Anti-SARS-CoV-2-specific antibodies in human breast milk following SARS-CoV-2 infection during pregnancy: a prospective cohort study

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Abstract

Background

While the presence of SARS-CoV-2 in human breast milk is contentious, anti-SARS-CoV-2 antibodies have been consistently detected in the human breast milk. However, it is uncertain when the antibodies become present and for how long they last.

Methods:

This was a prospective cohort study including all consecutive pregnant women with confirmed SARS-CoV-2 infection during pregnancy, recruited at six maternity units in Spain and Hong Kong over a one-year period. Colostrum and mature milk were prospectively collected by manual expression with strict contact precautions and paired maternal blood samples were also collected. Colostrum samples were first tested with rRT-PCR-SARS-CoV-2 and skimmed acellular milk and maternal sera were tested against SARS-CoV-2 specific immunoglobulin (Ig) M, A and G reactive to receptor binding domain of SARS-CoV-2 spike protein 1 to determine the presence of immunoglobulins. Then, we examined how the presence of each immunoglobulin type in the colostrum was related to the time when infection was acquired by logistic regression analysis and how they were correlated to the immunoglobulins present in maternal serum and mature milk by Cohen's kappa statistic.

Results

187 pregnant women with confirmed SARS-CoV-2 infection during pregnancy or at delivery were recruited. No SARS-CoV-2 was found in the human breast milk. IgA and IgG were present in 129/162 and 5/163 colostrum samples and in 19/74 and 3/74 mature milk samples, respectively. IgA was the predominant immunoglobulin found in the breast milk and its levels were significantly higher in the colostrum than in the mature milk. We did not find that the presence of immunoglobulins in the colostrum was correlated to their presence in maternal serum, the severity of the disease or the time when the infection had occurred.

Interpretation

Since antibodies are found in the colostrum irrespective of the time of infection and SARS-CoV-2 is not detected in human breast milk, all women should be encouraged to breastfeed, undertaking contact precautions when there is active disease.

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Background

On March 11 2020 the COVID-19 pandemic was declared by the World Health organization (WHO)(5). Since then, extensive efforts have focused on evaluating the effects of the new coronavirus on pregnancy. At the very beginning of the pandemic, newborns were separated from their mothers with confirmed SARS-CoV-2 infection in order to protect them against the virus and breastfeeding was avoided because it was unknown if the virus could be transmitted via human breast milk. To date, some studies have reported the presence of SARS-CoV-2 in the human breast milk(1–4) while others have not(6–8), but the sample size of these studies is small.

Currently, most healthcare systems and international organizations such as the Centers for Disease Control and Prevention (CDC) recommend breastfeeding for all mothers with active or past infection of SARS-CoV-2, as there appear to be more benefits of breastfeeding than the potential risk of transmission through human breast milk. One of the most important reasons to recommend breastfeeding is the possible passive immunization in the newborns against SARS-CoV-2(9). Several studies have reported the presence of anti-SARS-CoV-2 antibodies(10–15) in the human breast milk. Pace et al. have demonstrated that the anti-SARS-CoV-2 antibodies in human breast milk can effectively neutralize SARS-CoV-2 infectivity(16). However, it is uncertain when the antibodies become present and for how long they last in the human breast milk.

The aims of this study were first, to determine the presence of anti-SARS-CoV-2 virus and antibodies in colostrum and mature human breast milk in women who had SARS-CoV-2 infection during pregnancy or at the time of delivery; second, to investigate the correlation between the anti-SARS-CoV-2 antibodies in human milk with the levels of anti-SARS-CoV-2 antibodies in maternal blood, severity of SARS-CoV-2 infection and the time interval from active illness; and third, to evaluate how each immunoglobulin type evolved from the colostrum to the mature milk.

Methods

Study population

This was a prospective cohort study including all consecutive pregnant women with laboratory confirmed SARS-CoV-2 infection by deep throat saliva (DTS) or nasopharyngeal swab (NPS) real-time reverse-transcriptase-polymerase-chain-reaction (rRT-PCR) test or by rapid antigen-detection tests (Panbio™ Covid-19 Ag Rapid Test Device)(17), during pregnancy or at the time of delivery, who consented to participate in the study. Additionally, for suspected cases of COVID-19 where rRT-PCR was negative, if the symptoms had started within seven days of testing, the rRT-PCR was repeated 24 hours after the first test, and if the symptoms had started beyond seven days of testing, a serology test (ELISA) was performed(18) and women with positive results by either test were also offered participation.

