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SARS-CoV-2 RNA and antibody detection in breast milk from a prospective multicentre study in Spain

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ABSTRACT

Objectives To develop and validate a specific protocol for SARS-CoV-2 detection in breast milk matrix and to determine the impact of maternal SARS-CoV-2 infection on the presence, concentration and persistence of specific SARS-CoV-2 antibodies.

Design and patients This is a prospective, multicentre longitudinal study (April–December 2020) in 60 mothers with SARS-CoV-2 infection and/or who have recovered from COVID-19. A control group of 13 women before the pandemic were also included.

Setting Seven health centres from different provinces in Spain.

Main outcome measures Presence of SARS-CoV-2 RNA in breast milk, targeting the N1 region of the nucleocapsid gene and the envelope (E) gene; presence and levels of SARS-CoV-2-specific immunoglobulins (Igs)—IgA, IgG and IgM—in breast milk samples from patients with COVID-19.

Results All breast milk samples showed negative results for presence of SARS-CoV-2 RNA. We observed high intraindividual and interindividual variability in the antibody response to the receptor-binding domain of the SARS-CoV-2 spike protein for each of the three isotypes IgA, IgM and IgG. Main Protease (MPro) domain antibodies were also detected in milk. 82.9% (58 of 70) of milk samples were positive for at least one of the three antibody isotypes, with 52.9% of these positive for all three Igs. Positivity rate for IgA was relatively stable over time (65.2%–87.5%), whereas it raised continuously for IgG (from 47.8% for the first 10 days to 87.5% from day 41 up to day 206 post-PCR confirmation).

Conclusions Our study confirms the safety of breast feeding and highlights the relevance of virus-specific SARS-CoV-2 antibody transfer. This study provides crucial data to support official breastfeeding recommendations based on scientific evidence.

Trial registration number NCT04768244.

INTRODUCTION

Breast feeding is considered the gold standard for infant feeding and is of crucial importance in influencing both infant growth and development. Epidemiological studies have demonstrated that breast feeding decreases risk of infections in infants.^{1–4}

What is already known on this topic?

- Breast feeding provides optimal nutrition in infants.
- Data are conflicting on whether SARS-CoV-2 is present in breast milk of infected mothers.
- Breast milk of infected mothers contains antibodies to SARS-CoV-2, especially IgA.

What this study adds?

- SARS-CoV-2 RNA was not detected in any of the breast milk samples from our study.
- There is high intra- and inter-individual variability in the antibody response against the receptor-binding domain of the SARS-CoV-2 spike protein for the three antibody isotypes (IgA, IgM and IgG) and also against non-structural proteins, like MainProtease (MPro).
- Most of the breast milk samples (82.9%) had antibodies after SARS-CoV-2 infection for at least one of the three isotypes, with 52.9% of these positive for all three immunoglobulins.

Due to its beneficial effects, international organisations including the WHO recommend exclusive breast feeding for the first 6 months of life, and continuing breast feeding while complementary foods are introduced until 2 years of age or beyond.⁵

The COVID-19 global pandemic caused by SARS-CoV-2 has increased concerns about potential mother-to-infant transmission, including via breast feeding. While some studies reported the presence of SARS-CoV-2 in breast milk,^{6,7} although its potential for infection is unclear,⁸ other studies found no presence of the virus.^{9–11} In general, these studies showed several limitations, with the most relevant being the lack of targeted and validated protocols for viral detection in milk matrix. Furthermore, a strong antibody response is induced after maternal SARS-CoV-2 infection, with higher presence of neutralising secretory IgA in breast milk.^{7,12,13} However, several questions remain unanswered, including a specific and reliable method to detect SARS-CoV-2 in human milk, the extent of



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the response, the persistence of maternal antibodies in milk and their potential protective role in infants. Under this scenario, our main objectives were (1) to provide a specific and reliable detection method for SARS-CoV-2 in breast milk; and (2) to determine the levels of reactive IgA, IgG and IgM antibodies against structural and non-structural SARS-CoV-2 proteins in breast milk collected during the COVID-19 pandemic.

MATERIALS AND METHODS

Study population

This is a prospective, observational, longitudinal and multicentre study in mother–infant pairs with confirmed SARS-CoV-2 infection. Participants were recruited from seven health centres from different provinces in Spain (Valencia, Barcelona, Granada and Zaragoza). The recruitment period was from April to December 2020. Participants were pregnant women intending to breast feed and nursing women with positive PCR for SARS-CoV-2 on nasopharyngeal swabs or presence of SARS-CoV-2 antibodies in serum determined in hospitals. Women were excluded when COVID-19 symptomatology required specific treatment and/or hospitalisation in intensive care units. Exclusion criteria included women unable to breast feed due to severe symptomatology that required intensive care unit and/or mother's need for drugs with potential adverse effects on the infant and/or impossibility to obtain milk. All participants received oral and written information about the study and written consent was obtained. Extended details on the control group are described in online supplemental text S1.

Breast milk collection and processing

Breast milk was collected following a standardised protocol described elsewhere.¹⁴ Details on collection, sampling and storage are described in online supplemental text S1. Whole milk was used for SARS-CoV-2 RNA detection and whey milk was

used for antibody determination. Further details are provided in online supplemental text S1.

Validation of SARS-CoV-2 RNA extraction, detection and quantification in breast milk samples

A manual column-based commercial kit (referred to as MN) and an automated assisted method based on magnetic beads (referred to as Max) were adapted following previous recommendations¹⁵ and were compared to assess their sensitivity in detecting viral particles in breast milk. Details related to RNA extraction procedures, viral recoveries with different virus and limits of detection (LoD_{95%} and LoD_{50%}) are provided in online supplemental text S1.

Breast milk SARS-CoV-2-specific antibody detection

Levels of antibodies directed to structural (receptor-binding domain (RBD) of the SARS-CoV-2 spike protein) and non-structural (the main protease MPro or 3C-like protease (3CLpro)) viral proteins were analysed (online supplemental text S1). RBD-specific antibodies were determined by ELISA as previously described.^{16 17} MPro-reactive antibodies were quantified using a commercial ELISA kit (ImmunoStep, Salamanca, Spain).

RESULTS

Study population characteristics

Maternal demographic and clinical characteristics of women from the COVID-19 pandemic period group (n=60) and the prepandemic group (n=13) are described in table 1. Among the 60 mothers, 52 were diagnosed with SARS-CoV-2 PCR test on nasopharyngeal swabs while 8 were seropositive (IgG-positive). Most PCR tests (38 of 52, 73.1%) were performed as part of routine surveillance before labour (online supplemental text S2,

Table 1 Characteristics of the volunteers included in the study

	COVID-19 (n=60)	Prepandemic control (n=13)	P value
Maternal characteristics			
Age	34.8±4.6*	33.8±4.2	0.483†
Gestational age (weeks)‡	39.2 (38.1–40.6)§	39 (39.0–40.0)	0.963†
Delivery mode, n (%)¶			0.306**
Vaginal	42 (76.4)	8 (61.5)	
Caesarean section	13 (23.6)	5 (38.5)	
Infant characteristics			
Birth weight (g)	3247±519††	3323±475.7	0.630†
Birth length (cm)	49.8±2.4‡‡	50.5±1.6	0.296†
Breastfeeding status§§, n (%)			0.756**
Exclusive	35 (66.0)	8 (61.5)	
Mixed feeding	18 (34.0)	5 (38.5)	
Gender¶¶, n (%)			0.533**
Male	24 (44.4)	4 (30.8)	
Female	30 (55.6)	9 (69.2)	

*Missing data from 4 individuals.

†Unpaired t-test.

‡Values are given as median and 25th and 75th percentile.

§Missing data from 10 individuals.

¶Missing data from 5 individuals.

**Fisher's exact test (two-sided).

††Missing data from 8 individuals.

‡‡Missing data from 14 individuals.

§§Missing data from 7 individuals.

¶¶Missing data from 6 individuals.

table S1). Longitudinal samples from 12 women were available (two time points at <7 days and 15 days after delivery, approximately). Of the women, 38 (63.3%) were asymptomatic, and the rest reported mild COVID-19 symptoms (pain, fatigue or headache, among others). No other effects or medical problems were reported. All neonates were negative for SARS-CoV-2 and in good health.

Validation of SARS-CoV-2 RNA extraction and detection methods in breast milk

Breast milk SARS-CoV-2 viral RNA detection was optimised with Porcine Epidemic Diarrhoea Virus (PEDV) strain CV777 and Mengovirus (MgV) vMCO strain recoveries, and limits of detection (LoD_{95%} and LoD_{50%}) from spiked prepandemic breast milk samples using manual (MN) and an automated (Max) extraction method were tested (online supplemental text S2). These results suggested comparable analytical performance of both extraction methods for enveloped viruses; thus, the Max extraction method was further characterised using gamma inactivated SARS-CoV-2 and human coronavirus (HCoV) 229E (ATCC-VR740) (online supplemental figure S1), along with PEDV and MgV, as the method intended to be used for screening breast milk samples from women with COVID-19. LoD_{95%} values were as low as 36 gc/100 µL, 209 gc/100 µL, 13 gc/100 µL and 7 gc/100 µL, and LoD_{50%} values were 8 gc/100 µL, 48 gc/100 µL, 3 gc/100 µL and 2 gc/100 µL, for SARS-CoV-2, HCoV 229-E, PEDV and MgV, respectively (online supplemental figure S2). Based on these analytical results, the Max method was selected to screen the 72 breast milk samples for presence of SARS-CoV-2 RNA. Targeting the N1 and E regions, all samples resulted negative for presence of SARS-CoV-2 RNA. The RP gene used as quality control excluded false negative results (Cq=27.98±3.04). No remaining volume was available from 2 out of the 72 samples for the following analyses.

