detections**[a]	**First detection**[b]
		RD[c,d] (%)	RR[d]	RD[e] (%)	RR[e]	RR[d]	RR
Viruses	AdV non-F	2.1 (-0.0, 4.2)	**1.49 (1.05, 2.12)**	1.2 (-0.8, 3.3)	1.24 (0.87, 1.76)	**1.75 (1.15, 2.67)**	**2.02 (1.07, 3.84)**
	AdV 40/41	6.5 (-0.6, 13.6)	**2.52 (1.33, 4.77)**	5.9 (-1.1, 12.9)	**2.22 (1.16, 4.22)**	**2.83 (1.36, 5.89)**	**3.58 (1.96, 6.56)**
	AstV	3.6 (-1.7, 9.0)	1.83 (0.92, 3.62)	3.2 (-2.1, 8.5)	1.60 (0.81, 3.16)	2.30 (0.94, 5.66)	**2.16 (1.06, 4.42)**
	HBoV	1.5 (-0.7, 3.8)	1.35 (0.91, 2.00)	1.6 (-0.7, 3.9)	1.23 (0.83, 1.82)	1.28 (0.72, 2.29)	1.78 (0.94, 3.38)
	EV	**2.7 (0.3, 5.2)**	**1.65 (1.16, 2.33)**	2.1 (-0.2, 4.3)	1.38 (0.98, 1.94)	**2.04 (1.38, 3.02)**	1.65 (0.85, 3.19)
	NoV GI	1.1 (-6.6, 8.9)	1.26 (0.31, 5.08)	-0.2 (-8.1, 7.7)	1.05 (0.25, 4.35)	n/c	1.62 (0.41, 6.34)
	NoV GII	**12.8 (6.9, 18.7)**	**4.10 (2.79, 6.02)**	**12.4 (6.5, 18.3)**	**3.75 (2.57, 5.48)**	**4.25 (2.60, 6.94)**	**5.76 (3.76, 8.82)**
	PeV	**3.8 (0.5, 7.0)**	**1.92 (1.25, 2.95)**	**3.7 (0.6, 6.9)**	**1.82 (1.20, 2.77)**	1.70 (0.94, 3.09)	**2.09 (1.12, 3.90)**
	RV wild	**23.4 (2.8, 44.0)**	**6.36 (3.04, 13.3)**	**22.7 (2.1, 43.3)**	**5.82 (2.81, 12.0)**	**8.63 (3.81, 19.6)**	**6.36 (3.04, 13.3)**
	SaV GI/II/IV/V	**9.6 (4.6, 14.7)**	**3.30 (2.28, 4.77)**	**9.1 (4.1, 14.0)**	**2.86 (2.00, 4.10)**	**3.19 (1.89, 5.39)**	**3.80 (2.36, 6.13)**

Bactcteria	*Camp.*	n/c	n/c	n/c	n/c	n/c	n/c
	C. diff.	1.6 (-1.6, 4.7)	1.36 (0.79, 2.36)	1.4 (-1.6, 4.5)	1.32 (0.76, 2.31)	1.19 (0.54, 2.60)	0.95 (0.37, 2.44)
	Salmonella	18.7 (-1.0, 38.4)	**5.26 (2.20, 12.6)**	17.9 (-1.7, 37.5)	**4.35 (1.69, 11.2)**	**12.0 (5.60, 25.8)**	**6.84 (2.63, 17.8)**
Protozoa	*Blast.*	11.0 (-6.6, 28.6)	**3.48 (1.10, 11.0)**	11.5 (-6.0, 28.9)	3.12 (0.87, 11.2)	2.32 (0.34, 15.6)	**5.65 (1.69, 18.9)**
	Crypto.	9.9 (-3.3, 23.0)	**3.25 (1.28, 8.25)**	9.6 (-3.5, 22.7)	**2.85 (1.11, 7.30)**	3.09 (0.82, 11.6)	**3.50 (1.40, 8.75)**
	D. frag.	0.1 (-2.0, 2.1)	1.01 (0.64, 1.60)	0.1 (-1.8, 2.1)	0.87 (0.54, 1.41)	0.68 (0.32, 1.46)	1.03 (0.45, 2.36)
	Giardia	4.7 (-9.3, 18.7)	2.05 (0.44, 9.64)	2.0 (-12.5, 16.5)	1.68 (0.37, 7.52)	n/c	n/c

Shigella and Yersinia species are not shown as they were not detected during the ORChID study; 95% confidence intervals calculated using generalized linear models; ADI = acute diarrheal illness; AdV = adenovirus; AstV = classic human astrovirus; Blast. = Blastocystis; Camp. = Campylobacter; CI = confidence interval; C. diff. = Clostridioides difficile A/B; Crypto. = Cryptosporidium; D. frag. = Dientamoeba fragilis; HBoV = human bocavirus; EV = enterovirus; n/c = not calculated or cannot be calculated; NoV = norovirus; PeV = parechovirus A; RD = risk difference in %; RR = relative risk; RV = rotavirus; SaV = sapovirus GI/II/IV/V; ***bold*** *indicates statistical significance at p<0.05;* [a] *defined as a new detection where co-detections with other pathogens within ± 7 days were excluded;* [b] *defined as the first ever detection of that organism for that child;* [c] *positive results indicate a higher risk of ADI when exposed to a new detection;* [d] *adjusted for repeated measurements per child;* [e] *adjusted for repeated measurements per child, age, older child at home, exclusive breastfeeding, and formal childcare attendance.*

FIGURE LEGEND

Incidence rates of new potentially pathogenic organisms and rotavirus vaccine-type strain detections in stool samples during the Observational Research in Childhood Infectious Diseases study. X-axis: Age in months. Y-axis: Incidence rate (95% confidence interval) per child-year. Markers are slightly offset along the X-axis on shared plots for clarity. Note: differing scales and maximum values on Y-axes. AdV n-F = non-group F adenovirus; AdV 40/41 = group-F adenovirus types 40/41; AstV = classic human astrovirus; HBoV = human bocavirus; EV = enterovirus; NoV GI = norovirus genogroup I; NoV GII = norovirus genogroup II; PeVA = parechovirus A; RV (vacc) = vaccine-type rotavirus; RV (wild) = wild-type rotavirus; SaV GI/II/IV/V = sapovirus genogroups I/II/IV/V; *C. diff* = *Clostridioides difficile*; *D. fragilis* = *Dientamoeba fragilis*.

