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Original article

Intestinal shedding of enteric agents in histo-blood group antigen-secretor children in an Australian community-based birth cohort study

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Abstract

Histo-blood group antigens are inherited polymorphic glycans expressed on mucosal epithelial cell surfaces and shed in secretions. They act as receptors for gastrointestinal pathogens. Those expressing these antigens are categorized as secretors and have an increased susceptibility to gastrointestinal infections, particularly from some norovirus and rotavirus genotypes. However, high-level evidence of association between secretor status and susceptibility is lacking for many other gastrointestinal pathogens. We compared differences in detection rates for 8 viruses, 5 bacteria, and 4 protozoa in children under 2 years of age enrolled in the Australian Observational Research in Childhood Infectious Diseases birth cohort study. Their secretor status was determined by phenotyping (ABO, Lewis, UEA-I) and genotyping test results. Data from polymerase chain reaction assay testing of weekly stool samples collected from birth

as well as daily symptom and illness diaries were analyzed. Incidence rates were calculated by secretor status and compared using incidence rate ratios for each pathogen. Additional comparisons included symptomatic/asymptomatic detections, illness severity, and healthcare utilization by secretor status. Eighty-four children (77% secretors) participated for an exposure period of 157 child-years. The incidence rates of norovirus GI, GII, wild-type rotavirus, and *Campylobacter* detections were more than 50% higher in secretors than non-secretors, but lower for *Blastocystis*. For these pathogens, secretor status was not associated with symptomatic illness, severity, or healthcare use. Our results confirmed the previously known higher susceptibility to norovirus GII infections in secretors but displayed variations in susceptibility to other pathogens. This study strengthens the evidence for norovirus susceptibility in Australian children.

Keywords

Infection, host susceptibility, histo-blood group antigens, secretor phenotype, norovirus, *Blastocystis*.

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Introduction

Diarrheal disease is a leading cause of morbidity and mortality. In 2016, there were an estimated 1.7 million deaths and 4.5 billion acute diarrheal episodes globally [1]. That year diarrheal disease was the eighth leading cause of death across all age groups and the fifth in children under 5 years of age. Rotavirus, followed by *Shigella*, were the major etiologic pathogens [1]. In children under 5 years of age rotavirus caused an estimated 128,500 deaths and more than 258 million episodes of diarrhea [2]. Nevertheless, rotavirus vaccines, improvements in sanitation, food/water quality, and nutrition, as well as increased availability of oral rehydration solutions have all contributed to the decline in diarrhea mortality since 1990 [3]. As rotavirus vaccines are incorporated into more national immunization programs [4] and candidate *Shigella*, *Vibrio cholerae*, and norovirus vaccines undergo further assessment in clinical trials [5-7], more attention is being devoted to identifying population-level and individual host susceptibility factors for gastrointestinal infection. One such factor is a group of mucosal surface glycans, known as histo-blood group antigens (HBGAs), that can act as receptors for selected pathogens [8].

HBGAs are polymorphic glycans present on mucosal epithelial cell surfaces and in body fluids, such as saliva and breast milk, where they may also function as decoy receptors. The HBGAs include the ABH and Lewis antigens and their expression in tissues is controlled by specific fucosyltransferase (*FUT*) genes, specifically the *FUT2* (secretor) and *FUT3* (Lewis) genes [9]. The simplest ABH antigens are the H antigens, whose precursor type I H antigen is fucosylated at the α (1,2) position by *FUT2* to form the mature type I H antigen or secretor molecule. The type I H antigen can then be converted into blood group A or B antigens by A or B glycosyltransferases respectively, or if these are absent remain as the H antigen, characteristic of blood group O. The type I H antigen is fucosylated by *FUT3* at the α (1,4) position to become either the Lewis a antigen when the H antigen persists in its precursor form or the Lewis b antigen when the mature H antigen has had two fucose residues added by the combined actions of *FUT2* and *FUT3* enzymes. Thus, the Lewis b antigen is found only in secretors.

Homozygous inactivating mutations in *FUT2* result in the non-secretor phenotype, and although individuals with these mutations can still produce Lewis a antigens by *FUT3* fucosylation of the precursor type I H antigen, they do not express blood group antigens in tissues or secretions. In contrast, while those with *FUT3* mutations have the Lewis-negative phenotype, if they have an intact *FUT2* gene they can still express blood group antigens in both tissues and secretions and have the secretor phenotype. At a population level the prevalence of the secretor phenotype varies between different ethnicities, being present in approximately 70% to 80% of Caucasians, but in only 55% of African populations [10, 11].

People with the secretor phenotype express HBGAs on their gut mucosal epithelial cell surfaces, which act as cellular receptors for several gastrointestinal pathogens, including some norovirus strains (especially GII.4) and genotype P[8] rotaviruses [12-14]. Volunteer challenge and epidemiological studies show that secretors are significantly more susceptible to infections by these viruses than non-secretors [15-20].

Much less is known about the susceptibility to other pathogens shed in stools [21, 22]. Given the greatest burden of diarrheal disease is in young children [1], longitudinal birth cohort studies are the ideal method to examine the association between HBGAs and gastrointestinal pathogens. However, to date such studies are few and have been conducted in low- and middle-income countries of Latin America, Africa, and South Asia, focusing mainly on norovirus or rotavirus, and sampling primarily at the times of acute diarrheal episodes or following rotavirus vaccination with only one study taking regular monthly stool samples irrespective of symptoms [12, 23-26].

