

ARTICLE



Detection of SARS-CoV-2 IgA and IgG in human milk and breastfeeding infant stool 6 months after maternal COVID-19 vaccination

Lauren Stewart Stafford^{1,8}, Vivian Valcarce^{2,8}, Matthew Henry², Josef Neu², Leslie Parker³, Martina Mueller⁴, Valeria Vicuna¹, Taylor Gowen², Emilee Cato⁵, Ivan Kosik⁶, Jonathan Wilson Yewdell⁶, Mark Atkinson⁷, Nicole Cacho², Nan Li² and Joseph Larkin III¹✉

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OBJECTIVE: Assess presence, durability, and neutralization capacity of SARS-CoV-2-specific antibodies in breastfeeding infants' stool, mother's plasma and milk following maternal vaccination.

DESIGN: Thirty-seven mothers and 25 infants were enrolled between December 2020 and November 2021 for this prospective observational study. All mothers were vaccinated during lactation except three, which were vaccinated during pregnancy. Milk, maternal plasma, and infants' stool was collected pre-vaccination and at periods up to 6 months following COVID-19 vaccine series initiation/completion. SARS-CoV-2 antibody levels and their neutralization capacities were assessed.

RESULTS: SARS-CoV-2-specific IgA and IgG levels were higher in infant stool post-maternal vaccination amongst milk-fed compared to controls. Maternal SARS-CoV-2-specific IgA and IgG concentrations decreased over 6 months post-vaccination but remained higher than pre-vaccination levels. We observed improved neutralization capacity in milk and plasma after COVID-19 vaccination.

CONCLUSIONS: The presence of SARS-CoV-2-specific antibodies in infant stool following maternal vaccination offers further evidence of the lasting transfer of these antibodies through breastfeeding.

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INTRODUCTION

The grievous consequences of COVID-19 infection are widely known. With the upsurge of new variants [1], we have witnessed how COVID-19 has affected the US (United States) population, including pregnant women [2] and children [3].

Vaccination during pregnancy is known to generate functional anti-spike IgG antibodies in maternal circulation that are detectable in umbilical cord blood at birth and can protect the infant from COVID-19 [4–7]. Further, recent studies have demonstrated that COVID-19 infection, as well as vaccination against COVID-19, induces secretion of neutralizing SARS-CoV-2 IgA and IgG in human milk (HM) [8–16]. However, little is known about the transfer of neutralizing HM antibodies to breastfeeding infants.

HM has antimicrobial properties, evidenced by the decreased rate of infections and hospitalization among breastfeeding infants [17]. This can be attributed to molecules with immuno-protective functions within HM: immunoglobulins, lactoferrin, oligosaccharides, and cytokines, to name a few [18]. HM antibodies, in particular, sIgA, prevent the entry of microorganisms into the tissue by decreasing surface colonization and dampening penetration of potentially harmful organisms [19].

SARS-CoV-2 relies on its main receptor, angiotensin-converting enzyme 2 (ACE2), to enter cells, which is abundantly present in the human epithelia of the lung, small intestine, and colon [20, 21]. Reports have shown that a notable proportion of patients with COVID-19 develop gastrointestinal symptoms and nearly half of patients have detectable SARS-CoV-2 RNA in their fecal samples [22, 23].

To protect against SARS-CoV-2 infection, the immune system must mount a robust and specific response. In addition to SARS-CoV-2-specific antibody concentration, antibody specificity, affinity, and neutralizing capacity are also necessary to elicit a proper immune response. As such, we hypothesize that there is a transfer of neutralizing HM SARS-CoV-2 specific IgA and IgG to the intestinal tract of breast-fed infants that can protect them from COVID-19 infection. Together our results provide interesting insight into the relationship between the durability of maternal antibodies subsequent to vaccination, and the potential protection of breastfed infants.

