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Approval Sheet

Acquired immunity to enteric viruses in an Indian birth cohort

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Abstract Cover Page

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Abstract

Acquired immunity to enteric viruses in an Indian birth cohort

By Izzy Kates

Diarrheal disease causes widespread morbidity and mortality among children under 5 globally; enteric viruses contribute to this burden. Acquired immunity due to initial infection has been shown to protect against re-infection by some enteric viruses, but the extent and duration of such immunity remains to be fully explored. We obtained a dataset of 373 children and 1,831 diarrheal episodes from an Indian birth cohort and examined natural immunity for astrovirus, sapovirus, norovirus GI and norovirus GII. We fit a Cox Proportional Hazards Model and obtained hazard ratios for virus-specific diarrheal episodes, comparing time to episode among children who had already experienced one virus-specific episode and those who had not yet experienced one. We also analyzed protection against overall diarrheal episodes (not virus-specific) as well as cross-protection between viruses, and lastly fit a frailty model to adjust our results for differential baseline risk. We found that a prior episode did not confer substantial protection against subsequent episodes associated with the same virus. Compared to those without a previous episode of astrovirus, children who had already experienced one had a slightly lower hazard of subsequent episodes of astrovirus (HR=0.83, 95% CI: 0.55, 1.25). Children who had already experienced an episode of norovirus GI had a 65% higher hazard of subsequent episodes involving norovirus GI (HR=1.65, 95% CI: 0.64, 4.25), although this effect was mitigated in the frailty analysis (HR=1.28, 95% CI: 0.53, 3.07). Sapovirus (HR=1.03, 95% CI: 0.77, 1.38) and norovirus GII (HR=1.08, 95% CI: 0.79, 1.48) did not show substantial protection. Children who experienced one diarrheal episode (regardless of pathogen presence) had a 30% higher hazard of subsequent episodes (HR=1.30, 95% CI: 1.10, 1.52); however, this was reduced in the frailty analysis (HR=0.86, 95% CI: 0.73, 1.02). Limited evidence of immunity may result from our episode-centered study design and small sample size. Results indicate host-specific factors that impact an individual's risk may affect acquisition of natural immunity. Further research, including longitudinal seroprevalence and multi-site cohort studies, is needed to disentangle the extent and duration of acquired immunity to enteric viruses.

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Chapter 1: Literature Review

Diarrheal disease

Diarrheal disease affects millions of children around the world, causing widespread illness and mortality and putting a burden on health systems.[1] As of 2016, it is the 8th leading global cause of mortality, causing more than 1.6 million deaths.[2] The number of episodes far exceeds even this number. It can be caused by bacterial, viral, protozoal, and fungal infection, and its effects are often most severe among children under the age of 5.

Common etiologies of diarrheal disease

In 2013, a highly influential study, the Global Enteric Multicenter Study (GEMS), identified the major contributors to diarrheal disease among children in low- and middle-income countries (LMICs), noting that rotavirus, *Cryptospiridium*, Enterotoxin producing *e. coli*, *Shigella*, adenovirus, *Aeromonas*, and *Campylobacter jejuni* are the major pathogens (among others) that cause moderate-to-severe diarrhea among children in these locales.[3] In another study, the Malnutrition and Enteric Disease Study (MAL-ED), birth cohorts from eight countries were followed for 24 months, with fecal samples taken twice-weekly. Attributable fractions were highest for norovirus GII, rotavirus, campylobacter, astrovirus, and cryptosporidium in the first year of life, while the highest disease burden in second year of life was associated with infection by campylobacter, norovirus GII, rotavirus, astrovirus, and Shigella, with substantial heterogeneity across sites.[4]

Burden and etiology of diarrheal disease in India

Much like children in other LMICs, Indian children are affected by diarrheal disease. According to the Institute for Health Metrics and Evaluation, 66 million Indian children between the ages of 1 and 4 suffered from diarrheal disease in 2019, with a mortality rate of 17.9 deaths per 100,000 children.[5] According to the National Family Health Survey, conducted in India between 2015 and 2016, 9% of children under the age of 5 reported experiencing diarrhea in the last two weeks, roughly unchanged from the number ten years prior.[6] Studies seeking to estimate prevalence of different enteropathogens among Indian children have shown a high prevalence of bacterial disease, as well as rotavirus, astrovirus, and adenovirus.[7] The GEMS study determined that norovirus GII was responsible for between 1.3% and 8.1% of all moderate-to-severe diarrheal episodes among Indian children between 12 and 23 months old.[3]

Natural immunity against enteric pathogens

Infection with a pathogen can confer acquired immunity, wherein the host is protected against re-infection with that same pathogen for a period. Mucosal immunity of the gut is not well-understood, but observational studies point to relatively short-lived immunity for some enteric pathogens after natural infection, while it lasts longer for others.[8,9]

Biology of acquired immunity

The gastrointestinal mucosa is the largest organ in the immune system, containing an estimated trillion lymphocytes and a greater concentration of antibodies than any other tissue in the body.[10] Both arms of the adaptive immune system (humoral and cell-mediated) appear to be active in controlling and eliminating enteric pathogens.[11,12] Commensal gut flora have been shown to play a role in enhancing enteric viral

infections through mechanisms including enhanced binding, modulated host immune response, and expanded host cell targets.[13] Exact immune responses vary by pathogen, as does the level to which immune mechanisms have been explored and elucidated. In 10 healthy children followed until 4 years of age, norovirus- and rotavirus-specific IgG antibodies and T cells were detected, with no correlation found between cell-mediated and antibody responses.[12]

Immuno-epidemiology of enteric viruses

Rotavirus

Studies in humans suggest that rotavirus infection confers short-lived immunity against re-infection among children and that protection builds with each subsequent episode. A 2002 study in young children in Guinea-Bisseau found 34% protection against re-infection in a different rotavirus epidemic, in contrast to 66% protection against re-infection in the same epidemic, suggesting either that natural immunity is higher for the latter than the former or that immunity is short-lived.[14] Along similar lines, protection in an Indian birth cohort followed from birth to 3 years of age appeared to be short-lived, as 91% of enrolled children had more than one documented rotavirus infection.[15] By contrast, a 1996 study in Mexican infants found that 52% of 316 documented rotavirus infections were a first infection, there were no instances of moderate-to-severe diarrhea after two infections, and secondary infections were significantly less severe than primary infections.[16] From these studies, a picture emerges of incomplete protection from natural immunity due to rotavirus infection.