To address some of these limitations, the primary aim of the current study was to explore the association between secretor status and the shedding of several viruses, bacteria, and protozoa in stools collected weekly from an unselected Australian birth cohort until their second birthday. This included organisms recognized as genuine gastrointestinal pathogens, others that can be shed in stools, but are more commonly associated with extra-gastrointestinal symptoms, and others whose pathogenic status in healthy children has not been established. A secondary aim was to explore differences in illness severity and healthcare use during symptomatic detection episodes by secretor status.

Methods

Study characteristics

Study participants were enrolled in the Observational Research in Childhood Infectious Diseases (ORChID) study, which is registered on clinicaltrials.gov (NCT01304914). This was a prospective, community-based birth cohort study of unselected healthy children born at term and conducted in the subtropical city of Brisbane, Australia between 2010 and 2014 [27]. The ORChID study focused upon respiratory and gastrointestinal infections during the first 2 years of life. At its completion, children were invited to attend annual clinic visits from ages 3 to 7 years as part of the Early Life Lung Function (ELLF) study [28]. The Children's Health Queensland, the Royal Brisbane and Women's Hospital, and The University of Queensland Human Research Ethics Committees approved the studies.

Child and family characteristics were collected at baseline, while breastfeeding, formal childcare attendance (defined as regulated care outside the child's home), and presence of other children in the household were collected progressively. Parents recorded a daily diary of pre-defined symptoms, including fever and the daily number of loose stools and vomits. Diarrhea was defined as 3 or more loose (liquid or looser than normal) stools within a 24-hour period [29], while vomiting was categorized as any or none within 24 hours. Acute diarrheal/vomiting episodes consisted of 1 or more days of diarrhea/vomiting, separated by 3 or more days without loose stools/vomiting. Parents also kept a separate illness burden diary, where healthcare utilization associated with a gastrointestinal illness was recorded. The modified Vesikari score of clinical severity for acute gastroenteritis was calculated for each symptomatic episode associated with a new pathogen detection. The score ranges from 0 to 20, with higher scores indicating more severe illness (**Supplementary Tab. 1**) [30].

Laboratory studies

Parents collected stool samples (from soiled diapers) weekly, which were sent by surface mail to the research laboratory where they were processed and stored at -80°C, a median 3 (interquartile range 2 to 4) days after their collection [31]. Samples were later thawed and batch-tested by real-time polymerase chain reaction (PCR) assays [27]. Viruses investigated were generic adenovirus, group-F adenovirus (types 40/41), classic human astrovirus, human bocavirus, enterovirus, norovirus genogroup (G) I, norovirus GII, parechovirus A, wild-type rotavirus, and sapovirus (groups I/II/IV/V combined). Rotavirus wild-type and vaccine strains were differentiated from one another by strain-specific real-time PCR assays [32]. Bacteria comprised *Campylobacter jejuni/coli*, *Clostridioides difficile* (toxins A/B), *Salmonella sp.*, *Shigella*, and *Yersinia*. Protozoa were *Blastocystis sp.*, *Cryptosporidium sp.*, *Dientamoeba fragilis*, and *Giardia intestinalis*. All virus detections with cycle threshold values ≤ 40 were considered positive.

New detection episodes were either the detection of a new pathogen or, if the same pathogen, if the subsequent detection was separated by a refractory period of at least two negative test results or, if intervening samples were not returned, at least 28 days from when the original pathogen was last detected. New pathogen detections were categorized as symptomatic if the positive sample was collected within 7 days before or 7 days after the onset of diarrhea or vomiting.

Saliva samples were collected at the 3-year clinic visit for children who participated in the ELLF extension study. As detailed further in the **Supplementary Methods**, the saliva samples underwent ELISA testing for A, B and H (O) HBGAs and Lewis a and b antigens [33]. The *Ulex Europaeus* (UEA-I) agglutinin was used as an additional confirmatory test of secretor status. To confirm the phenotyping results for secretor status, saliva samples were analyzed for 2 *FUT2* genetic variant (rs6013338 and rs602662) single-nucleotide polymorphisms, which are non-functional if the mutant allele is present in the homozygous state (**Supplementary Tab. 2** and **Supplementary Tab. 3**).

Children were categorized as secretors if one or more of A, B, AB, Lewis b, or UEA-1 were positive by ELISA. Those negative for A and B, with either a Lewis a+/b- or Lewis a-/b- phenotype were labelled non-secretors. Similarly, the presence of Lewis a and/or b antigens identified infants with a Lewis-positive phenotype, and their absence a Lewis-negative phenotype.

