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Norovirus-associated diarrhea and asymptomatic infection in children aged under 4 years: a community-cohort study in the Philippines

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ABSTRACT

Objectives: This study aimed to estimate the incidence of norovirus (NoV)-associated diarrhea and asymptomatic infections in children under 4 years of age and identify the genotypes of multiple NoV infections.
Methods: A community-based cohort study was conducted in Tarlac, Philippines. Children aged 0-2 years were followed up for 2 years. The prevalence and incidence rates of NoV-associated diarrhea and asymptomatic infections were calculated. Risk factors were assessed using the Cox proportional hazards model. The genotypes and immunotypes of repeated infections were tabulated.
Results: A total of 338 children aged 6208 child-months were analyzed. NoV was detected in 17.4% (84 of 527, 95% confidence interval [CI]: 12.7-19.7%) of diarrheal episodes and 10.8% (219 of 2031, 95% CI: 9.4-12.3%) of asymptomatic stool samples. The highest incidence of NoV-associated diarrhea occurred in children aged 6-11 months (2.31 per 100 child-months, 95% CI: 1.30-3.32) and 18-23 months (2.34 per 100 child-months, 95% CI: 1.57-3.12), whereas the highest incidence of asymptomatic NoV infection was observed in children aged 12-23 months (4.49 per 100 child-months, 95% CI: 3.41-5.56). Repeated NoV infections were detected between different genotypes, except in two children who had repeated NoV GI.3 and two children with GI.9 infections.
Conclusions: Children had the highest risk of NoV-associated diarrhea during their first year of life, whereas asymptomatic NoV infections persisted after the second year. Repeated NoV infections suggest genotype-specific immunity after NoV infection.

Introduction

Norovirus (NoV) is a major cause of viral gastroenteritis worldwide [1], with 30-70% of children under 2 years of age experiencing NoV-associated diarrhea [2]. An estimated 50,000 child deaths occur annually [3], mostly in low- and middle-income countries.

NoV exhibits high genetic diversity, with 10 genogroups (GI-GX) and over 49 genotypes identified [4]. The most common genogroups in human is GII, followed by GI [5], with repeated infections by multiple genotypes observed in the same child [6]. However, repeated infections of the same genotype are rare, except for GII.4 variants [4,6]. In a hospital-based cohort study in Chile, repeated infections in children were often asymptomatic [7], and a birth cohort study in Peru showed some protection from previous NoV infection [6]. Furthermore, immunotypes were proposed classifying 28 NoV genotypes of GI, GII, and GIX into 12 types based on the cluster and pairwise distance of the NoV strains supported by six studies and natural history patterns, hypothesized to function as “immunotypes” that influence vaccine antigen selection [8]. These findings suggest partial cross-immunity between genotypes with accumulated immunity from multiple infections providing protection against symptomatic infections.

Previous studies indicate that NoV infection rates vary with age and are influenced by maternal immunity, breastfeeding, weaning, and previous infection [2]. The highest incidence of NoV-associated diarrhea occurred in the first year of life and decreased in the second year [2,9]. Asymptomatic NoV infections are common in children [10,11]. A multicounty birth cohort study, The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Study (MAL-ED) found a 19.0% prevalence of asymptomatic NoV infections in children aged 0-24 months [12]. De-

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spite multiple cohort studies over the past decade, few have investigated NoV infection incidence in children over 2 years old. Most studies focused on children up to 2 years of age [6,7,13], with only two studies in India and Nicaragua including older children; however, neither assessed asymptomatic infection over 2 years of age [14,15]. Asymptomatic infections in young children may be crucial to household and community NoV transmission. To build on these findings, we aimed to investigate the prevalence and incidence of asymptomatic NoV infection in children aged 0-4 years. We also analyzed genotypes of multiple NoV infections. To the best of our knowledge, no cohort studies on NoV infections have been conducted in Southeast Asia. In addition, we investigated the risk factors for NoV-associated diarrhea. Understanding the patterns of natural infections and acquired immunity is essential for ongoing vaccine development.

Methods

Study design

A community-based cohort study was conducted in two communities in Tarlac province, located approximately 150 km north of Metro Manila, Philippines, from July 2014 to June 2019: La Paz (Community A) and San Nicolas (Community B). Community A is in the rural area surrounded by rice fields, whereas Community B is an urban setting in Tarlac City. Despite their contrasting environments, both communities have access to primary health care units and community hospitals. Drinking water is sourced from owned or shared tap water or commercially supplied water, and approximately 80% have a latrine.

This study includes children under 2 years of age whose parents provided written informed consent and followed up for 2 years. Children with severe or chronic condition were excluded (Supplementary Method). Children were randomly recruited by block, which is the geographic unit of the community, ensuring an equal proportion from the list provided by the community. Field workers visited the household daily to monitor diarrhea-associated symptoms and breastfeeding status. (Supplementary Material). Nutritional status was evaluated bimonthly using anthropometric measurements.

Sample collection

Stool samples were collected from the cohort every 3 months, regardless of symptoms. During diarrhea episodes, additional stool samples were requested twice within the first week and again on days 14, 30, and 60 post-episode.

Definition of diarrhea and asymptomatic infection

A new diarrheal episode was defined by the presence of three or more liquid or semi-liquid stools per day without diarrhea in the previous 2 days. NoV-associated diarrhea was identified by detecting NoV in samples collected during an episode or within 7 days. Diarrhea severity was assessed using a modified Vesikari score (Supplementary Method). Asymptomatic samples were defined as stools from children without gastroenterologic symptoms, including diarrhea, vomiting, anorexia, or abdominal pain, in the past 7 days. Asymptomatic NoV infection was defined as the presence of NoV in an asymptomatic stool sample from a child who had no diarrhea episodes within the last 30 days. If diarrhea occurred within 30 days or if any of the aforementioned symptoms were observed within 7 days, the episode was classified as “undefined.” If a NoV-positive sample was untypable (i.e. genotypes could not be classified) but belonged to the same genogroup within 30 days or if the same genotype was detected in any later samples collected within 60 days, those later samples were considered to indicate viral shedding, regardless of whether the samples were collected during a diarrhea episode or when asymptomatic.