Norovirus

Human challenge studies conducted in the 1970s and 1980s suggested that duration of immunity against infection with the same norovirus is between 6 months and 2 years; however, since the viral dose given in these trials was much greater than is necessary to cause infection and disease in a natural setting, and the studies used a norovirus genotype (GI.1) rarely found in wild-type infections anymore, the ability to extrapolate these results to natural infection can be questioned.[17,18] An analysis of the MAL-ED study conducted by Rouhani et al observed acquired immunity among children infected with Norovirus GII, who had a 27% lower hazard of re-infection and a 26% lower hazard of diarrhea upon re-infection. There was a statistically insignificant trend of lower hazard of symptomatic infection with increasing infections.[19] While there have not been many field studies conducted of norovirus immunity, a mathematical model estimated the duration of protective immunity conferred by natural infection to be between 5 and 9 years, depending on differing assumptions and parameterizations.[20] More trials, with longer follow-up, are needed to determine the average duration of protective immunity among children infected with norovirus.

Astrovirus and sapovirus

While rotavirus and norovirus have been the subject of several observational studies and modelling experiments dedicated to determining the duration and extent of protective immunity, these characteristics remain relatively unexplored for sapovirus and astrovirus. However, some research has been done in this area. A study in Peruvian children in an urban community detected sapovirus reinfection in a short time span in 8 children, representing 16 out of 862 diarrheal episodes.[21] Another study in Peru of 100 children randomly selected from a birth cohort found 59 children had

repeated sapovirus infections.[22] While observational studies of astrovirus are rare, animal models and human challenge trials have demonstrated the importance of both humoral and cellular immunity in astrovirus infection, and observational studies have pointed to a high prevalence of anti-astrovirus antibodies in child populations.[23]

Negative control to adjust for confounding by host- and location-specific factors

Using the same MAL-ED data that Rouhani et al analyzed, Rogawski-McQuade et al examined protection against infections by a range of enteropathogens including rotavirus, norovirus, sapovirus, and astrovirus. An important feature of this analysis was the use of exposure to other enteropathogens to control for confounding by common exposures and host-specific risks, for instance to reduce the downward biasing effect on natural immunity of exposure to an infected reservoir. This negative control strategy revealed biased associations for astrovirus- and sapovirus-caused diarrheal episodes (14% and 12%, respectively), but not for norovirus GII, which indicates that the protective effect of primary infection on subsequent infection for the former two are biased toward the null. Adjustments for important covariates and calibration for negative controls increased estimates of protection. A single prior infection reduced hazard of a diarrheal episode associated with reinfection by 38% (95% CI: 48 – 82) and 33% (95% CI: 49 – 91) against astrovirus and norovirus GII respectively; minimal protection was observed for astrovirus. There was a trend of increased reduction in hazard across all three infections as the number of prior infections increased. Two prior infections with astrovirus reduced the hazard of subsequent diarrhea caused by astrovirus by a staggering 48%.[24]

Writing in comment, Lopman and Baker point out some sources of uncertainty and potential issues in the Rogawski-McQuade analysis. Virus-specific attribution is difficult in cases of diarrhea where there are multiple pathogens present in stool, and Rogawski-McQuade‘s approach relies on the assumption that pathogen quantity in stool can proxy viral etiology. The negative control strategy used, while innovative, assumes that the distribution of confounders will be the same for the enteropathogens intended as the causal exposures and those serving as the negative controls. This assumption may not hold in the case of norovirus and rotavirus, for which susceptibility is known to have a strong genetic component. Lastly, Rogawski-McQuade relies on intra-pathogen categorization, which may break down in the case of enteropathogens like norovirus that exhibit diverse genotypes and low levels of cross-immunity.[25] Although advances have been made in understanding natural immunity for certain pathogens, gaps still remain to be filled.

Description of viruses

Our dataset includes test results for four viruses: astrovirus, sapovirus, norovirus G1 and norovirus G2.

Astrovirus

Astroviruses are a single-stranded, positive-sense RNA virus with no envelope. In stool, some particles exhibit a characteristic five- or six-pointed star when viewed through an electron microscope.[26] Clinical features are limited mostly to diarrhea with a median duration of 3 days, with fever, bloody diarrhea, and vomiting being less common symptoms. Lanata et al conducted a systematic review estimating attributable fractions

for gastroenteritis burden in children under 5 years of age, as part of their work with the Child Health Epidemiology Reference Group (CHERG), a WHO- and UNICEF-funded research group in pediatric disease. They estimated that astrovirus caused 15,000 deaths in 2011 (95% CI: 6,000 – 25,000), or 2.1% of global diarrheal mortality.[27] Although national estimates of this virus' burden in India are scarce, one study examining 1,340 cases of acute gastroenteritis (including 1,240 children) in Western India found that 3.1% of specimens tested were positive for astrovirus, ranging geographically from 2.9 – 4%; the highest prevalence was found in children less than 1 year of age.[28] Commonly affecting children, astroviruses have been found in approximately 2.5-9% of pediatric patients hospitalized with diarrhea.[29] A 7-year Chinese study in children with diarrhea under 5 years old that tested negative for both rotavirus and calicivirus detected astrovirus in 5.5% of specimens; over 95% of infections were found in children under 2 years old.[30] The age distribution may vary by setting, as a study in France found a higher rate of infection among children older than 3 years, relative to younger children.[31] No vaccines against these viruses exist, and the different antigenicity of the multiple genotypes has not been fully explored.