Statistical methods

The at-risk (exposure) period was calculated as the period between the first and last submitted stool samples. The exposure period excluded all but the first day of new detection episodes and a refractory period of two or more negative PCR results for that organism or, if samples were not returned, 28 or more days from when the pathogen was last detected. Incidence rates were calculated using Poisson regression with the at-risk period entered as an exposure-time variable and robust variance estimates. Incidence rate ratios and 95% confidence intervals (CIs) comparing secretors and non-secretors were calculated. When incidence rate ratios < 0.67 and > 1.5 were identified they were interpreted as possibly signaling a clinical effect. The modified Vesikari score summary value was reported as a median with inter-quartile range, and the association between secretor status and modified Vesikari score was tested using median regression. A difference in the modified Vesikari scores of at least 3 was considered clinically significant [30]. Healthcare utilization was coded as any or none. To compare the risk of healthcare use during symptomatic detections by secretor status, absolute (risk difference) and relative (relative risk) measures were calculated. When risk difference was $\geq 30\%$ or relative risk was < 0.67 or > 1.5 this too was interpreted as possibly signaling a clinical effect. Two-tailed p-values < 0.05 were considered statistically significant. Stata software (StataCorp, 2019, Stata® Statistical Software: Release 16; College Station, TX: StataCorp LLC) was used.

Results

Of the 158 children returning stool samples in the first 2 years of life during the ORChID study, 86 participated in the ELLF follow-up study and 84 provided saliva. Their characteristics are outlined in **Tab. 1** and are similar to those of the total ORChID cohort. Eighty-two percent (69/84) of children provided stool samples until at least 23 months of age, for a total observation period of 157 child-years. Just over half (56%) the children were introduced to food other than breastmilk by 4 months of age and breastfeeding ceased completely for most (55%) children in this cohort by age 12 months. Most (60%) had attended formal childcare by 18 months of age and almost all (93%) were fully vaccinated against rotavirus by 32 weeks of age (**Tab. 1**).

Tab. 2 shows that non-group F adenovirus had the greatest number of new detection episodes in stool samples (n = 367), followed by enterovirus (n = 316), *D. fragilis* (n = 239), and human bocavirus (n = 225). Of the established gastrointestinal pathogens associated with diarrheal disease, sapovirus was the most common (n = 129), followed by norovirus GII (n = 120), classic human astrovirus (n = 72), and enteric adenovirus 40/41 (n = 56). In this highly vaccinated cohort (**Tab. 1**), there were just 11 wild-type rotavirus infections, involving 8 P[8] and 3 P[4] genotypic strains.

Seventy-seven percent (65/84) of participants were categorized as secretors, while all 19 non-secretors were also homozygous for the 2 *FUT2* inactivating single-nucleotide polymorphisms. Secretors had a substantially higher rate of new detections for norovirus GI and GII, wild-type

Table 1. Characteristics of study participants.

Characteristics [a]		Current study (n = 84)	ORChID cohort (n = 158)
Sex: female		44 (52%)	83 (53%)
Season of birth	**Spring**	23 (27%)	43 (27%)
	Summer	23 (27%)	42 (27%)
	Autumn	16 (19%)	30 (19%)
	Winter	22 (26%)	43 (27%)
Delivery method: vaginal		55 (65%)	107 (68%)
Mother's highest level of education	**High school**	30 (36%)	58 (37%)
	University	54 (64%)	99 (63%)
	Missing data	-	1
Household income [b]	**≥ $115,000**	51 (61%)	86 (55%)
	$67,500-$114,999	28 (34%)	52 (34%)
	< $67,500	4 (5%)	17 (11%)
	Missing data	1	3
Older sibling(s) at home		30 (36%)	55 (35%)
Age at end of breastfeeding (exclusive/any) [c]	**1 month**	37% / 2%	46% / 7%
	3 months	46% / 8%	56% / 17%
	6 months	99% / 21%	99% / 28%
	12 months	100% / 55%	100% / 60%
Age of formal childcare attendance [d]	**6 months**	12%	13%
	12 months	43%	47%
	18 months	60%	64%
	24 months	67%	72%
Rotavirus vaccination [e]		78/84 (93%)	122/136 [f] (90%)
ABO profile	**A-secretor**	27 (32%)	n/a
	B-secretor	3 (4%)	n/a
	AB-secretor	1 (1%)	n/a
	O-secretor	34 (40%)	n/a
	Non-secretor	19 (23%)	n/a
Lewis a/b profile	**a- b-**	5 (6%)	n/a
	a- b+	59 (70%)	n/a
	a+ b-	18 (21%)	n/a
	a+ b+	2 (2%)	n/a
Secretor/Lewis phenotype	**Se+ Le+**	61 (73%)	n/a
	Se+ Le-	4 (5%)	n/a
	Se- Le+	18 (21%)	n/a
	Se- Le-	1 (1%)	n/a
Secretor		65 (77%)	n/a

n/a: not available.
[a] frequencies and percentages shown unless otherwise noted; [b] gross, per year, Australian Dollars, 2012; [c] calculated using life tables; [d] formal childcare was regulated care outside the child's home; [e] n/N (%) shown, fully vaccinated if received 3 doses by < 32 weeks of age of the pentavalent human-bovine reassortant vaccine (RotaTeq; Commonwealth Serum Laboratories/Merck and Co Inc), which was Queensland's publicly funded rotavirus vaccine during the ORChID study and administered in a 3-dose schedule at 6 weeks, 4, and 6 months of age, with the upper age limit for the third dose being 32 weeks; [f] 136 of the initial 158 children remained in the study at the end of the 32nd week of age.