SARS-CoV-2 reactive antibodies in breast milk

We tested the reactivity of breast milk IgA, IgM and IgG antibodies to the RBD of the spike glycoprotein. Prepandemic milk samples (n=13) served as controls and to determine positive cut-off values (online supplemental figure S3). Strong reactivity was found for IgA, IgM and IgG in milk samples from COVID-19 infected/recovered women, and low levels of non-specific binding were observed in the prepandemic samples (online supplemental figure S4a-c). When applying positive cut-off levels, 84.5% (49 of 58) of the milk samples were positive for the RBD antigen for at least one of the three antibody classes (online supplemental figure S4d). When analysing the 70 collected samples, 58 (82.9%) were positive at least for one of the three antibody classes (IgA, IgM or IgG). Thirty-seven milk samples (52.9%) were positive for all three immunoglobulins (Igs), whereas 12 samples (17.1%) did not show reactivity to RBD for any of the three antibody classes (online supplemental figure S4e). We corroborated our results using the MPro antigen.¹⁸ Milk samples from COVID-19 infected and recovered donors still showed significantly higher reactivity to the MPro antigen than the prepandemic samples (online supplemental figure S5). Noteworthy, the positivity rate using this antigen decreased from 67.6% to 42.3% for IgA and from 64.2% to 31.3% for IgG.

Antibody response was analysed as a function of time from diagnosis with PCR test (online supplemental figure S6). The positivity rate for IgA was relatively stable over time (65.2%–87.5%). Most positive samples for IgM were detected when collected at 11–20 days after PCR confirmation (83.3%), and

then the levels consistently declined to 62.5%. IgG positivity rate continuously raised from 47.8% to 87.5% from day 41 up to day 206 post-PCR confirmation. RBD-specific IgA response in symptomatic COVID-19 cases tended to be higher than in the asymptomatic group, although differences did not reach significance and no changes were detected in virus-specific IgM and IgG (online supplemental figure S7).

We compared endpoint titres of positive samples between the different antibody isotypes and observed that the magnitude of the response was similar for all three Igs (online supplemental figure S8). Furthermore, all three Igs significantly correlated with each other, particularly IgA and IgM (r=0.7812, p<0.0001), but also IgA and IgG (r=0.6100, p<0.0001) and IgG and IgM (r=0.5708, p=0.0001).

A positive correlation (r=0.5527, p=0.0001) was also observed between the total IgA levels and the SARS-CoV-2-specific antibody response (online supplemental figure S9a). In fact, the total IgA levels were significantly higher in the COVID-19 group compared with the prepandemic controls (online supplemental figure S9b) and could be part of the response to infection. In a subset of longitudinal milk samples collected within the first 20 days after birth, we observed a generalised decrease in IgA and endpoint titres for RBD except in one mother, which exhibited low but rising antibody titres in breast milk (online supplemental figure S10). Generally, total IgA concentrations correlated negatively with lactation stage (r=-0.3357, p=0.0045), similar to RBD-specific IgA (r=-0.3088, p=0.0093) and IgM (r=-0.4334, p=0.0002), while the RBD-specific IgG response was independent of lactation stage. Furthermore, there was high interindividual and intraindividual variability in the antibody response to the virus for each of the three isotypes (online supplemental figure S11). In most of the samples, lactation stage and post-PCR detection coincided in a narrow time period; in fact, for 40 of the positive tested samples in online supplemental figure S11a, the difference between PCR detection and birth was not more than 5 days. Seven out of the eight milk samples from seropositive women showed positive antibody responses for all three antibody classes, except one sample that tested negative for IgM (online supplemental figure S11b). The remaining sample tested negative for all three isotypes and was from a mother diagnosed with SARS-CoV-2 infection by serological testing 226 days prior to sample collection for our study.

DISCUSSION

During the current COVID-19 pandemic, science has primarily focused on providing solutions and treatments against SARS-CoV-2 infection to reduce mortality. However, specific vulnerable populations including pregnant and lactating mothers as well as infants have not been widely considered, resulting in a big gap in knowledge on maternal-infant health regarding COVID-19.

Breast feeding is considered the most relevant postnatal link between mothers and infants. However, the lack of understanding of SARS-CoV-2 vertical transmission¹⁹ has considerably reduced breastfeeding practice. Even mothers with SARS-CoV-2 infection were recommended to temporarily separate from their infants.²⁰

Being a rapid and sensitive technique, RNA detection by reverse transcription quantitative PCR (RT-qPCR) is the gold standard for both clinical diagnosis and viral food contamination.^{21 22} However, milk components might affect nucleic acid isolation and quantification, as demonstrated by the variable recovery of contaminating microorganisms and the occurrence of (partial/total) inhibitory effect during amplification, which may cause subestimated and false negative results,^{23–25} as we

have also observed. Thus, it is of primary importance to include appropriate quality controls for extraction, detection and quantification of molecular targets while defining the analytical performance of the overall workflow.²⁶ In our study, whole milk was used to test for viral RNA presence. LoD_{95%} and LoD_{50%} for gamma inactivated SARS-CoV-2 resulted in values as low as 36 gc/100 µL and 8 gc/100 µL, respectively. These data are in line with the detection limit suggested by Chambers and colleagues,²⁷ where samples with >25 gc/100 µL of SARS-CoV-2 RNA would be considered positive although a higher limit of ca. 10³⁻⁴ gc/100 µL was informed elsewhere.⁶

Available data show that around 2%–6% of milk samples would harbour viral RNA. A recent systematic review (n=37 articles with 68 lactating mothers with COVID-19) showed that SARS-CoV-2 RNA was detected in nine of the samples (9 of 68, 13.2%).²⁸ Another systematic review reported that SARS-CoV-2 RNA detection in breast milk was 2.16%.²⁹ The biggest study to date included 110 women in the USA (n=65 testing positive for SARS-CoV-2) and showed that SARS-CoV-2 RNA was present in 6% of the milk samples; however, no infectious viral particles could be isolated by cell culture.⁸ By using SARS-CoV-2, SARS-CoV-2 surrogates and a non-enveloped viral model (MgV), we define the analytical performance (eg, recovery and LoD) of a specific protocol able to efficiently isolate and detect SARS-CoV-2 RNA in breast milk. We further validate the protocol using appropriate quality controls in whole breast milk.

In our study, we have not detected SARS-CoV-2 RNA in any of the breast milk samples, contributing to the evidence that there is no vertical transmission during breast feeding.⁹ There are still many open questions: when are SARS-CoV-2 antibodies produced after maternal infection, when can they be detected in breast milk, and how long do they persist? While different studies reported the presence of SARS-CoV-2-specific IgA antibodies,^{7 12 13 30} limited information is available on IgG and IgM. Our results showed the presence of anti-SARS-CoV-2 antibodies in milk, primarily IgA but also IgG and IgM targeting RBD. High intraindividual and inter-individual variability was observed in antibody presence, and significant differences for all three antibody classes were identified when compared with the pre-pandemic samples.¹⁸ We did not detect time-dependent quantitative differences in endpoint titres for the different antibody classes, most likely due to high interindividual variability. However, we found a time-dependent increase in IgG-positive samples collected from day 41 up to day 206 post-PCR diagnosis. These data are in line with findings in human serum samples from a Chinese cohort showing persistence of IgG up to 6 months.³¹ To date, only a few studies have quantified virus-specific antibodies in pre-pandemic samples to establish cut-off values to discriminate between positive and negative samples,^{7 12 32} which allows comparison of results between studies. We found a similar proportion of positive samples for both IgA (from 72.9% in our study to 80.0%^{7 12}) and IgM (72.9% in our study compared with 55.0% in the study reported by Peng *et al*³²). Of the samples, 17.1% did not present virus-specific antibodies, which is in accordance with a previous study (Fox Iscience). Also, accumulating data report the lack of SARS-CoV-2 IgG in serum after previous infection in some individuals; in fact, in a cohort study (n=2547),³³ 6.3% were reported to be IgG seronegative.

In this study, we also assessed the presence of antibodies against other non-structural viral proteins, specifically the viral cysteine-like protease, also known as 3CLPro or main viral

protease (MPro).¹⁸ Our data showed the presence of anti-MPro IgA and IgG antibodies in milk samples from the COVID-19 group compared with pre-pandemic samples, although the sensitivity was lower than when using the RBD antigen for detection of virus-specific antibodies. MPro is a viral antigen not exposed on the viral particle like the spike protein; however, strong and similar reactivities were found for both MPro and RBD, and the nucleocapsid protein in serum and saliva samples.¹⁸ Our study is the first to use MPro for detection of SARS-CoV-2 antibodies in breast milk, and the reactivities to the different viral antigens, also in function of isotype, have been previously reported in breast milk.^{7 34}

Our results are in agreement with previous data showing higher levels of antibodies against SARS-CoV-2 in milk from infected/recovered mothers compared with samples from women before the pandemic.^{30 35} However, pre-pandemic samples showed some reactivity to SARS-CoV-2, particularly in RBD-reactive IgA, which may be explained by cross-reaction with other seasonal coronavirus (HCoV) in breast milk samples before 2020, as previously reported.³⁶ Women with COVID-19 symptoms showed slightly higher virus-specific IgA levels in milk compared with women with asymptomatic infection, although differences were not significant. Moreover, no differences in IgM or IgG levels were found, possibly due to minor COVID-19 symptoms (pain, headache, etc) in this data set. Despite these observations, further analyses including a bigger sample size and different symptoms as well as severe COVID-19-infected donors are warranted.

In summary, our study demonstrates (1) the absence of SARS-CoV-2 RNA in breast milk from women with COVID-19 and (2) the high intervariability and intravariability in the SARS-CoV-2 antibody response. Women with COVID-19 exhibited IgA, IgG and IgM antibodies in breast milk not only against structural proteins like RBD but also against non-structural proteins like MPro. The presence of Igs suggests that breast milk might have a protective effect in newborns. Interestingly, positive associations between total IgA and specific antibodies (IgA, IgG and IgM) were observed, although their persistence and stability differed between mothers and antibody type. Our study endorses the safety of breast feeding during the pandemic and highlights the potential relevance of virus-specific SARS-CoV-2 antibodies providing passive immunity to breastfeeding infants, protecting them against COVID-19. Our study supports official recommendations stating the safety of breast feeding during the COVID-19 pandemic, indicating that breast feeding should be a priority with potential benefit for both mothers and neonates.

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Correction notice This paper has been corrected since it was published online. The Collaborators section was inadvertently omitted.