Sapovirus

Like astroviruses, sapoviruses are a small, single-stranded, positive-sense RNA virus with no envelope. They are a diverse group of viruses, being divided into five antigenically distinct genogroups based on the molecular structure of their VP1 protein.[32] This is important for the purposes of analyzing protective immunity, as studies in Japan have found evidence of re-infection from different genogroups.[33] The illness, often not severe, is characterized by diarrhea and vomiting, as well as nausea, cramps,

chills, headache, and myalgia. As with astrovirus, the global disease burden of sapovirus remains to be fully estimated, but one systematic review of studies done in LMICs (two-thirds of which studied patients hospitalized for acute gastroenteritis) estimated a prevalence of 6.2% (range: 0.2%, 39%).[34] Indian national estimates are similarly light when it comes to sapovirus burden. A Delhi-based study of children with acute gastroenteritis detected sapovirus in 39% of samples.[35] It affects all ages, but primarily the children and elderly: sapovirus-positives percentages in gastroenteritis patients under 5 years old range from 5.4% in an American study to 12.7% in a U.K. study, and sporadic sapovirus infection has been detected globally.[36,37] It appears sapovirus infection is common in early childhood, as cumulative infections in school-age children can exceed 90%.[38]

Norovirus

Noroviruses (formerly known as Norwalk-like viruses) are another small, single-stranded, positive-sense RNA virus with no envelope. Only 3 genotypes can infect humans: G1, G2, and G4. They are primarily transmitted through the fecal-oral pathway. They have an exceedingly low infectious dose, as just one virus particle has a 50% chance of causing infection[39], and are extraordinarily persistent in the environment, with one challenge experiment finding infectivity of the virus after as much as 61 days in water.[40] In contrast to the other viruses mentioned, global estimates of norovirus burden are more plentiful and rigorous. World Health Organization's Global Estimates of the Burden of Foodborne Disease, released in 2010, showed norovirus to be one of the most common causes of diarrheal disease globally, with nearly 685 million cases (approx. 95% CI: 491 million, 1.1 billion) resulting in 212,000 deaths (approx. 95% CI:

160,600, 278,000).[41] The extent of local surveillance of norovirus prevalence varies geographically, which is a challenge for the development of local and national disease burden estimates (especially in LMICs), but those estimates are increasingly becoming available from both studies done in individual countries and systematic reviews.[42] A systematic review that pooled studies of norovirus prevalence in patients with gastroenteritis globally found that 18% of such patients tested positive for norovirus by PCR.[43] As is the case with astrovirus and sapovirus, national estimates of norovirus' burden are lacking in India; however, Rouhani et al's analysis of data from the global MAL-ED study found an incidence of 5.8% (95% CI: 4.1, 8.3) for norovirus GI and an incidence of 10.2% (95% CI: 7.8, 13.2) for norovirus GII in Vellore, India.[19]

Public health significance of proposed study

Astrovirus, sapovirus, and norovirus are major drivers of episodes of acute gastroenteritis among children.[22,29,44] Diarrheal deaths among children aged 0-6 years in India have been estimated to exceed 150,000 annually.[45] As coverage of the rotavirus vaccine increases in Indian birth cohorts[46], diarrheal episodes caused by other gut viruses will make up an increasing proportion of total episodes. This shifting burden of diarrheal illness, and the increasing rates of rotavirus vaccination around the world, means a greater role will be played in global morbidity and mortality by norovirus, astrovirus, and sapovirus. Additionally, the immune protection provoked by live vaccines to enteric pathogens (e.g. polio, rotavirus) is, for unknown reasons, often inferior in LMICS than in developing countries.[47] This fact serves as a reminder of how much remains to be learned about acquired immunity to enteric viruses and how such information can help inform future vaccine research. As such, it is important to explore

epidemiological questions around these viruses, such as the role of acquired immunity in host susceptibility to infection and disease and the immune mechanisms and public health impact of vaccination.

Description of analysis and dataset

In this study, I examine natural immunity conferred upon infection with astrovirus, sapovirus, and norovirus. Using survival analysis, I explore time to re-infection after initial infection and estimate hazard ratios. The analytic dataset (provided by partners at Christian Medical College in Vellore, India) includes 373 children aged 0-3 years, from low-income housing in Vellore. Out of 452 infants recruited at birth between March 2002 and August 2003, these children completed a three year follow-up period, with home visits twice a week and surveillance samples taken every two weeks. Stool samples were collected every two weeks and every other day during diarrheal episodes. Children were followed from 2002 through 2006. Diarrhea was defined as three or more watery stools in a 24 hour period. 48 hours of normal bowel movements post-episode marked two separate episodes.

Chapter 2: Manuscript

Introduction

Diarrheal disease affects millions of children around the world, causing widespread illness and mortality and putting a burden on health systems.[1] Diarrheal disease is a major public health concern, particularly in low and middle income countries.[48] In some settings, higher incidence of diarrhea is associated with young age, low socioeconomic status, low birth weight, inadequate breastfeeding, poor sanitation and hygiene, and poor maternal literacy, as well as significant household and societal financial costs.[49,50] The gastrointestinal pathogens causing the highest morbidity and mortality burden globally include rotavirus, *Shigella, Cryptosporidium*, *Shiga toxin-producing E. coli*, *Aeromonas hydrophila*, *Campylobacter jejuni,* rotavirus, norovirus GII, and *Vibrio cholerae*.[3]

In India, more than 77,000 children died from diarrheal disease in 2015.[51] Etiologic studies among Indian children have shown a high prevalence of bacterial disease, as well as rotavirus, astrovirus, and adenovirus.[7] Additional pathogens such as norovirus are a major cause of viral gastroenteritis, due to their low infectious dose and high rates of viral shedding.[52] Astrovirus is also known to be an important cause of acute diarrhea in Indian children, having been detected in ~5% of diarrheal samples.[53] As coverage of the newly introduced rotavirus vaccine increases in Indian birth cohorts[46], diarrheal episodes caused by other enteric viruses will make up an increasing proportion of total episodes. This shifting burden of diarrheal illness, and the increasing rates of rotavirus vaccination around the world, means norovirus, astrovirus, and sapovirus could play an increasing role in the burden of diarrheal disease in India and other settings.