SUPPLEMENTARY METHODS

Cohort study: introduction and recruitment

The Observational Research in Childhood Infectious Disease (ORChID) study was a community-based, prospective longitudinal, dynamic birth cohort study conducted between 2010 and 2014 [1]. Its objectives were to describe acute respiratory and acute diarrheal illnesses and their associated microbiology in children living in an urban environment during the first 2 years of life. Recruitment took place from September 2010 to October 2012, and all children were followed until their second birthday. As a dynamic cohort study, families could leave the study temporarily (such as during holidays) and re-join at a later date.

The Children's Health Queensland (HREC/10/QRCH/16), the Royal Brisbane and Women's Hospital (HREC/10/QRBW125), and The University of Queensland (2010000820) Human Research Ethics Committees approved the study. Parents provided informed consent for study participation. Griffith University Ethics Committee (2020/197) approved the secondary data analysis.

The study was conducted in the subtropical city of Brisbane, in the state of Queensland, Australia which, at the time of the study, had a population of approximately 2.2 million. Pregnant women were approached for enrolment of their newborn infants at antenatal clinics of two metropolitan hospitals in Brisbane: one government funded (the Royal Brisbane and Women's Hospital) and one private (the Northwest Private Hospital). These hospitals had approximately 6,100 and 1,700 deliveries per year, respectively, at the time of recruiting for the ORChID study.

The mother and child were visited shortly after birth, while they were still in hospital. The following were completed during this visit: a written consent for participation was obtained, a stool sample was collected from the child, parents were taught how to collect stool specimens from diapers, study paperwork (e.g., diaries) was reviewed, and a demographic, social, and a health questionnaire was completed by the parent.

Children were eligible if born healthy and at term (36 to 42 weeks) without congenital abnormalities or underlying chronic disorders. The exclusion criteria for enrolment and ongoing participation were: parents unable to converse in English, and living outside the Brisbane metropolitan region or planning to move from the area within the following 2 years.

Cohort study: observations

Parents were requested to perform the following regular tasks from the day of birth: recording the daily number of loose (defined as liquid or looser than normal) stools in the symptom diary, weekly collection and mailing of stool samples, and participation in telephone interviews every 3 months to record immunization status and changes in breastfeeding and childcare attendance. The completed diary pages were returned by mail at the end of each month.

Weekly SMS text messages were sent to parents reminding them to collect stool samples from their study child's diapers using a plastic-shaft, rayon-budded swab, which was placed in a transport tube with a foam pad reservoir (Virocult MW950, Medical Wire & Equipment, 38 Wiltshire, England). Specimens were sent to the Queensland Paediatric Infectious Disease research laboratory by surface mail taking a median 3 (inter-quartile range: 2–4) days to reach

the laboratory. The stool samples were stored in a -80°C freezer upon their receipt in the laboratory.

Research staff managed the study recruitment, cohort maintenance, specimen collection/transport, and recording of socio-demographic and clinical data. Study coordinators supplied parents with stool sample swabs, a specimen collection instruction sheet, sealable bags, pre-addressed envelopes, and adhesive labels at the commencement of the study and at regular intervals.

Formal childcare was defined as regulated care outside the child's home, and informal childcare as non-regulated care provided by family or friends. The duration of exclusive breastfeeding was defined as the period from birth to the time of introducing milk formula or solids, whichever was sooner. Rotavirus vaccination details were collected from the Australian Childhood Immunisation Register.

Sample testing

The specimens were batch tested for viruses, bacteria, and protozoa using previously validated and reported real-time polymerase chain reaction (PCR) assays specific for individual organisms [1], or PCR assays developed specifically for this study. A PCR assay specific for the rotavirus vaccine (Rotateq; Merck Sharpe & Dohme [Australia]) strains was also included because many laboratories do not differentiate between wild-type and live-attenuated rotavirus vaccine, and our previous data showed frequent detection of vaccine strains in stools from infants submitted to a diagnostic laboratory [2]. Each stool sample was homogenized in approximately 2.5mL 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 nucleic

acid extraction. In brief, 200µl of stool suspension was processed on the Roche MagNApure 96 automated platform using manufacturer recommended conditions and the DNA and Viral NA Small Volume Kit (Roche Diagnostics, Australia). Whole equine herpesvirus (EHV) spiked into each sample determined nucleic acid extraction quality and presence of PCR inhibitors using a EHV real-time PCR [3]. Any extract having a >3 cycle threshold (Ct) difference to that of the expected value by EHV real-time PCR assays was considered to have failed quality control and the sample was re-extracted. Positive and negative controls were included in every run [3-5]. Viral real-time PCR assays were described previously [3-5] with bacterial and protozoal PCRs using similar conditions to those of the DNA virus PCRs and previously published oligonucleotides and probes (Supplementary Table 1). In brief, for each reaction, 4pmol of forward and reverse primer, 1.6pmol of probe, 5µl of Bioline SensiMix II Probe (Bioline, Australia), and 2µl of template in a final 10µl reaction volume were run in a 384 well format on Viia7 PCR instruments (Applied Biosystems, Australia) using the following cycling conditions: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

The individual results of the PCR assays were not conveyed to parents/caregivers. This was because the testing of stool swabs was delayed and conducted in batches. Importantly, we did not wish to influence parental healthcare seeking behavior for illness in their children as this was an important component of the ORChID study and has been reported elsewhere [6].

Measures of association

In addition to the relative risk (RR) and the risk difference (RD), the odds ratio, attributable risk, and the attributable fraction in the exposed were also calculated to allow comparison with findings from other studies.

The odds ratio (OR) is defined as the fold increase in odds of outcome from an unexposed to an exposed state and has been shown recently to be less affected by outcome prevalence than RR [7]. When there is no association between exposure (e.g., a new detection of a potentially pathogenic organism) and outcome (e.g., acute diarrheal illness [ADI]), OR = 1. The OR ranges from 0 (exposure is never associated with outcome) to infinity (exposure is always associated with outcome).

Attributable risk (AR) is defined as the proportion of ADI cases that can be attributed to an exposure and is interpreted as a measure of preventable disease. AR was calculated as:

AR = (prevalence of organism) × (RR - 1) / [1 + (prevalence of organism) × (RR - 1)].

AR was presented as a percentage and was not calculated when the univariable RR was <1 (i.e., when the percentage of new detections with ADI was lower than the percentage of non-detections with ADI). When there was no association between a new detection of a potentially pathogenic organism and ADI, AR = 0%, and conversely when RR tends to infinity, AR tends to 100%. However, note that the interpretation of AR is limited, as it is dependent upon the prevalence of the exposure. AR also has numerous synonyms, such as the ‘attributable fraction for the population’ or the ‘population attributable fraction’, which further confuse its application [8, 9].