Table 2. Incidence rates per child-year and incidence rate ratios with their corresponding 95% confidence intervals for new pathogen detections by secretor status (Poisson regressions, n = 84).

Pathogen [a]	Freq.	Secretor (n = 65)	Non-secretor (n = 19)	Incidence rate ratio (secretors/non-secretors)
Adenovirus (non-F)	367	3.02 (2.69-3.39)	3.29 (2.65-4.08)	0.92 (0.72-1.17)
Adenovirus (40/41)	56	0.34 (0.25-0.46)	0.48 (0.29-0.78)	0.70 (0.39-1.26)
Enterovirus	316	2.33 (2.05-2.64)	2.69 (2.15-3.37)	0.86 (0.67-1.12)
Human astrovirus	72	0.48 (0.37-0.62)	0.45 (0.27-0.74)	1.08 (0.61-1.91)
Human bocavirus	225	1.53 (1.32-1.78)	1.90 (1.47-2.46)	0.81 (0.60-1.09)
Norovirus GI	23	0.17 (0.11-0.26)	0.09 (0.03-0.27)	1.90 (0.57-6.40)
Norovirus GII	120	0.90 (0.74-1.09)	0.48 (0.29-0.78)	**1.89 (1.12-3.20)** [c]
Parechovirus A	179	1.41 (1.20-1.66)	1.07 (0.76-1.51)	1.32 (0.90-1.92)
Rotavirus (wild-type [b])	11	0.08 (0.04-0.15)	0.03 (0.00-0.21)	2.85 (0.36-22.3)
Sapovirus (I/II/IV/V)	129	0.85 (0.70-1.03)	0.96 (0.67-1.36)	0.88 (0.59-1.33)
Campylobacter sp.	9	0.07 (0.03-0.13)	0.03 (0.00-0.21)	2.28 (0.28-18.2)
Clostridoides difficile	163	1.19 (1.00-1.42)	1.40 (1.04-1.90)	0.85 (0.60-1.21)
Salmonella sp.	8	0.07 (0.03-0.14)	n/c	n/c
Blastocystis hominis	10	0.04 (0.02-0.10)	0.15 (0.06-0.36)	**0.28 (0.08-0.96)** [c]
Cryptosporidium sp.	23	0.15 (0.09-0.24)	0.15 (0.06-0.35)	1.02 (0.38-2.74)
Dientamoeba fragilis	239	1.67 (1.44-1.93)	1.99 (1.55-2.56)	0.84 (0.63-1.12)
Giardia intestinalis	5	0.03 (0.01-0.09)	0.03 (0.00-0.21)	1.10 (0.12-9.88)

Freq.: frequency; n/c: not able to be calculated; sp.: species.
[a] *Shigella* and *Yersinia* excluded as they were not detected in the cohort; [b] wild-type rotavirus P[8] genotype was detected in 73% (8/11) of all wild-type rotavirus detections; [c] statistically significant (at $p < 0.05$).

rotavirus, and *Campylobacter*, although this was only significant for norovirus GII (**Tab. 2**). Similarly, *Salmonella* was detected only in secretors, although the number of new detection episodes (n = 8) was small. In contrast, secretors had a significantly lower rate of new detections of *Blastocystis*.

Most detection episodes were asymptomatic. The incidence rate ratios of new asymptomatic detections comparing secretors with non-secretors were, except for classical human astrovirus, enterovirus, and *Cryptosporidium*, in the same direction as symptomatic detections (**Supplementary Tab. 4**). Secretors were significantly less likely than non-secretors to have symptomatic adenovirus 40/41 infections and their modified Vesikari scores were substantially lower during symptomatic adenovirus 40/41 and human bocavirus detection episodes, with the difference being significant for human bocavirus (**Supplementary Tab. 5**). Healthcare utilization also occurred less frequently for secretors during symptomatic adenovirus 40/41 and *D. fragilis* episodes, but numbers were small, and no differences were statistically significant (**Supplementary Tab. 6**).

Discussion

In our study of 84 children living in a subtropical state capital city in Australia, 77% of children were found to be HBGA secretors on analysis of their saliva samples. This is similar to the proportion of secretors reported in other Caucasian populations (70-80%) [10, 11]. The incidence rate ratios were at least 50% greater in secretors for norovirus GI and GII, wild-type rotavirus, and *Campylobacter* detection, while they were lower for *Blastocystis*. The difference in incidence rates between secretors and non-secretors was significant for norovirus GII and *Blastocystis*, while illness severity and healthcare use with infection were similar.

Our findings of elevated susceptibility in secretors for norovirus, the wild-type rotavirus, and *Campylobacter*, all important causes of

acute diarrheal illness in Australia [34], agree with the previous literature [12, 14, 35]. The elevated susceptibility in secretors followed the same pattern for symptomatic and asymptomatic detections in our study, and is consistent with earlier observations [13, 36]. Similar to reports from Europe, North and Central America, and Asia, where the population is also more likely to be secretor and Lewis-positive, the wild-type P[8] and P[4] rotaviruses predominated in our study [9]. Although there were only 8 *Salmonella* infections, these were confined to secretor children, which was unexpected as non-secretors have been deemed to be more susceptible [8].