Given the burden associated with diarrheal disease, the subject of acquired immunity has been explored as a means of understanding different GI pathogens, analyzing epidemiologic trends, and predicting future burden of disease. Rotavirus and norovirus are the diarrheal diseases for which acquired immunity has been most thoroughly explored. For both of these viruses, studies using observational, experimental, and modelling data suggest that infection confers virus-specific immunity at least temporarily, though observations vary by setting and study type. The extent and duration of rotavirus immunity can vary based on the population and setting in which it is being studied.[14,15] Modeling of norovirus immunity suggests a longer duration of protection than had been suggested by observations from both cohort studies and human challenge experiments conducted in the 1970s and 1980s.[17–20] Other diarrheal diseases, like astrovirus and sapovirus, have been studied infrequently and in limited settings.[21–24]

As the relative distributions of diarrheal diseases shifts, it is important to explore epidemiological questions around these viruses, including the role of acquired immunity in potentially preventing future events. Here we test whether diarrheal illness caused by astrovirus, sapovirus, norovirus GI and norovirus GII confers protection against subsequent episodes involving those same viruses in a cohort of Indian children. Secondarily, we test 1) whether overall episodes of diarrheal illness (with or without a pathogen detected) confer protection against subsequent diarrheal episodes and 2) whether episodes associated with one virus confer protection against subsequent episodes with a different virus.

Methods

For this analysis, we obtained data on a community-based cohort of 373 children from urban slums in Vellore, India. Detailed descriptions of the cohort and follow-up methods are provided by Gladstone et al.[15] Out of 452 infants recruited at birth between March 2002 and August 2003, 373 children completed a three year follow-up period, with home visits twice a week and surveillance stool samples taken. An episode of diarrhea was defined as three or more watery stools in a 24-hour period or, in breast-fed children, an increased number of daily stools considered to be diarrhea by the mother, and additional stool samples were taken during each diarrheal episode. Our dataset included each episode of diarrhea recorded, with a unique subject identifier, the age of the subject (in days), and the results (in cycle threshold (Ct) values) of molecular testing of diarrheal specimens for astrovirus, sapovirus, norovirus GI, and norovirus GII. Ct values of 40 or below were considered positive for a given pathogen.

A Cox Proportional Hazards model was fit with the Andersen-Gill extension, which can model recurrent events where age is an important underlying contributor to disease prevalence.[54,55] Generalized estimating equations with clustered standard errors were used to estimate hazard ratios (HRs) for each virus-specific model separately, comparing time-to-diarrheal-episode among those with 1 pathogen-specific episode of diarrhea compared to those without a previous episode. The predictor variable was previous occurrence of virus-specific diarrhea (dichotomous), while the outcome variable was time until a subsequent virus-specific diarrheal episode. For more on the model, including rationale and implementation details, see Appendix A.

We also conducted several supplementary analyses. We assessed whether having a previous diarrheal episode (from any cause) impacted the hazard of subsequent episodes as well as the effect of diarrheal illness with one virus on the hazard of subsequent illness with a different virus. In conducting these analyses, we aimed to understand the extent of intra-subject episodic correlation and underlying risk, as well as to explore the hazards associated with prior episodes and prior episodes associated with different viruses. We also restricted our dataset and refit our models to examine a) episodes occurring before 1 year of age, b) episodes occurring before two years of age, and c) episodes for which recorded Ct values were 35 or lower. Lastly, in order to further adjust our estimates for intra-subject correlation, we fit a frailty model, which incorporates random effects into the model to induce dependence in recurrent event times and account for heterogeneity not accounted for by observed covariates.[54]

This project did not require Emory IRB review because it did not meet the definition of “human subjects research”. All data were deidentified and no demographic information beyond age in days at time of diarrheal episode was accessible.

Results

346 out of 373 (92.8%) children had at least one diarrheal episode recorded over the course of follow-up, while 142 (38.1%) had at least 5 diarrheal episodes recorded (Table 1). Among the 1,823 diarrhea stool samples collected, the most commonly detected pathogen was sapovirus (n=257, 14.1%), followed by norovirus GII (n=227, 12.5%), astrovirus (n=178, 9.8%) and lastly norovirus GI (n=57, 3.1%). Half (49.9%) of children had at least one episode of sapovirus-associated diarrhea. Norovirus GII and

astrovirus associated diarrhea episodes were also common with 44.8% (n=167) and 39.4% (n=147) of children having at least one episode of norovirus GII and astrovirus, respectively. A small portion of children had multiple episodes caused by the same pathogen with up to 3 (astrovirus, norovirus GI) or 4 (sapovirus, norovirus GII) pathogen-specific episodes total. The age distribution of episodes was right skewed, with nearly half (45.0%) of episodes occurring between 0 and 11 months and half (55.0%) occurring between 12 and 36 months. No meaningful differences by episode number were found between Ct values.