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Contributors Study concept and design: CM-C and MCC. Drafting of the manuscript: CB, WR, CM-C and MCC. Clinical aspects including recruitment of the participants, collection of samples and medical records: CM-C, LM, AP-L, EGV, CL, VF, FCr, GR, CC and GR-R. Sample management, processing and analysis: CB, WR, MS-R, GS, FJP-C and MCC. All authors read and approved the final manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All protocols performed in the study were in accordance with the ethical standards approved by the Ethical Committee of the Hospital Clínico Universitario de Valencia (ref 2020/133). A control group of women not exposed to SARS-CoV-2 and from pre-pandemic time were included. These women were randomly selected from the MAMI birth cohort in Spain (ClinicalTrials.gov identifier: NCT03552939). This protocol was approved by the Ethical Committee of the Hospital Clínico Universitario de Valencia (ref 2015/0024) and by the local Ethical Committee of Atención Primaria-Generalitat Valenciana (CEIC-APCV).

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Text S1. Extended Material and Methods

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Initially, to characterize the viral recovery of both methods, porcine epidemic diarrhoea virus (PEDV) strain CV777 (an enveloped virus member of the *Coronaviridae* family and surrogate for SARS-CoV-2), and also, mengovirus (MgV) vMCO (CECT 100000, non-enveloped member of the *Picornaviridae* designated in the ISO 15216-1:2017 standard method as process control) were spiked in phosphate buffer solution (PBS) and in breast milk. Detection ratios and limits of detection ($\text{LoD}_{95\%}$ and $\text{LoD}_{50\%}$) were also characterized for both extraction methods by spiking serial dilutions of PEDV.

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All RT-qPCR assays were performed in duplicate on a LightCycler 480 instrument (Roche Diagnostics, Germany). Positive (genomic RNA), negative (nuclease-free water), and inhibition (either 10-fold diluted RNA or RP gene) controls were included in each assay.

Breast milk SARS-CoV-2-specific antibody detection

Levels of antibodies directed to structural proteins like the RBD of the SARS-CoV-2 spike protein and to non-structural viral proteins like the cysteine-like protease, also known as the main protease (Mpro) or 3CLpro, were analyzed.

RBD-specific antibodies were determined using a previously published and validated ELISA protocol for use in human plasma and serum samples [9,10], modified for its use in human milk samples. RBD protein was produced under HHSN272201400008C and obtained through BEI Resources, NIAID, NIH: Spike Glycoprotein RBD from SARS-CoV-2, Wuhan-Hu-1 with C-Terminal Histidine Tag, Recombinant from HEK293T Cells, NR-52946. Briefly, 96-well ELISA immunoplates (Costar) were coated with RBD protein at 2 µg/mL and incubated at 4 °C overnight. Coated plates were blocked in 3 % (w/v) milk powder in PBS containing 0.1 % Tween 20 (PBS-T) for 1 h. Then, 4-fold dilution of samples in 1 % (w/v) milk powder in PBS-T were added, incubated for 2 h at room temperature and washed with PBS-T before addition of horseradish peroxidase-conjugated secondary antibodies. For detection of the different antibody isotypes, anti-human IgA (α-chain-specific) HRP antibody (Thermo-Fisher Scientific; A18781; 1:6.000), anti-human IgM (µ-chain-specific) HRP antibody (Sigma-Aldrich; A0420; 1:4.000), and anti-human IgG (Fc specific) HRP antibody (Sigma-Aldrich; A0170; 1:4.000) were used and incubated for 1 h in 1 % (w/v) milk powder in PBS-T. Bound antigen-specific antibodies were detected with 100 µL 3,3',5,5'-Tetramethylbenzidine (TMB) and reactions were stopped with 50 µL of 2M sulfuric acid. Absorbance at 450 nm was read in a ClarioStar (BMG Labtech) microplate reader using the path length correction mode. For detection of MPro-reactive antibodies, a commercial ELISA Kit (ImmunoStep, Salamanca, Spain) was used. Samples were incubated 1:4 diluted, and remaining steps of the protocol were performed according to manufacturer's instructions. For ELISA studies, milk samples were considered positive when OD values from undiluted samples exceeded the positive cut-off values for each assay and isotype calculated from prepandemic control samples and defined as the mean + two standard deviations (SD). Values from dilution curves were used for determining the area under the curve (AUC) to get a better quantitative impression between COVID-19 and control group. Endpoint titers were calculated from log-transformed titration curves using 4-parameter non-linear regression function in GraphPad Prism 8.0 and the positive cut-off values obtained from the prepandemic control group for each antigen and isotype.

Breast milk Total IgA quantification

Total IgA, including secretory IgA (sIgA), was measured in whey milk using a sandwich ELISA quantitation kit from Bethyl Laboratories (Montgomery, TX) following manufacturer's instructions as previously detailed [11]. Briefly, an anti-human IgA antibody pre-adsorbed to the plate allowed to capture the IgA, which was later detected by the addition of a biotinylated detection antibody and streptavidin-conjugated horseradish peroxidase that catalyzed the colorimetric reaction with the chromogenic substrate TMB. All whey milk samples were analyzed at a 1:8,000 dilution rate. Data were expressed as mg/L of milk. Duplicate determinations were performed on each plate.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 8.0. After Shapiro-Wilk normality test, non-parametric t-test (Mann-Whitney) was used to detect significant differences between groups and Spearman correlation analysis to assess correlations between variables.

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Text S2: Extended Results

Study population characteristics

Among the 60 mothers, 52 were diagnosed with a SARS-CoV-2 PCR test in nasopharyngeal swabs while 8 mothers were seropositive (IgG positive). Most PCR tests (38/52, 73.1%) were performed as part of routine surveillance before labor. Table S1 specifies when SARS-CoV-2 was diagnosed either by PCR or by serology.

Validation of SARS-CoV-2 RNA extraction and detection methods in breast milk

To optimize SARS-CoV-2 viral RNA detection in breast milk samples, an initial analytical comparison aimed to determine the recovery of PEDV and MgV from spiked pre-pandemic breast milk samples using either a manual (MN) or an automated (Max) extraction method. Compared to spiked PBS, PEDV and MgV were recovered at 30 % (27-33 %) and 132 % (94-188 %) when extracted from whole milk samples with MN, respectively. Conversely, better recoveries (>100 %) were observed for both viruses extracted by Max. No significant inhibitions due the milk matrix were observed as depicted by the cycle threshold (Ct) values of 10-fold diluted RNA or RP gene reactions.

By spiking PEDV serial dilutions in milk samples, we further defined the detection ratios and limits of detection (LoD_{95%} and LoD_{50%}), which are shown in Table S2. Results demonstrated similar sensibility of both extraction methods being 10 and 13 PEDV gc/100µL the limit of detection with 95 % confidence for MN and Max, respectively. These results suggest comparable analytical performances of MN and Max extraction methods for enveloped viruses.

Table S1: Type of COVID-19 diagnosis (PCR/serology) and average time to sample collection

COVID-19 diagnosis:		n=60
Positive nasopharyngeal PCR confirmation		n=52
confirmation 48 h before delivery		38
confirmation 48 h after delivery		2
confirmation > 48 h before delivery		9
confirmation > 48 h after delivery		3
Positive serology confirmation		n=8
confirmation 48 h before/after delivery		3
confirmation > 48 h before delivery		1
confirmation > 48 h after delivery		4
COVID-19 symptomatology		
Symptomatic prior or at enrollment		22
Average time from COVID-19 diagnosis to breast milk sample collection		
Days post-PCR confirmation:	median	15.0
	minimum and maximum	1 - 206
	25 th and 75 th percentile	6.0 – 29.8
Days post-serology confirmation:	median	15.5
	minimum and maximum	1 - 226
	25 th and 75 th percentile	(2.0 – 40.5)

Table S2. Concentrations, detection ratios, and limits of detection (LoD_{95%} and LoD_{50%}) characterizing the analytical performances of a manual commercial kit (MN) and an automated assisted method (Max) to extract porcine epidemic diarrhoea virus (PEDV) spiked in breast milk

Levels of inoculated PEDV (gc/100uL)	MN				Max			
	Back calculated concentration (gc/100uL ± SD)	Detection ratio (+/total)	LoD ₉₅ (gc/100 uL)	LoD ₅₀ (gc/100 uL)	Back calculated concentration (gc/100uL ± SD)	Detection ratio (+/total)	LoD ₉₅ (gc/100 uL)	LoD ₅₀ (gc/100 uL)
10e3	1348.84 ± 152.84	6/6			3137.92 ± 756.22	6/6		
10e2	196.52 ± 10.10	6/6			575.86 ± 346.03	6/6		
10e1	25.82 ± 5.03	6/6	10.0	2.96	92.94 ± 44.48	6/6	13.38	3.10
10e0	2.05 ± 0.25	2/6			16.39 ± 4.45	3/6		
10e-1	-	0/6			-	0/6		

Supplementary Material

Primers and probe, reaction mix, thermal cycling conditions and calibration curve used to detect and quantify HCoV 229-E.

Reaction mix (10 μ L) consisted of 5.00 μ L 2X One Step RT-PCR Buffer III, 0.20 μ L PrimeScript RT enzyme Mix II, 0.20 μ L ROX, 0.20 mL TaKaRa Ex Taq HS, 0.30 μ L 229E-F and 229E-R primers (10mM), 0.15 μ L 229E-P probe (10mM). The cycling parameters were as RT at 48 $^{\circ}$ C for 30 min, preheating at 95 $^{\circ}$ C for 10 min and 45 cycles of amplification at 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 1 min.

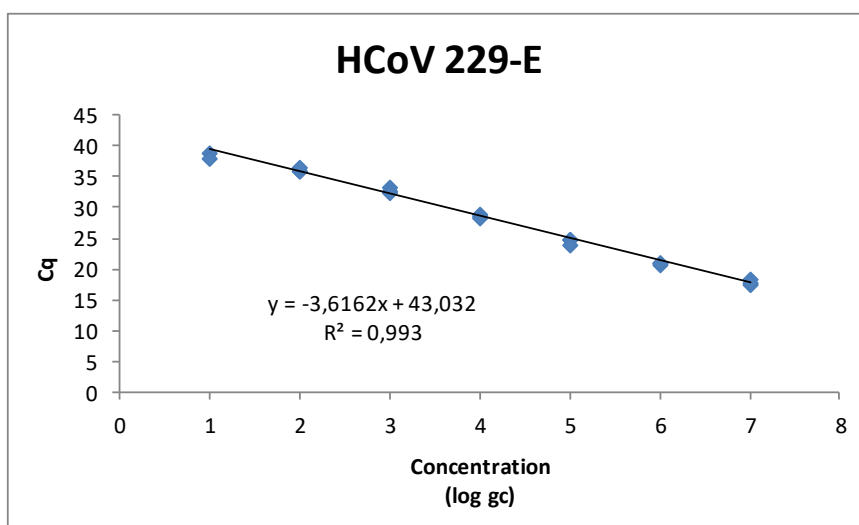


Figure S1. Standard curve generated to quantify HCoV 229-E performed with 10-fold dilutions (10^0 - 10^7 gc/reaction) of genomic RNA.