The attributable fraction in the exposed (AFE) is similar to AR as it is defined as the proportion of episodes of ADI that can be attributed to a new detection of a potentially pathogenic organism, but AFE is calculated only among the subjects with a new detection of the potentially pathogenic organism of interest. AFE was calculated as:

$$\mathbf{AFE = (RR - 1) / RR}.$$

AFE was presented as a percentage and was not calculated when univariable RR <1. Similar to AR, AFE = 0% when there is no association between a potentially pathogenic organism and ADI, while AFE tends to 100% as RR tends to infinity. While it does not take the prevalence of exposure into account, this limitation also provides the advantage of portability between studies [10].

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Supplementary Table 1. Oligonucleotide names and sequences used for the Observational Research in Childhood Infectious Disease study

Pathogen and oligonucleotide name	Oligonucleotide sequences 5'-3'	Reference
Adenovirus (generic)		[1]
AdenoHeim-F	GCCACGGTGGGGTTTCTAAACTT	
AdenoHeim-R	GCCCCAGTGGTCTTACATGCACAT	
AdenoHeim-TaqAP	Quasar670-TGCACCAGACCCGGGCTCAGGTACTCCGA-BHQ	
Adenovirus (serotype 40/41)		[2]
Feen-Ad40-41-hexon-F	CCGACCCACGATGTAACCA	
Feen-Ad40-41-hexon-R	GCGGTCGACGGGCACGAA	
Feen-Ad40-41-hexon-TM	FAM- ACAGGTCRCAGCGACTGACGC-BHQ1	
Human Classic Astrovirus		[3, 4]
Astrovirus F	GACTGCWAAGCAGCTTCGTGA	
Astrovirus R	GCTAGCCATCACACTTCTTTGGTCCT	
Astrovirus TM	FAM-TCACAGAAGAGCAACTCCATCGCATTTG-BHQ1	
Human bocavirus		[5]
Pan-Boca-1F	CCTATATAAGCTGCTGCACTTCCTG	
Pan-Boca-1R	AAGCCATAGTAGACTCACCACAAG	
Pan-Boca-234F	GCACTTCCGCATYTCGTCAG	
Pan-Boca-3R	GTGGATTGAAAGCCATAATTTGA	
Pan-Boca-24R	AGCAGAAAAGGCCATAGTGTCA	
Pan-Boca-FAM	FAM-CCAGAGATGTTCACTCGCCG-MGB	
Enterovirus		[6]
AN190	CCTGAATGCGGCTAATCC	
AN50	TTGTCACCATWAGCAGYCA	
AN234pr	FAM-CCGACTACTTTGGGWGTCCGTGT-BHQ1	
Norovirus 1		[7]
NORO-Kagey-COG1-F	CGYTGGATGCGNTTYCATGA	
NORO-Kagey-COG1-R	CTTAGACGCCATCATCATTYAC	
NORO-Kag- RING 1a TM	FAM-AGATYGCGATCYCCTGTCCA-BHQ1	
NORO-Kag RING 1b TM	FAM-AGATCGCGGTCTCCTGTCCA-BHQ1	
Norovirus 2		[7]
NORO-Kagey-COG2-F	CARGARBCNATGTTYAGRTGGATGAG	
NORO-Kagey-COG2-R	TCGACGCCATCTTCATTCACA	

Noro RING2 TM	Quasar670-TGGGAGGGCGATCGCAATCT–BHQ3	
Parechovirus A		[8]
PeV_For	CTGGGGCCAAAAGCCA	
PeV_Rev	GGTACCTTCTGGGCATCCTTC	
PeV_Probe	FAM- AAACACTAGTTGTAWGGCCC-MGB	
Rotavirus – vaccine type strains		[9]
RotaTeq-VP6F	GCGGCGTTATTTCCAAATGCACAG	
RotaTeq-VP6R	CGTCGGCAAG CACTGATTCA CAAA	
RotaTeq-VP6P	FAM-ATCACGCAA“C” AGTAGGACT“C” ACGCTT-BHQ1 “C” = C-5 propynyl-dC.	
Rotavirus – wild type strains		[10, 11]
Rota-pang-NVP3-F1	ACCATCTACACATGACCCTC	
Rota-pang-NVP3-F2	ACCATCTTCACGTAACCCTC	
Rota-pang-NVP3-R	GGTCACATAACGCCC	
Rota-pang-NVP3-TM	FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-BHQ1	
Sapovirus (genogroups I, II, IV and V)		[3, 4]
Sapovirus F1	TTGGCCCTCGCCACCTAC	
Sapovirus F2	GAYCASGCTCTCGCYACCTAC	
Sapovirus R	CCCTCCATYTCAAACACTA	
Sappovirus TM	VIC-CCRCCTATRAACCA-MGB	
Campylobacter jejuni/coli		[12]
CAMP 221F	CTGCTAAACCATAGAAATAAAATTTCTCAC	
CAMP 221R	CTTTGAAGGTAATTTAGATATGGATAATCG	
CAMP Pb FAM	FAM-CATTTTGACGATTTTTGGCTTGA-BHQ1	
Clostridioides difficile A		[13]
C.DIFF-A-FOR	TTCAAGCAGAAATAGAGCACTC	
C.DIFF-A-REV	TATCAGCCCATTGTTTTATGTATTC	
C.DIFF-A-PROBE-FAM	FAM-TCACTGACTTCTCCACCTATCCATACAA-BHQ1	
Clostridioides difficile B		[13]
C.DIFF-B-FOR	GGTATTACCTAATGCTCCAAATAG	
C.DIFF-B-REV	TTTGTGCCATCATTTTCTAAGC	
C.DIFF-B-PROBE-FAM	HEX-ACCTGGTGTCCATCCTGTTTCCCA-BHQ1	
Salmonella spp		[12]
SALM 178F	TGCATAATGCCAGACGAAAGAG	