METHODS

Study design

This prospective observational study was conducted at the University of Florida under institutional review board approval (#IRB202003255). Thirty-

¹Department of Microbiology and Cell Science, University of Florida, Gainesville, FL, USA. ²Department of Pediatrics, University of Florida, Gainesville, FL, USA. ³College of Nursing, University of Florida, Gainesville, FL, USA. ⁴College of Nursing, Medical University of South Carolina, Charleston, SC, USA. ⁵Emerging Pathogens Institute, University of Florida, Gainesville, FL, USA. ⁶Laboratory of Viral Diseases, National Institutes of Health/National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA. ⁷Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida Diabetes Institute, Gainesville, FL, USA. ⁸These authors contributed equally: Lauren Stewart Stafford, Vivian Valcarce. ✉email: jlarkin3@ufl.edu

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seven breastfeeding mothers were recruited before or after COVID-19 vaccination with Pfizer/BioNTech, Moderna, or Johnson & Johnson from December 2020 to November 2021. All mothers were vaccinated during lactation except three, who were vaccinated during pregnancy. In June 2021, we added breastfeeding infant enrollment for stool collection. A total of 25 infants were enrolled from June to November 2021. Mothers contributed data at various time points up to 6 months after completing the series (8 mothers provided longitudinal HM and 7 provided plasma samples at pre-vaccination, post-1st dose, 7–30 days, and 6 months post-vaccination). Inclusion criteria included breastfeeding women 18 years and older, pre-or-post-COVID-19 vaccination, and providing informed consent.

Participants and procedures

Recruitment methods included the University of Florida's institutional e-mail listserv and advertising flyers posted in University of Florida facilities and the surrounding community. Up to 37 mothers and 25 infants were recruited at different time points; of those, 3 mother's and 1 infant samples were not included in the analysis (1 mother stopped breastfeeding prior to first vaccination; and two participants received the J&J vaccine). Given significant differences in effectiveness and antibody response with J&J compared to mRNA vaccines, those two mother-infant dyads were excluded from the analysis. 96 maternal serum, 101 maternal milk, and 31 infant stool samples were collected up to 6-time points relative to COVID-19 vaccination completion: pre-vaccination, 15–30 days after the first vaccine dose (for participants receiving two-dose mRNA vaccines), and then at 7–30 days, 60–75 days, 90–105 days, and 6 months following 2-dose vaccination series completion. Not all participants contributed samples at every listed collection time point (Supplementary Table 1).

Participants completed a questionnaire collecting maternal/infant demographics, medical and family history, and vaccination side effects upon agreeing to participate.

Sample collection and processing

Maternal blood samples were collected via venipuncture or finger prick in ethylenediaminetetraacetic acid-coated (EDTA) tubes at the designated time points. Plasma was separated from cellular matter by centrifugation at $2000 \times g$ for 10 min at 4 °C and then stored undiluted at –20 °C.

For milk, mothers were instructed to express samples of 10–30 ml. Samples were stored at –20 °C within 4 h after collection. The samples were aliquoted into 2 mL tubes, then centrifuged at 500 g for 15 min at 4 °C. Using a 21 G needle, the aqueous layer was separated from the fat layer and placed in a clean tube. This aqueous layer was then centrifuged at $3000 \times g$ for 15 min at 4 °C. The final aqueous layer was removed and stored undiluted at –20 °C.

Stool samples were collected in diapers and dropped off either the same day or kept refrigerated overnight by participants before their collection appointments, then stored at –80 °C until further processing. Stool samples were then diluted with 2 mL DPBS, vortexed vigorously to achieve a homogenized sample, and centrifuged at $1500 \times g$ for 20 min. The supernatant was then placed in a clean tube, vortexed, and centrifuged at $10,000 \times g$ for 10 min. Supernatant was removed and stored undiluted at –20 °C.

SARS-CoV-2-specific IgA and IgG concentrations were measured in HM, plasma, and infant stool using previously-validated COVID-19 Human IgA and IgG ELISA kits (RayBiotech Life, Peachtree Corners, Georgia, USA) [10]. SARS-CoV-2-specific IgA samples were run at 1:500 dilution in plasma. SARS-CoV-2-specific IgG samples were run at 1:1000 dilution in plasma. SARS-CoV-2-specific IgA samples were run at 1:3 dilution in HM. SARS-CoV-2-specific IgG samples were run undiluted (1:1) in HM. SARS-CoV-2-specific IgA and IgG samples were run undiluted (1:1) in infant stool. We used 10 pre-COVID-era and 1 maternal pre-vaccination infants stool samples as negative controls. All samples were run in duplicate.

