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Research Paper

Norovirus-specific immunoglobulin A in breast milk for protection against norovirus-associated diarrhea among infants

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ABSTRACT

Background: Norovirus (NV) causes acute gastroenteritis in infants. Humoral and fecal immunoglobulin A (IgA) responses have been correlated with protection against NV; however, the role of breast milk IgA against NV infection and associated diarrhea is still unknown. This study aimed to evaluate the protective role of NV-specific IgA (NV-IgA) in breast milk.

Methods: Ninety-five breast milk samples collected from mothers enrolled in a 2016–2017 Peruvian birth cohort study were tested for total IgA and NV-IgA by ELISA using GII·4 variants and non-GII·4 genotype virus-like particles (VLPs). Breast milk samples were grouped according to the NV infection and diarrheal status of infants: NV positive with diarrhea (NV+D+, n=18); NV positive without diarrhea (NV+D-, n=37); and NV negative without diarrhea (NV-D-, n=40). The percent positivity and titer of NV-IgA were compared among groups. The cross-reactivity was estimated based on the correlation of ratio between NV-IgA against GII·4 variants and non-GII·4 genotype VLPs.

Findings: NV-IgA had high positivity rates against different VLPs, especially against GII (89–100%). The NV+D- group had higher percent positivity (89% vs. 61%, p=0·03) and median titer (1:100 vs 1:50, p=0·03) of NV-IgA than the NV+D+ group against GI·1 VLPs. A relatively high correlation between different GII·4 variants (0·87) and low correlation between genogroups (0·23–0·37) were observed.

Interpretation: Mothers with high positivity rates and titers of NV-IgA in breast milk had NV infected infants with reduced diarrheal symptoms. Antigenic relatedness to the genetic diversity of human norovirus was suggested.

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Introduction

Norovirus (NV), which belongs to family *Caliciviridae*, is a genetically and antigenically diverse RNA virus [1]. Norovirus is a major cause of gastroenteritis in children, only second to rotavirus (RV), and associated with about 200,000 deaths annually worldwide, mostly children from developing countries [2]. In areas where

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Research in context

Evidence before this study

Norovirus (NV) is the leading cause of acute gastroenteritis in areas where the rotavirus vaccine is effectively implemented. Breastfeeding protects against diarrheal infections caused by non-viral pathogens like enterotoxigenic *Escherichia coli* (ETEC), Campylobacter, Salmonella, and Giardia but not in rotavirus. The varying levels of breast milk components may be influenced by the endemicity of diarrheal pathogen and health status, genetics, and immune history of the mother. Previous studies of IgA in breast milk showed protection against non-viral pathogen causing diarrhea but not in viral pathogens like rotavirus. Norovirus-specific IgA (NV-IgA) has been detected in mothers who recently gave birth; however, as far as we know, the protective role of NV-IgA against NV infection and associated diarrhea was not known. We tested the breast milk of Peruvian mothers taken before the first NV infection of infants against different GII·4 variants and non-GII·4 genotype VLPs.

Added value of this study

At present, NV vaccines are still under development. NV primarily infects infants aged 6–23 months and at this age, innate immunity of infants comes from the maternal antibodies through breastfeeding. Our research may provide evidence of the role of breastfeeding against the first NV infection of the infants at the time they are primarily nourished by breast milk.

Implications of all the available evidence

The high levels of NV-IgA did not prevent the infant from having NV infection, however, NV symptoms were reduced which may have protected the infant from having NV-associated diarrhea. We believe this research will be valuable in the implementation of NV vaccines. The high levels of breast milk rotavirus-specific IgA seemed to decrease rotavirus vaccine efficacy hence, we think that NV-IgA may also affect the future NV vaccines. Our study reinforced the need to continue breastfeeding practice up to one year to promote the health of both mothers and infants.

rotavirus vaccines are effectively implemented, NV has become the leading cause of acute gastroenteritis [3],[4]. Approximately 70% of NV pediatric infections occur within 6–23 months, suggesting protection conferred from maternal antibodies or low NV exposure during the first six months of life [5].

Maternal antibodies like immunoglobulin G (IgG) and IgA are transmitted in infants either in utero or through breastfeeding. In a longitudinal study conducted in the Philippines, longer periods of exclusive breastfeeding showed a decrease in diarrheal morbidity [6]. Breastfeeding was proven to protect infants against diarrheal infections caused by enterotoxigenic *Escherichia coli* (ETEC), Campylobacter, Salmonella, and Giardia [7]. The observed effects of breastfeeding against rotavirus were inconsistent [8]. The variations in the protective role of breastfeeding may be attributed to the health status of the mother, the endemicity of diarrheal pathogens, or the varying composition of breast milk, which is influenced by genetics and immune history of mothers [9].

Breast milk contains a variety of bioactive components including IgA antibodies that are part of the maternal immune system, where the increased level is related to the protection against non-viral pathogens causing diarrhea like *Vibrio cholerae, Campylobacter jejuni,* and *Giardia lamblia* [7]. However, high levels of rotavirus-specific IgA in breast milk do not always reflect protection against rotavirus infection in infants [10,11]. In previous studies, the presence of high levels of rotavirus-specific IgA in breast milk from Zambian mothers correlated with a lower rotavirus vaccine seroconversion than the New Zealand mothers, who had no evidence of association in their IgA response following rotavirus vaccination [12,13].

Previous studies showed that breast milk collected postpartum reacted to various NV virus-like particles (VLPs), but the protective ability of NV-specific IgA (NV-IgA) is yet to be established [14–16]. In this study, we evaluated the linkage of NV-IgA in breast milk with protection against NV and found that (i) NV-IgA reacted against GII·4 variants and non-GII·4 genotype VLPS, and (ii) high titer of NV-IgA was associated to the reduction of diarrheal symptoms in NV infected infants.