Detection and genotyping for norovirus

NoV was detected using quantitative real-time reverse transcription polymerase chain reaction (PCR), as previously described [16]. Briefly, RNA was extracted from stool suspensions using the QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany), and complementary DNA was synthesized using M-MLV RT and random primers (Invitrogen; Waltham, MA, USA). Amplification was performed using the TaqMan Fast Advanced Master Mix on a 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). NoV-positive samples were amplified using Ex Taq (TAKARA BIO INC., Japan) and sequenced using Sanger method with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a Genetic Analyser 3500 (Applied Biosystems). Partial polymerase and capsid regions were sequenced using primer sets of GI4871F, 5′-GATGATGARATHGTBTCHAC-3′, and GISKR [17] for GI and GII4766F, 5′-CCDGCHGGNTGGTTYGGVAARYT-3′, and GIISKR for GII [18]. When the sequence was not available, forward primers (GISKF and GIISKF) were replaced for partial capsid region amplification. Genotypes were determined using NoroNet (http://www.noronet.nl) [19].

Statistical analysis

Background characteristics were compared between the two communities using the chi-square test. Nutrition status was assessed with Z-scores for length-for-age and height-for-age [20]. NoV prevalence in diarrheal and asymptomatic samples was analyzed across age groups, excluding repeat samples with viral shedding. The attributable fraction (AF) was calculated as AF = Pr x (OR − 1)/OR [21], where Pr is the prevalence of NoV-associated diarrhea, and OR is the odds ratio for diarrhea vs asymptomatic cases. Incidence rates (IRs) were calculated as episodes per 100 child-months. Cycle threshold values were compared between symptomatic and asymptomatic samples using the Mann-Whitney *U* test. Cox proportional hazard models assessed adjusted hazard ratios (aHRs) for NoV-associated diarrhea and asymptomatic infections, using factors such as age group, community, income, monthly electricity expenses, sanitation, water sources, household assets, and animal presence in the household. Variables with $P < 0.1$ in univariable analyses were included in the multivariable models. For children aged 0-5 months, NoV infection rates were compared between those receiving only breastfed 4 days or more in the past 7 days and those who were not. The prevalence and AF were analyzed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA), whereas IRs and HRs were analyzed using Stata version 15.0 (StataCorp, College Station, TX, USA). Statistical significance was set at $P < 0.05$. In addition, pairwise comparisons of repeated NoV infections were summarized in a genotype and immunotype matrix [8]. For children with three or more infections, pairwise comparisons were made, with earlier episodes classified as previous infections relative to later ones (e.g. one vs two, one vs three, two vs three). The severity of diarrhea episodes in the repeated infections was compared by calculating the mean difference using heatmap (Supplementary Methods).

Results

Characteristics of cohort children

Of the 390 children enrolled in this study, 32 were excluded due to having less than 90 days of follow-up, and 20 were excluded due to incomplete sample collection (<50%), leaving 338 children with 6208 child-months of follow-up. The median age at recruitment and follow-up time was 9 months (interquartile range [IQR]: 5-14 months), with a median follow-up of 729 days (IQR: 482-730 days) (Table 1). Malnutrition (height-for-age Z-score [SD] <−2) affected 36% (79 of 222) of children at 1 year of age, increasing to 40% (109 of 273) by age 2, with higher rates in La Paz (36% vs 29%, $P = 0.005$). Rotavirus vaccine coverage

Table 1
Demographic characteristics of the cohort children.