SALM 178R	ATCATTTCTATGTTCGTCATTCCA	
SALM 178 PB FAM	FAM-GAGGATTCTGTCAATGTAGAACGACCC-BHQ1	
Shigella spp		[14]
SHIG.SPP FOR	CCTTTTCCGCGTTCCTTGA	
SHIG.SPP REV	CGGAATCCGGAGGTATTGC	
SHIG.SPP PROBE Q670	QUASAR670-CGCCTTTCCGATACCGTCTCTGCA-BHQ3	
Yersinia enterocolitica		[15]
YER.ENT-FOR	ATGATAACTGGGGAGTAATAGGTTCG	
YER.ENT-REV	CCCAGTAATCCATAAAGGCTAACATAT	
YER.ENT-PB Q670	QUASAR670-TCTATGGCAGTAATAAGTTTGGTCACGGTGATCT-BHQ3	
Blastocystis spp		[16]
Blasto -Sten-F	GGTCCGGTGAACACTTTGGATTT	
Blasto-Sten-R	CCTACGGAAACCTTGTTACGACTTCA	
Blasto-Sten-TM FAM	FAM-TCGTGTAAATCTTACCATTTAGAGGA-MGB	
Dientamoeba fragilis		[17]
D-frag-Stark-DF3	GTTGAATACGTCCCTGCCCTTT	
D-frag-Stark-DF4	TGATCCAATGATTTCACCGAGTCA	
D-frag-Stark-TM-FAM	FAM-CACACCGCCCGTCGCTCCTACCG-BHQ1	
Cryptosporidium spp		[18]
Liu-crypto-F	GGGTTGTATTTATTAGATAAAGAACCA	
Liu-crypto-R	AGGCCAATACCCTACCGTCT	
Liu-crypto-TM FAM	FAM-TGACATATCATTCAAGTTTCTGAC-BHQ1	
Giardia intestinalis		[19]
Giardia-Haq-Gd-80F	GACGGCTCAGGACAACGGTT	
Giardia-Haq-Gd-127R	TTGCCAGCGGTGTCCG	
Gd-FT-pb Q670	QASAR670-CCCGCGGCGGTCCCTGCTAG-BHQ3	

Supplementary Table 2. Number and percentage of positive and negative stool samples, new detections, and detections associated with acute diarrheal illness (ADI) during the Observational Research in Childhood Infectious Disease study (column headers as per Supplementary Figure 1).

	A	C	E	H	I	J	K	N	Q
AdV non-group F	11,111	1,993 (18)	544 (27)	8,771 (96)	504 (93)	372 (4)	32 (6)	8,325 (99)	470 (100)
AdV 40/41	11,111	180 (2)	81 (45)	10,488 (96)	74 (91)	447 (4)	8 (11)	9,960 (99)	66 (100)
AstV	11,111	218 (2)	106 (49)	10,449 (96)	100 (94)	454 (4)	8 (8)	9,914 (99)	92 (100)
HBoV	11,111	870 (8)	338 (39)	9,824 (96)	326 (96)	420 (4)	19 (6)	9,326 (99)	307 (100)
Enterovirus	11,111	1,339 (12)	491 (37)	9,396 (96)	460 (94)	394 (4)	32 (7)	8,923 (99)	428 (100)
NoV GI	11,111	69 (1)	40 (58)	10,597 (96)	36 (90)	465 (4)	2 (6)	10,051 (99)	34 (100)
NoV GII	11,111	399 (4)	186 (47)	10,288 (96)	171 (92)	422 (4)	29 (17)	9,789 (99)	142 (100)
Parechovirus A	11,111	1,423 (13)	276 (19)	9,309 (96)	256 (93)	378 (4)	20 (8)	8,869 (99)	235 (100)
RV (wild-type)	11,077[a]	41 (0)	19 (46)	10,589 (96)	18 (95)	459 (4)	5 (28)	10,049 (99)	13 (100)
SaV GI/II/IV/V	11,111	458 (4)	196 (43)	10,232 (96)	183 (93)	425 (4)	25 (14)	9,732 (99)	156 (99)
Campylobacter	11,111	35 (0)	13 (37)	10,623 (96)	13 (100)	465 (4)	0 (0)	10,078 (99)	12 (92)
C. difficile A/B	11,111	1,602 (14)	256 (16)	9,124 (96)	240 (94)	390 (4)	14 (6)	8,665 (99)	225 (100)
Salmonella	11,111	41 (0)	14 (34)	10,620 (96)	13 (93)	462 (4)	3 (23)	10,077 (99)	10 (100)
Shigella	11,111	0 (0)	0 (n/c)	10,658 (96)	0 (n/c)	467 (4)	0 (n/c)	10,110 (99)	0 (n/c)
Yersinia	11,111	0 (0)	0 (n/c)	10,658 (96)	0 (n/c)	467 (4)	0 (n/c)	10,110 (99)	0 (n/c)
Blastocystis	11,111	106 (1)	14 (13)	10,565 (96)	13 (93)	464 (4)	2 (15)	10,020 (99)	11 (100)
Cryptosporidium	11,111	85 (1)	32 (38)	10,580 (96)	28 (88)	462 (4)	4 (14)	10,037 (99)	24 (100)
D. fragilis	11,111	904 (8)	350 (39)	9,807 (96)	334 (95)	436 (4)	15 (4)	9,293 (99)	316 (99)
Giardia intestinalis	11,111	166 (1)	11 (7)	10,499 (96)	11 (100)	462 (4)	1 (9)	9,956 (99)	10 (100)
Total	211,075	9,929 (5)	2,967 (30)	193,077 (96)	2,780 (94)	8,311 (4)	219 (8)	183,284 (99)	2,551 (100)
RV (vaccine-type)	11,077[a]	909 (8)	233 (26)	9,744 (96)	225 (97)	438 (4)	6 (3)	9,245 (99)	217 (99)

[a] 34 positive samples were not genotyped; AdV = adenovirus; AstV = classic human astrovirus; G = genogroup; HBoV = human bocavirus; NoV = norovirus; RV = rotavirus; SaV = sapovirus; column headers as per Supplementary Figure 1: 'A' number of stool samples, 'C' number of positive test results, 'E' number of new detections, 'H' number of negative test results with recorded symptoms, 'I' number of new detections with recorded symptoms, 'J' number of negative test results with symptoms, 'K' number of new detections with symptoms, 'N' number of negative test results without new onset of an ADI and no pre-existing ADI at sampling, 'Q' number of new detections without new onset of an ADI and no pre-existing ADI at sampling.