Methods

Ethical Statement

The mothers included in this study were selected from a Peruvian birth cohort study carried out in 2016–2017 which was approved by the Institutional Review Boards of Asociacion Benefica PRISMA, Universidad Peruana Cayetano Heredia, Johns Hopkins University, and University of California-San Francisco. Informed consent was obtained from the mothers or caretakers of the infants. The analysis of archived specimen was approved by the ethics committee of the Tohoku University, Graduate School of Medicine.

Study site, design, and population

The samples used in this study were from mother-infant pairs who participated in a birth cohort study done in Villa El Salvador, Lima, Peru. Infants were monitored daily for the presence or absence of diarrhea. A diarrhea episode was defined as the presence of three or more liquid or semiliquid stools within 24 hours. For infants younger than two months, the definition was based on the assessment of the mother or the caretaker of the infant. Socio-demographic and socio-economic data, birth weight of infants, age of gestation, and type of delivery were collected upon enrollment. History of rotavirus vaccination was confirmed based on the card given by the health center. Daily breastfeeding practice and monthly anthropometric measurements were also collected. Exclusive breastfeeding was defined as no other food, or drink, nor water, except for breast milk.

For this study, we included breastfeeding mothers whose infants had $\geq$75% of stool samples collected with $\geq$70% of samples evaluated during the first six months postpartum. Non-breastfeeding mothers were excluded in this study.

Sample collection, transport, and storage

Stool samples from infants were collected weekly, except when an infant had a diarrhea episode, the samples were collected within 48 hours of the diarrhea episode, while the stool samples from the mother were collected monthly. Saliva samples of mother-infant pairs were collected upon recruitment. Breast milk samples were collected initially at recruitment and then monthly. All samples were transported and stored at -80°C until processing. A subset of breast milk samples from 95 mothers collected during the first six months postpartum were selected according to the criteria described below.

Criteria for selection and grouping of mothers

The mothers were grouped according to the first NV infection and diarrhea status of infants during the first six months postpartum. The NV positive with diarrhea (NV+D+) group was composed of mothers whose infants had NV positive stool taken during or within seven

days at the start or end of the diarrheal episode of infants. The NV positive without diarrhea (NV+D-) group was comprised of mothers whose infants had no diarrhea episodes within seven days after the stool sample was NV positive. Finally, the NV negative without diarrhea (NV-D-) group consisted of mothers whose infants had no diarrhea episodes and had NV negative stool samples. We excluded mothers whose infants were NV positive but were co-infected with other diarrheal pathogens such as sapovirus or ETEC. We also excluded infants who were NV negative but positive for other diarrheal pathogens.

Detection and genotyping of norovirus and other pathogens by real-time RT-PCR

All diarrheal samples and one monthly routine stool sample were tested for NV as previously described.[17]·[18] Genotypes and GII·4 variants were identified using NoroNet (https://www.rivm.nl/en/noronet). Sapovirus, rotavirus, and ETEC were detected and genotyped as previously described [19–22].

Detection of norovirus-specific IgA by indirect ELISA

To determine the presence of NV-IgA in breast milk, we developed an in-house ELISA using VLPs from two GII·4 variants (GII·4 MD2004–3/2004 and GII·4 RockvilleD1/2012) and four commonly circulating non-GII·4 genotypes (GII·6 BethesdaD1/2012, GII·17 GaithersburgD1/2014, GI·1 Norwalk/1968, and GI·9 Vancouver730/2004). Virus-like particles were produced as described elsewhere. [23,24] Wells of polystyrene microtiter-plates (BM6001; BMBIO, Tochigi, Japan) were coated with 100 μL/well of 0·5 μg/ml (GII) or 0·25 μg/ml (GI) VLPs in carbonate-bicarbonate buffer (pH 9·6). Following blocking with 5% Blotto (37530; Thermo Scientific, Massachusetts, USA), the wells were incubated with 100 μL of 1:50, 1:100, 1:500, 1:1000, 1:5000, and 1:10,000 dilutions of breast milk serum for two hours. The VLPs and breast milk IgA interaction was detected using horse raddish peroxidase-conjugated anti-human IgA goat polyclonal IgG (109–035–011; Jackson Immuno Research Laboratories, Pennsylvania, USA). After one-hour incubation and washing, o-phenylenediamine (34005; Thermo Fisher Scientific, Massachusetts, USA) substrate was added; the reaction was stopped by adding 100 μL/well of 0·1N H_2SO_4. All washes were performed with 0·1% phosphate buffer saline-Tween 20. Plates were read at 490 nm using an ELISA plate reader (Tecan Multiplate Reader Rainbow Thermo, Grodig, Austria). Pooled known positive samples from NV positive mothers and samples with low positive over negative (P/N) ratio, served as positive and negative controls respectively. Percent positivity was defined as a sample having a P/N ratio of $\geq$3·0 at 1:50 sample dilution, while titers were defined as the reciprocal of the highest final dilution with a P/N ratio of $\geq$3·0.

Detection of total IgA by ELISA

The detection of total IgA was like NV-IgA except for the coating step, where anti-human IgA goat polyclonal IgG (109–005–011; Jackson Immuno Research Laboratories, Pennsylvania, USA) was used. Known amounts of purified-IgA (I1010: Sigma-Aldrich, Missouri, USA) and diluted breast milk serum were used as standards and samples. To compute for the ratio of NV-IgA to total IgA, the values of NV-IgA and total IgA were calculated from the IgA standards using a nonlinear regression curve. Norovirus-specific IgA was divided by total IgA and multiplied by 1×10^6 to ensure all values were positive [25].

Determination of H-antigen by direct UEA ELISA

The secretor status of mother-infant pairs were determined by testing their saliva samples for the presence of H-antigen using *Ulex europaeus* (UEA-I) lectin (361029–2; EY Laboratories, California, USA), as previously described [26]. An absorbance value four times the negative control was considered as secretor-positive (secretor).