To determine the neutralization capability of SARS-CoV-2 antibodies, we used an assay containing vesicular stomatitis virus (VSV) expressing the SARS-CoV-2 Wuhan-hu-1 wild type strain spike protein and green fluorescent protein (GFP) (VSV-gfp-SARS-CoV-2-S-gp) described in a previously published study [24]. Milk, plasma, or stool samples were mixed with VSV and later incubated with baby hamster kidney (BHK) cells expressing human ACE2 receptors. GFP frequency was measured by flow cytometry to quantify BHK-ACE2 cell infectivity. The raw GFP+ values of each sample were normalized to a scale of 0–100% infectivity using negative and positive controls (Supplementary Fig. 2). The normalized values were then used to make non-linear regression curves of infectivity, providing IC50 values for each sample. All samples were run in duplicate.

Statistical analysis

The demographics and clinical features of the study sample were characterized via descriptive statistics. Medians with 25th and 75th percentiles (IQR1 and IQR3) were provided for log-transformed [10] SARS-CoV-2 specific milk, plasma, and stool IgA and IgG levels at all time points (Supplementary Table 1).

Mann–Whitney U-tests (unpaired, nonparametric, two-tailed) were used to compare antibody concentrations in infant stool after mother's COVID-19 vaccination vs. pre-COVID era infant stool samples across available time points; and IC50 in milk and plasma pre-vaccination vs. 7–30 days post-2nd dose and six months post-vaccination series completion. Spearman correlation (two-tailed) was carried out to explore the relationship between log-transformed [10] SARS-CoV-2 specific IgA and IgG in milk, plasma, and infant stool at 6 months; and IgA and IgG in milk, plasma with IC50 in milk and plasma at three-time points (pre-vaccination, 7–30 days post-2nd dose, and six months post-vaccination series completion). Statistical test results were reported as p-values. Statistical analyses were performed using SPSS statistics software and GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). All figures were created using GraphPad Prism 9. Center values of each figure were shown as geometric mean with geometric mean SD.

RESULTS

Thirty-seven lactating women and 25 infants were enrolled in the study. Of those, 34 women and 24 infants were included in the statistical analysis (please refer to methods section); 8 women completed longitudinal samples for HM and 7 for plasma at pre-vaccination, post-1st dose, 15–30 days post-second dose, and 6 months after 2-dose vaccination series completion (Supplementary Fig. 1). Pre-vaccination samples consisted of 25 HM and 16 plasma samples; 6-month samples included 16 HM and 19 plasma samples. Thirty-one infant's stools samples were collected up to 6 months post-vaccination. The study population consisted primarily of White non-Hispanic women in their mid-30s and their infants with a median infant age of 10 months at enrollment (Table 1).

SARS-CoV-2 IgA and IgG are present in the stool of breastfeeding infants after maternal COVID-19 vaccination and stool contents can neutralize the pseudovirus in vitro

We detected SARS-CoV-2 IgA and IgG in the stool of breastfeeding infants whose mothers received COVID-19 vaccination, with a statistically significant increase in IgG relative to negative controls ($p = 0.08$ and <0.0001 in IgA and IgG, respectively) (Fig. 1). Infant stool IgG concentrations were positively correlated with maternal plasma and HM IgG concentrations at six months post vaccination ($p = 0.0475$, $R = 0.59$ and $p = 0.0092$, $R = 0.73$, respectively) (Supplementary Table 2).

Although breastfeeding infant's stool, obtained both prior and subsequent to maternal vaccination could neutralize pseudovirus activity in vitro; the ability to neutralize pseudovirus activity was enhanced after maternal vaccination. Neutralization capacity is measured by the half-maximal inhibitory concentration (IC50): hence the lower the IC50, the stronger the neutralization capability. The geometric mean IC50 for infant stool samples after mother's vaccination was 0.94 compared to a geometric mean IC50 of 1.90 in pre-COVID negative control samples ($p = 0.4$) (Fig. 1).