Supplementary Table 3. New potentially pathogenic organism co-detections in stool samples during the Observational Research in Childhood Infectious Diseases study and their symptomatic (diarrheal) status

Organism	New detections with merged symptoms (column I[a])	Co-detection with another organism	Symptomatic co-detection with another organism	AdV non-group F	AdV 40/41	AstV	HBoV	Enterovirus	NoV GI	NoV GII	Parechovirus A	RoV (wild-type)	SaV GI/II/IV/V	*Campylobacter*	*C. difficile* A/B	*Salmonella*	*Blastocystis*	*Cryptosporidium*	*D. fragilis*	*Giardia*
AdV non-group F	504	133 (26%)	9 (7%)		0	0	0	2	1	4	2	0	1	0	1	0	0	1	1	0
AdV 40/41	74	14 (19%)	2 (14%)	0		0	2	0	0	0	1	0	1	0	0	0	0	0	0	0
AstV	100	39 (39%)	3 (8%)	10	0		1	0	0	0	0	1	1	0	0	0	0	0	0	0
HBoV	326	103 (32%)	9 (9%)	27	5	5		0	0	2	2	0	3	0	0	0	0	0	1	0
Enterovirus	460	116 (25%)	7 (6%)	21	5	10	18		0	2	1	0	1	0	0	0	0	0	2	0
NoV GI	36	11 (31%)	2 (18%)	6	0	0	0	3		1	0	0	0	0	1	0	0	0	0	0
NoV GII	171	57 (33%)	12 (21%)	11	3	2	10	17	1		2	0	1	0	2	0	0	1	2	0
Parechovirus A	256	79 (31%)	10 (13%)	19	1	5	18	14	1	10		0	3	0	2	0	0	0	2	0
RV (wild-type)	18	5 (28%)	1 (20%)	0	1	1	0	1	0	0	0		0	0	0	0	0	0	0	0
SaV GI/II/IV/V	183	49 (27%)	10 (20%)	14	1	3	6	11	2	3	7	0		0	0	0	0	0	3	1
Campylobacter	13	2 (15%)	0 (0%)	1	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0
C. difficile A/B	240	75 (31%)	7 (9%)	23	3	3	17	10	2	5	11	2	3	1		0	1	1	0	0
Salmonella	13	6 (46%)	0 (0%)	4	0	0	1	0	1	0	1	0	0	0	1		0	0	0	0
Blastocystis	13	1 (8%)	1 (100%)	0	0	0	0	0	0	0	0	0	0	0	1	0		0	0	0
Cryptosporidium	28	10 (36%)	2 (20%)	3	0	0	0	4	0	2	0	0	1	0	2	1	0		0	0
D. fragilis	334	92 (28%)	9 (10%)	23	1	6	17	25	1	6	12	1	12	0	5	0	0	1		0
Giardia	11	3 (27%)	1 (33%)	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	

lower triangle: pairwise frequency of all co-detections; upper triangle: pairwise frequency of co-detections that were symptomatic; AdV = adenovirus; AstV = classic human astrovirus; G = genogroup; HBoV = human bocavirus; NoV = norovirus; RV = rotavirus; SaV = sapovirus; no detections for Shigella *and* Yersinia*; [a] of Supplementary Table 2.*

Supplementary Table 4. Univariable associations and 95% confidence intervals between new potentially pathogenic organism detection episodes and acute diarrheal illness (ADI), as observed during the Observational Research in Childhood Infectious Diseases study

		N	**crude odds ratio**[a] (95% CI)	**attributable risk**	**attributable fraction in the exposed** (95% CI)
Viruses	Adenovirus non-group F	9,199	**1.52 (1.05, 2.21)**	2.6%	**33% (5, 53)**
	Adenovirus 40/41	10,481	**2.70 (1.32, 5.53)**	1.1%	**60% (23, 79)**
	Classic human astrovirus	10,468	1.90 (0.90, 3.99)	0.8%	45% (-7, 72)
	Human bocavirus	10,072	1.37 (0.91, 2.08)	1.1%	26% (-16, 53)
	Enterovirus	9,777	**1.69 (1.16, 2.47)**	2.9%	**39% (14, 57)**
	Norovirus GI	10,552	1.27 (0.29, 5.58)	0.1%	20% (-207, 79)
	Norovirus GII	10,382	**4.74 (3.02, 7.43)**	4.9%	**76% (66, 83)**
	Parechovirus A	9,502	**2.00 (1.26, 3.17)**	2.4%	**48% (20, 66)**
	Rotavirus (wild-type)	10,526	**8.42 (3.04, 23.3)**	0.9%	**84% (67, 93)**
	Sapovirus GI/II/IV/V	10,338	**3.67 (2.40, 5.60)**	3.9%	**70% (56, 79)**
Bact.	*Campylobacter*	10,555	n/c	0.1%	100% (n/c)
	Clostridioides difficile A/B	9,294	1.38 (0.80, 2.38)	0.9%	26% (-23, 56)
	Salmonella	10,552	**6.54 (1.94, 22.1)**	0.5%	**81% (49, 93)**
Protozoa	*Blastocystis*	10,497	3.93 (0.00, 15.8)	0.3%	71% (-3, 92)
	Cryptosporidium	10,527	**3.62 (1.31, 10.0)**	0.6%	**69% (23, 88)**
	Dientamoeba fragilis	10,060	1.01 (0.60, 1.70)	n/c	n/c
	Giardia intestinalis	10,429	2.15 (0.00, 13.1)	0.1%	51% (-217, 92)

Shigella *and* Yersinia *are not shown as they were not detected during ORChID; 95% confidence intervals calculated using generalized linear models;* ***bold*** *indicates statistical significance at p<0.05; CI = confidence interval; G = genogroup; N = number of stool samples tested; bact. = bacteria; n/c = not calculated or cannot be calculated;* [a] *adjusted for repeated measurements per child, age, older child at home, exclusive breastfeeding, and formal childcare attendance.*