Data analysis

Background characteristics of mother and child pairs, secretor status, breastfeeding practice were compared within groups. Z-scores for height for age and weight for age were calculated according to the World Health Organization Anthro Survey Analyzer (https://whonutrition.shinyapps.io/anthro/). The diarrhea severity was determined using Community Diarrhea Assessment (CODA) score based on the symptoms reported by the mother or the caregiver. For each diarrhea episode, a severity score of a maximum of 15 points was assigned based on the presence and duration of fever, vomiting, anorexia, liquid stools, and the maximum number of stools taken during a 24-hour period [27].

Absolute frequencies and percentages were used to describe groups. Continuous variables were described using the median and interquartile range (IQR). The socio-demographic characteristics, the titer of NV-IgA, the ratio of NV-IgA to total IgA, and other continuous variables were compared between groups using the Kruskal-Wallis test. Categorical variables, including socio-demographic and clinical characteristics, socio-economic status, and percent positivity of NV-IgA were analyzed using the Chi-square test or Fisher's exact test. In addition, multiple pairwise comparison tests were performed when the p-value for comparison among three groups was $<$ 0·1, using the Dunn test for continuous variables and Fisher's exact tests for categorical variables. The Spearman correlation coefficient was used to assess the pairwise correlation and cross-reactivity between NV-IgA against GII·4 variants and non-GII·4 genotype VLPs. A non-parametric two-way ANOVA was employed to compare the titer of total IgA levels taken during different collection periods. A p-value of $<$0·05 was considered statistically significant for these analyses. The statistical analysis was performed using R version 3·3·3 and GraphPad Prism version 6·7.

Role of the funding source

The sponsors of this study had no role in the study design, data, sample collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all samples and data and had final responsibility for the decision to submit for publication.

Results

Clinical and sociodemographic characteristics of mother-infant pairs

In total, 307 mothers were enrolled in the birth cohort study, after applying the inclusion criteria, a subset of 95 mother-infant pairs was selected and classified as 18 NV+D+, 37 NV+D-, and 40 NV-D- groups (Figure 1). Within 55 mothers in the NV+ groups, only one mother in NV+D- group had secretor-negative status (non-secretor). The mothers in the NV-D- group had a lower proportion of secretor individuals than those in the NV+D+ (85% vs. 100%, p=0·16) and NV+D- (85% vs. 97%, p=0·11) groups, although it was not statistically significant. There was no difference in the duration of breastfeeding and duration of exclusive breastfeeding. However, the NV-D- group had a higher percentage of days of exclusive breastfeeding practice than the NV+D + group [100·0 (IQR: 86·3–100·0) vs. 97·4 (IQR: 37·0–100·0), p=0·06] with borderline significance and NV+D- [100·0 (IQR: 86·3–100·0) vs.

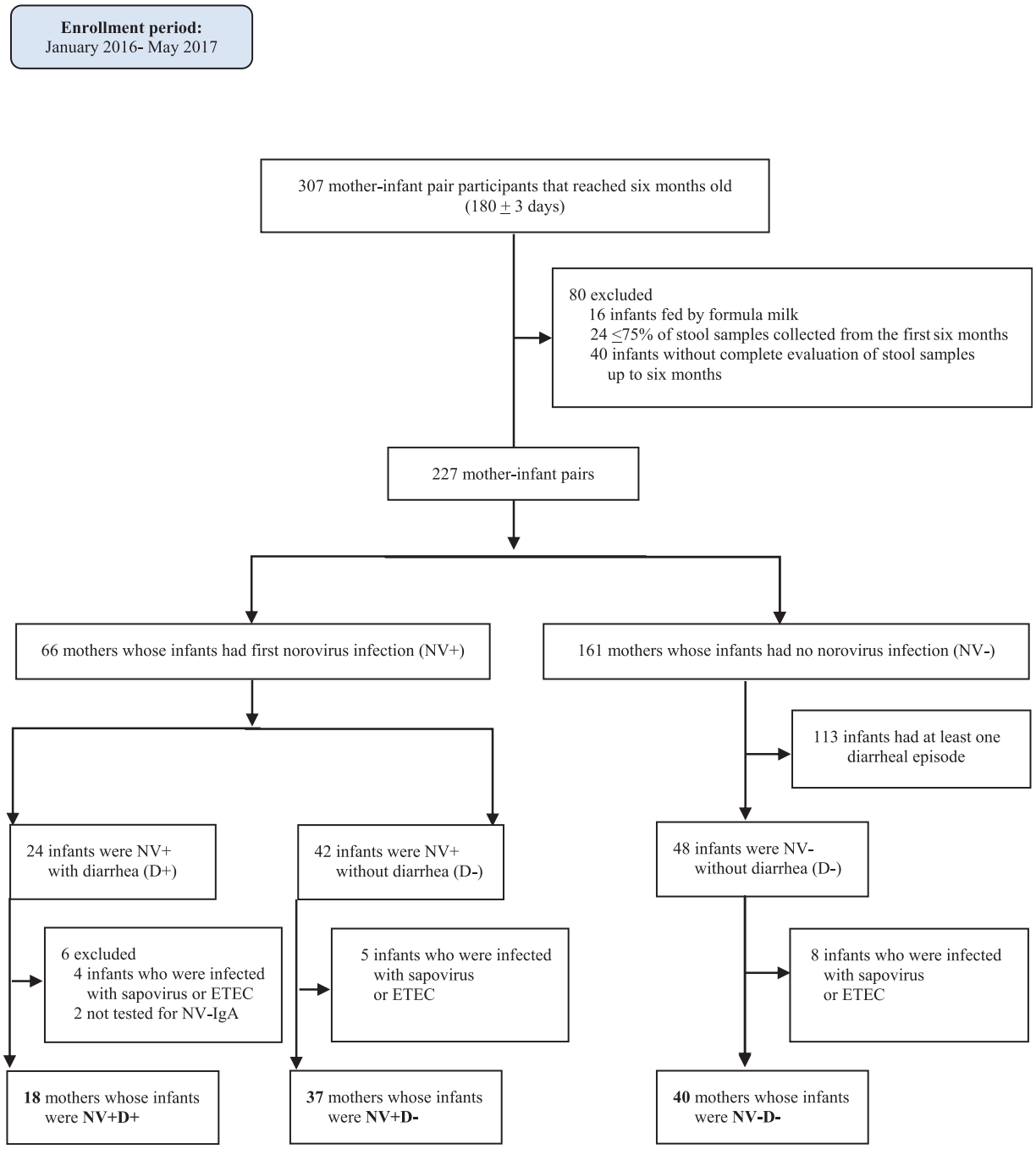

Figure 1. Study profile
Notes: NV+D+: norovirus positive with diarrhea group. NV+D-: norovirus positive without diarrhea group. NV-D-: norovirus negative without diarrhea group.