Our results showed increased susceptibility in non-secretors for approximately half of the pathogens assessed. Despite their relatively low incidence (10 detections), the higher rates in non-secretors compared to secretors was significant for *Blastocystis*, a suspected intestinal commensal in immunocompetent hosts [37]. Nevertheless, for other shed pathogens, the effect sizes in non-secretors were small and non-significant. These included the recognized gastrointestinal pathogens, group-F adenovirus types 40/41 and sapovirus, as well as pathogens commonly shed in stools, but more associated with respiratory and/or systemic symptoms, such as other adenovirus types and enterovirus, while like *Blastocystis*, *D. fragilis* is still regarded as a bowel commensal rather than a genuine pathogen [37, 38]. In general, our results are consistent with the MAL-ED multi-national birth cohort study from Bangladesh, Peru, and Tanzania, which found an association between HBGA status and infections with norovirus, rotavirus, and *Campylobacter*, but did not identify similar associations with group-F adenovirus, classical human astrovirus, or sapovirus [12].

The positive association between secretors and the risk of norovirus GII infection has been well documented in challenge and outbreak studies [13, 14]. However, it is also important to recognize that resistance to norovirus infections by non-secretors is not absolute. As shown in the current study, non-secretors can still shed noroviruses in their stools and develop symptoms. This is because with the absence of HBGAs and resulting decreased competition for glycosyltransferase, non-secretors have elevated levels of sialylated glycans in their mucosal tissue and secretions, and these can act as receptors for secretor-independent GI and GII norovirus strains [8, 39]. Although the results of the ORChID birth cohort cannot be directly compared with previous evidence as participants of prospective studies could have differing exposure durations, our results align with a 2016 meta-analysis of the association between secretor status and norovirus infections [14]. The ORChID study was novel as it observed asymptomatic detections while most cohort studies to date adopted clinical symptoms as an outcome [13, 23-26]. Asymptomatic shedders may nevertheless play an important role in outbreaks, and should be identified to fully understand their transmissibility and potential for harm within the community [40]. Finally, to help inform disease prevention measures, local community-level evidence is required, which is what studies such as ORChID seek to achieve [13].

The regular sampling applied during the ORChID study is the ideal method for detecting pathogen shedding in stools and capturing any associated symptoms [41, 42]. Our findings benefited from good participant retention and protocol adherence. There are limited data on the role of HBGA expression and infections with a broader spectrum of gastrointestinal pathogens [21]. The inclusion of a wider range of gastrointestinal viruses, bacteria, and protozoa also makes this study unique. We presented illness severity and healthcare use associated with symptomatic norovirus GII detections by secretor status, for which there is limited information, but this could be useful for evaluating health-economic benefits of future norovirus vaccination.

Nevertheless, there are important limitations. These include not typing noroviruses beyond the genogroup level. However, at the time of the ORChID study norovirus G II predominated in the Australian community and this coincided with the rise of the pandemic variant GII.4, Sydney 2012 [43, 44], which is strongly linked with secretor status in children [12, 14, 23, 25, 26]. The results were affected by having a homogenous population with few Lewis-negative individuals and the relatively low incidence of detections for many pathogens, resulting in insufficient power despite moderate sized effects being present. We also did not determine the maternal HBGA status, which in secretor positive women may influence breastmilk and infant gut microbiota composition and also increase susceptibility to gastrointestinal infection in early life [12]. Although exclusive breastfeeding in the study cohort was very common in the first few weeks of life, this virtually

ceased by 6 months of age, and during this period detection of viral gastrointestinal pathogens was uncommon [45]. Finally, symptomatic episodes associated with more than one new pathogen detection were attributed equally to each of these agents.

In conclusion, our study confirmed that secretors in our study population were more likely to have norovirus GII infections, but a novel finding was that they were less likely to have *Blastocystis* detections than non-secretors during the first 2 years of life. Investigation of a range of other viruses, bacteria, and protozoa shed in stools did not provide definitive results. Symptomatic and asymptomatic detections, illness severity, and healthcare utilization were generally similar between secretor and non-secretor children. Our results help strengthen evidence of increased norovirus-susceptibility in young secretor Australian children.

Data availability

To discuss accessing ORChID data please contact the corresponding author.

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Declaration of interest

The Authors declare that there is no conflict of interest.

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Supplementary Methods.

ELISA for ABH (ABO) histo-blood group, Lewis a, and Lewis b antigens

ELISA assays detected histo-blood group antigens (HBGAs) and Lewis antigens in saliva according to published methods with modifications [33].