Breast milk samples were collected from six maternity units, five in Spain (Hospital Universitario de Torrejón and Hospital Universitario Príncipe de Asturias in Madrid, Hospital Universitario Vall d'Hebrón in

Barcelona, Hospital Clínico Universitario San Cecilio in Granada and Hospital Clínico Universitario Virgen de la Arrixaca in Murcia) and one in Hong Kong SAR, China (The Chinese University of Hong Kong COVID-19 collaborative network), from March 2020 to March 2021. All participants were unvaccinated against SARS-CoV-2, and it was their first SARS-CoV-2 infection. The study was approved by each of the Local Research Ethics Committees at the participating centers. All women gave written informed consent.

Participants had one sample of colostrum (between the day of delivery until day 4 postpartum) collected and stored at -80ºC. Maternal blood for serological analysis was also collected at the same time, serum was separated and stored at -80ºC. Whenever possible, one sample of mature milk (from day 7 postpartum until 6 weeks postpartum) was also collected and stored.

Clinical data, including maternal age, body mass index (BMI) at the beginning of pregnancy, gestational age at the time of SARS-CoV-2 infection and severity of the disease, were recorded for every participant, pseudo-anonymized and entered into a secured common database. The COVID-19 severity was classified as asymptomatic, mild (when no hospitalization was required) and pneumonia (when the diagnosis of pneumonia was established and needing hospitalization)(19). Gestational age was determined by first trimester sonographic assessment of fetal crown-rump length(20) or conception date in *in vitro* fertilization pregnancy.

Biological sample collection and analysis

Breast milk (from 0·1 to 1·0 mL) was collected by manual expression with strict contact precautions to avoid contamination (facial mask and hand cleaning). Blood samples were collected in serum sep clot activator 8 mL tubes, which were then centrifuged for five minutes at 3500g and then serum was collected. Both serum and breast milk samples were divided into 0·5 mL aliquots (when possible) in separate Eppendorf tubes, which were labelled with a unique patient identifier and stored at -80ºC until subsequent analysis. Stored samples from Barcelona were analyzed locally at the end of the recruitment period. Samples from all other sites were sent without any further processing overnight on dry ice to Synlab Diagnósticos Globales Laboratory in Madrid on monthly basis from Spanish sites and in a single batch after rt-RT-PCR testing performed locally at the end of the recruitment period from Hong Kong.

At the laboratory, breast milk samples were thawed, centrifuged at 800g for 15 minutes, fat was removed, and supernatant transferred to a new tube. Centrifugation was repeated twice to ensure removal of all cells and fat(12). Skimmed acellular milk was then tested against SARS-CoV-2 specific immunoglobulin M (IgM), immunoglobulin A (IgA) and immunoglobulin G (IgG) reactive to the receptor binding domain (RBD) of the SARS-CoV-2 spike protein 1 (protS1)(12,15). Serum samples were thawed and tested against SARS-CoV-2 specific antibodies. Determination of IgA and IgG antibodies were performed by ELISA method (Enzyme-Linked Immunosorbent Assay) providing semi-quantitative serology results against the S1 domain of the spike protein of SARS-CoV-2 in serum samples (Anti-SARS-CoV-2 ELISA IgG and Anti-SARS-CoV-2 ELISA IgA, Euroimmunn Medizinische Labordiagnostika AG, Lubeck, Alemania)(21). IgM determination was performed by chemiluminescencent microparticle immunoassays, using spike protein specific (Abbott test, SARS-CoV-2 IgM Abbott, Abbott Ireland Diagnostics Division Finisklin, Ireland)(22).

IgA and IgG were considered positive, indeterminate and negative when results were > 1·1, 0·8 to 1·1 and < 0·8, respectively; IgM was considered positive, indeterminate and negative when results were > 1·1, 0·9 to 1·1 and < 0·9, respectively.