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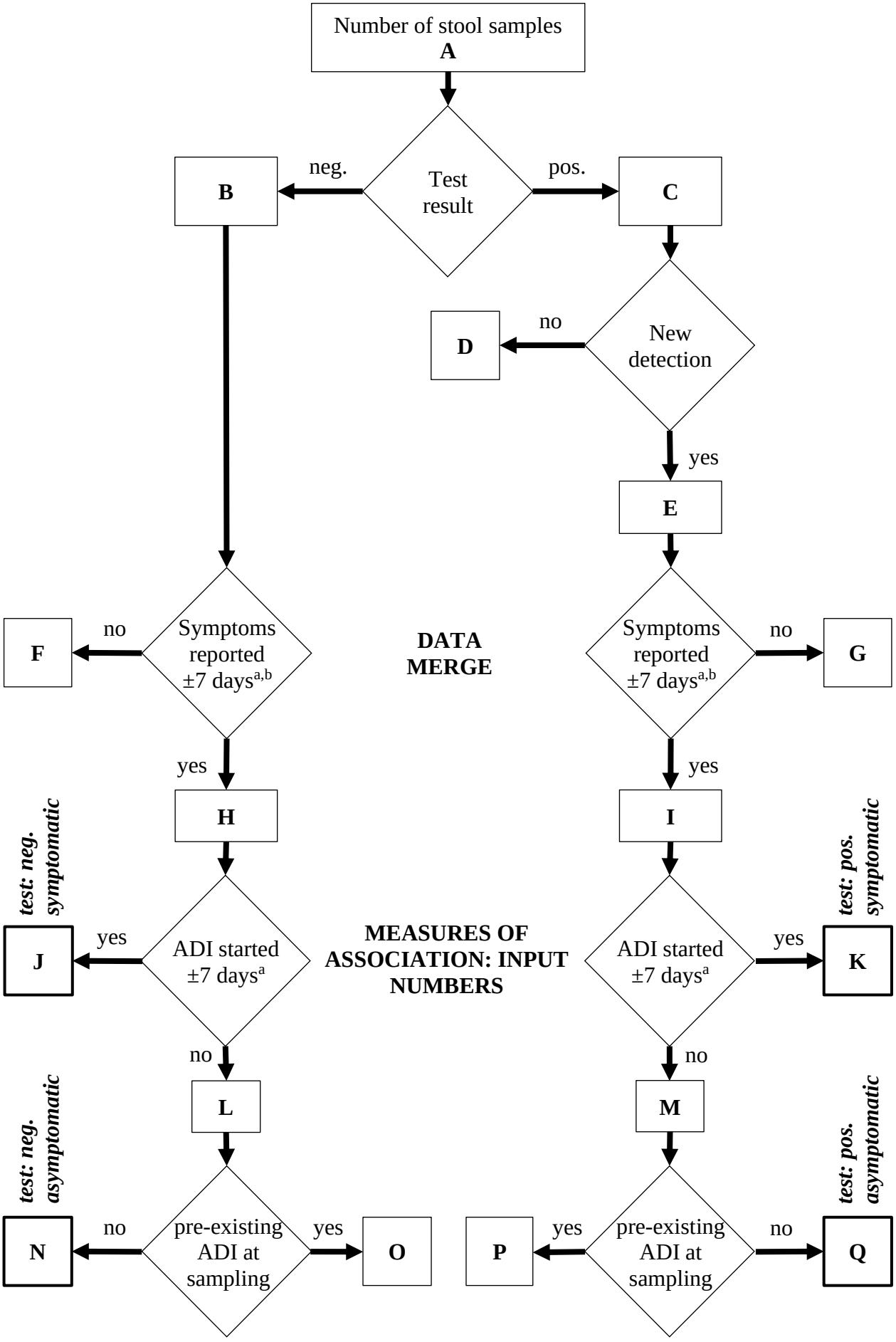
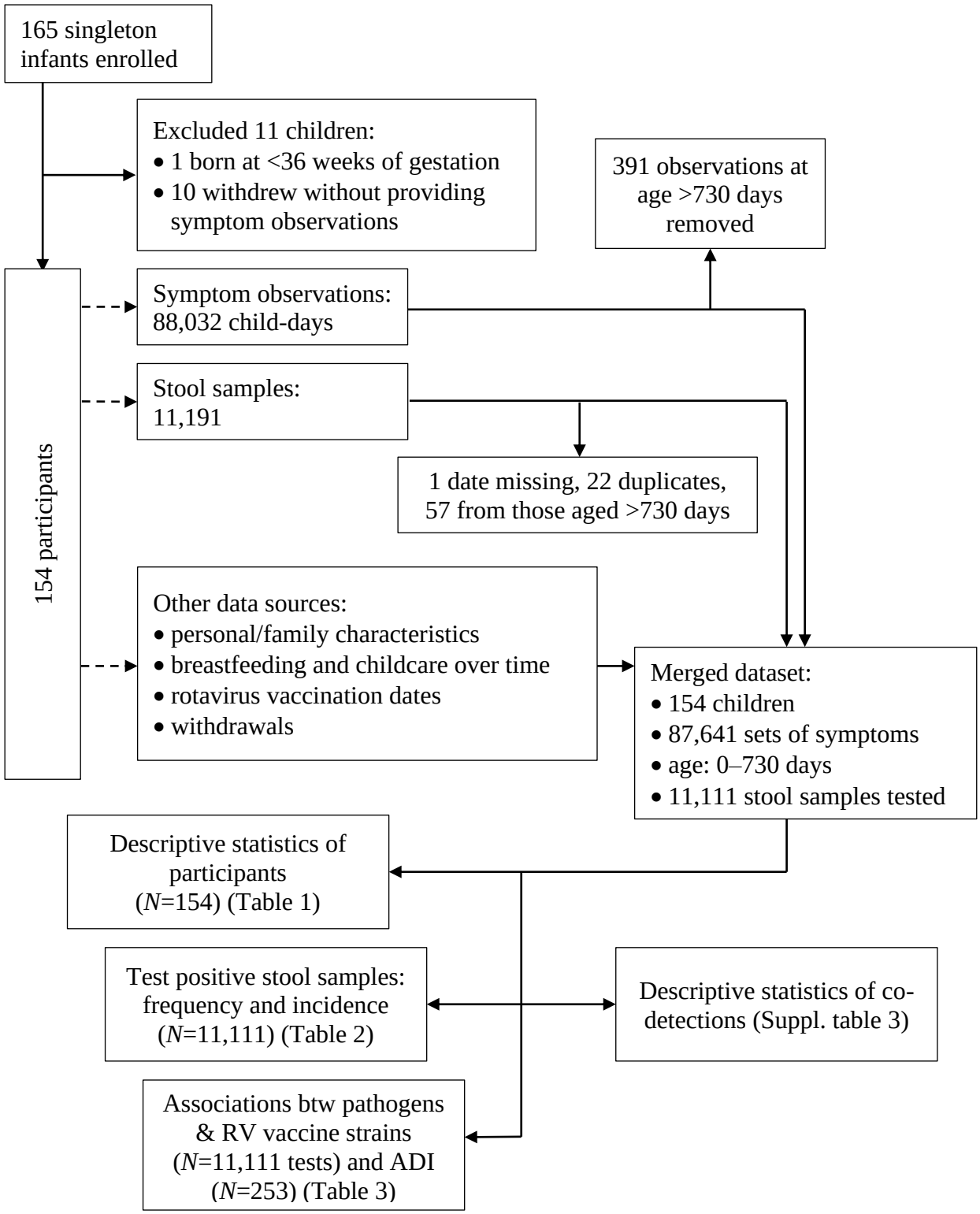
Supplementary Figure 1: Algorithm for calculating the number of positive/negative fecal samples that were symptomatic/asymptomatic. *"A", "B", "C", etc. = frequencies (see Supplementary Table 2 for actual numbers); neg. = negative; pos. = positive; ADI = acute diarrheal illness; [a] day of sampling and +7 days in the first 7 days of life; [b] 'no' if study participation ended in the next 7 days.*