85·5 (IQR: 48·9–100·0), p=0·02] groups with statistical significance. No significant differences were observed in the age, civil status, education, number of previous pregnancies, type of delivery, and socioeconomic status of mothers. A higher proportion of mothers who used garbage trucks for waste disposal against elimination of trash in an open field or container was observed in the NV+D- group than the NV+D+ group (97% vs. 78%; p=0·04). Significantly lower proportion of mothers who always performed handwashing before food preparation was observed in the NV+D+ group than the NV-D- group (72% vs. 95%; p=0·02) (Table 1, Supplemental Table 1).

The median z-scores for height for age before the first NV infection of infants was lower in the NV+D+ group than the NV+D- [NV+D+: -0·8 (IQR: -1·3–0·0) vs. NV+D-: +0·02 (IQR: -0·7–1·0), p=0·05] and NV-D- [NV+D+: -0·8 (IQR: -1·3–0·0) vs NV-D-: +0·2 (IQR: -0·6–0·5), p=0·10] groups although it was not statistically significant. Based on CODA, 28% and 72% of infants of the NV+D+ group had mild and moderate diarrhea, respectively. No significant differences were observed in the sex, birth weight, gestational age, rotavirus vaccination history, secretor status, blood type, age at the time of the first NV infection, z-scores for height for age after the first NV infection, Z-scores for weight for age before and after the first NV infection, and cycle quantification (Cq) value of NV real-time PCR when we compared the infants in all groups (Table 2).

Norovirus genotypes in stool samples of infants

Norovirus GII·4 Sydney was the most commonly detected capsid genotype in the NV+D+ (56%) and NV+D- (24%) groups. In the NV+D+

Table 1
Socio-demographic and clinical characteristics of mothers

Characteristics	Group			*P*-value*			
	A: NV+D+(n= 18)	**B: NV+D-**(n= 37)	**C: NV-D-**(n= 40)	A vs. B vs. C	A vs. B	A vs. C	B vs. C
Age in years median (IQR)	32·1 (26·9–35·5)	32·5 (27·4–39·2)	31·4 (26·9–38·0)	0·91	–	–	–
Civil status							
Single (%)	1 (6)	3 (8)	5 (12)				
Married (%)	17 (94)	34 (92)	35 (88)	0·73	–	–	–
Education							
Primary (%)	3 (17)	2 (6)	5 (12)				
High school (%)	11 (61)	29 (7)	24 (60)				
Technical/ University (%)	4 (22)	6 (16)	11(28)	0·42	–	–	–
Number of previous pregnancies	9 (50)						
1–2 (%)	9 (50)	12 (32)	20 (50)				
> 3 (%)		25 (68)	20 (50)	0·24	–	–	–
Type of delivery							
Normal spontaneous delivery (%)	8 (44)	27 (73)	29 (73)				
Caesarian section (%)	10 (56)	10 (27)	11 (27)	0·07	0·07	0·07	1·00
Secretor status							
Secretor (%)	18 (100)	36 (97)	34 (85)	0·08	1·00	0·16	0·11
Non-secretor (%)	0 (0)	1 (3)	6 (15)				
Duration of breastfeeding before NV infection of infant (months)† median (IQR)	3·3 (2·3–4·5)	2·9 (2·2–4·4)	2·6 (1·6–4·9)	0·63	–	–	–
Duration of exclusive breastfeeding before NV infection of infant (months)‡ median (IQR)	2·2 (0·8–3·5)	2·2 (0·7–3·1)	2·6 (1·6–4·3)	0·27	–	–	–
Percentage (%) of days of exclusive breastfeeding before NV infection of infant¶ median (IQR)	97·4 (37·0–100·0)	85·5 (48·9–100·0)	100·0 (86·3–100·0)	**0·02**	1·00	0·06	**0·02**

NV: norovirus, D: diarrhea, IQR: interquartile range.
* P-values were computed using either Kruskal-Wallis test, Chi-square test or Fisher exact test for comparisons of three groups.
† Duration of breastfeeding (exclusive or non-exclusive) from the time the mothers were recruited for the cohort study to the time the infant had a NV infection.
‡ Duration from the time the mother and children participated into the study to the time the infant had a NV positive stool sample.
¶ Number of days of exclusive breastfeeding over the total number of breastfeeding days before the infant had a NV positive stool sample.