Saliva samples were collected using eye spears (Defries Industries, Dandenong South, Victoria, Australia). Eye spears were centrifuged for 5 minutes at 3,000 × g. Supernatants were stored at -80°C until batch testing for HBGA. Thawed samples were heated for 5 minutes at 95°C and then diluted 1:250 in 0.05 M Carbonate-Bicarbonate coating buffer pH 9.6 (Sigma, Merck, North Ryde, NSW, Australia). A 100 µL aliquot of the diluted sample was coated onto flat bottom, high binding ELISA plates (Jet Biofil, DKSH, Hallam, Victoria, Australia) and incubated at 37°C for 2 hours before being left at 4°C overnight. The next day, the coated plates were washed 4 times in the wash buffer; 0.01 M of Phosphate buffered saline (NaCl 0.138M, KCl-0.0027M) with 0.05% Tween 20, pH 7.4 (Sigma). The washed plates were then either incubated with primary antibodies or negative control reagents for 90 minutes at room temperature. Primary antibodies used were anti-A, anti-B, anti-AB, anti-Le^b from Diagast (Loos, France; Paragon Care Group Company, Chatswood, NSW, Australia), and anti-Le^a from Seraclone (Biorad Laboratories). Negative control reagents were from Diagast. Primary antibodies were diluted 1:5,000 in SuperBlock T20 (PBS) Blocking buffer (Thermofisher Scientific) for anti-A, anti-B, anti-AB, and anti-Le^b; 1:1,000 for anti-Le^a and the same dilutions of negative control reagents were used as for the corresponding primary antibodies. Plates were then washed 4 times with the wash buffer and incubated with 100 μL of the secondary antibody Goat-anti-mouse (H+L) IgG EIA grade Horse Radish Peroxidase (Biorad) at room temperature for 90 minutes (1:3,000). After 4 washes in wash buffer, color was developed at room temperature for 30 minutes using 100 μL of the chromogenic substrate 3,3'5,5'-Tetramethylbenzidine (TMB) (Thermofisher Scientific). Color development was stopped by adding 100 µL of 2M H_2SO_4 and signals were read at 450 nm with a chromogenic plate reader (FLUOstar Omega, BMG Labtech, Mount Eliza, Victoria, Australia).

UEA-I ELISA assay

The plant lectin UEA-I (Sigma) was used as additional confirmatory test for the secretory status of samples. The UEA-I ELISA aided in distinguishing between secretory Group O and non-secretory, non-Group O cases in a subset of Lewis negative (a-/b-) participants. In addition, UEA-I could also confirm secretory status where participants appeared concurrently positive for anti-A, anti-B, and anti-AB.

One hundred µL aliquots of heated and diluted (1:250) saliva supernatant were coated onto flat bottom, high-binding ELISA plates (Jet Biofil, DKSH) at 37°C for 2 hours, then left overnight at 4°C before being washed the next day as described above in the HBGA and Lewis ELISA assays. PBS-reconstituted Horse Radish Peroxidase conjugated UEA-I (1 mg/mL) was diluted 1:1,000 in Superblock T20 (PBS) Blocking buffer and added to the reaction wells and incubated for 90 minutes at room temperature. After 4 further washes in wash buffer, TMB was added for color development, the reaction was stopped with 100 µL of 2M H_2SO_4 and signals read at 450 nm on a plate reader as described above.

FUT2 genetic variants genotyping

Two fucosyltransferase 2 (*FUT2*) genetic variants with single-nucleotide polymorphisms (rs601338 and rs602662 on chromosome 19) associated with the non-secretor phenotype were investigated in this study [46]. Fifty μL of saliva collected from children underwent nucleic acid extraction using QIAmp DNA Mini kit (Qiagen, Chadstone, VIC, Australia) following the manufacturer's protocol: 'DNA Purification from Blood or Body Fluids'. DNA eluate was subsequently analysed by polymerase chain reaction (PCR) to amplify the *FUT2* genetic region (~423 bp) containing the two polymorphisms. Each 25 μL PCR reaction consisted of 12.5 μl of the 2× QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.3 μM of forward and reverse primers (**Supplementary Tab. 2**) and 5 μl of DNA extract. PCR was performed in a Rotorgene Q instrument (Qiagen) under the following conditions: 15 minutes incubation at 95°C, followed by 45 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. A final step of melt curve analysis was added with a melting profile of 55° to 95°C at 1°C per second. PCR amplicons with a single melt curve peak at 88 to 89°C were submitted to the Australian Genome Research Facility for Sanger sequencing. Sequences from each child were aligned with reference sequences retrieved from dbSNP Short Genetic Variation database (NCBI) using Geneious R10 (Biomatters Ltd., New Zealand) to determine either the wildtype (G) or the mutant allele (A) associated with rs601338 and rs602662 on the *FUT2* gene (**Supplementary Tab. 3**).

Supplementary Table 1. The modified Vesikari score of illness severity used in this study, adapted from Schnadower, et al. [30].

Scoring points	1	2	3
Duration of diarrheal illness (days)	1 to 4	5	≥ 6
Highest daily number of loose stools	1 to 3	4 or 5	≥ 6
Duration of vomiting illness (days)	1	2	≥ 3
Highest daily number of vomiting episodes	1	2 to 4	≥ 5
Max. recorded body temperature [a, b]	37.1-38.4°C	38.5-38.9°C	≥ 39°C
Healthcare use	None	Primary care physician	ED
Treatment	Intravenous rehydration	Hospitalization	-

ED: Emergency Department.
[a] Temperature readings ≤ 37.5°C were not recorded in symptom diaries; [b] missing temperature values were assumed to be in the 37.1 to 38.4°C range for severity scoring purposes.

Supplementary Table 2. Oligonucleotide sequences for *FUT2* genotyping.

Name	Oligonucleotide sequences (5' to 3')
Forward primer	AACTACCACCTGAACGACTGG
Reverse primer	ACACTGTGTGAGTAGAGCAAAATC

Supplementary Table 3. rs601338 and rs602662 polymorphisms and associated amino acid and phenotype changes.