Characteristics		Total n (%)	Community A n (%)	Community B n (%)	*P*-value
Total number of children		338 (100)	200 (100)	138 (100)	NS
Median follow-up days		729	729	729	NS
(25^{th}-75^{th} % range)		(482-730)	(528-730)	(388-729)	
Sex					
Male		154 (46)	94 (47)	60 (43)	
Female		184 (54)	106 (53)	78 (57)	NS
Age enrolled to the study (months)					
0-5		99 (29)	62 (31)	37 (27)	
6-11		103 (30)	62 (31)	41 (30)	
12-17		93 (27)	50 (25)	43 (31)	
18-23		42 (12)	25 (12)	17 (12)	
24-36		1 (1)	1 (1)	0 (0)	NS
Height-for-age Z-score					
1 year old	>−2	143 (64)	82 (59)	61 (73)	
	≤−2 to −3	47 (22)	33 (24)	14 (17)	
	≤−3	32 (14)	24 (17)	8 (10)	
2 years old	>−2	164 (60)	91(54)	73 (71)	
	≤−2 to −3	82 (30)	58 (34)	24 (23)	
	≤−3	27 (10)	21(12)	6 (6)	**0.005**
Rotavirus vaccination (dose)					
None		329 (97)	193 (97)	136 (98)	
One		4 (1)	3 (1)	1 (1)	
Two		4 (1)	3 (1)	1 (1)	
Three		1 (1)	1 (1)	0 (0)	NS
Total number of family members[a]					
2		12 (4)	10 (5)	2 (1)	
3		79 (23)	45 (22)	34 (25)	
4		89 (26)	42 (21)	47 (34)	
5		73 (22)	49 (25)	24 (17)	
≥6		85 (25)	54 (27)	31 (23)	**0.027**
Having siblings <5 years old					
No		188 (56)	124 (62)	64 (46)	
Yes		150 (44)	76 (38)	74 (54)	**0.005**
Mother's education level					
<7 grades		130 (39)	74 (37)	56 (41)	
7-12 grades		187 (55)	109 (55)	78 (56)	
>12 grades		21 (6)	17 (8)	4 (3)	0.506
Land area of household					
<40 m^2		167 (49)	82 (41)	85 (62)	
≥40 m^2		171 (51)	118 (59)	53 (38)	**<0.001**
House status					
Owned		183 (54)	159 (80)	24 (17)	
Not owned		155 (46)	41 (20)	114 (83)	**<0.001**
Water source for drinking					
Owned tap water		172 (51)	107 (54)	65 (47)	
Common tap water		95 (28)	50 (25)	45 (33)	
Commercial water		71 (21)	43 (21)	28 (20)	0.302
Heating water[b]					
No		201 (59)	108 (54)	93 (67)	
Yes		137 (41)	92 (46)	45 (33)	**0.014**
Storing water					
No		207 (61)	116 (58)	91 (66)	
Yes		131 (39)	84 (42)	47 (34)	0.141
Septic tank distance from water source[c]					
<7 m		162 (48)	94 (47)	68 (49)	
≥7 m		174 (51)	104 (52)	70 (51)	0.745
Restroom					
None		60 (18)	42 (21)	18 (13)	
One or more		278 (82)	158 (79)	120 (87)	0.060
Refrigerator					
Owned		73 (22)	45 (22)	28 (20)	
Not owned		265 (78)	155 (78)	110 (80)	0.627
Washing machine					
Owned		126 (37)	77 (39)	49 (36)	
Not owned		212 (63)	123 (61)	89 (64)	0.576
Family monthly income					
≤6000 PHP		116 (34)	84 (42)	32 (23)	
>6000 PHP		191 (57)	88 (44)	103 (75)	**<0.001**
Don't know		31 (9)	28 (14)	3 (2)	

(*continued on next page*)

Table 1 (*continued*)

Characteristics	Total n (%)	Community A n (%)	Community B n (%)	*P*-value
Monthly electricity fee				
≤600 PHP	197 (58)	139 (70)	58 (42)	
>600 PHP	135 (40)	57 (28)	78 (57)	**<0.001**
Don't know	6 (2)	4 (2)	2 (1)	
Domestic animals				
Owned	174 (51)	116 (58)	58 (42)	
Not owned	164 (49)	84 (42)	80 (58)	**0.004**
Animal manure inside house				
No	300 (89)	172 (86)	128 (93)	
Yes	38 (11)	28 (14)	10 (7)	0.053
Dogs enter house				
No	209 (62)	107 (54)	102 (74)	
Yes	69 (20)	57 (28)	12 (9)	**<0.001**
Don't know	60 (28)	36 (18)	24 (17)	
Cats enter house				
No	290 (86)	177 (89)	113 (82)	
Yes	35 (10)	16 (8)	19 (14)	0.081
Don't know	13 (4)	7 (3)	6 (4)	

A total of 338 children with 6208 child-months of follow-up were included in this study. Approximately 36% (79 of 222) of children were malnourished (height-for-age Z-score [SD] < −2) at 1 year of age, increasing to 40% (109 of 273) by 2 years of age. Children in La Paz were more likely to be malnourished (36% vs 29%, $P = 0.005$). Only one child completed all three doses of the rotavirus vaccine. Among the 338 households, 191 (57%) had a family income of over 6000 Philippine pesos (approximately 104 US$) per month, which was below the national median level. Household characteristics were compared between two communities using chi-square test. Categories of "Don't know" were excluded from the analysis.

[a] Total number of family members included in the cohort of children from each family.

[b] Heating water: Water used to prepare food for children was heated.

[c] Septic tank distance from the water source: Two households had no data on septic tank distance from the water source.NS: not significant.

was minimal, with only one child completing all three doses. The median family size was four in both communities. Community A had more households with larger land areas (>40 m^2) than Community B (59% vs 38%, P <0.001), whereas household income was higher in Community B (>6000 Philippine pesos/month, about 104 US$) than in Community A (44% vs 75%, P <0.001). In addition, 61% of mothers (208 of 338) had high school education or higher.

Prevalence and incidence rate of norovirus-associated diarrhea and asymptomatic infection

A total of 527 diarrhea episodes were reported during the study period, of which 84 (17.4%, 95% confidence interval [CI]: 12.7-19.7%) were identified as NoV-associated diarrhea. Among these, 14 (2.7%, 95% CI: 1.4-4.5%) episodes were NoV GI– and 72 (13.7%, 95% CI: 10.7-17.2%) were NoV GII–associated diarrhea (GI and GII co-infection n = 2). Therefore, most NoV-associated diarrhea cases were due to GII (85.7%, 72 of 84). All NoV-associated diarrhea episodes were mild, scoring 2-4. A total of 219 asymptomatic NoV infections were identified among 2031 non-diarrheal stool samples (10.8%, 95% CI: 9.4-12.3%). Among the 219 NoV asymptomatic infections, 28% (62 of 219) were infected with GI and 74% (161 of 219) were infected with GII (GI and GII co-infection, n = 4). The cycle threshold value of NoV-associated diarrhea samples was significantly lower than asymptomatic samples (24.0 vs 28.0, P <0.001). Among 109 undefined NoV infections, 20% (22 of 109) were GI, and 80% (88 of 109) were GII.

The prevalence of NoV in diarrheal episodes was highest in children aged 6-11 months (23.0%, standard error range (SER): 18.5-27.5%) and 18-23 months (23.5%, SER: 20.0-27.0%). In contrast, the prevalence of NoV in asymptomatic samples peaked in children aged 36-47 months (16.9%, SER: 12.8-21.0%) (Figure 1a). The AF for NoV-associated diarrhea was 16% in the first year of life, 6% in the second year, −1% in the third and fourth years of life.