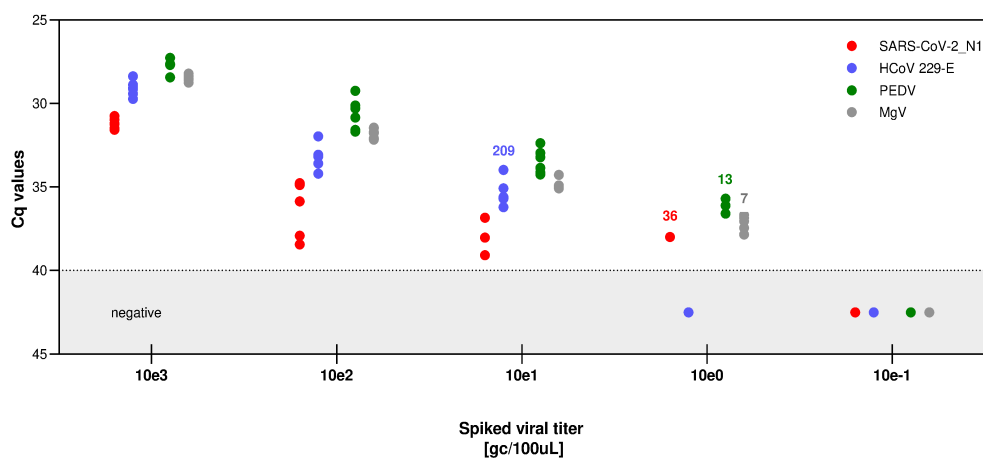


Figure S2. Distribution of cycle threshold values (Cq) characterizing the limits of detection of the automated assisted method (Max) to extract viral RNA form breast milk samples: severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), human coronavirus 229E (HCoV 229-E), porcine epidemic diarrhea virus (PEDV), and mengovirus (MgV) spiked in breast milk. The numbers above viral series denotes the $LoD_{95\%}$ (gc/100uL) for each virus.

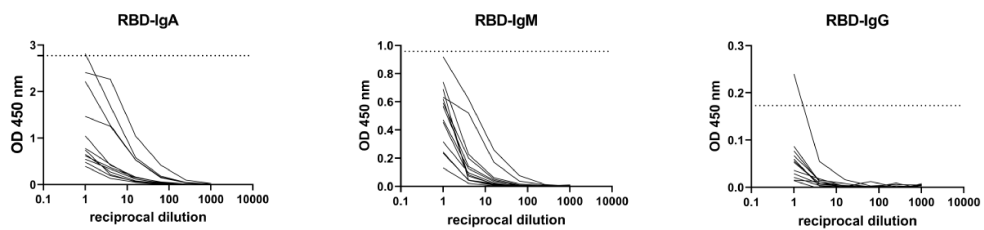


Figure S3: Titration curves of pre-pandemic Control samples. Serial dilutions of pre-pandemic Controls to determine cut-off values in order to discriminate between negative and positive samples in COVID-19 collected breastmilk samples, calculated as the mean + 2SD.

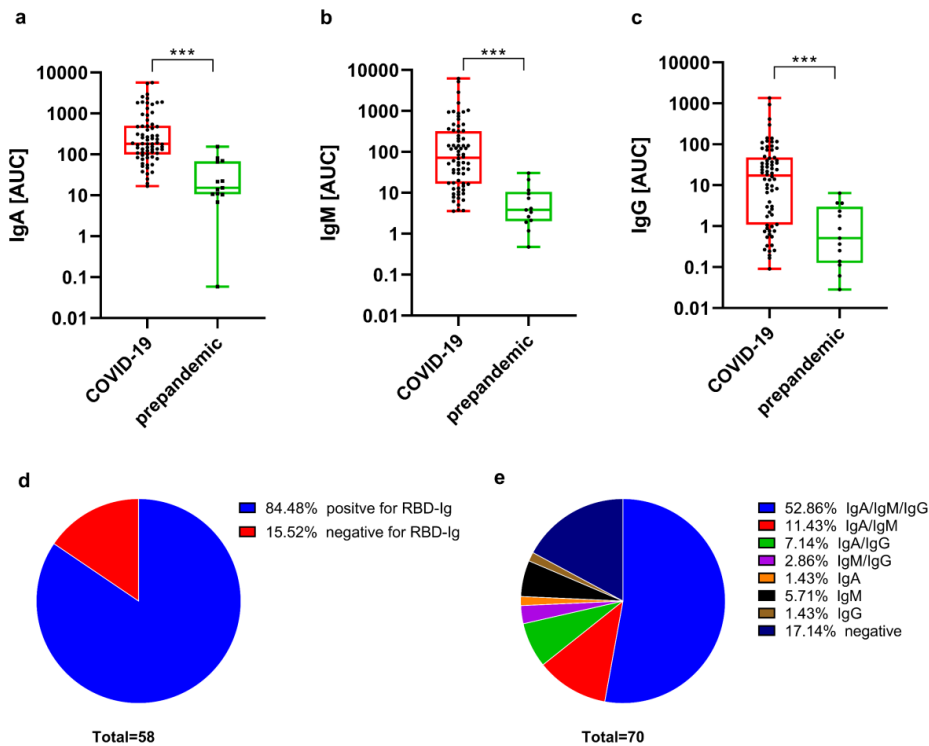


Figure S4: Breast milk from COVID-19 infected and/or recovered mothers show significantly higher antibody binding to the RBD antigen of SARS-CoV-2 than prepandemic controls and are detectable in 82.9 % of samples. AUC were calculated from titration curves for RBD-reactive (a) IgA, (b) IgM, and (c) IgG in order to get a better graphical impression. Asterisks show statistically significant differences between groups (***) $p < 0.0001$ using the Mann–Whitney test (unpaired nonparametric test). (d) Proportion of human milk donors who had positive RBD-reactive Igs in at least one milk recollection point, and negative samples (e) Proportion of RBD-reactive positive and negative human milk samples subdivided according to different isotypes.

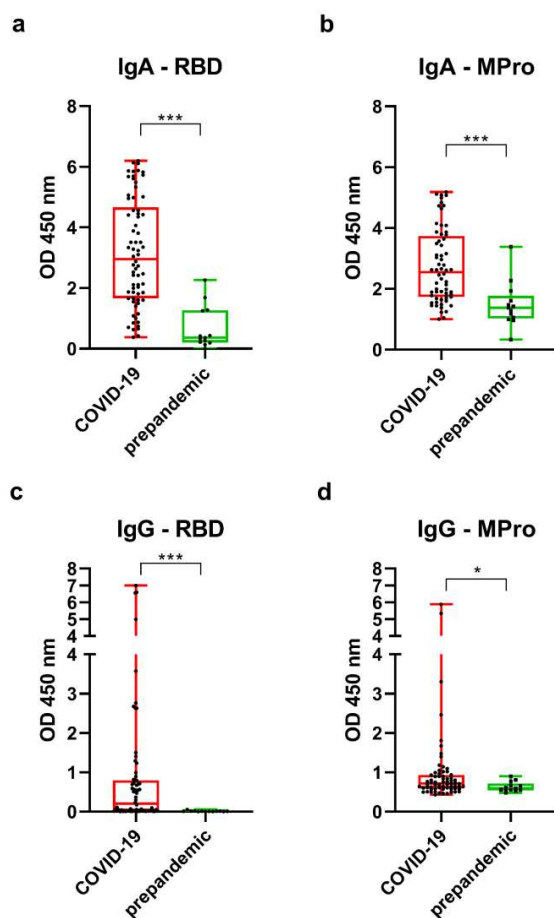


Figure S5: Samples from infected milk donors show significantly higher binding to SARS-CoV-2 antigens RBD and Mpro compared to prepandemic control. Grouped OD values of 1:4 diluted samples of RBD- (a) and MPro- (b) reactive IgA, and RBD- (c) and MPro- (d) reactive IgG, respectively. Asterisks show statistically significant differences between groups (** $p < 0.0001$, * $p < 0.05$) using the Mann-Whitney test (unpaired nonparametric test).

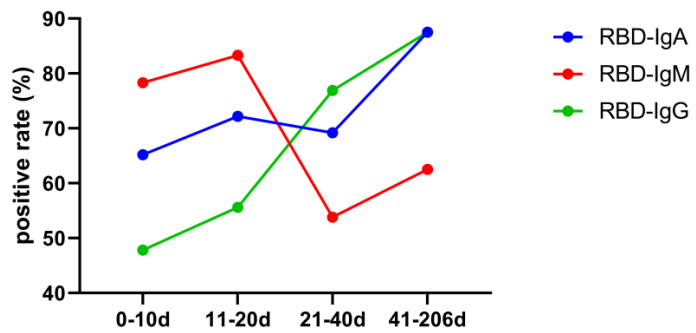


Figure S6: Graph of positive rates of RBD-specific IgA, IgM and IgG versus days after positive PCR diagnosis.