Table 2
Socio-demographic and clinical characteristics of infants

Characteristics	Group			P-value*			
	A: NV+ D+ (n= 18)	B: NV+ D−(n= 37)	C: NV− D−(n= 40)	A vs. B vs.C	A vs. B	A vs. C	B vs. C
Sex					–	–	–
Male (%)	10 (55)	19 (51)	18 (45)	0·73			
Female (%)	8 (45)	18 (49)	22 (55)				
Age in month† median (IQR)	4·4 (2·8–5·1)	3·4 (2·6–4·9)	3·5 (2·5–5·4)	0·56	–	–	–
Birth weight (kg) median (IQR)	3·4 (3·0–3·8)	3·6 (3·2–4·0)	3·6 (3·3–3·9)	0·20	–	–	–
Gestational age (week) median (IQR)	39·0 (38·5–40·0)	39·0 (39·0–40·0)	40·0 (39·0–40·0)	0·42	–	–	–
History of rotavirus vaccine							
Yes (%)	16 (89)	37 (100)	37 (93)	0·13	–	–	–
No (%)	2 (11)	0 (0)	3 (7)				
Secretor status							
Positive (%)	18 (100)	37 (100)	40 (100)	1·00	–	–	–
Negative (%)	0 (0)	0 (3)	0 (0)				
Blood type							
A (%)	5 (28)	4 (13)	9 (23)	0·28			
B (%)	1 (5)	3 (8)	3 (7)		–	–	–
O (%)	12 (67)	30 (79)	28 (70)				
Height for age (Z-score) before the infection median (IQR)	-0·8 (-1·3–0·0)	0·02 (-0·7–1·0)	0·2 (-0·6–0·5)	**0·04**	0·05	0·10	1·00
Height for age (Z-score) after the infection median (IQR)	-0·2 (-1·0–0·4)	0·0 (-0·6–0·9)	0·02 (-0·5–0·8)	0·32	–	–	–
Weight for age (Z-score) before the infection median (IQR)	0·5 (-0·2–1·2)	0·7 (-0·1–1·6)	0·6 (-0·1–1·2)	0·80	–	–	–
Weight for age (Z-score) after the infection median (IQR)	1·0 (-0·4–1·4)	0·7 (0·1–1·5)	0·8 (0·0–1·3)	0·94	–	–	–
Cq value of NV real-time RT-PCR result median (IQR)	26·9 (24·2–31·7)	26·8 (23·0–32·5)	–	0·82	–	–	–
Severity of diarrhea							
Mild (%)	5 (28)						
Moderate (%)	13 (72)	–	–	–	–	–	–
Severe (%)	0 (0)						

NV: norovirus, D: diarrhea, IQR: interquartile range. Cq value: cycle quantification value, RT-PCR: reverse transcription PCR.
* P-values were computed using either Kruskal-Wallis test, Chi-square test or Fisher exact test for comparisons of three groups.
† Age of the infant when NV was positive in the stool sample. The samples from group C (NV-D-) were age-matched with NV+ (A and B) groups.

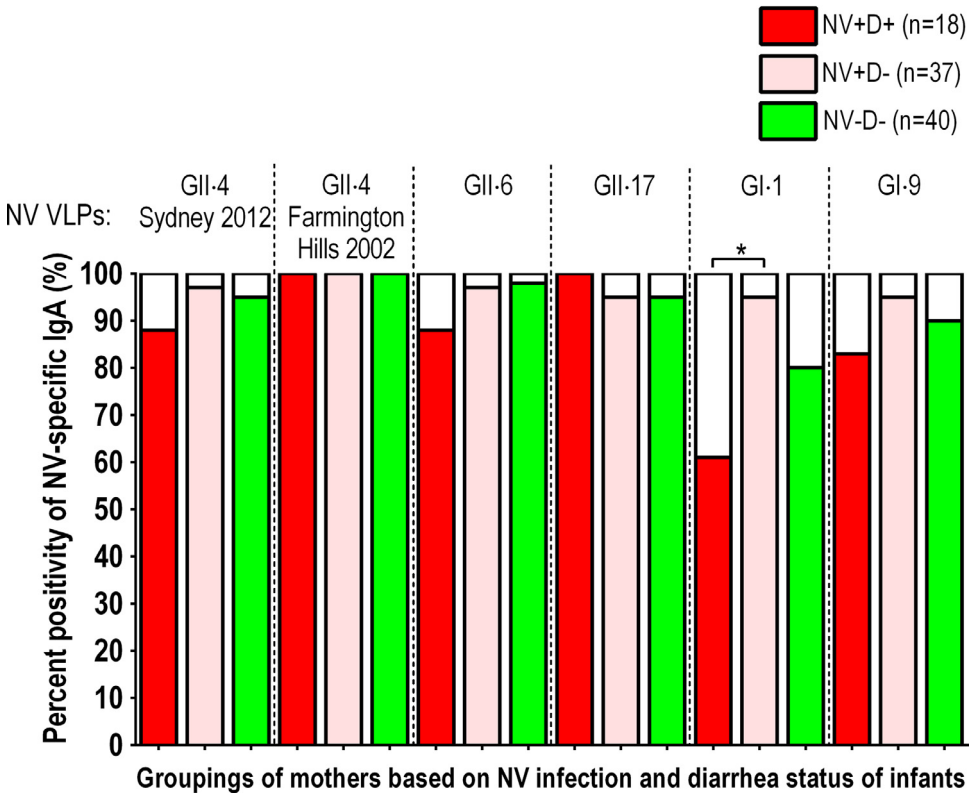

Figure 2. Percent positivity (%) of NV-specific IgA against and GII·4 variants and non-GII·4 genotype VLPs in breast milk taken before the first NV infection of infants.
Notes: Breast milk samples from NV-D- were age-matched with NV+D+ and NV+D- groups.
NV+D+: norovirus positive with diarrhea group. NV+D-: norovirus positive without diarrhea group.
NV-D-: norovirus negative without diarrhea group. NV: norovirus. VLPs: virus-like particles.
IgA: immunoglobulin A. Sample size (n) per group: NV+D-, n=18; NV+D-, n=37; NV-D-, n=40.
*$p<0{\cdot}05$ (Chi-square test or Fisher's exact test for comparison among three groups and Fisher's exact test for pairwise comparison).

group, the next most frequently detected genotypes were GII·1 (17%) and GII·17 (12%); while GII·1 (11%), GII·6 (11%), and GII·17 (11%) were the next most commonly detected genotypes in the NV+D- group (Supplemental Table 2).