SARS-CoV-2 IgA concentrations in human milk and plasma appear to decline over time but remain higher than pre-vaccination 6 months after

SARS-CoV-2-specific IgA concentrations in milk and plasma declined 6 months after vaccination, but levels remain higher than the pre-vaccination value (Fig. 2). For milk, the median for log-transformed [10] SARS-CoV-2-IgA concentration was 1.3 unit/ml pre-vaccination versus 1.6 unit/ml six months post-vaccination. In plasma, the median for log-transformed [10] SARS-CoV-2-IgA concentrations was 3.3 unit/ml pre-vaccination, compared to 3.5

Table 1. Study participants characteristics^a.

Maternal characteristics (n = 34)	N (%) or mean ± standard deviation ^a
Age (years)	33.5 ± 3.9
Race	
White	33 (97)
Asian	1 (3)
Ethnicity	
Non-Hispanic	24 (69)
Hispanic	7 (19)
Not disclosed	3 (11)
Body mass index (kg/m ²) ^{b,c}	24.3 ± 3.9
Normal/healthy weight	21 (61)
Overweight	8 (25)
Obese	4 (11)
History of allergies ^c	9 (25)
History of asthma ^c	4 (11)
History of inadequate immune response to vaccine ^c	2 (5)
History of known COVID-19 infection ^d	1 (3)
Family history of cancer ^c	19 (53)
Family history of autoimmune disorder ^c	3 (8)
Antibiotic use in 6 months before enrollment ^c	10 (29)
Time postpartum (months)	5.2 ± 6.2
Decreased human milk supply after COVID vaccine ^c	
No	27 (84)
Yes	2 (6)
Transient	3 (9)
Vaccine brand ^c	
Moderna	13 (36)
Pfizer	20 (56)
Infant characteristics (n = 24)	N (%) or mean ± standard deviation ^a
Infant gender	
Female	10 (42)
Male	14 (58)
Infant age at the time of stool collection (months)	10 ± 6
Number of stools in each infant age group ^e	
<6 months	5 (23)
6–12 months	12 (46)
13–24 months	6 (23)
>24 months	2 (8)

^aCategorical data are given as the number of participants and, in parentheses, the percentage of the total. Continuous data are provided as means ± standard deviations.

^bDefinitions put forth by the U.S. Centers for Disease Control and Prevention were used for body mass index categories [16].

^cMissing data from 1 subject.

^dPre-vaccination sample data unavailable from a subject with known prior COVID-19 infection.

^en = 24 due to one infant being in different age groups on dates of different sample collections.

units/ml six months post-vaccination (Supplementary Table 1). Furthermore, the positivity rate decreased to 53% in milk and 77% in plasma at 6 months, from 88 and 87%, respectively, at 7–30 days post-vaccination (Supplementary Table 1).

SARS-CoV-2 IgG concentration appears to decline in plasma and to a lesser degree in human milk 6 months after COVID-19 vaccination

Although SARS-CoV-2 IgG decreased in HM 6 months after vaccination, values remained higher than pre-vaccination levels (Fig. 3). Median log-transformed [10] SARS-CoV-2-IgG concentrations in HM pre-vaccination were 0.08 units/ml, versus 0.6 units/ml six months post-vaccination (Supplementary Table 1). In plasma, we observed a steep decline in SARS-CoV-2 IgG six months after vaccination compared to immediately post second dose; however, the positivity rate remained 100% above the positive cutoff in milk and in plasma after 6 months (Supplementary Table 1).

For both milk and plasma, we noticed an overall decline in SARS-CoV-2 antibody levels at six months compared to 7–30 days post-vaccination. IgG concentration in HM and plasma appears to have a more dynamic predominant response to vaccination in comparison to IgA.