The overall IR of NoV-associated diarrhea and asymptomatic infection were 1.35 (95% CI: 1.08-1.68) and 3.53 (95% CI: 3.08-4.03) per 100 child-months, respectively. The IR of NoV-associated diarrhea for GI and GII were 0.23 (95% CI: 0.12-0.38) and 1.16 (95% CI: 0.91-1.46) per 100 child-months, respectively (Table 2). Comparing the IR of NoV-associated diarrhea and asymptomatic infection among the age groups, both rates increased as age increased from 0-5 and 6-11 months. However, the difference in the incidence rate of NoV-associated diarrhea between 0-5 and 6-11 months was not statistically significant (Figure 1b). The highest incidence rate of NoV-associated diarrhea was observed in children aged 6-11 months (IR: 2.31, 95% CI: 1.30-3.32) and 18-23 months (IR: 2.34, 95% CI: 1.57-3.12), which was about three to four times higher than the rates in children aged 24-35 (IR: 0.72, 95% CI: 0.39-1.21) and 36-47 months (IR: 0.51, 95% CI: 0.00-1.22). In contrast, the incidence rate of NoV asymptomatic infections was the highest in children aged 18-23 months (IR: 4.49, 95% CI: 3.41-5.56), which was 1.9 times higher than that in children aged 6-11 months (IR: 2.42, 95% CI: 1.39-3.46); however, this difference was not statistically significant.

Risk factors for norovirus-associated diarrhea and asymptomatic infection

Children aged 24-47 months had a significantly lower risk for NoV-associated diarrhea than those aged 0-11 months (aHR: 0.36, 95% CI: 0.19-0.66, $P = 0.001$), whereas for NoV asymptomatic infection, the risk in those aged 12-23 months was significantly higher than in those aged 0-11 months (aHR: 1.97, 95% CI: 1.29-3.02, $P = 0.002$).

When incorporating household characteristics along with age group, family size, and communities, only age group was significantly associated with NoV-associated diarrhea in the Cox proportional hazard model (Table S1). Children drinking commercial water had a lower HR for NoV-associated diarrhea than those drinking tap water, although this was not statistically significant (HR: 0.57, 95% CI: 0.31-1.03, $P = 0.064$). The children with monthly family income of over 6000 Philippine pesos had a lower risk than those with <6000 Philippine pesos (HR: 0.63, 95% CI: 0.39-1.03, $P = 0.066$). The children with height-for-age Z-score of −2 had higher HRs than those of 0 or more than 0 (HR: 1.66, 95% CI: 0.93-2.97, $P = 0.086$). After adjusting for these four factors, only the age group remained significant, with children aged 0-11 months having a significantly higher risk than those aged 24-47 months ($P = 0.001$) (Table 2). The difference between children aged 0-11 months and those aged 12-23 months was not significant ($P = 0.574$).

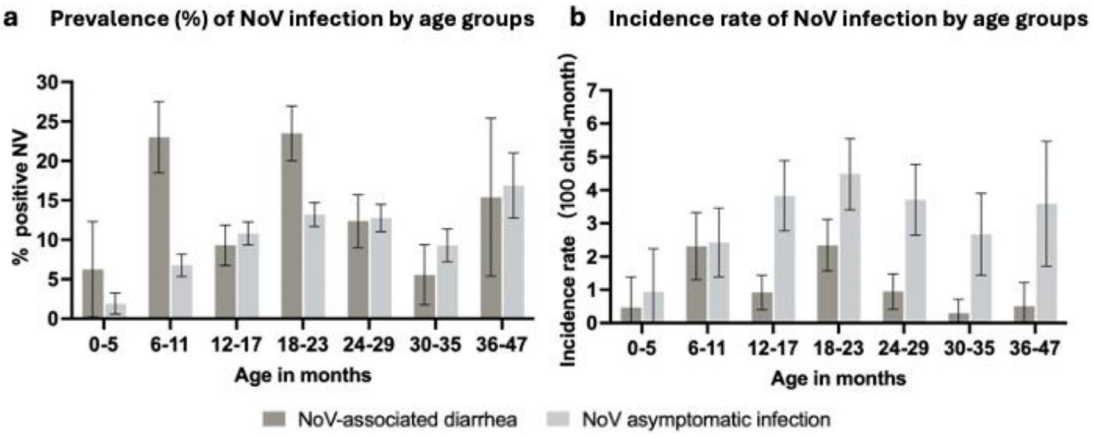

Figure 1. Prevalence and incidence rate of NoV infection by age.
(a) Prevalence (%) of NoV in diarrheal episodes and non-diarrheal stool samples by age group. In total, 2558 stool samples were included from 338 cohort children aged less than 2 years, including 527 diarrheal stool samples and 2031 non-diarrheal stool samples. Error bars represent standard error of the prevalence. (b) Incidence of NoV infection every 6 months of age. The overall incidence rates for NoV-associated diarrhea and asymptomatic infection were 1.35 and 3.53 per 100 child-months, respectively. Error bars represent the 95% confidence interval of incidence.
NoV, norovirus.

Table 2
Age-specific incidence rate per 100 child-months of NoV-associated diarrhea and asymptomatic infection.