SNP	Wild-type to mutant	AA change	Phenotype association	Mutation type
rs601338	TGG[W] to TAG[stop]	Trp143ter	Non-secretor	Non-sense
rs602662	GGT[G] to AGT[S]	Gly258ser	Non-secretor	Mis-sense

AA: amino acid; SNP: single nucleotide polymorphism.
Table adapted from dbSNP Short Genetic Variations database (NCBI).

Supplementary Table 4. Incidence rates per child-year, incidence rate ratios, and corresponding 95% confidence intervals for new detection episodes of various pathogens by secretor phenotype and symptomatic status in cohort children who returned contemporaneous symptom diaries.

Pathogen [a]		Freq.	IR in secretors (n = 63)	IR in non-secretors (n = 21)	Incidence rate ratio (secretors/non-secretors)
Adenovirus (non-F)	**Symptomatic**	44	0.35 (0.25, 0.49)	0.44 (0.24, 0.79)	0.80 (0.41, 1.59)
	Asymptomatic	303	2.47 (2.17, 2.81)	2.82 (2.23, 3.55)	0.88 (0.67, 1.14)
Adenovirus (40/41)	**Symptomatic**	9	0.03 (0.01, 0.09)	0.15 (0.06, 0.36)	**0.23 (0.06, 0.84)** [b]
	Asymptomatic	43	0.28 (0.20, 0.39)	0.30 (0.16, 0.55)	0.93 (0.46, 1.88)
Enterovirus	**Symptomatic**	51	0.39 (0.29, 0.53)	0.38 (0.21, 0.69)	1.01 (0.52, 1.97)
	Asymptomatic	251	1.81 (1.57, 2.09)	2.27 (1.78, 2.90)	0.80 (0.60, 1.06)
Human astrovirus	**Symptomatic**	14	0.08 (0.05, 0.16)	0.12 (0.04, 0.32)	0.71 (0.22, 2.26)
	Asymptomatic	56	0.38 (0.28, 0.51)	0.33 (0.18, 0.59)	1.16 (0.60, 2.25)
Human bocavirus	**Symptomatic**	27	0.19 (0.12, 0.29)	0.20 (0.09, 0.45)	0.96 (0.39, 2.37)
	Asymptomatic	194	1.31 (1.12, 1.55)	1.67 (1.26, 2.20)	0.79 (0.57, 1.09)
Norovirus GI	**Symptomatic**	5	0.04 (0.02, 0.10)	n/c	n/c
	Asymptomatic	17	0.12 (0.07, 0.20)	0.09 (0.03, 0.27)	1.33 (0.38, 4.63)
Norovirus GII	**Symptomatic**	52	0.40 (0.30, 0.53)	0.18 (0.08, 0.40)	2.23 (0.95, 5.23)
	Asymptomatic	59	0.42 (0.32, 0.56)	0.30 (0.16, 0.55)	1.43 (0.72, 2.82)
Parechovirus A	**Symptomatic**	21	0.16 (0.10, 0.26)	0.13 (0.05, 0.35)	1.27 (0.43, 3.76)
	Asymptomatic	149	1.16 (0.97, 1.39)	0.94 (0.65, 1.35)	1.23 (0.82, 1.85)
Rotavirus (wild-type)	**Symptomatic**	4	0.03 (0.01, 0.09)	n/c	n/c
	Asymptomatic	6	0.04 (0.02, 0.10)	0.03 (0.00, 0.21)	1.42 (0.17, 12.19)
Sapovirus (I/II/IV/V)	**Symptomatic**	37	0.24 (0.17, 0.35)	0.28 (0.14, 0.53)	0.87 (0.41, 1.85)
	Asymptomatic	88	0.57 (0.45, 0.73)	0.68 (0.45, 1.03)	0.84 (0.52, 1.36)
Campylobacter species	**Symptomatic**	0	n/c	n/c	n/c
	Asymptomatic	9	0.07 (0.03, 0.13)	0.03 (0.00, 0.21)	2.28 (0.28, 18.21)
Clostridioides difficile	**Symptomatic**	15	0.10 (0.05, 0.18)	0.17 (0.07, 0.40)	0.59 (0.20, 1.72)
	Asymptomatic	143	1.04 (0.86, 1.26)	1.24 (0.90, 1.71)	0.84 (0.58, 1.23)
Salmonella species	**Symptomatic**	2	0.02 (0.00, 0.07)	n/c	n/c
	Asymptomatic	5	0.04 (0.02, 0.10)	n/c	n/c
Blastocystis hominis	**Symptomatic**	3	0.01 (0.00, 0.06)	0.06 (0.01, 0.24)	0.14 (0.01, 1.53)
	Asymptomatic	7	0.03 (0.01, 0.09)	0.09 (0.03, 0.28)	0.37 (0.08, 1.65)
Cryptosporidium species	**Symptomatic**	6	0.04 (0.02, 0.10)	0.03 (0.00, 0.21)	1.41 (0.17, 12.1)
	Asymptomatic	15	0.09 (0.05, 0.16)	0.12 (0.04, 0.31)	0.78 (0.25, 2.44)
Dientamoeba fragilis	**Symptomatic**	22	0.15 (0.09, 0.24)	0.20 (0.09, 0.44)	0.77 (0.30, 1.96)
	Asymptomatic	209	1.44 (1.23, 1.69)	1.79 (1.38, 2.34)	0.80 (0.59, 1.09)
Giardia intestinalis	**Symptomatic**	0	n/c	n/c	n/c
	Asymptomatic	5	0.03 (0.01, 0.09)	0.03 (0.00, 0.21)	1.10 (0.12, 9.88)

Freq.: frequency; IR: incidence rate per child-year; n/c: cannot be calculated.
[a] *Shigella* and *Yersinia* excluded due to no detections; [b] statistically significant at $p < 0.05$.