Human milk neutralizing activity

Human milk obtained from pre- and post-COVID-19 vaccinated individuals neutralized the viral binding of the pseudovirus VSV-gfp-SARS-CoV-2-S-gp to the BHK21-ACE2 cell line in vitro. The geometric mean IC₅₀ (indicative of viral adherence to cells) for HM pre-vaccination was 0.14, compared to 0.03 six months post-vaccination (Fig. 4). The increase in HM neutralization capability after vaccination was statistically significant with a *p*-value of 0.05. There was heterogeneity in response, where roughly half of the participants had increased neutralization at six months compared to immediately post 2nd dose, and the other half had decreased neutralization at six months compared to post 2nd dose (Supplementary Fig. 3). This work supports current, published data that shows milk can neutralize SARS-CoV-2 in vitro after COVID-19 mRNA vaccination [8, 25] and highlight a novel finding: HM neutralizing capability to SARS-CoV-2 increased over the 6-month time span, even as SARS-CoV-2 specific antibody concentrations decreased.

Plasma neutralizing activity

We detected a statistically significant increase in plasma neutralization capability after COVID-19 vaccination (*p* < 0.0001) (Fig. 4). Before vaccination, the geometric mean IC₅₀ in plasma was 0.5. The highest neutralization occurred 7–30 days after the second vaccine dose (IC₅₀ = 0.001) and diminished six months after vaccination (IC₅₀ = 0.036) while remaining above pre-vaccination levels. Neutralization capacity increased by 99% in plasma immediately post COVID-19 vaccination completion and remained statistically, significantly higher than pre-vaccination levels six months after vaccination (*p* = 0.002). The decline in neutralization is also observed in other studies that show neutralization in blood peaks roughly two weeks following the second dose but falls in vaccinated participants over time [26, 27].

Plasma neutralization (IC₅₀) was statistically significantly correlated to plasma IgA concentrations 7–30 days post 2nd dose (*R* = −0.79, *p* = 0.009) (Supplementary Table 3). This means the higher the plasma SARS-CoV-2 IgA concentration, the higher the plasma neutralization.

DISCUSSION

Infants are born with an immature immune system, and it has been well-established that breastfeeding protects them against respiratory infections [17–19]. Prior studies have shown a significant increase in neutralizing SARS-CoV-2 specific antibodies

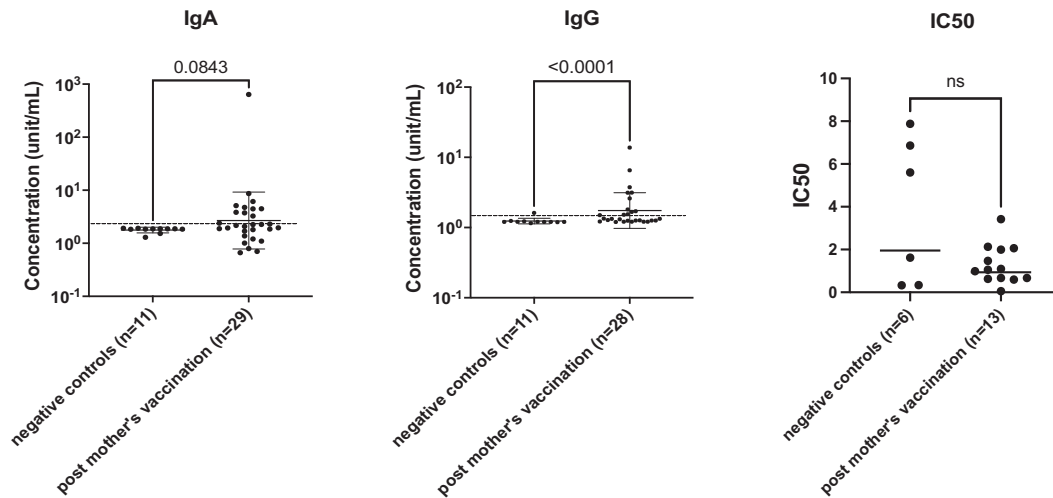


Fig. 1 SARS-CoV-2 specific antibodies (IgA and IgG) and neutralization capacity (IC50) in infant stool up to 6 months after mother's COVID-19 vaccination. IC50: the lower the IC50, the stronger the neutralization capability. Concentration (unit/mL) listed in log10 form. Both antibody concentration comparison and IC50 statistical calculations are measured using Mann-Whitney unpaired, nonparametric test; the figure is shown as geometric mean and geometric SD.