	Child-months	NoV-associated diarrhea (n = 84)			Asymptomatic infection (n = 219)		
		n	IR (95% CI)	aHR (95% CI)	n	IR (95% CI)	aHR (95% CI)
Total	6208	84	1.35 (1.08-1.68)	NA	219	3.53 (3.08-4.03)	NA
Genogroup							
GI	6208	14	0.23 (0.12-0.38)	NA	62	1.00 (0.77-1.28)	NA
GII	6208	72	1.16 (0.91-1.46)	NA	161	2.59 (2.21-3.03)	NA
Age in months							
0-11	1079	21	1.95 (1.20-2.98)	Ref.	23	2.13 (1.35-3.20)	Ref.
12-23	2799	47	1.68 (1.23-2.23)	0.87 (0.52-1.43)	117	4.18 (3.46-5.01)	1.97[a] (1.29-3.02)
24-47	2330	16	0.69 (0.39-1.15)	0.36[a] (0.19-0.66)	79	3.39 (2.68-4.22)	1.60[b] (1.01-2.55)
Refrigerator							
Not Owned	4901	71	1.45 (1.13-1.82)	Ref.	188	3.80 (3.30-4.43)	Ref.
Owned	1306	13	0.99 (0.594-1.70)	0.70 (0.40-1.21)	31	2.38 (1.61-3.37)	0.62[b] (0.41-0.92)

Age groups were combined into 0-11, 12-23, and 24-47 months for the hazard model because the number of NoV-associated diarrhea episodes was limited to younger and older age groups. Incidence rates for NoV GI and GII, including co-infections with NoV GI and GII: Two co-infections were in NoV-associated diarrhea and four co-infections were in asymptomatic NoV infection. Hazard ratios (aHRs) were adjusted for age groups and with or without refrigeration using the Cox hazard model. In this model, infections in the same child were treated as clusters.
aHR, adjusted hazard ratio; CI, confidence interval; GI, genogroup I; GII, genogroup II; IR, incidence rate; mo., months; NA: not applicable; NoV, norovirus; Ref., reference.
[a] P <0.01
[b] P <0.05.

Three factors, including age group (12-23 months: HR: 1.96, 95% CI: 1.28-3.01, P = 0.002), owning a refrigerator (HR: 0.62, 95% CI: 0.42-0.92, P = 0.018), land area of the household (HR: 0.75, 95% CI: 0.57-1.00, P = 0.046), and owning the house or not (HR: 0.76, 95% CI: 0.58-1.00, P = 0.047), were significantly associated with NoV asymptomatic infection (Table S1). Four factors with P <0.1, including storing water or not (HR: 1.30, 95% CI: 0.98-1.71, P = 0.066) and owning a washing machine or not (HR: 0.76, 95% CI: 0.56-1.03, P = 0.073), were also adjusted for HR. Age group (12-23 months:(aHR: 1.97, 95% CI: 1.29-3.02, P = 0.002) and owning a refrigerator (aHR: 0.62, 95% CI: 0.41-0.92, P = 0.017) were identified as factors significantly associated with NoV asymptomatic infection after adjustment (Table 2).

No significant difference was observed in the hazard ratio for NoV-associated diarrhea or asymptomatic infection between the two communities or between children who were receiving only breastfed 4 days or more in the past 7 days and those less than 4 days.

Distribution of genotypes in norovirus infection and repeated infection

NoV genotypes were identified in 305 of 412 infections, including 84 NoV-associated diarrhea, 219 asymptomatic infections, and 109 undefined infections. Of the 84 NoV-associated diarrheal episodes and 219 asymptomatic infections, polymerase and capsid region genotypes were available for 56 (67%) and 142 (65%) cases, respectively. Only capsid

Table 3
Distribution of genotypes in NoV infection.

	NoV-associated diarrhea (n = 84)		Asymptomatic infection (n = 219)		Undefined infection (n = 109)	
	n	(%)	n	(%)	n	(%)
Total	84	(100)	219	(100)	109	(100)
Genogroup I	14	(17)	62	(28)	22	(20)
GI.1[P1]	0	(0)	5	(2)	1	(1)
GI.2[P2]	0	(0)	1	(< 0.5)	0	(0)
GI.3[P3]	4	(5)	12	(5)	1	(1)
GI.3[P10]	0	(0)	1	(< 0.5)	2	(2)
GI.3[P13]	1	(1)	7	(3)	3	(3)
GI.3	1	(1)	4	(2)	3	(3)
GI.4	1	(1)	1	(< 0.5)	0	(0)
GI.5[P4]	0	(0)	1	(< 0.5)	0	(0)
GI.5[P5]	0	(0)	1	(< 0.5)	1	(1)
GI.5	0	(0)	1	(< 0.5)	0	(0)
GI.6[P6]	2	(2)	1	(< 0.5)	1	(1)
GI.6[P11]	0	(0)	3	(1)	1	(1)
GI.6	0	(0)	4	(2)	1	(1)
GI.7[P7]	0	(0)	5	(2)	0	(0)
GI.9[P9]	1	(1)	3	(1)	1	(1)
GI.9	0	(0)	2	(1)	0	(0)
Unidentified	4	(5)	11	(5)	7	(6)
Genogroup II	72	(86)	161	(74)	87	(80)
GII.2[P16]	7	(8)	23	(10)	6	(6)
GII.2[P31]	1	(1)	3	(1)	1	(1)
GII.2	3	(4)	3	(1)	1	(1)
GII.3[P12]	7	(8)	6	(3)	8	(7)
GII.3	0	(0)	0	(0)	1	(1)
GII.4 Sydney[P16]	9	(11)	5	(2)	7	(6)
GII.4 Sydney[P31]	5	(6)	6	(3)	5	(5)
GII.4 Sydney (combined P16 and P31)	14	(17)	11	(5)	12	(11)
GII.4 Hong Kong[P31]	3	(4)	5	(2)	1	(1)
GII.4[P16]	0	(0)	0	(0)	1	(1)
GII.4[P31]	2	(2)	4	(2)	1	(1)
GII.6[P7]	5	(6)	13	(6)	11	(10)
GII.6	1	(1)	2	(1)	2	(2)
GII.7[P7]	4	(5)	19	(9)	10	(9)
GII.7	0	(0)	1	(< 0.5)	0	(0)
GII.13[P16]	0	(0)	3	(1)	2	(2)
GII.13	1	(1)	1	(< 0.5)	2	(2)
GII.14[P7]	0	(0)	1	(< 0.5)	0	(0)
GII.17[P17]	4	(5)	12	(5)	1	(1)
GII.17	3	(4)	4	(2)	0	(0)
GIX.1[P15]	1	(1)	2	(1)	0	(0)
GIX.1	0	(0)	0	(0)	1	(1)
Unidentified	16	(19)	48	(22)	26	(24)