Percent positivity and titer of norovirus-specific IgA against GII·4 variants and non-GII·4 genotype VLPs

All 95 breast milk samples contained IgA that reacted to GII·4 Sydney 2012, GII·4 Farmington Hills 2002, GII·6, GII·17, GI·1, and GI·9 VLPs. A significantly higher percent positivity of NV IgA was observed against GII than in GI [(89–100%) vs (61–92%), p=0·03] VLPs. Among the three groups, the NV+D- group had the highest percent positivity of NV IgA, except for GII·4 Farmington Hills and GII·17. Significantly higher percent positivity of NV IgA was observed in the NV+D- group than in the NV+D+ group against GI·1 (89% vs. 61%, p=0·03) (Figure 2). Like the breast milk samples collected before the first NV infection of the infants, breast milk samples collected during the first NV infection of infants contained different NV-IgA types. The percent positivity of NV-IgA against GII was still higher when compared to GI VLPs although it was not statistically significant [(86–100%) vs. (57–100%), p=0·50]. Among the three groups, the NV+D- group had higher percent positivity of NV-IgA against GII·6 (100% vs. 86%, p=1·00), GI·1(100% vs. 57%, p=0·19) and GI·9 (100% vs. 86%, p=1·00) than the NV+D+ group although it was not statistically significant (Supplemental Figure 1).

A higher median titer of NV IgA was observed in the NV+D- group than the NV+D+ group using GI·1[1:100 (IQR: 1:50–1:500) vs. 50 (IQR: 0–1:100), p=0·01]. Similar titers were observed in NV IgA against GII·4 Farmington Hills, GII·6, and GII·17 VLPs in all groups (Figure 3). When we analyzed the breast milk samples collected during the first NV infection of infants, the median titer of NV-IgA against GI and GII VLPs was still lowest in the NV+D+ group than the NV+D- and NV-D- groups. However, it was statistically significant when we compared the NV+D+ and NV-D- groups in the NV-IgA against GII·17 [1:100 (IQR: 1:100–1:100) vs. 1:1000 (IQR: 1:550–1:1000), p=0·03] (Supplemental Figure 2).

Cross-reactivity of norovirus-specific IgA against GII·4 variants and non-GII·4 genotype VLPs

Breast milk NV-IgA was assessed for potential cross-reactivity where NV-IgA against intra-variants showed a high ρ value (0·87, $p<0.001$), whereas against intragenogroup showed moderate to moderately high (0·49–0·77, $p<0.001$) and against intergenogroup showed low (0·23–0·37, $p<0.01$) ρ values respectively (Figure 4).

Monthly levels of norovirus-specific IgA and total IgA

For each of the NV+D+ or NV+D- group, we selected one mother who had an NV-positive stool sample to determine the dynamics of NV-IgA and total IgA in breast milk. The mother (SP301Y) from the NV+D+ group with a GII·4 Sydney positive stool, had a 50-fold increase in the NV-IgA titer against GII·4 variants but not against non-GII·4 genotypes and 96 days later, she had a 50–200-fold increase of NV-IgA against GI VLPs. While the mother (SP305Y) from the NV+D- group, who had a GII·6 positive stool sample, had a 10-fold increase in the titer of NV-IgA against GII·6 but not against other non-GII·4 genotypes and GII·4 variants and 93 days later, she had a 20-fold increase in NV-IgA against GII·4 variants. Finally, the mother (SP067Y) from the NV-D- group had similar titers of NV-IgA against