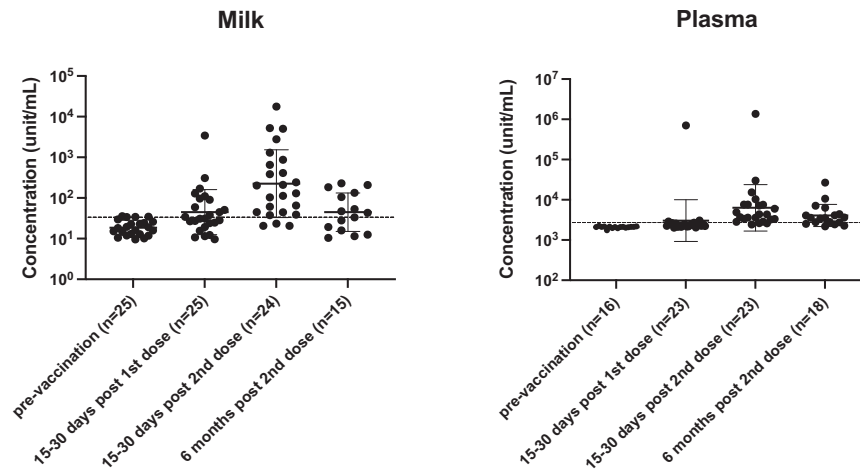


Fig. 2 SARS-CoV-2 specific IgA in milk and plasma up to six months after COVID-19 vaccination. Concentration (unit/mL) is listed in log10 form. Figures are shown as geometric mean and geometric mean SD.

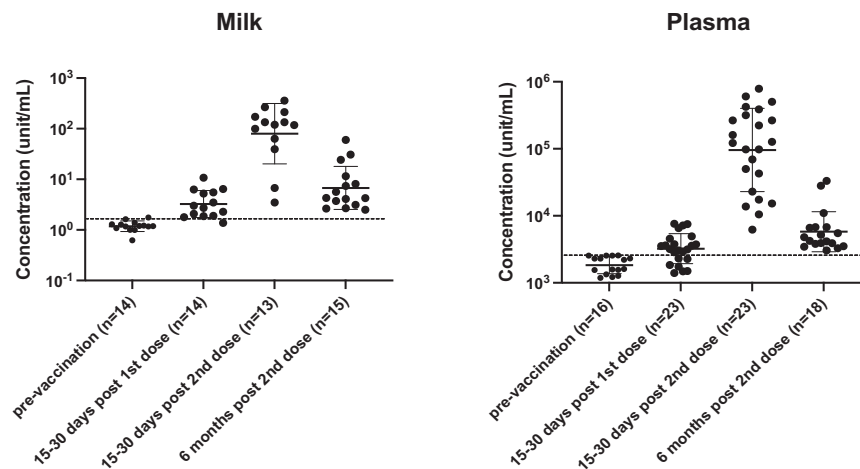


Fig. 3 SARS-CoV-2 specific IgG in milk and plasma up to six months after COVID-19 vaccination. Concentration (unit/mL) is listed in log10 form. Figures are shown as geometric mean and geometric mean SD.