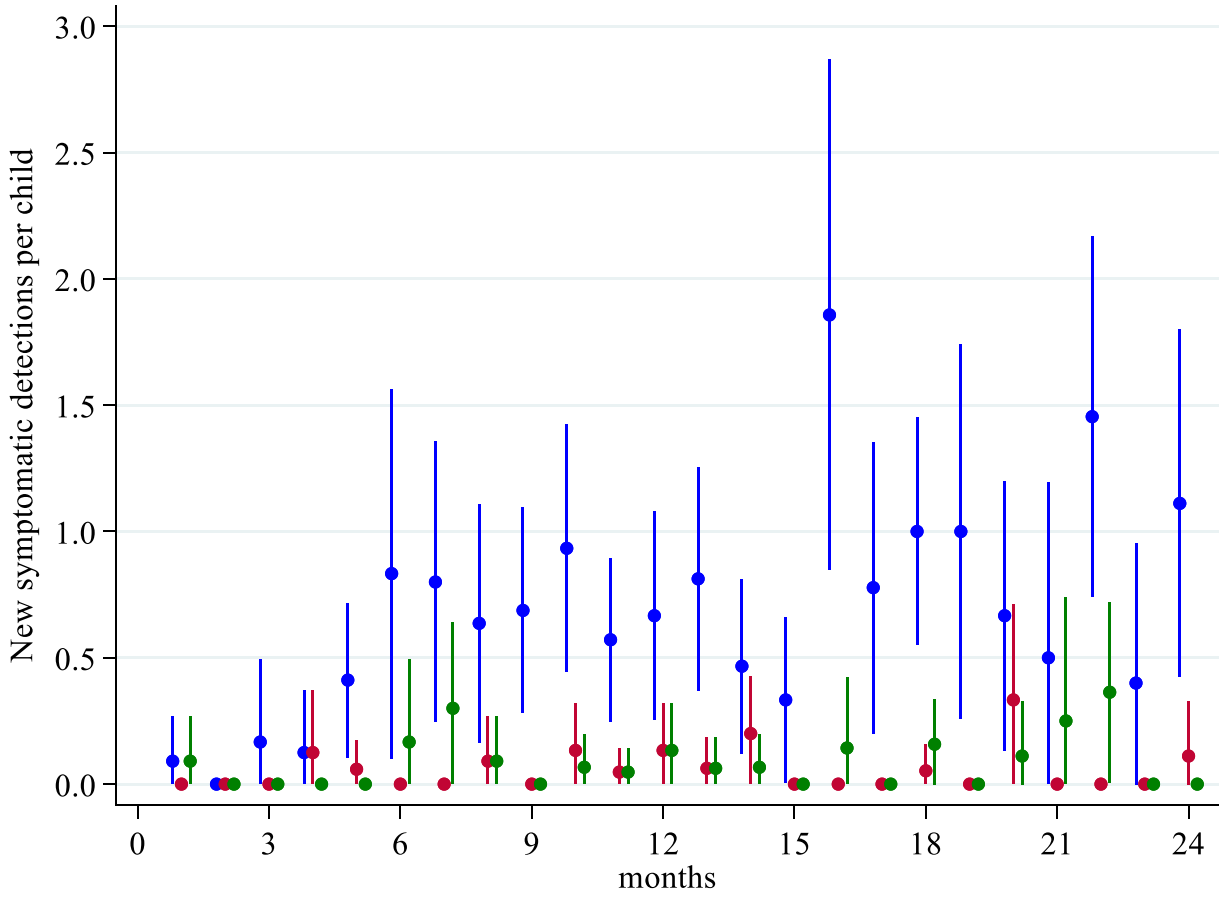


Supplementary Figure 2: Flow chart of observations collected on children participating in the Observational Research in Childhood Infectious Diseases study



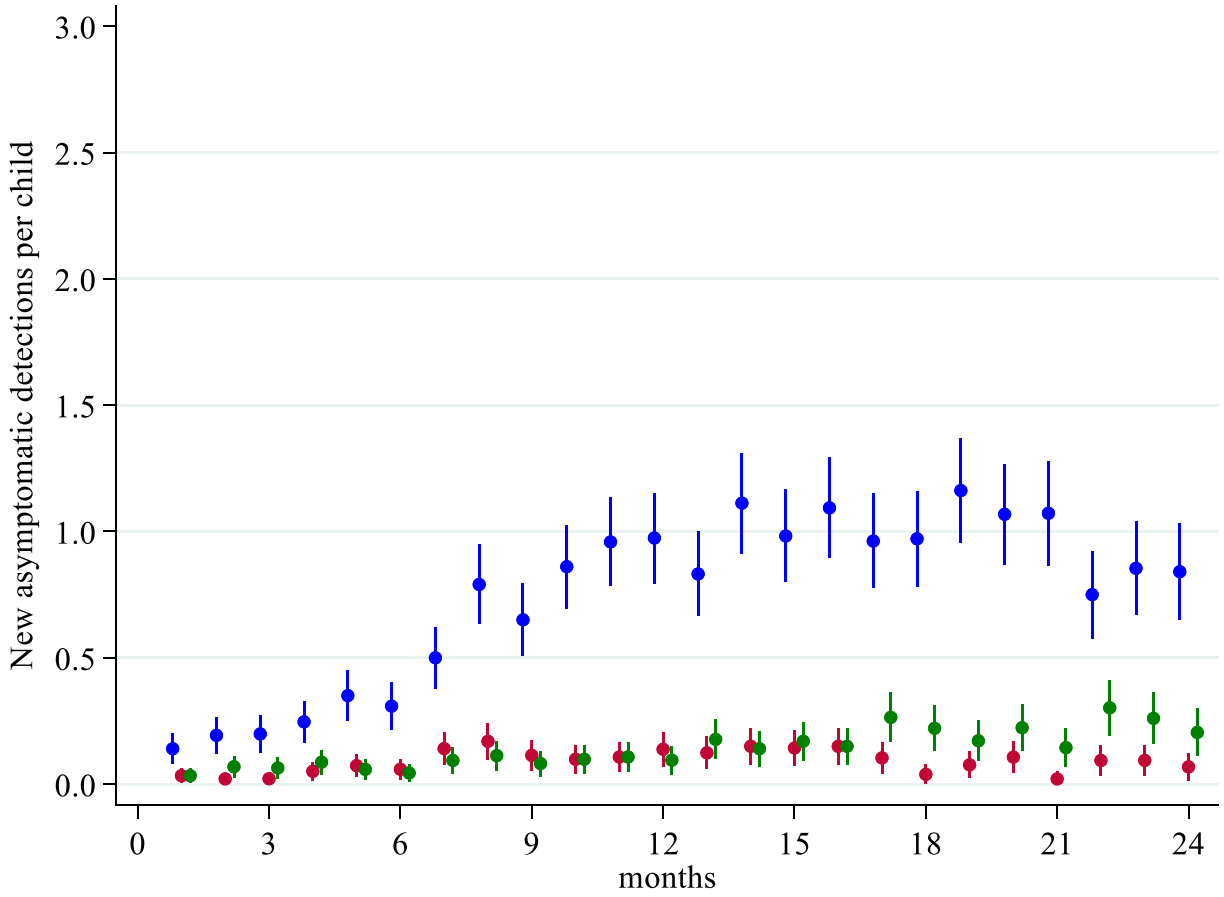
ADI = acute diarrheal illness; btw = between; RV = rotavirus; Suppl. = supplementary.