Compared to those without a previous astrovirus-related diarrheal episode, children who had already experienced an astrovirus-related diarrheal episode had a slightly lower hazard of subsequent episodes (HR = 0.83, 95% CI: 0.55, 1.25), while no marked difference was observed between the two groups for sapovirus and norovirus GII (Table 2). Children who had already experienced an episode of norovirus GI had a 65% higher hazard of subsequent episodes involving the same virus, although the small size of this group does not allow for precise parameter estimates (HR = 1.65, 95% CI: 0.64, 4.25). Sensitivity analyses (Appendix Table 1) restricting the dataset to children 1 year of age and below and to children 2 years of age and below found no marked differences in hazards for astrovirus, sapovirus, and norovirus GII, but for norovirus GI the HR indicated an increased hazard of subsequent norovirus GI episodes among children with a previous episode (for 1 year of age and below: HR = 3.13, 95% CI: 1.17, 8.39; for 2 years of age and below: HR = 2.55, 95% CI: 1.08, 6.01). Restricting virus positives in the main dataset (Table 2) to those with Ct values of 35 or lower resulted in higher hazards of reinfection for each virus but none substantially so. When frailty

models were used for these analyses, the hazard of norovirus GI-associated diarrheal recurrence was reduced (HR = 1.28, 95% CI: 0.53, 3.07).

Supplementary analyses of diarrheal episodes and cross-virus protection are shown in Appendix Tables 1 and 2. Children who experienced one diarrheal episode had a higher hazard of experiencing another (HR = 1.30, 95% CI: 1.10, 1.52), and each subsequent diarrheal episode further raised the hazard (Appendix Table 1). After five episodes, the hazard of having another episode was 52% higher compared to children who had not had a previous infection (HR = 1.52, 95% CI: 1.25, 1.86). Compared to children who had not had a previous diarrheal episode related to norovirus GII, children who had experienced a norovirus GII-related episode had a 26% higher hazard for subsequent diarrheal episodes involving sapovirus (HR = 1.26, 95% CI: 0.97, 1.64), and a 52% higher hazard for subsequent diarrheal episodes involving Norovirus GI (HR = 1.52, 95% CI: 0.85, 2.70) (Appendix Table 2). The higher hazard for subsequent diarrheal episodes (without considering virus) virtually disappears when using a frailty model (HR at five episodes = 0.97, 95% CI: 0.81, 1.16). Of note, fitting the frailty model to the data for diarrheal episodes occurring in only the first year of life resulted in a drastically reduced hazard for norovirus GII-associated diarrheal recurrence (HR = 0.27, 95% CI: 0.16, 0.46).

Discussion

Here we analyzed the hazard of recurrent diarrheal episodes connected with astrovirus, sapovirus, and norovirus GI and GII gastroenteritis in a dataset of 1,831 diarrheal episodes among 373 children from an Indian birth cohort. We found that having a

previous virus-specific episode of diarrhea did not provide substantial protection against subsequent episodes from the same pathogen. Children who experienced an astrovirus-related diarrheal episode were slightly less likely to experience another astrovirus-related diarrheal episode than were children without such a previous episode. Our analysis of all diarrheal episodes (regardless of pathogen presence) indicated that the higher hazards for reoccurrence of diarrhea increased with each subsequent episode; however, HRs were greatly reduced when computed with the frailty model.

Research into immunity from enteric viruses has produced varying results in different populations. Rogawski-McQuade et al analyzed cross-country birth cohort data from the global MAL-ED study, using a more sensitive negative control method to calibrate for intra-subject correlations and differential baseline risk, and found a 33% lower hazard for norovirus GII re-infection, and 38% lower hazard for astrovirus re-infection.[24] Conversely, limited studies have shown a high incidence of sapovirus reinfection in cross-sectional and cohort samples of Peruvian youth, although these may have not been able to adequately adjust for within-subject correlation due to inadequate sample sizes.[21,22] The live oral rotavirus vaccine provides yet another example of differential immunity by population providing strong protection against rotavirus gastroenteritis among children in low burden settings and substantially poorer protection among children in high burden settings.[56]

Our finding that a previous virus-specific diarrheal episode does not provide substantial protection against subsequent virus-specific diarrheal episode further demonstrates the complexity and heterogeneity of pediatric enteric immunity and epidemiology. Studies of immunity to enteric pathogens in different settings and using

different designs have produced different results; for instance, a re-analysis of rotavirus studies done in India and Mexico found that different levels of baseline susceptibility in the two cohorts (due to child- and location-specific factors) contributed to differences in the final estimates of natural immunity produced by those studies.[57] The problem of differential baseline risk, and the related issue of intra-subject correlation, are common challenges in recurrent events analysis of diarrheal disease.[54] Essentially, individuals may have heterogenous risk depending on factors specific to them or to their setting, which will affect estimates of survival. For instance, children living near a contaminated water source may have a higher baseline risk of infection, which might spuriously increase the estimated hazard.

The importance of fully adjusting for differential baseline risk and intra-subject correlation when analyzing diarrheal recurrence is underlined by our findings of increased vulnerability to re-infection by norovirus GI, and substantially increased hazard for recurrence of diarrheal episodes (regardless of pathogen presence) that is then greatly reduced by fitting the frailty model. Our sample size limited our ability to more fully resolve this complication using the negative control method employed by Rogawski-McQuade[24], as very few individuals tested positive for the same virus more than twice. We used clustered standard errors and frailty modeling to compute our parameter estimates, mitigating the problem somewhat; despite these adjustments, however, additional within-subject correlation could remain. Our findings are therefore consistent with previous studies indicating the importance of within-subject correlation and differential baseline risk in recurrent enteric viral infections.[24,54]

This study has limitations and assumptions that may affect interpretation of its results. These include the episode-based study design, relatively small sample size, and the unavailability of important demographic data. Data were limited to positive tests taken upon report of diarrheal illness. Infection (not disease) is what causes immunity, and infections outnumber disease for all the analyzed viruses, for several to a great extent[58–60]; not having data on infections thus limits the number of events in our dataset and, more importantly, prevents us from directly analyzing enteric immunity. Longitudinal studies combining measurements of seroprevalence and incidence in high-risk populations could provide the high-resolution data needed to explore the question of acquired immunity from infection, instead of needing to speak only in terms of diarrheal episodes. Our data, although they represent over 1,000 episodes of diarrhea across hundreds of children, lacks enough recurrent episodes for each virus to draw meaningful inferences. For instance, only 4 children experienced a second episode associated with norovirus GI. Additional, larger birth cohort studies would increase the number of events available for analysis, improving precision in parameter estimates and providing ample space to apply novel methods such as the negative control method. Diarrheal episodes were not accompanied by severity data, which decreases the resolution with which we are able to analyze levels of protection against disease. Lastly, we did not have access to data the study investigators compiled on sex, specific location, or socio-economic status, so we could not determine the effects of these and other demographic factors on acquired immunity from enteric viruses.