Genotypes of NoV infection, including asymptomatic, symptomatic, and undefined infection, were identified in 305 of 412 infections. Genotypes without the polymerase type indicated that only the capsid genotypes were available. Genotype IX.1 was previously named as GII.15 and recently redesigned as GIX.1. There were two cases of co-infection with GI and GII in NoV-associated diarrhea and four cases in asymptomatic infections.
NoV: norovirus; GI: genogroup I; GII: genogroup II.

region genotypes were available for 10 (12%) diarrheal episodes and 23 (11%) asymptomatic infections. Four capsid genotypes were identified in NoV GI, and eight were identified in NoV GII. In addition, GI.1, GI.2, GI.5, GI.6, GI.7, and GII.14 were only identified in asymptomatic NoV infections (Table 3). GII.4 was the most common genotype in diarrheal episodes (23%, 19 of 84) and the second most common in asymptomatic infections (9%, 20 of 219). Three variants—GII.4 Sydney [P16], GII.4 Sydney [P31], and GII.4 Hong Kong [P31]—were found in diarrheal episodes and asymptomatic infections. NoV GII.4 Sydney accounted for 17% (14 of 84) of NoV-associated diarrhea episodes, which was significantly higher than that of 5% (11 of 219) observed in asymptomatic infections (OR: 3.8, 95% CI: 1.5-9.6, $P = 0.001$). However, such a significant difference was not observed for the GII.4 Hong Kong variants, which accounted for 4% (three of 84) of NoV-associated diarrhea episodes and 2% (five of 219) of asymptomatic infections. GII.2 was the most frequently detected genotype in asymptomatic infections (13%, 29 of 219) and the second most commonly detected genotype (13%, 11 of 84) in NoV-associated diarrhea.

In a total of 118 children, with a median follow-up of 729 days (IQR: 643-730 days), 316 samples, including 64 children were identified as having two, 38 with three, 11 with four, three with five, and two with six NoV infections. Repeated infections within the same genotype were rare, occurring in two children from community B (GI.9[P9], >6 months apart) and two from community A (GI.3, 11 months and 62 days apart), with negative samples in between, and were, therefore, considered separate infections (Figure 2a, b). No repeated infections with GII.4 variants were observed. Comparing the immunotypes, we found two repeated infections within the same immunotype of C (NoV GI.7, 93 days and 6 months after GI.3) and three repeated infections within the same immunotype of J (NoV GII.17[P17] infections occurred over 6 months after NoV GII.13[P16]). Other typable repeated infections pairwise in our study involved different immunotypes (95%, 158 of 167).