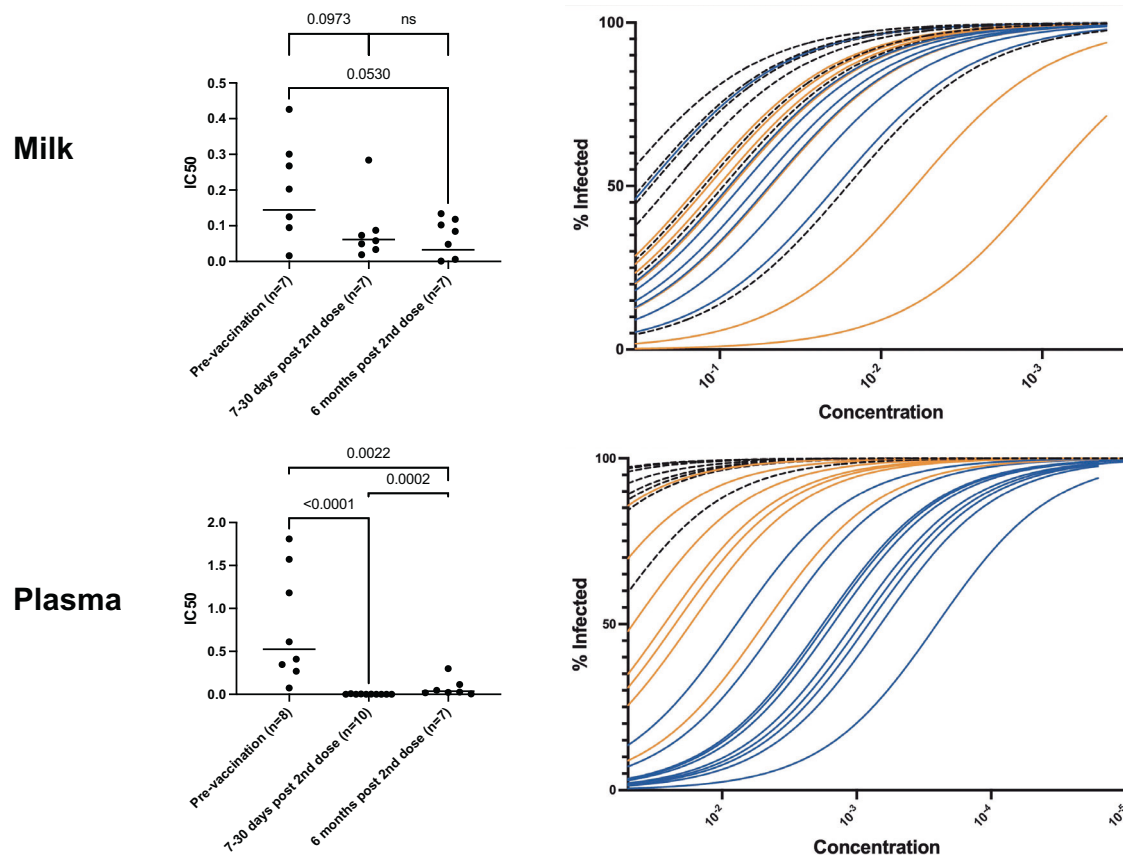


Fig. 4 Neutralization of VSV-gfp-SARS-CoV-2-S in milk (top) and plasma (bottom). In milk and plasma, neutralization is compared pre-vaccination, 7–10 days post 2nd dose, and six months post vaccination using the half-proximal inhibitory concentration (IC₅₀). IC₅₀ statistical calculations are measured using Mann–Whitney unpaired, nonparametric tests. Figures are shown as geometric mean. Line graphs represent non-linear regression models of inhibition at pre-vaccination (dotted, black line), 7–10 days post 2nd dose (solid, blue line), and six months post vaccination (solid, orange line).

in HM after COVID-19 vaccination or infection, suggesting a possible transfer of these antibodies to breastfeeding infants [10, 11, 14–16, 28]. Our study is among the first to demonstrate the presence of SARS-CoV-2 antibodies in breastfeeding infants' stools with improvement in infant's stool SARS-CoV-2 specific neutralization capabilities following maternal SARS-CoV-2 vaccination. Despite not being statistically significant, in part due to a small sample size, our results further support the maternal/infant passive transfer of SARS-CoV-2 antibodies through HM.

SARS-CoV-2, like other coronaviruses, invades intestinal cells as they express high levels of ACE2, their receptor, causing inflammation, malabsorption, intestinal dysfunction, and diarrhea [21, 22]. The high sensitivity of epithelial cells to coronaviruses increases their replicative capacity. Furthermore, the pediatric population can shed SARS-CoV-2 RNA for up to several weeks in the stool after confirmed respiratory clearance [29]. Although additional confirmatory studies will be necessary, our results make it tempting to speculate that SARS-CoV-2 specific IgA and IgG, present in the human milk of vaccinated individuals, potentially block viral-ACE2 receptors' interactions within the infant's intestinal mucosa.