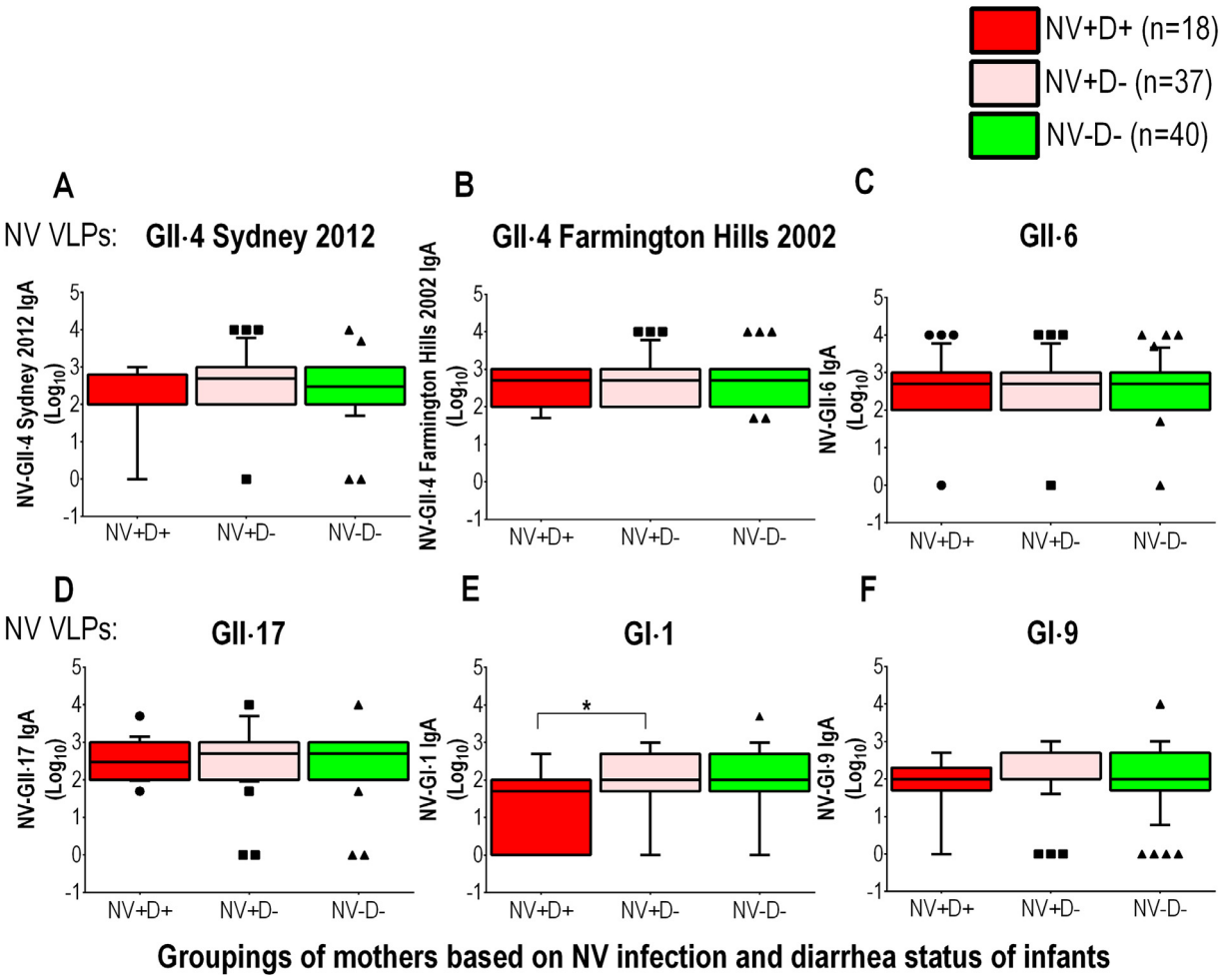

Figure 3. Titer of NV-specific IgA against GII·4 variants and non-GII·4 genotype VLPs in breast milk taken before the first NV infection of infants.
Notes: Breast milk samples from NV-D- were age-matched with NV+D+ and NV+D- groups.
Values are 10 and 90% percentile and median. NV+D+: norovirus positive with diarrhea group.
NV+D-: norovirus positive without diarrhea group. NV-D-: norovirus negative without diarrhea group.
NV: norovirus. VLPs: virus-like particles. IgA: immunoglobulin A.
Sample size (n) per group: NV+D-, n=18; NV+D-, n=37; NV-D-, n=40.
*p<0·05 (Kruskal-Wallis test for comparison among three groups and Dunn test for pairwise comparison).

all VLPs in all collection periods. All three mothers used in this analysis had secretor status (Supplemental Figure 3).

The total IgA (ng/ml) in breast milk during the first six months postpartum were analyzed in 20 secretor- mothers including seven NV+D+, eight NV+D-, and five NV-D- mothers. The lowest total IgA level was observed in breastmilk samples during 5th month postpartum when compared with 0th [$1{\cdot}2 \times 10^6$ (IQR:$0{\cdot}4 \times 10^6$–$1{\cdot}9 \times 10^6$) vs. $0{\cdot}5 \times 10^6$ (IQR:$0{\cdot}3 \times 10^6$–$0{\cdot}7 \times 10^6$), p=0·03] and 6th [$1{\cdot}1 \times 10^6$ (IQR:$0{\cdot}5 \times 10^6$–$1{\cdot}4 \times 10^6$) vs. $0{\cdot}5 \times 10^6$ (IQR:$0{\cdot}3 \times 10^6$–$0{\cdot}7 \times 10^6$), p=0·03] months postpartum in the mothers from the NV+D+ group (Supplemental Figure 4).

Discussion

Breastfeeding is known to contribute to providing adequate nutrition and demonstrates a dose-response relationship in protection against diarrheal morbidity and mortality in infancy [28]. The effectiveness of breastfeeding against diarrhea can be attributed to its active components such as IgA antibodies, which are vertically transferred to the infants.

We showed that breast milk samples collected before and during the first NV infection of infants contained multiple NV-IgA using an in-house ELISA-based method which was concordant with the previous studies on postpartum breast milk [14–16]. In our study, Peruvian mothers had a higher percent positivity (61–100%) of NV-IgA in breast milk than mothers in these studies. This may be related to the antigens used for testing or the high proportion of secretor-positive mothers, which may have had more history of NV exposure [25].

Mothers with breast milk that contained higher titer of NV-IgA against GII·4 Sydney, GI·1, and GI·9 appeared to have infants with reduced NV symptoms. We also observed that mothers with NV infected infants had a lower percentage of days of exclusive breastfeeding from the recruitment. These results suggest the possibility of a protective effect of NV-IgA in the breast milk against NV infection and associated diarrhea. The protective mechanism of the mucosal IgA has been reported as IgA temporarily coats the gastrointestinal microbiota [29] or block the binding site of NV to human cells [30]; therefore it may consequently reduce diarrheal symptoms in infants. However, we were not able to measure NV-IgA levels in the infant stool nor serum in this study. Thus, the detailed protective mechanisms need to be further investigated including the presence of other bioactive components in breast milk such as IgG, human milk oligosaccharides, and lactoferrin. The level of exposure, immunological, nutritional status of infants, or secretor-positive status which can increase the susceptibility of infants to NV infection [31,32] are also needed to be adjusted in a large-scale study.