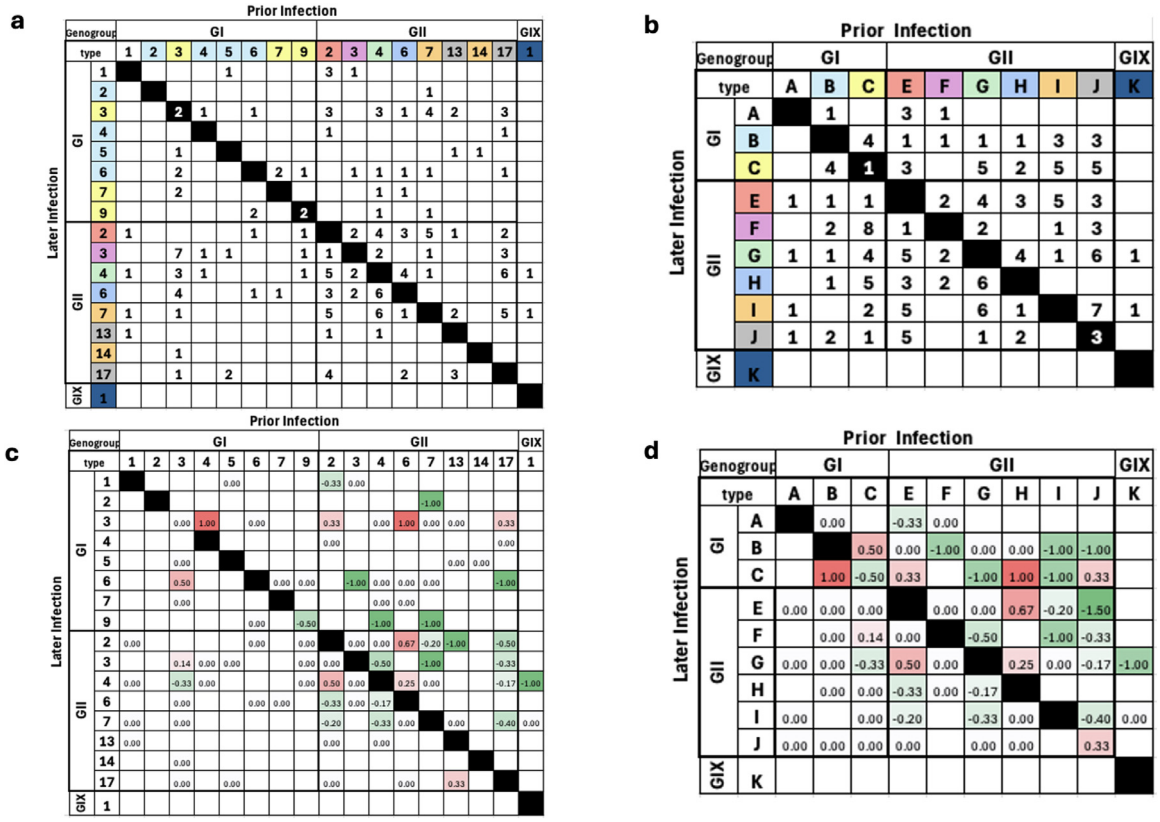

Figure 2. Pairwise comparison of NoV repeated infection by capsid genotype and immunotype.
(a) The matrix shows the pairwise comparison of NoV genotypes in previous and later infections. The columns represent previous infections and rows represent later infections in the repeated infection pairs. The numbers in the cells show the number of infections observed. (b) Matrix tabulated by immunotype in previous and later infections. Three repeated infections were observed in immunotype C and three repeated infections in immunotype J. (c) The difference in symptom between previous and later infections by genotypes. Red represents symptom in later infection increased, whereas green stands for symptom in later infection reduced. The symptom in the repeated infections was compared by computing the difference from clinical score. Severity of infection was evaluated based on the Vesikari clinical score. The severity of asymptomatic infections was considered as 0. Nine infections without fulfilling diarrhea criteria but with other gastroenterological symptoms within 7 days were scored 1 in the clinical score. The changes of symptom increased, reduced, or the same in the later infection were marked as +1, −1, or 0, respectively. The mean of the sum of differences were computed to describe the pattern of symptom changing in repeated infections using heatmap. (d) The difference in symptom between previous and later infections by immunotypes.
Same color in panel a and b represents same immunotype: immunotype A (white), GI.1; immunotype B (light blue), GI.2, GI.4, GI.5 and GI.6; immunotype C (yellow), GI.3, GI.7 and GI.9; immunotype E (red), GII.2; immunotype F (purple), GII.3; immunotype G (green), GII.4; immunotype H (indigo), GII.6; immunotype I (brown), GII.7 and GII.14; immunotype J (grey), GII.13 and GII.17; immunotype K (deep blue), GIX.1.
Genotype IX.1 was previously named as GII.15 and recently redesigned as GIX.1.
GI: Genogroup I; GII: Genogroup II; NoV: norovirus.

Among NoV infections reported initially in the study period, 20% (24 of 118) were symptomatic and 18% (35 of 195) of the second or later infections were symptomatic. Majority of the infection pairs did not increase the severity in the later infection (88%, 73 of 83) (Figure 2c, d). Most later GI infections did not increase severity, except for GI.3 and GI.6. Severity increased with children with GI.3 after GI.4, GII.2, GII.6, and GII.17, with GI.6 following GI.3. Severity decreased after GII.3, GII.4, and GII.7 infections but not after GII.6. Infections after GII.17 did not increase severity, except with GI.3. Although severity increased with GI.3, this pattern was absent in GI.7 and GI.9 (both immunotype C). Severity decreased more after GII infections than GI (6% vs 36% [18 of 50], $P = 0.002$).

Discussion

In this study, we investigated the prevalence and incidence of NoV-associated diarrhea and asymptomatic infections in children aged 0-4 years. The prevalence of asymptomatic NoV infection reached 12% in the second year and remained relatively stable (12-17%) until age 4. Limited data exist on asymptomatic NoV infections in children from Southeast Asia; however, a multicentered hospital-based study including four countries in Southeast Asia (Philippines and Thailand) and Latin America (Brazil and Chile) reported a similar prevalence of 12% in non-diarrheal outpatients aged 1-2 years, which dropped to 0-4% in Latin American countries but maintained 10% in Philippines and Thailand in a relatively small number of children aged 3-5 years [22]. The prevalence of NoV-associated diarrhea in children aged 6-11 months was similar to that reported in a birth cohort study in Peru. However, the prevalence of asymptomatic NoV infection in this study (7%) was almost half that in the Peruvian study (approximately 15-20%), which resulted in higher AF in children aged 0-11 months in our study. Nevertheless, the prevalence and incidence of asymptomatic NoV infection remain high in the third and fourth years of life, indicating that older children carrying NoV may serve as a source of transmission to younger children and vulnerable populations.

The incidence of NoV-associated diarrhea varied by age, starting at 0.5 per 100 child-months in children aged 0-5 months, increasing five-fold at 6-11 months, declining in the third and fourth years, and returning to early levels. This finding suggests that children may develop some degree of immunity to NoV-associated diarrhea early in life, al-

though this may not be complete. Children experiencing the highest risk of NoV-associated diarrhea in the first year of life were consistent with studies in India [14] and Ecuador [23] but contrasted with findings from Peru, where the highest risk was observed in the second year of life [6]. The incidence rate of NoV-associated diarrhea in children under 1 year old in this study was similar to that reported in India (2.2 per 100 child-months), whereas the rate in Peru was three times (6.6 per 100 child-months) for children aged 6-11 months. In Ecuador, the rate was 2.9 for 100 children per month for the same age group. This discrepancy could be due to differences in NoV detection methods because Peru and Ecuador used one-step PCR assays that are more sensitive. Furthermore, stool sampling was less frequent in asymptomatic children, and stool samples with NoV shedding were excluded from the analysis. This may be due to the different susceptibilities of Asian and South American children to NoV. The proportions of secretors vary among different populations [24], and secretors are known to have a higher susceptibility to NoV infections [12]. Previous studies have reported that the non-secretory phenotype constitutes approximately 41% of the population in the Philippines [25], whereas the secretor takes up about 93% in Peru [26]. In addition, the children in our study had better nutrition status than the Peruvian and Indian cohorts; this may also explain the lower incidence of NoV-associated diarrhea in our study.