As diarrheal disease remains a common global contributor to morbidity and mortality, it is important to fully explore factors that impact prevalence, including host

susceptibility. Acquired immunity to viruses is often an important predictor of infection- and disease-related outcomes, on both an individual and community level; elucidation of the extent of acquired immunity in enteric viruses therefore furthers our understanding of enteric disease transmission, illuminates routes of inquiry worth further exploring in study design, prevalence, and disease mechanisms, and enables the development of more effective interventions for reducing the burden of disease. This study adds to the extant literature on acquired immunity to enteric viruses, finding little effect of hazard reduction in already-infected children and reinforcing the importance of adjusting for differential baseline risk when analyzing diarrheal recurrence. Future studies should enroll larger numbers of participants, conduct repeated seroprevalence surveys, and/or collect frequent asymptomatic samples to adjust for differential baseline risk and better understand the role of acquired immunity in preventing infection.

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Table 1. Characteristics of study participants (N = 373) and episodes of diarrheal illness (N = 1,823)					
	All Episodes (N = 1,823)	Astrovirus-associated episodes (N = 178)	Sapovirus-associated episodes (N = 257)	Norovirus GI-associated episodes (N = 57)	Norovirus GII-associated episodes (N = 227)
Patients with at least one episode, n (% of all individuals)	346 (92.8)	147 (39.4)	186 (49.9)	51 (13.7)	167 (44.8)
Number of episodes per individual, n (% of all individuals)					
0	27 (7.2)	226 (60.6)	188 (50.4)	322 (86.3)	206 (55.2)
1	49 (13.1)	120 (32.2)	131 (35.1)	46 (12.3)	118 (31.6)
2	41 (11.0)	23 (6.2)	40 (10.7)	4 (1.1)	40 (10.7)
3	66 (17.7)	4 (1.1)	14 (3.8)	1 (0.3)	7 (1.9)
4	48 (12.9)	--	1 (0.3)	--	2 (0.5)
5	50 (13.4)	--	--	--	--
6	40 (10.7)	--	--	--	--
7	20 (5.4)	--	--	--	--
8	12 (3.2)	--	--	--	--
9	11 (2.9)	--	--	--	--
>10	9 (2.4)	--	--	--	--
Number of diarrheal episodes, mean (SD)	4.2 (2.4)	1.21 (0.47)	1.38 (0.65)	1.12 (0.38)	1.36 (0.62)
CT for all positive diagnoses, median (IQR)	30.4 (24.4, 35.2)	34.1 (25.3, 36.9)	30.2 (24.1, 36.2)	28 (24.2, 32.7)	28.5 (24.4, 33.9)
CT for positive diagnosis, by number episode, median (IQR)					
1	--	34.5 (26.6, 37.1)	30.0 (23.9, 36.1)	27.9 (22.2, 32.1)	28.9 (24.5, 34.1)
2 or more	--	33.4 (24.1, 35.0)	30.6 (26.2, 36.4)	33.9 (29.4, 34.5)	27.3 (24.3, 31.8)
Number of diarrheal episodes, by age at time of episode, n (%)					
0 – 5 m	411 (22.5)	51 (28.6)	53 (20.6)	16 (28.1)	60 (26.4)
6 – 11 m	410 (22.5)	52 (29.2)	76 (29.6)	15 (26.3)	59 (26.0)
12 – 23 m	662 (36.3)	32 (18.0)	53 (20.6)	10 (17.5)	28 (12.3)
24 – 36 m	340 (18.7)	43 (24.2)	75 (29.2)	16 (28.1)	80 (35.3)
All ages	1,823 (100.0)	178 (100.0)	257 (100.0)	57 (100.0)	227 (100.0)
Median age of episode, days (IQR)					
Episode 1	97.5 (46, 200)	324 (154, 628)	314 (170, 591)	308 (166, 620)	251 (136, 407)
Episode 2	223 (138, 356)	388 (196, 644)	518 (326, 736)	315 (180, 320)	426 (308, 617)
Episode 3	352.5 (230.8, 639.3)	435 (337, 628)	616 (350, 854)	477 (NA)	458 (359, 556)
Episode 4	451.5 (313.75, 701.25)	--	462 (NA)	--	464 (418, 510)
Episode 5	615 (428.5, 764.8)	--	--	--	--
Episode 6	740 (528.8, 837)	--	--	--	--
Episode 7	804.5 (647.8, 910)	--	--	--	--
Episode 8	793.5 (660.5, 938.3)	--	--	--	--
Episode 9	807.5 (704.5, 1002.8)	--	--	--	--
Episode 10 or greater	897 (826.5, 962)	--	--	--	--

Fig. 1: cumulative probability of infection, by episode number, for astrovirus, sapovirus, norovirus GI, and norovirus GII