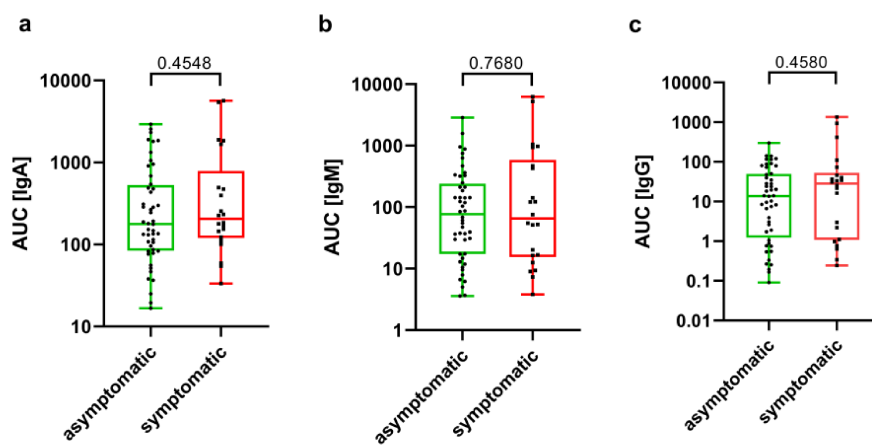


Figure S7: : Reactivity of breast milk samples from asymptomatic and symptomatic COVID-19 infected and/or recovered mothers to RBD antigen. AUC were calculated from titration curves for RBD-reactive (a) IgA, (b) IgM and (c) IgG in order to get a better graphical impression. Mann–Whitney test (unpaired nonparametric test) was used to assess for statistical significance.

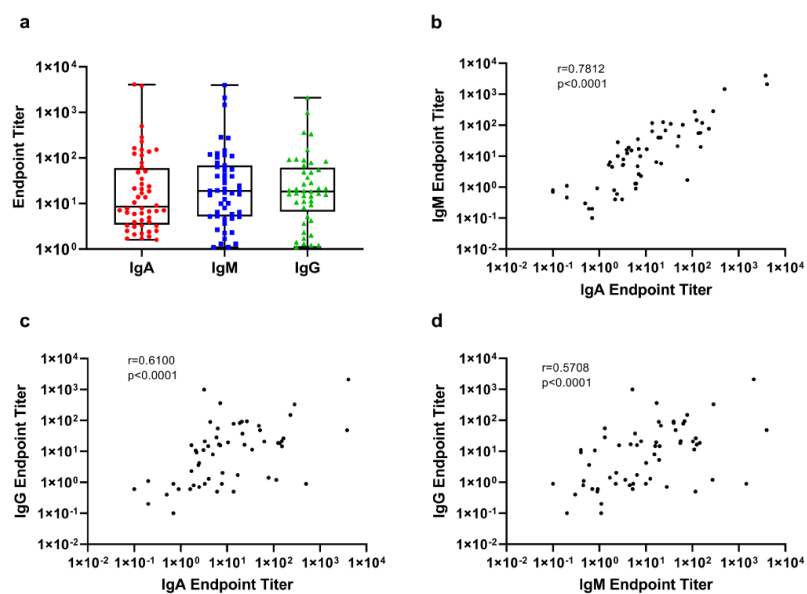


Figure S8: Endpoint titers for RBD-specific IgA, IgM and IgG and correlation analysis between antibody subclasses. (a) Grouped endpoint titers of the three different isotypes. Spearman's correlation analysis of endpoint titers between (a) RBD-specific IgA and RBD-IgM, (b) RBD-IgA and RBD-IgG, and (c) RBD-IgM and RBD-IgG.

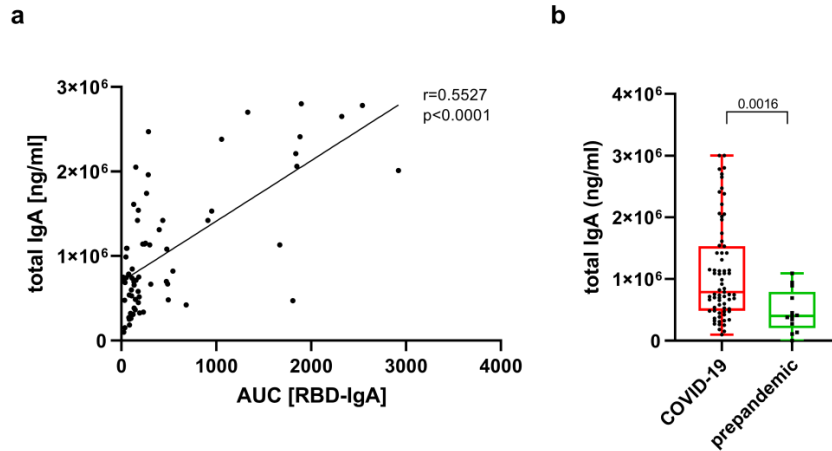


Figure S9: Total IgA concentration and virus specific antibody response.

(a) Spearman's correlation analysis of total IgA concentration and virus specific IgA response expressed as AUC. (b) Total IgA in COVID-19 infected and recovered and pre-pandemic control samples. Mann-Whitney test (unpaired nonparametric test) was used to assess for statistical significance.

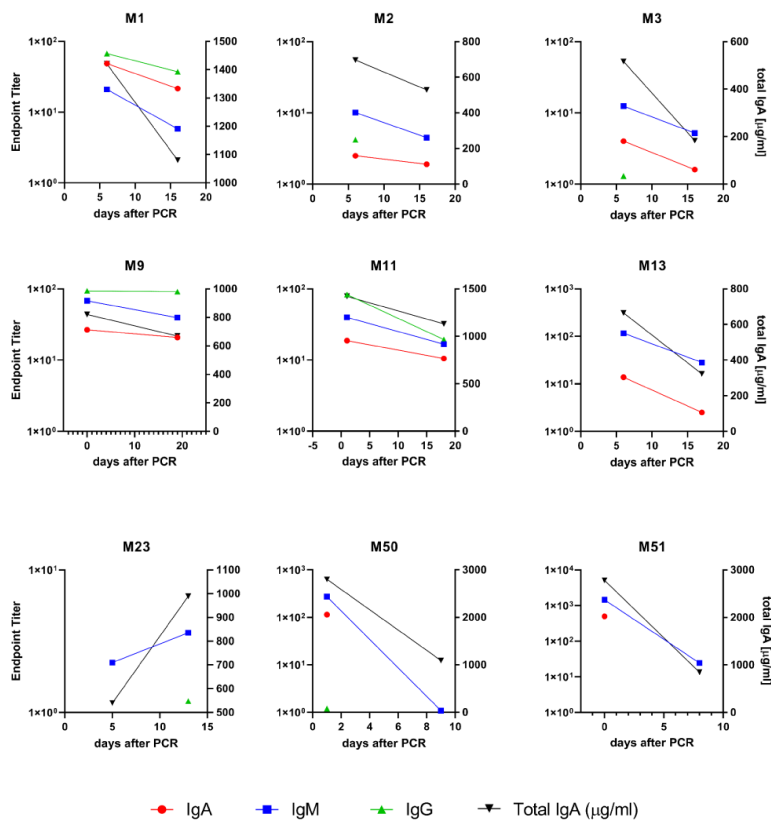
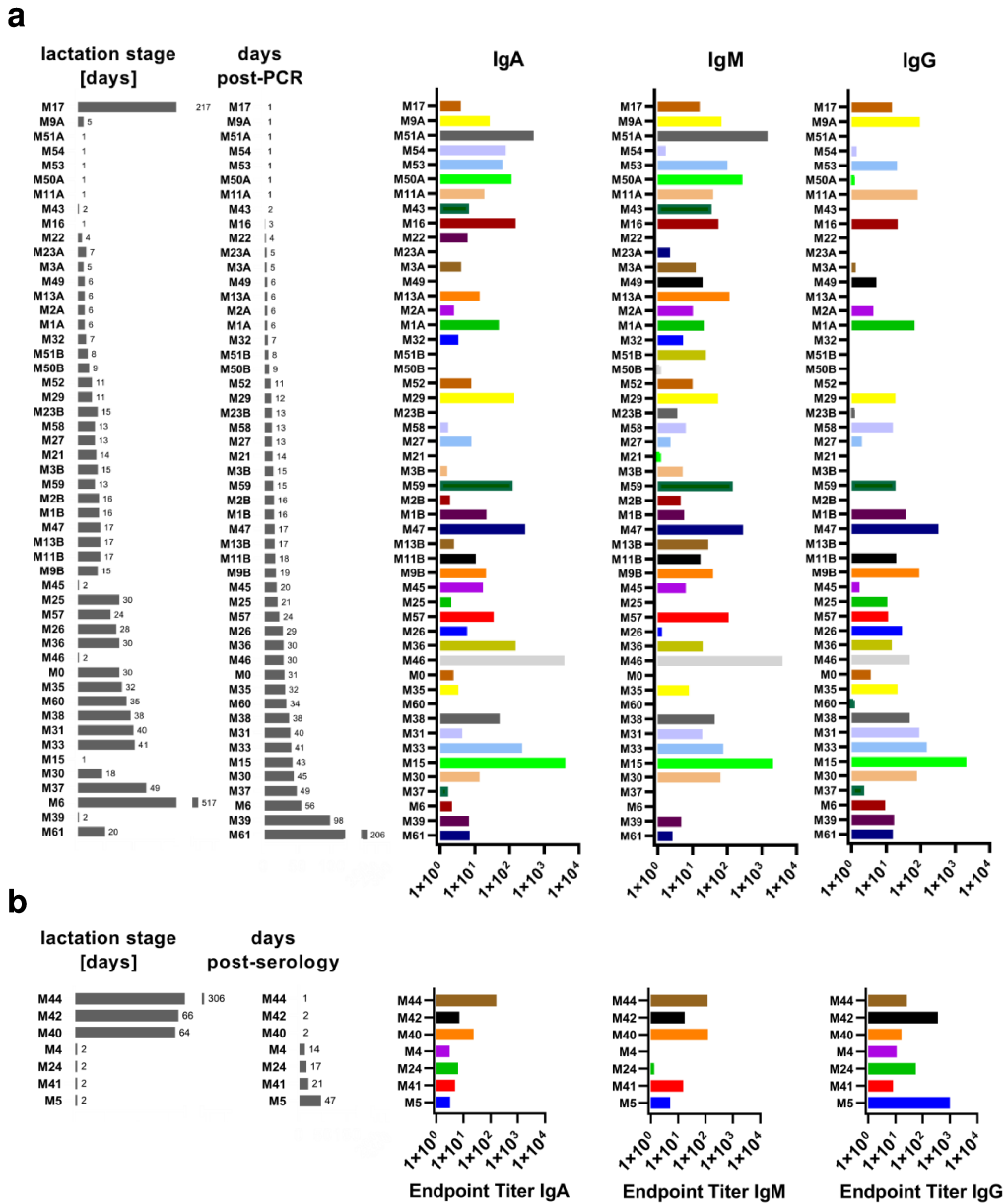


Figure S10: Temporal dynamic changes of endpoint titers in longitudinal samples for RBD-specific Igs. Longitudinal samples at two time points from 12 mothers were available, samples from 3 mothers were negative at both times and not represented. Virus-specific IgA (red circles), IgM (blue squares), IgG (green triangles) and total IgA (black triangles). All isotypes were tested for RBD binding in both time points, only positive endpoint titer are drawn in the graphs, the absence of data in a given time point indicates samples that were below the Cut-off values and considered negative.