It should be noted that HM was able to neutralize the pseudovirus in vitro even before vaccination. It has been established that antibodies can possess cross-reactivity to different human coronaviruses [30, 31]. Additionally, the extensive array of immune-protective molecules within HM (immunoglobulins, IgA secretory component, lactoferrin, oligosaccharides, blood-derived leukocytes) are also likely to contribute [17, 32, 33]. Once vaccination is administered to breastfeeding mothers, we observed an increase in HM neutralization capability in 50% of

the samples at 6 months post-vaccination, despite a decrease in antibody levels; this could result from the specialization and affinity maturation of secreted antibodies in HM, as seen for SARS-CoV-2 IgG in plasma in a prior study [34].

These results lead us to hypothesize that secreted HM SARS-CoV-2 IgA and IgG may serve to neutralize the virus and offer immunologic protection to breastfeeding infants, potentially preventing SARS-CoV-2-ACE2 interaction and reducing the severity of infection-related symptoms. However, up to date, no clinical correlation has been established between HM antibodies concentration and breastfeeding infants' protection. Establishing a correlation between antibody levels/antibody neutralization and clinical efficacy is confounded by a number of factors including viral mutation rates, cellular (T cell-mediated) immunity, genetic factors, age, and the environment. A recent article, published by the laboratories of Drs. Triccas and Davenport in 2021 developed a modeling system to predict a correlation between in vitro neutralization levels and observed protection from SARS-CoV-2 infection [35]. In this paper, using modeling strategies, it was estimated that 20 and 3 percent of the mean convalescent SARS-CoV-2 specific antibody levels were capable of providing 50% protection from detectable infection and severe infection respectively. It would be exciting to utilize such modeling strategies to establish correlates between in vitro neutralization assays using infant stool and clinical outcomes. Additionally, the true functionality of stool derived SARS-CoV-2 specific antibodies remains to be determined. Further studies, utilizing larger, more diverse, populations will bring clarity to these important clinical considerations.

LIMITATIONS

This study is among the first to suggest an effective transfer of HM SARS-CoV-2 IgA and IgG to breastfeeding infants' gastrointestinal tract. There are limitations to some of the results. First, there was a small sample size and limited diversity in this cohort of mothers. Although participants contributed data at various time points, no subjects contributed on all occasions. Furthermore, whether infants or mothers might have had a previously undiagnosed COVID infection, and how this may have interfered with results, cannot be known. We did not test IgA secretory component, rather we assumed that IgA found in milk is majority secretory IgA. The mean age of our infant subjects was 10 months, with many of them having solid food their main source of nutrition and breastfeeding as supplementation; this in combination with small sample size might have interfered with the stool antibodies concentration and neutralization capacity results, since exclusively breastfeeding infants may have higher antibodies levels.

DATA AVAILABILITY

The datasets generated during this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

LSS, MS: First author, literature search, study design, methodology, data collection, figures, data analysis, statistics, writing-original draft. VV, MD: First author, literature search, study design, project administration, data collection, data curation, writing-original draft, funding acquisition. MH, MD: Literature search, data collection, data analysis, writing original draft. JN, MD: Literature search, study design, supervision, writing-review and editing, funding acquisition. LP, PhD: Study design, writing-review and editing. MM, PhD: Formal analysis, writing-review and editing. VV: Data analysis. Taylor Gowen, MD: Data collection, writing-original draft, writing-review and editing. EC: Data collection. Ivan Kosik, PhD: Methodology, provided critical reagents, data interpretation. JWY, MD PhD: Methodology, provided critical reagents, data interpretation. MA, PhD: Provided critical reagents and equipment, assisted in data acquisition. NC, DO: Study design, writing-review and editing. NL, PhD: Data collection, data analysis. JLI, PhD: Senior author. Literature search, study design, methodology, supervision, funding acquisition, writing-review and editing. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was performed in accordance with the Declaration of Helsinki and was approved by the University of Florida Institutional Review Board (#IRB202003255).

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Correspondence and requests for materials should be addressed to Joseph Larkin III.

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