The prevalence of NoV in diarrheal episodes showed two peaks at 6-11 and 18-23 months of age, with high AF. However, it was lower in children aged 12-17 months. A similar trend was observed in a birth cohort study conducted in Ecuador, which also had active surveillance on diarrhea in community children. Although they performed one-step real-time PCR for NoV detection that resulted in higher positivity, they showed similar pattern of a relatively low prevalence of NoV in diarrheal stool samples collected from children aged 16-19 months [23]. The duration of immunity against subsequent NoV infection in children is not yet known; however, the immunity acquired in the first year of life might have protected the children for approximately 6 months, which may wane over time by 16-18 months of age. Other possible explanations for the two peaks pattern might be children not receiving protection from breast milk after 6 months and increasing exposure from activities and contact after 18 months.

Repeated NoV infections were observed in approximately one-third of the children in this study. Symptomatic repeated infections were observed in 18% of these cases, which is lower than the 42% in Peruvian [6], 40% in Chilean [7] studies, and 25% in Indian birth cohort [14]. This lower proportion of symptomatic repeated infections in our study was probably due to the older age of children with repeated infections than other birth cohort studies. Most of the repeated infection episodes in our study occurred after the age of 2, possibly missing the first infection.

Repeated infections within the same genotypes were observed only in GI.3 and GI.9 (both immunotype C), possibly due to antigenic differences within genotypes [27]. Although immunotypes were defined by pairwise genetic distance, our findings align with previous study [8], except for repeated infections in immunotype J. Rare repeated infections suggest genotype- or immunotype-specific immunity, indicating the potential to reduce genotypes in future vaccine development.

In addition, our study found that most repeated infection pairs showed no increase in severity and tended to decrease after GII infections, especially GII.4, except for previous GII.6 infections, which may have distinct immunogenicity. Tohma *et al.* observed a distinct evolutionary pattern of GI.3, GII.6, and GII.17, potentially explaining GII.6's unique infection pattern. Our finding suggests that previous NoV GII infection protects against severe symptoms in later infections. However, further studies are required to confirm these hypotheses.

Other studies have reported repeated infections with the same genotypes, mostly GII.4 variants [6,8]. However, no repeated GII.4 infections were observed in our study, which identified three variants: Sydney[P16], Sydney[P31], and 10 strains close to Hong Kong[P31] (NoroNet classification). GII.4 Sydney was significantly more frequent in diarrheal episodes than in asymptomatic infections, suggesting high pathogenicity or incomplete herd immunity, 2 or more years after the global outbreak. The GII.4 Hong Kong variant, first reported in the Philippines in May 2017 and later in the Netherlands, China, and the United Kingdom (2017-2019) [28] was observed in our study earlier (October 2016 to December 2017), which predates GenBank records. Besides GII.4, GII.2 and GII.17 were also prevalent, consistent with reports from China (GII.2: October to December 2016; GII.17: in October 2014 to January 2015) and Thailand (GII.2: October 2016 to February 2017; GII.17: November 2015 to February 2016) during our study period [29,30].

Our study found that, besides age, owning a refrigerator served as a protective factor for NoV asymptomatic infection. Although this association may be confounded by other indicators of high socioeconomic status, the model showed that it was not significantly influenced by other socioeconomic factors. Although refrigeration may not affect the viral load in food, people who use refrigerators may be more aware of food safety and environmental hygiene.

This study was limited by not being a birth cohort, recruiting few children under 6 months, and having a limited follow-up period for older children, potentially underrepresenting these age groups. In addition, the lack of data on previous NoV infections may have underestimated the impact of repeated infections. Moreover, stool samples collected 3-monthly may have missed some asymptomatic infections, which probably would lead to lower IR and reduce statistical power, and excluding vomiting without diarrhea might have missed some symptomatic episodes. In addition, whole genome sequencing was not conducted to verify possible antigenic differences. Lastly, the analysis of repeated infection did not have the pre-study, not having enough number of each pairwise combination of genotypes or immunotypes to evaluate the symptomatic patterns and excluding infection with untyped sequence.

Conclusion

To the best of our knowledge, this is the first cohort study in Southeast Asia to estimate the incidence rate of NoV-associated diarrhea and asymptomatic infection in children under 4 years. Children had the highest risk of NoV-associated diarrhea in the first year of life, whereas asymptomatic infections persisted after the second year. Repeated infections suggest that children may acquire genotype- and immunotype-specific immunity, with age being a key factor for NoV-associated diarrhea.

Declarations of competing interest

The authors have no competing interests to declare.

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Ethical statement

This study was approved by the institutional review board of Tohoku University Graduate School of Medicine and Research Institute for Tropical Medicine. Informed consent was obtained from all the participants involved in this study. The protocol was reviewed and approved by the ethics committee of Tohoku University Graduate School of Medicine, and all procedures complied with the ethical standards of the institutional committee and the 1963 Helsinki Declaration.

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Author contributions

M.S., H.O., and J.M.B. conceived and designed the study. M.C.A.C. managed sample collection and laboratory assays. M.O., C.D., and M.S.O. oversaw sampling logistics and laboratory management. M.K., T.I., and Y.S. aided in molecular analysis and result interpretation. C.Y. drafted the manuscript, with support provided by M.S. and H.O. All authors contributed to revising and finalizing the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijregi.2024.100549.

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