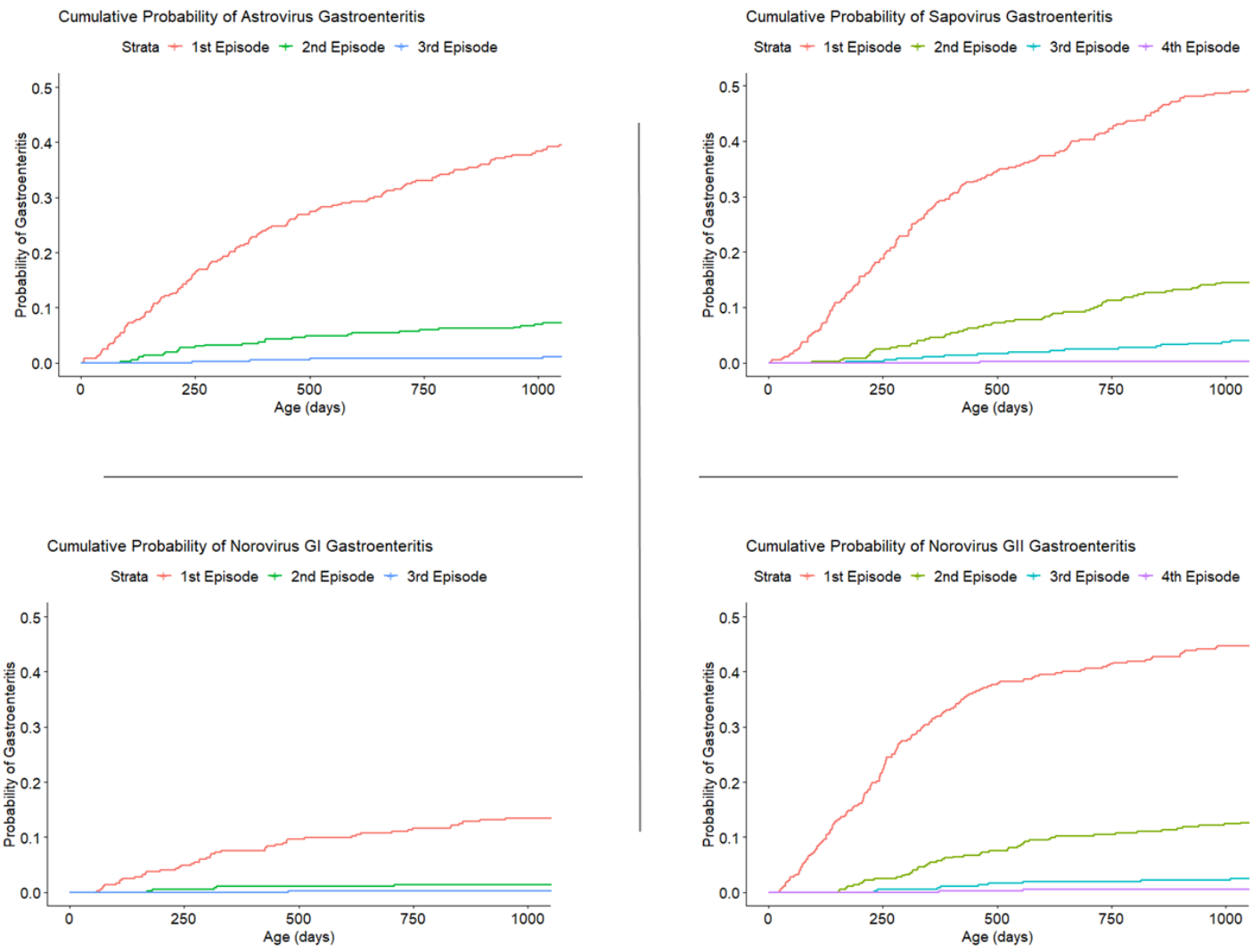


Table 2. Hazard of diarrheal episode associated with virus-specific reinfection in Indian children