Supplementary Table 5. Severity (modified Vesikari score) of new symptomatic pathogen detection episodes by secretor status.

Pathogens [a]	Secretors		Non-secretors		Median difference [b] (95% CI)
	n	median (IQR)	n	median (IQR)	
Adenovirus (non-F)	33	3 (2, 5)	11	4 (3, 7)	-1.0 (-3.1, 1.1)
Adenovirus (40/41)	4	5 (3, 6.5)	5	8 (7, 11)	-4.0 (-9.2, 1.2)
Enterovirus	40	3 (2.5, 5)	11	3 (3, 4)	0.0 (-1.7, 1.7)
Human astrovirus	10	2.5 (2, 6)	4	3.5 (2.5, 4)	0.0 (-3.9, 3.9)
Human bocavirus	21	3 (2, 5)	6	5.5 (3, 11)	**-5.0 (-8.0, -2.0)** [c]
Norovirus GI	5	3 (2, 3)	0	n/c	n/c
Norovirus GII	46	4 (3, 6)	6	3.5 (3, 8)	0.0 (-2.7, 2.7)
Parechovirus A	16	4.5 (3, 5)	4	3.5 (2.5, 6)	0.0 (-3.6, 3.6)
Rotavirus (wild-type)	4	6 (2.5, 9.5)	0	n/c	n/c
Sapovirus (I/II/IV/V)	28	4 (3, 5)	9	3 (3, 3)	1.0 (-0.8, 2.8)
Clostridoides difficile	10	3.5 (2, 6)	5	3 (2, 4)	1.0 (-1.8, 3.8)
Salmonella sp.	2	7.5 (5, 10)	0	n/c	n/c
Blastocystis hominis	1	2 (2, 2)	2	4.5 (2, 7)	n/c
Cryptosporidium sp.	5	4 (4, 6)	1	3 (3, 3)	n/c
Dientamoeba fragilis	16	3 (2, 4.5)	6	3 (3, 3)	0.0 (-2.0, 2.0)

CI: confidence interval; IQR: interquartile range; n: number; n/c: cannot be calculated; sp.: species.
[a] *Shigella*, *Yersinia*, *Campylobacter*, and *Giardia* excluded as there were no symptomatic detection episodes; [b] calculated with quantile (median) regression; [c] statistically significant at $p < 0.05$.

Supplementary Table 6. Risk of healthcare utilization (any type) during new symptomatic pathogen detection episodes by secretor status.

Pathogens [a]	Secretors		Non-secretors		Risk difference (95% CI)	Relative risk (95% CI)
	N	n (%)	N	n (%)		
Adenovirus (non-F)	33	7 (21%)	11	3 (27%)	-0.06 (-0.36, 0.24)	0.78 (0.24, 2.50)
Adenovirus (40/41)	4	1 (25%)	5	3 (60%)	-0.35 (-0.95, 0.25)	0.42 (0.07, 2.63)
Enterovirus	40	8 (20%)	11	3 (27%)	-0.07 (-0.36, 0.22)	0.73 (0.23, 2.31)
Human astrovirus	10	2 (20%)	4	0 (0%)	0.20 (-0.05, 0.45)	n/c
Human bocavirus	21	5 (24%)	6	2 (33%)	-0.10 (-0.51, 0.32)	0.71 (0.18, 2.80)
Norovirus GII	46	15 (33%)	6	2 (33%)	-0.01 (-0.41, 0.39)	0.98 (0.29, 3.27)
Parechovirus A	17	4 (24%)	4	0 (0%)	0.24 (0.03, 0.44)	n/c
Sapovirus (I/II/IV/V)	28	7 (25%)	9	1 (11%)	0.14 (-0.12, 0.40)	2.25 (0.32, 15.9)
Clostridoides difficile	10	4 (40%)	5	1 (20%)	0.20 (-0.26, 0.66)	2.00 (0.30, 13.5)
Blastocystis hominis	1	0 (0%)	2	1 (50%)	-0.50 (-1.19, 0.19)	n/c
Cryptosporidium sp.	5	2 (40%)	1	0 (0%)	0.40 (-0.03, 0.83)	n/c
Dientamoeba fragilis	16	3 (19%)	6	3 (50%)	-0.31 (-0.76, 0.13)	0.38 (0.10, 1.37)

CI: confidence interval; N: number of symptomatic episodes; n: number who sought healthcare; n/c: cannot be calculated; sp: species.
[a] Norovirus GI, wild-type rotavirus, *Campylobacter*, *Salmonella*, *Shigella*, *Yersinia*, and *Giardia* were excluded because of either no or insufficient number of symptomatic detection episodes.