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Breast milk SARS-CoV-2-specific antibody detection

Levels of antibodies directed to structural proteins like the RBD of the SARS-CoV-2 spike protein and to non-structural viral proteins like the cysteine-like protease, also known as the main protease (Mpro) or 3CLpro, were analyzed.

RBD-specific antibodies were determined using a previously published and validated ELISA protocol for use in human plasma and serum samples [9,10], modified for its use in human milk samples. RBD protein was produced under HHSN272201400008C and obtained through BEI Resources, NIAID, NIH: Spike Glycoprotein RBD from SARS-CoV-2, Wuhan-Hu-1 with C-Terminal Histidine Tag, Recombinant from HEK293T Cells, NR-52946. Briefly, 96-well ELISA immunoplates (Costar) were coated with RBD protein at 2 µg/mL and incubated at 4 °C overnight. Coated plates were blocked in 3 % (w/v) milk powder in PBS containing 0.1 % Tween 20 (PBS-T) for 1 h. Then, 4-fold dilution of samples in 1 % (w/v) milk powder in PBS-T were added, incubated for 2 h at room temperature and washed with PBS-T before addition of horseradish peroxidase-conjugated secondary antibodies. For detection of the different antibody isotypes, anti-human IgA (α-chain-specific) HRP antibody (Thermo-Fisher Scientific; A18781; 1:6.000), anti-human IgM (µ-chain-specific) HRP antibody (Sigma-Aldrich; A0420; 1:4.000), and anti-human IgG (Fc specific) HRP antibody (Sigma-Aldrich; A0170; 1:4.000) were used and incubated for 1 h in 1 % (w/v) milk powder in PBS-T. Bound antigen-specific antibodies were detected with 100 µL 3,3',5,5'-Tetramethylbenzidine (TMB) and reactions were stopped with 50 µL of 2M sulfuric acid. Absorbance at 450 nm was read in a ClarioStar (BMG Labtech) microplate reader using the path length correction mode. For detection of MPro-reactive antibodies, a commercial ELISA Kit (ImmunoStep, Salamanca, Spain) was used. Samples were incubated 1:4 diluted, and remaining steps of the protocol were performed according to manufacturer's instructions. For ELISA studies, milk samples were considered positive when OD values from undiluted samples exceeded the positive cut-off values for each assay and isotype calculated from prepandemic control samples and defined as the mean + two standard deviations (SD). Values from dilution curves were used for determining the area under the curve (AUC) to get a better quantitative impression between COVID-19 and control group. Endpoint titers were calculated from log-transformed titration curves using 4-parameter non-linear regression function in GraphPad Prism 8.0 and the positive cut-off values obtained from the prepandemic control group for each antigen and isotype.

Breast milk Total IgA quantification

Total IgA, including secretory IgA (sIgA), was measured in whey milk using a sandwich ELISA quantitation kit from Bethyl Laboratories (Montgomery, TX) following manufacturer's instructions as previously detailed [11]. Briefly, an anti-human IgA antibody pre-adsorbed to the plate allowed to capture the IgA, which was later detected by the addition of a biotinylated detection antibody and streptavidin-conjugated horseradish peroxidase that catalyzed the colorimetric reaction with the chromogenic substrate TMB. All whey milk samples were analyzed at a 1:8,000 dilution rate. Data were expressed as mg/L of milk. Duplicate determinations were performed on each plate.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 8.0. After Shapiro-Wilk normality test, non-parametric t-test (Mann-Whitney) was used to detect significant differences between groups and Spearman correlation analysis to assess correlations between variables.

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Text S2: Extended Results

Study population characteristics

Among the 60 mothers, 52 were diagnosed with a SARS-CoV-2 PCR test in nasopharyngeal swabs while 8 mothers were seropositive (IgG positive). Most PCR tests (38/52, 73.1%) were performed as part of routine surveillance before labor. Table S1 specifies when SARS-CoV-2 was diagnosed either by PCR or by serology.

Validation of SARS-CoV-2 RNA extraction and detection methods in breast milk

To optimize SARS-CoV-2 viral RNA detection in breast milk samples, an initial analytical comparison aimed to determine the recovery of PEDV and MgV from spiked pre-pandemic breast milk samples using either a manual (MN) or an automated (Max) extraction method. Compared to spiked PBS, PEDV and MgV were recovered at 30 % (27-33 %) and 132 % (94-188 %) when extracted from whole milk samples with MN, respectively. Conversely, better recoveries (>100 %) were observed for both viruses extracted by Max. No significant inhibitions due the milk matrix were observed as depicted by the cycle threshold (Ct) values of 10-fold diluted RNA or RP gene reactions.

By spiking PEDV serial dilutions in milk samples, we further defined the detection ratios and limits of detection (LoD_{95%} and LoD_{50%}), which are shown in Table S2. Results demonstrated similar sensibility of both extraction methods being 10 and 13 PEDV gc/100µL the limit of detection with 95 % confidence for MN and Max, respectively. These results suggest comparable analytical performances of MN and Max extraction methods for enveloped viruses.

Table S1: Type of COVID-19 diagnosis (PCR/serology) and average time to sample collection

COVID-19 diagnosis:	n=60
Positive nasopharyngeal PCR confirmation	n=52
confirmation 48 h before delivery	38
confirmation 48 h after delivery	2
confirmation > 48 h before delivery	9
confirmation > 48 h after delivery	3
Positive serology confirmation	n=8
confirmation 48 h before/after delivery	3
confirmation > 48 h before delivery	1
confirmation > 48 h after delivery	4
COVID-19 symptomatology	
Symptomatic prior or at enrollment	22
Average time from COVID-19 diagnosis to breast milk sample collection	
Days post-PCR confirmation: median	15.0
minimum and maximum	1 - 206
25 th and 75 th percentile	6.0 – 29.8
Days post-serology confirmation: median	15.5
minimum and maximum	1 - 226
25 th and 75 th percentile	(2.0 – 40.5)

Table S2. Concentrations, detection ratios, and limits of detection (LoD_{95%} and LoD_{50%}) characterizing the analytical performances of a manual commercial kit (MN) and an automated assisted method (Max) to extract porcine epidemic diarrhoea virus (PEDV) spiked in breast milk

Levels of inoculated PEDV (gc/100uL)	MN				Max			
	Back calculated concentration (gc/100uL ± SD)	Detection ratio (+/total)	LoD ₉₅ (gc/100 uL)	LoD ₅₀ (gc/100 uL)	Back calculated concentration (gc/100uL ± SD)	Detection ratio (+/total)	LoD ₉₅ (gc/100 uL)	LoD ₅₀ (gc/100 uL)
10e3	1348.84 ± 152.84	6/6			3137.92 ± 756.22	6/6		
10e2	196.52 ± 10.10	6/6			575.86 ± 346.03	6/6		
10e1	25.82 ± 5.03	6/6	10.0	2.96	92.94 ± 44.48	6/6	13.38	3.10
10e0	2.05 ± 0.25	2/6			16.39 ± 4.45	3/6		
10e-1	-	0/6			-	0/6		

Supplementary Material

Primers and probe, reaction mix, thermal cycling conditions and calibration curve used to detect and quantify HCoV 229-E.

Reaction mix (10 μ L) consisted of 5.00 μ L 2X One Step RT-PCR Buffer III, 0.20 μ L PrimeScript RT enzyme Mix II, 0.20 μ L ROX, 0.20 mL TaKaRa Ex Taq HS, 0.30 μ L 229E-F and 229E-R primers (10mM), 0.15 μ L 229E-P probe (10mM). The cycling parameters were as RT at 48 $^{\circ}$ C for 30 min, preheating at 95 $^{\circ}$ C for 10 min and 45 cycles of amplification at 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 1 min.

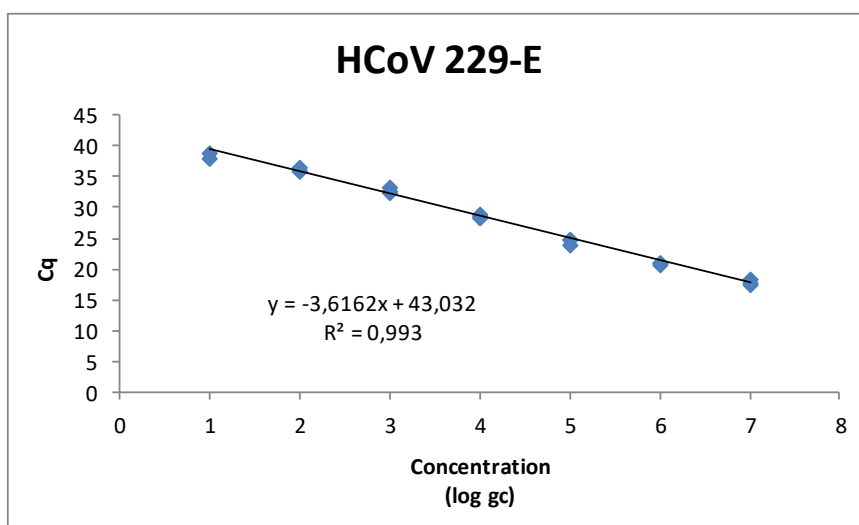


Figure S1. Standard curve generated to quantify HCoV 229-E performed with 10-fold dilutions (10^0 - 10^7 gc/reaction) of genomic RNA.