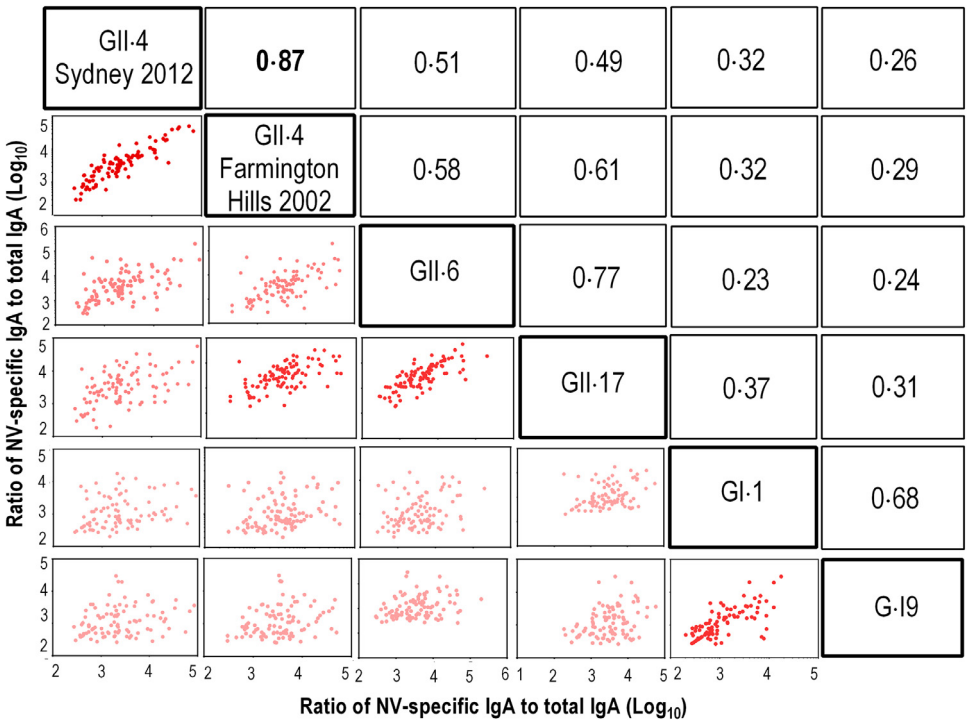

Figure 4. Cross-reactivity of NV-specific IgA against GII·4 variants and non-GII·4 genotype VLPs in breast milk taken before the first NV infection of infants.

Notes: Breast milk samples from NV-D- were age-matched with NV+D+ and NV+D- groups.

The lower left triangle is the multiple scattered plot of $\log_{10}$- transformed ratio of norovirus-specific immunoglobulin A to total immunoglobulin A while the top right triangle indicates Spearman correlation coefficient values (ρ) between each norovirus-specific immunoglobulin A pair with the highest ρ-values being highlighted. Sample size (n): 95. NV +D+: norovirus positive with diarrhea group.

NV+D-: norovirus positive without diarrhea group. NV-D-: norovirus negative without diarrhea group. VLPs: virus-like particles. IgA: immunoglobulin A.

In our study, NV-IgA antibodies in breast milk were cross-reactive which is concordant with a previous study that tested serum antibodies of children with NV infection [33], The level of correlations between the concentration of NV-IgA against different VLPs was associated with genotype classification of NV based on the genetic distances [34], which was also suggested in the patterns of repeated infection [1]. We also found some degree of cross-reactivity between different genogroups. A recent study showed that monoclonal antibodies against specific NV VLPs were able to bind with epitopes that are common in the capsid region of GI and GII genogroups despite having different genotypes. [35] Hence, the cross-reactivity of breast milk NV-IgA in our study highlighted the possibility of the NV-IgA to react to epitopes that are conserved in GI and GII that may affect the evaluation of the association between levels of antibody in the breast milk and infant diarrhea.

Norovirus-specific IgA antibodies were detectable in breast milk until six months postpartum, which is similar to the duration of rotavirus-specific and *Giardia lamblia*-specific IgA in breast milk [36,37]. We showed high titer of NV-IgA antibodies even before their infant had NV infection. This could be attributed to the mother's NV infection prior to the infant infection, or constant NV exposure of mothers in the community. Although we only observed the timeline of NV-IgA in three mothers, we need to have more direct and conclusive evidence to prove this hypothesis.

In the previous studies, humoral and salivary NV-IgA showed protection against severe NV infection among children but NV-IgA in breast milk against NV-associated diarrhea in infants has not been reported [38–40]. To our knowledge, this is the first study, which was able to collect samples from mother-infant pairs and evaluated for breast milk IgA against different NV VLPs when NV was detected for the first time in infants' life.

The limitations of this study were a relatively small number of mothers in the NV+D+ group, not having information on the past or current NV infections of mothers, and only a few mothers' stool samples collected. More than 85% of the participants were secretor-positive, therefore they are more susceptible to norovirus infection, which might account for the high positivity rates and titers of NV-IgA. To elucidate the protective role of breast milk IgA against NV infection, future studies must be conducted using a larger population, having more proportion of secretor- negative mothers, gathering information on the previous infection of the mothers, and evaluating serum and stool samples of infants.

In conclusion, mothers with high positivity rates and titers of breast milk NV-IgA had NV infected infants with reduced diarrheal symptoms. Antigenic relatedness to the genetic diversity of human norovirus was suggested. Further investigation regarding the protective mechanism of maternal IgA antibodies is needed. The presence and levels of NV-IgA in breast milk may need to be considered in the administration of NV vaccines once it is available. Our study reinforced the need to continue breastfeeding practice up to one year to promote the health of both mothers and infants.

Declaration of Competing Interest

HKML, MJP, KT, LAFS, RHG, LC, HM, GJS, ATC, CB, CD, TN, and GIP have no conflict of interest. HO reports grants from AMED and grants from the Ministry of Land, Infrastructure, Transport and Tourism, and Japan Science and Technology Agency, outside the submitted work. MS reports grants from National Institute of Health (NIH) and Japan Society for the Promotion of Science (Fostering Joint International Research B) during the conduct of the study; grants from the Ministry of Land, Infrastructure, Transport and Tourism, Japan Science and Technology Agency, Japan Society for the Promotion of Science, and Japan Agency for Medical Research and Development (AMED), outside the submitted work.

Authors Contributions

HKML designed, implemented, and managed this study; performed the laboratory assays and data analysis and develop the main manuscript. MJP designed, managed, coordinated fieldwork, sample collections, and data analysis in Peru. RHG acquired the grant funds; supervised the study. LC supervised the cohort study and sample and data collection in the field. CB contributed to the design of the study, acquisition of grant funds, and editing of the manuscript. HM, GJS and ATC did the laboratory assays and genomic analysis in Peru. KT, LAFS, and GIP provided VLPs, supervised the laboratory assays and interpretation, and edited the manuscript. CD oversaw the laboratory assays and analysis in Japan and edited the manuscript. TN and HO supervised the mucosal and virological aspects of this study respectively. MS designed, implemented, and managed the study and acquired the grant funds. All authors reviewed the draft and approved the final manuscript submitted for publication.

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Data Sharing Statement

The data that support the findings of this study are available from the corresponding author, M.S., upon reasonable request.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.eclinm.2020.100561.

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