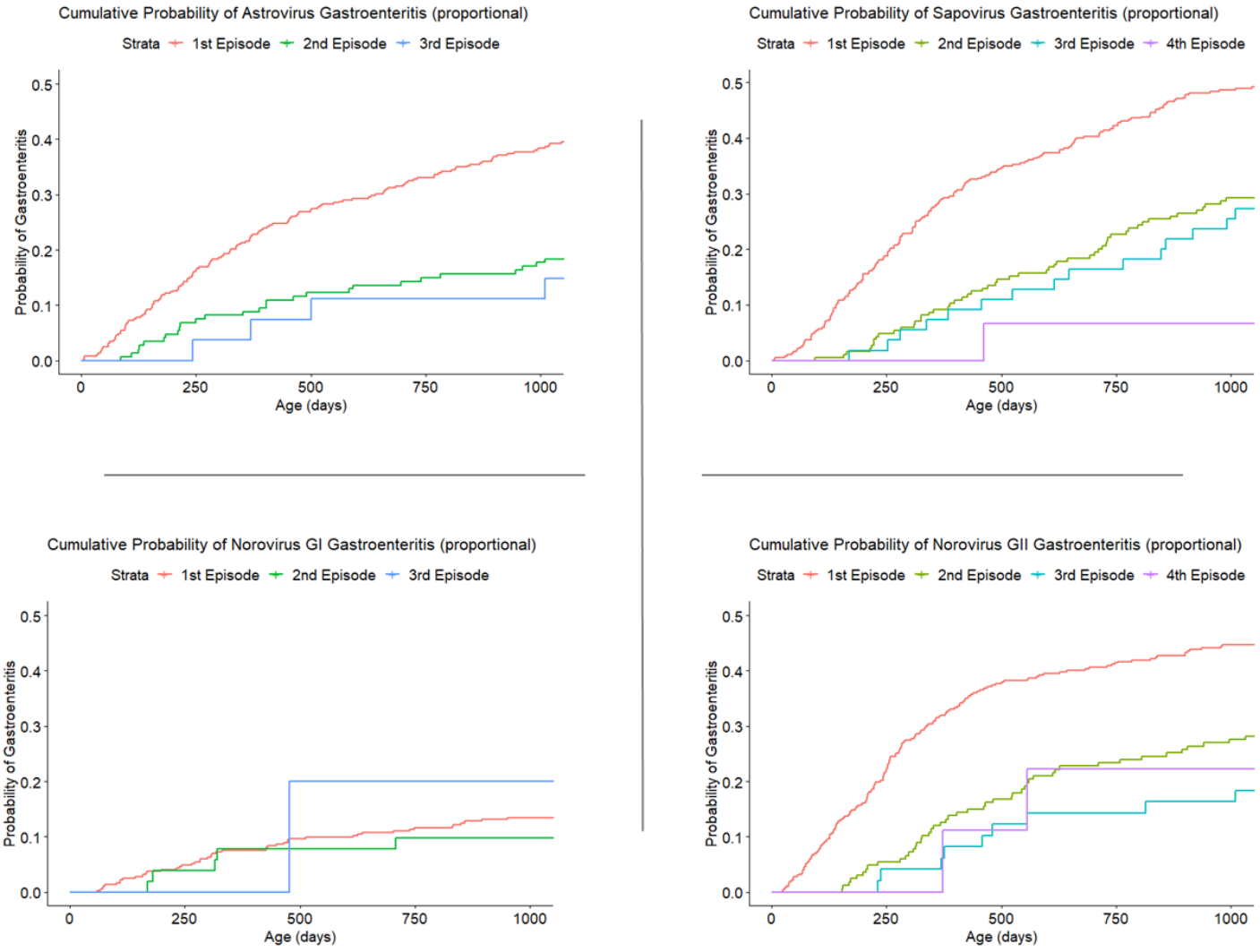
Andersen-Gill Model (Primary Analysis)			**Frailty Model**	
Results: all ages included				
	Hazard Ratio	**95% CI**	**Hazard Ratio**	**95% CI**
Astrovirus	0.83	(0.55, 1.25)	0.83	(0.55, 1.24)
Sapovirus	1.03	(0.77, 1.38)	1.03	(0.77, 1.38)
Norovirus GI	1.65	(0.64, 4.25)	1.28	(0.53, 3.07)
Norovirus GII	1.08	(0.79, 1.48)	1.08	(0.79, 1.47)
Sensitivity Analysis – Restricted to 1 year of age and below				
	Hazard Ratio	**95% CI**	**Hazard Ratio**	**95% CI**
Astrovirus	1.16	(0.66, 2.02)	1.16	(0.64, 2.08)
Sapovirus	1.00	(0.58, 1.71)	0.68	(0.40, 1.15)
Norovirus GI	3.13	(1.17, 8.39)	3.13	(1.08, 9.10)
Norovirus GII	0.77	(0.49, 1.21)	0.27	(0.16, 0.46)
Sensitivity Analysis – Restricted to 2 years of age and below				
	Hazard Ratio	**95% CI**	**Hazard Ratio**	**95% CI**
Astrovirus	1.01	(0.63, 1.63)	1.01	(0.65, 1.59)
Sapovirus	0.93	(0.65, 1.32)	0.93	(0.66, 1.30)
Norovirus GI	2.55	(1.08, 6.01)	2.54	(1.07, 6.09)
Norovirus GII	0.97	(0.68, 1.37)	0.82	(0.58, 1.17)
Sensitivity Analysis – Restricted to Ct < 35				
	Hazard Ratio	**95% CI**	**Hazard Ratio**	**95% CI**
Astrovirus	1.09	(0.66, 1.80)	1.09	(0.62, 1.90)
Sapovirus	1.08	(0.74, 1.57)	1.08	(0.74, 1.58)
Norovirus GI	1.73	(0.67, 4.47)	1.40	(0.58, 3.36)
Norovirus GII	1.21	(0.84, 1.73)	1.20	(0.85, 1.71)

Appendix Tables and Figures

Appendix Table 1. Hazard of further diarrheal episodes (regardless of pathogen presence), by episode number				
	Andersen-Gill Model		**Frailty Model**	
	Hazard Ratio	**95% CI**	**Hazard Ratio**	**95% CI**
1 episode	1.30	(1.10, 1.52)	0.86	(0.73, 1.02)
2 episodes	1.49	(1.30, 1.72)	1.14	(0.99, 1.32)
3 episodes	1.50	(1.30, 1.73)	1.02	(0.89, 1.19)
4 episodes	1.52	(1.30, 1.79)	1.01	(0.86, 1.18)
5 episodes	1.52	(1.25, 1.86)	0.97	(0.81, 1.16)

Appendix Table 2. Hazard of diarrheal episode associated with infection from a different virus, by etiology of prior diagnosis				
	Andersen-Gill Model		**Frailty Model**	
	Hazard Ratio	**95% CI**	**Hazard Ratio**	**95% CI**
Astrovirus, followed by:				
Sapovirus	0.95	(0.70, 1.30)	0.95	(0.70, 1.30)
Norovirus GI	1.04	(0.54, 2.00)	1.04	(0.54, 2.00)
Norovirus GII	0.98	(0.69, 1.38)	0.98	(0.69, 1.38)
Sapovirus, followed by:				
Astrovirus	1.24	(0.89, 1.72)	1.24	(0.89, 1.72)
Norovirus GI	1.06	(0.56, 1.98)	1.06	(0.56, 1.98)
Norovirus GII	0.95	(0.68, 1.33)	0.95	(0.68, 1.33)
Norovirus GI, followed by:				
Astrovirus	1.24	(0.89, 1.72)	1.24	(0.87, 1.76)
Sapovirus	1.03	(0.76, 1.38)	1.03	(0.77, 1.38)
Norovirus GII	0.95	(0.68, 1.33)	0.95	(0.68, 1.32)
Norovirus GII, followed by:				
Astrovirus	1.14	(0.82, 1.59)	1.14	(0.82, 1.59)
Sapovirus	1.26	(0.97, 1.64)	1.26	(0.97, 1.64)
Norovirus GI	1.52	(0.85, 2.70)	1.52	(0.85, 2.70)

Appendix Fig. 1: cumulative probability of infection (displayed proportionally), by episode number, for astrovirus, sapovirus, norovirus GI, and norovirus GII



Appendix Fig. 2 cumulative probability of infection (displayed proportionally as function of time difference between episodes), by episode number, for astrovirus, sapovirus, norovirus GI, and norovirus GII