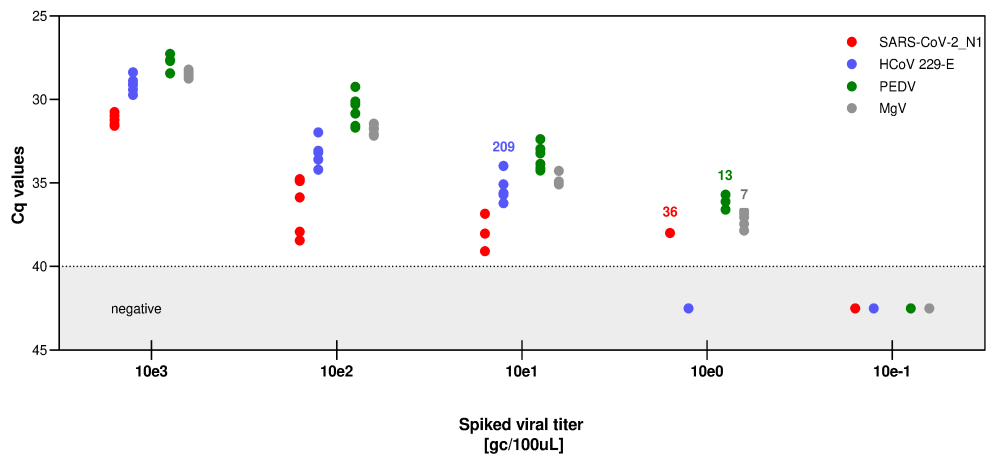


Figure S2. Distribution of cycle threshold values (Cq) characterizing the limits of detection of the automated assisted method (Max) to extract viral RNA form breast milk samples: severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), human coronavirus 229E (HCoV 229-E), porcine epidemic diarrhea virus (PEDV), and mengovirus (MgV) spiked in breast milk. The numbers above viral series denotes the $LoD_{95\%}$ (gc/100uL) for each virus.

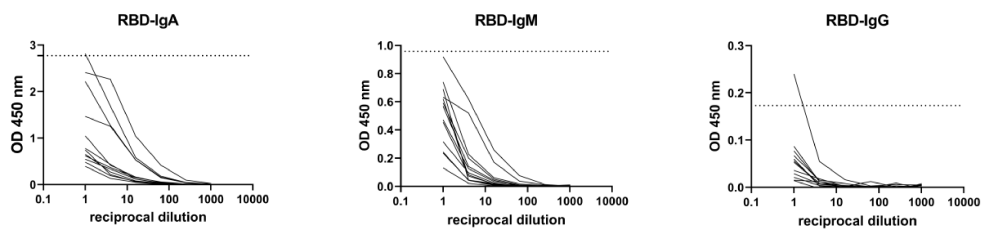


Figure S3: Titration curves of pre-pandemic Control samples. Serial dilutions of pre-pandemic Controls to determine cut-off values in order to discriminate between negative and positive samples in COVID-19 collected breastmilk samples, calculated as the mean + 2SD.

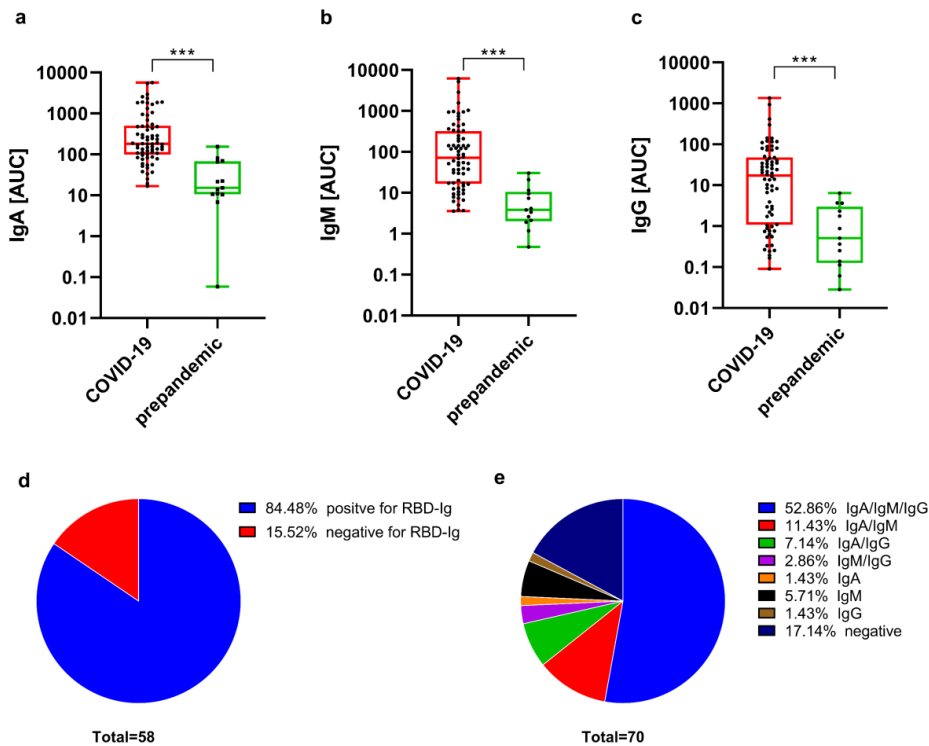


Figure S4: Breast milk from COVID-19 infected and/or recovered mothers show significantly higher antibody binding to the RBD antigen of SARS-CoV-2 than prepandemic controls and are detectable in 82.9 % of samples. AUC were calculated from titration curves for RBD-reactive (a) IgA, (b) IgM, and (c) IgG in order to get a better graphical impression. Asterisks show statistically significant differences between groups (***) $p < 0.0001$ using the Mann–Whitney test (unpaired nonparametric test). (d) Proportion of human milk donors who had positive RBD-reactive Igs in at least one milk recollection point, and negative samples (e) Proportion of RBD-reactive positive and negative human milk samples subdivided according to different isotypes.

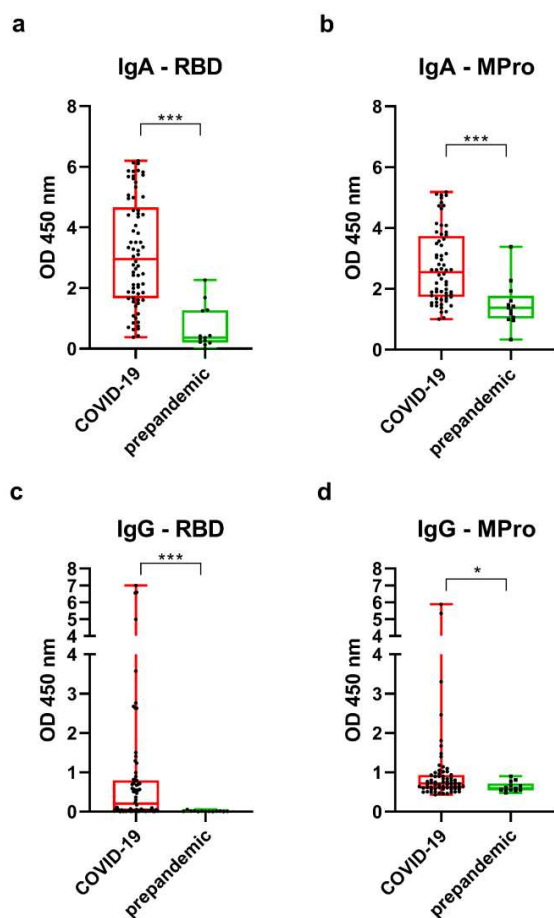


Figure S5: Samples from infected milk donors show significantly higher binding to SARS-CoV-2 antigens RBD and Mpro compared to prepandemic control. Grouped OD values of 1:4 diluted samples of RBD- (a) and MPro- (b) reactive IgA, and RBD- (c) and MPro- (d) reactive IgG, respectively. Asterisks show statistically significant differences between groups (** $p < 0.0001$, * $p < 0.05$) using the Mann-Whitney test (unpaired nonparametric test).

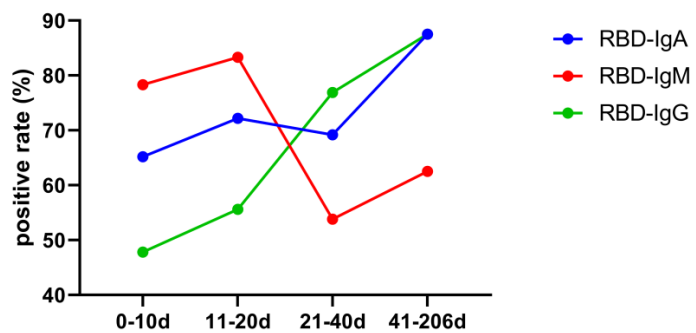


Figure S6: Graph of positive rates of RBD-specific IgA, IgM and IgG versus days after positive PCR diagnosis.

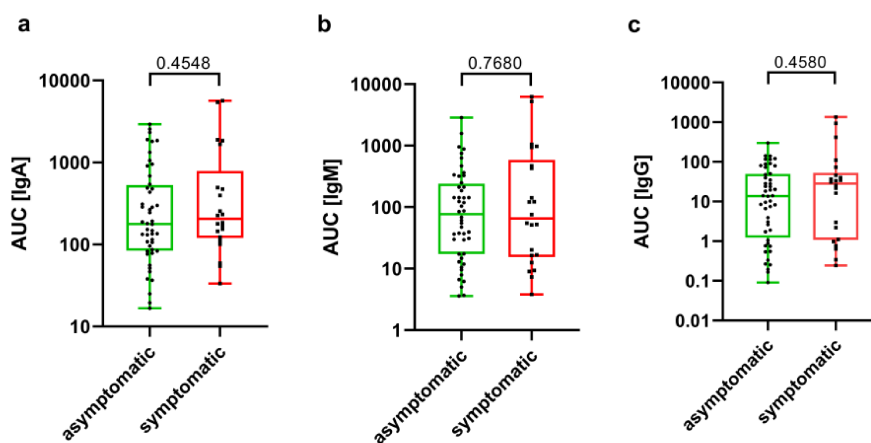


Figure S7: : Reactivity of breast milk samples from asymptomatic and symptomatic COVID-19 infected and/or recovered mothers to RBD antigen. AUC were calculated from titration curves for RBD-reactive (a) IgA, (b) IgM and (c) IgG in order to get a better graphical impression. Mann–Whitney test (unpaired nonparametric test) was used to assess for statistical significance.