Supplementary Figure 3a: Average monthly number of new symptomatic potentially pathogenic organism detections per child and 95% confidence intervals in stool samples during the Observational Research in Childhood Infectious Diseases study.



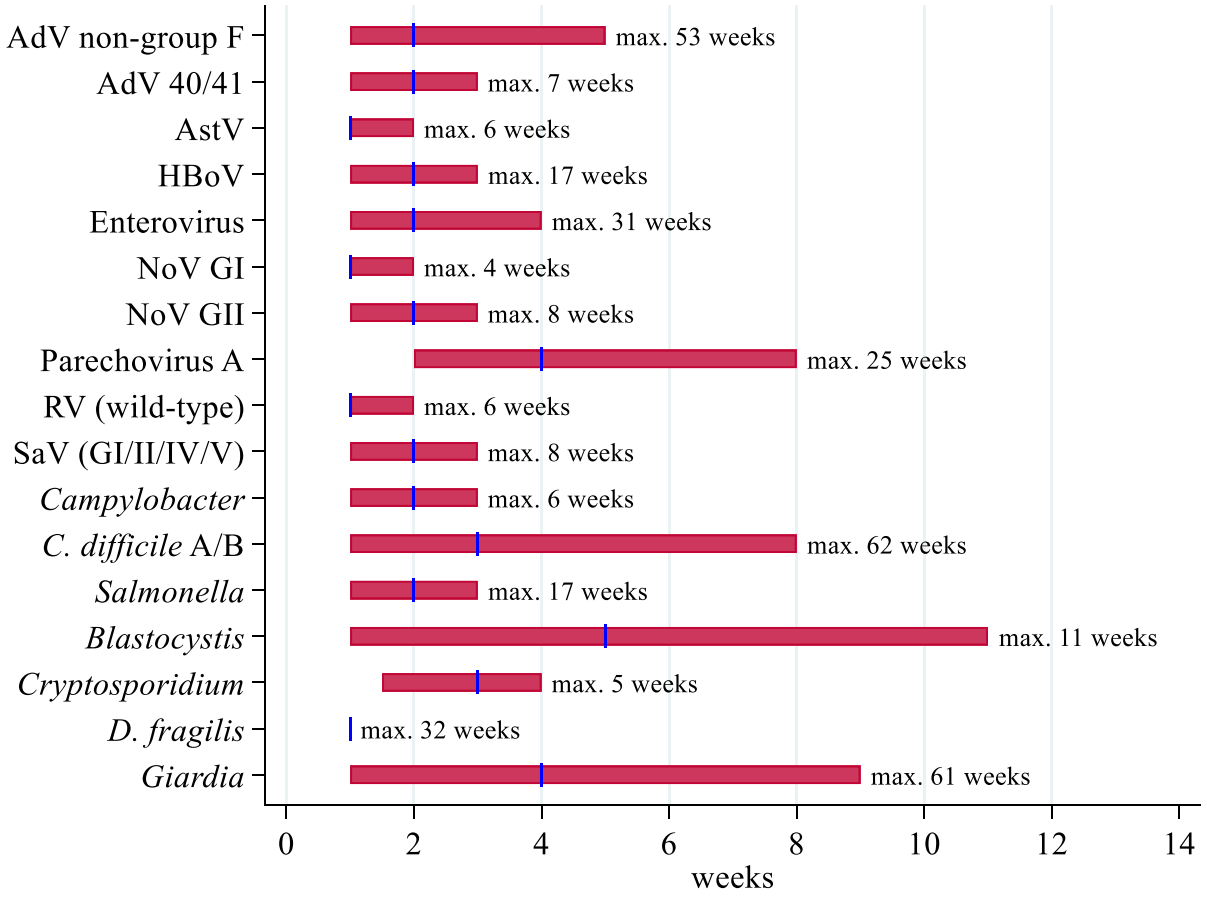
Markers and spikes are slightly offset along the x axis for clarity. Blue (offset to the left): viruses (excluding vaccine-type rotavirus strains). Red (middle): bacteria. Green (offset to the right): protozoa.

Supplementary Figure 3b: Average monthly number of new asymptomatic potentially pathogenic organism detections per child and 95% confidence intervals in stool samples during the Observational Research in Childhood Infectious Diseases study.



Markers and spikes are slightly offset along the x axis for clarity. Blue (offset to the left): viruses (excluding vaccine-type rotavirus strains). Red (middle): bacteria. Green (offset to the right): protozoa.

Supplementary Figure 4: Duration (median, 25th and 75th percentiles, and maximum) of pathogen detection episodes in stool samples during the Observational Research in Childhood Infectious Diseases study.



AdV = adenovirus; AstV = classic human astrovirus; BoV = human bocavirus; G = genotype; NoV = norovirus; RV = rotavirus; SaV = sapovirus; length of a new detection episode defined as the number of weeks from the first positive test until the last positive test (minimum value 1 week); red bar indicates 25th to 75th percentiles, blue vertical mark indicates median durations, maximum values shown; Shigella *and* Yersinia *not shown as there were no positive detections during the study.*