Whenever available, a second colostrum aliquot was tested for SARS-CoV-2 by rRT-PCR. In the Spanish samples, viral RNA was extracted with Chemagic Viral DNA/RNA Kit using the Chemagic 360 with integrated dispense, that includes lyophilized Poly(A) RNA, lyophilized Proteinase K and a lysis/binding buffer, and were analyzed with Euroinmune Kit (ORF1ab an N targets) and TaqMan™ 2019-nCov Assay kitv2 Thermofisher (s,ORF1ab and N targets). In the Hong Kong samples, viral RNA was extracted using RNeasy® Mini Kit (QIAGEN) and the detection of SARS-CoV-2 RNA was performed with the FDA-authorized CDC 2019-Novel Coronavirus (2019 nCoV) Real-Time RT-PCR Diagnostic Panel (EUA 200001). The N gene (both N1 and N2) was assayed, with the human RNase P (RP) as an endogenous reference control. In all cases, samples that contained organic or inorganic contaminants that interfered in the amplification of the PCR were considered inhibited.

For this study we included all women with available immunoglobulin results measured in the colostrum, additional samples or analyses were not mandatory for inclusion. Given the limited volume of colostrum and serum collected, not all tests could be carried out in all cases and for some laboratory analyses which failed at first attempt, repeat testing was not possible. Besides, due to the lockdown many women did not return to the clinic after delivery, therefore, we could not collect mature milk in these cases.

Statistical analysis

Descriptive data was expressed as median and interquartile range (IQR) and in proportions (absolute and relative frequencies). Cohen's Kappa was used to assess the concordance between the colostrum and the serum. The kappa statistic was calculated without weighting; very good levels of agreement were considered when it is > 0·80, good 0·80 – 0·60, moderate 0·60 – 0·40, poor 0·40 – 0·20 and very poor < 0·20(23). Logistic regression analysis was performed to assess if the presence of immunoglobulins in colostrum was associated with the presence of immunoglobulins in maternal serum, the severity of maternal symptoms, or the time passed from infection. Odds ratio (OR), 95% confidence interval (CI) and p-values were reported.(24) Lastly, McNemar test was used to evaluate how each immunoglobulin type evolved over time in all paired colostrum-mature milk samples. P-values were reported. Level of significance was set at 0·05.

The statistical software R version 4·1·2 (Vienna, Austria) was used for all data analyses(25).

Results

We included 187 women; 38 (20·3%), 65 (34·8%) and 84 (44·9%) women acquired the infection in the first (< 14 weeks), second (14–28^{+6}) and third trimester (> 28^{+6}) of pregnancy, respectively. From those diagnosed in the third trimester, 29 (34·5%) had active SARS-CoV-2 infection at the time of delivery (rRT-PCR-SARS-CoV-2 positive at delivery). Pregnancy and disease characteristics are shown in Table 1.

Table 1
Maternal, pregnancy and disease characteristics.

	1T (N = 38)	2T (N = 65)	3T (N = 57)	No active disease (N = 158)	Active infection at delivery (N = 31)	Overall (N = 189)
Maternal age (years)	33.0 [30.3, 37.0]	34.0 [30.0, 37.0]	31.0 [28.0, 36.0]	33.0 [29.0, 37.0]	33.0 [28.5, 37.0]	33.0 [29.0, 37.0]
Height (cm)	165 [159, 170]	162 [159, 168]	161 [156, 168]	163 [158, 169]	161 [156, 166]	163 [158, 168]
Weight (kg)	65.0 [56.3, 74.8]	69.0 [58.0, 76.0]	62.5 [54.0, 70.3]	65.0 [56.5, 74.0]	60.0 [54.4, 74.0]	64.5 [56.0, 74.0]
Missing	0 (0%)	0 (0%)	1 (1.8%)	1 (0.6%)	0 (0%)	1 (0.5%)
Body mass index (kg/m^2)	24.2 [21.5, 27.5]	24.9 [21.5, 28.7]	23.4 [21.7, 25.8]	24.1 [21.5, 27.6]	24.7 [21.0, 28.7]	24.2 [21.5, 28.0]
Covid-19 diagnosis with						
Antigens	9 (23.7%)	13 (20.0%)	7 (12.3%)	29 (18.4%)	3 (9.68%)	32 (16.9%)
RRT-PCR	20 (52.6%)	38 (58.5%)	38 (66.7%)	94 (59.5%)	26 (83.9%)	120 (63.5%)
Serology	9 (23.7%)	14 (21.5%)	12 (21.1%)	35 (22.2%)	2 (6.45%)	37 (19.6%)
Gestational age at diagnosis of COVID-19 (days)	65.5 [50.5, 82.8]	162 [134, 182]	240 [226, 254]	176 [105, 226]	268 [265, 280]	195 [115, 248]
COVID-19 symptoms						
Asymptomatic	6 (15.8%)	8 (12.3%)	17 (29.8%)	30 (19.0%)	17 (54.8%)	47 (24.9%)
Mild	31 (81.6%)	50 (76.9%)	33 (57.9%)	113 (71.5%)	12 (38.7%)	125 (66.1%)
Pneumonia	1 (2.63%)	7 (10.8%)	7 (12.3%)	15 (9.49%)	2 (6.45%)	17 (8.99%)
Gestational age at delivery (days)	279 [270, 284]	277 [272, 283]	278 [274, 283]	278 [273, 284]	275 [268, 286]	278 [272, 284]