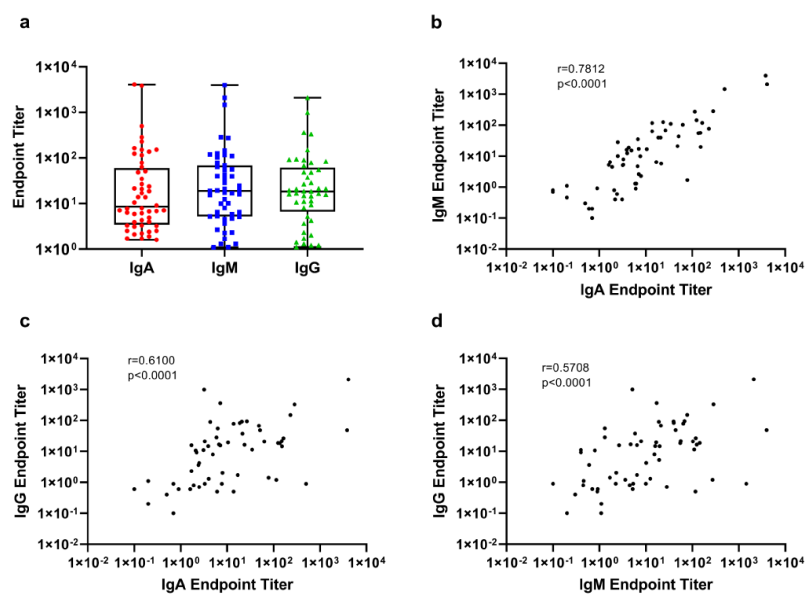


Figure S8: Endpoint titers for RBD-specific IgA, IgM and IgG and correlation analysis between antibody subclasses. (a) Grouped endpoint titers of the three different isotypes. Spearman's correlation analysis of endpoint titers between (a) RBD-specific IgA and RBD-IgM, (b) RBD-IgA and RBD-IgG, and (c) RBD-IgM and RBD-IgG.

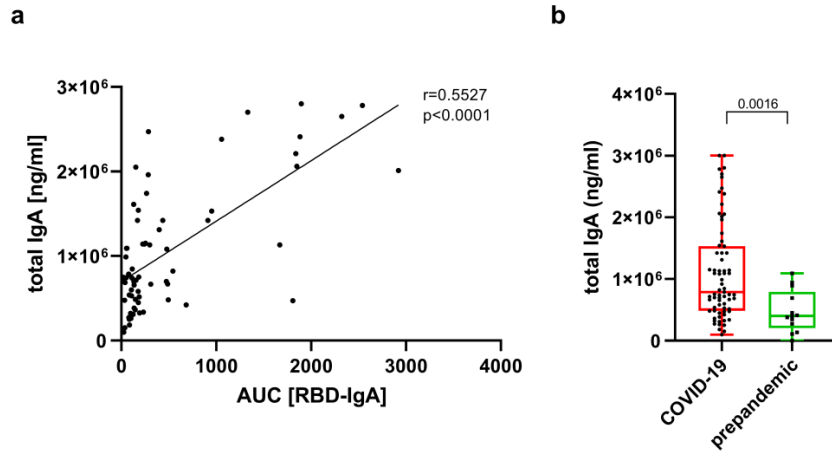


Figure S9: Total IgA concentration and virus specific antibody response.

(a) Spearman's correlation analysis of total IgA concentration and virus specific IgA response expressed as AUC. (b) Total IgA in COVID-19 infected and recovered and pre-pandemic control samples. Mann-Whitney test (unpaired nonparametric test) was used to assess for statistical significance.

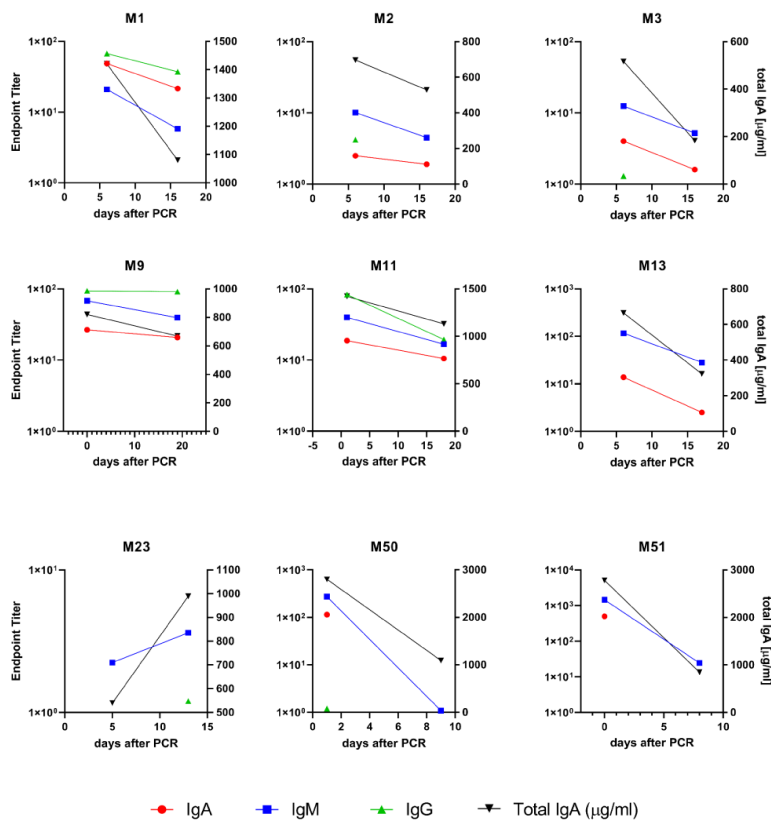


Figure S10: Temporal dynamic changes of endpoint titers in longitudinal samples for RBD-specific Igs. Longitudinal samples at two time points from 12 mothers were available, samples from 3 mothers were negative at both times and not represented. Virus-specific IgA (red circles), IgM (blue squares), IgG (green triangles) and total IgA (black triangles). All isotypes were tested for RBD binding in both time points, only positive endpoint titer are drawn in the graphs, the absence of data in a given time point indicates samples that were below the Cut-off values and considered negative.

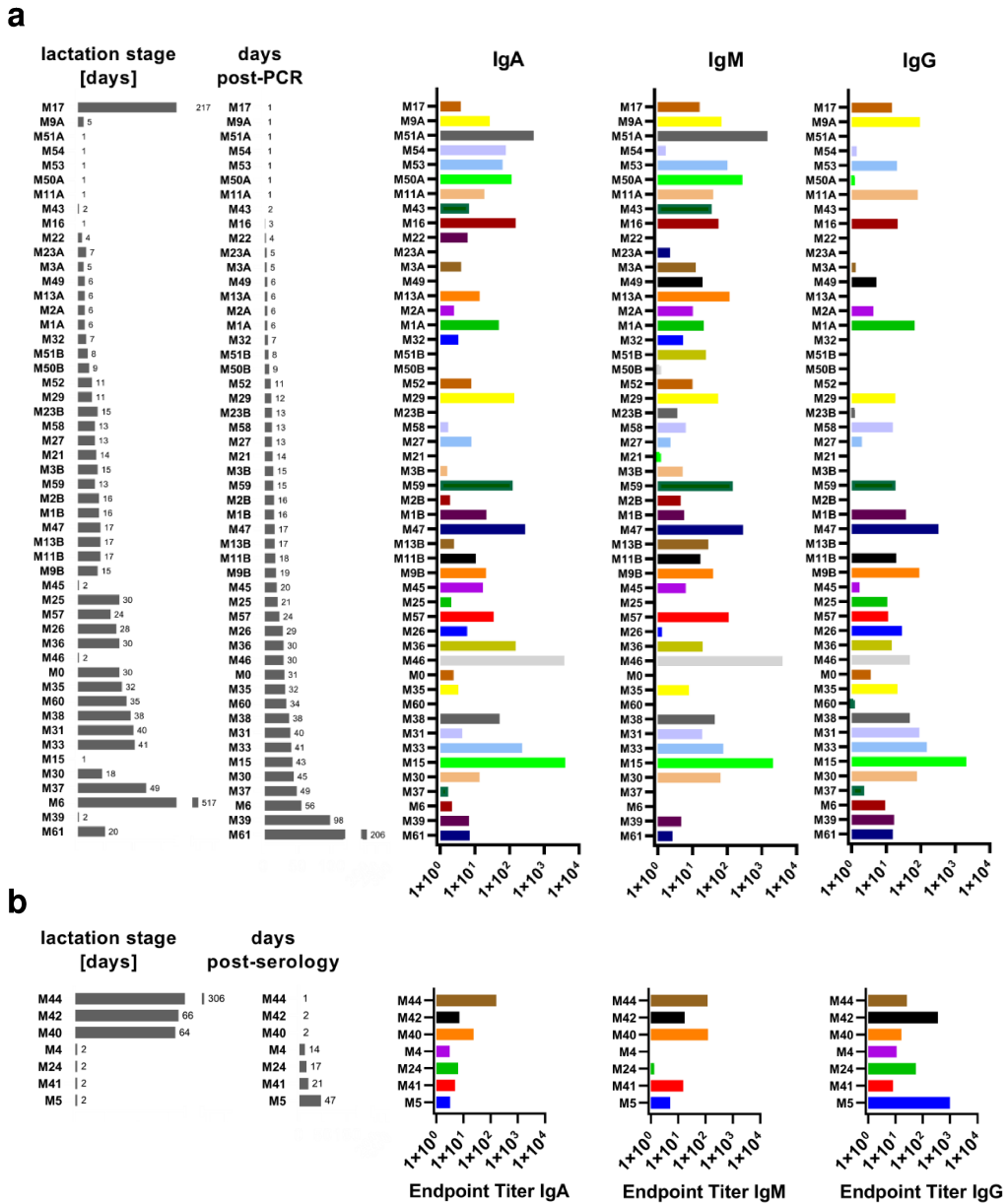


Figure S11: Individual endpoint titers of virus-specific antibodies in breast milk from SARS-CoV-2 infected and/or recovered mothers. (a) Endpoint titers of milk samples tested positive at least in one of the isotypes IgA, IgM and IgG are shown, ordered as days post-PCR. (B) Endpoint titers of milk samples tested positive at least in one of the isotypes IgA, IgM, and IgG are shown, ordered as days post-serology.