	1T (N = 38)	2T (N = 65)	3T (N = 57)	No active disease (N = 158)	Active infection at delivery (N = 31)	Overall (N = 189)
* Results are presented as median (interquartile range) or as n (%) as appropriate.						

The colostrum and blood samples were collected between the day of delivery until day 4 postpartum (median = 1; IQR 0 to 1). Mature milk samples were collected after day 7 postpartum (median = 39 days, IQR 25 to 44). Sample availability and serological status are displayed in Table 2.

Table 2
Human breast milk and maternal blood serology.

	Colostrum	Mature milk	Maternal blood at delivery
IgA			
Indeterminate	4 (2.14%)	0 (0%)	15 (12.5%)
Negative	29 (15.5%)	51 (75.0%)	42 (35.0%)
Positive	129 (69.0%)	15 (22.1%)	62 (51.7%)
Insufficient sample	25 (13.4%)	2 (2.94%)	1 (0.833%)
Missing	2 (1.1%)	121 (64.0%)	69 (36.5%)
IgG			
Indeterminate	3 (1.60%)	0 (0%)	9 (6.47%)
Negative	155 (82.9%)	63 (92.6%)	61 (43.9%)
Positive	5 (2.67%)	3 (4.41%)	68 (48.9%)
Insufficient sample	24 (12.8%)	2 (2.94%)	1 (0.719%)
Missing	2 (1.1%)	121 (64.0%)	50 (26.5%)
IgM			
Indeterminate	0 (0%)	0 (0%)	4 (4.26%)
Negative	61 (60.4%)	52 (96.3%)	59 (62.8%)
Positive	15 (14.9%)	0 (0%)	30 (31.9%)
Insufficient sample	25 (24.8%)	2 (3.70%)	1 (1.06%)
Missing	88 (46.6%)	135 (71.4%)	95 (50.3%)
PCR			
Negative	73 (90.1%)		
Positive	0 (0%)		
Inhibited	3 (3.70%)		
Insufficient sample	5 (6.17%)		
Missing	108 (57.1%)		

IgA, IgG and IgM were present in 129/162 (79·6%), 5/163 (3·1%) and 15/76 (19·7%) colostrum samples, respectively. 76 colostrum samples were tested for rRT-PCR-SARS-CoV-2, including 29 with active disease at the time of delivery. 73 tested negative and 3 were inhibited.

Concordance between colostrum and serum

118 women had at least one serology result. Concordance between the colostrum and serum measured with Cohen's kappa was 0·09 (IC95% -0·11 to 0·30) for IgA; 0·06 (IC95% -0·01 to 0·12) for IgG and 0·29 (IC95% 0·03 to 0·54) for IgM.

Factors related to colostrum positivity

There were no statistically significant differences between the immunoglobulin status in colostrum and the severity of the symptoms nor the time interval from the disease, either as a continuous variable or considering only active disease at delivery vs. no active disease at delivery (Table 3).

Table 3
Factors related to colostrum positivity. Results from three logistic regression models using immunoglobulin positivity in colostrum as dependent variable and a) symptoms, b) interval from disease to sample and c) active disease at delivery as independent variables.

Dependent: Ig positivity	**IgM**			**IgA**			**IgG**		
Predictors	**Odds Ratio**	**CI**	**p**	**Odds Ratio**	**CI**	**p**	**Odds Ratio**	**CI**	**p**
Asymptomatic	Reference			Reference			Reference		
Mild	0.80	0.21–3.97	0.761	1.27	0.50–3.04	0.602	1.22	0.15–25.01	0.866
Pneumonia	NA	NA	0.992	4.35	0.72–84.10	0.181	3.15	0.12–83.62	0.428
Interval from disease to sample – colostrum	1.00	0.99–1.00	0.227	1.00	0.99–1.00	0.423	1.00	0.98–1.01	0 .644
No active disease	Reference			Reference			Reference		
Active disease	1.19	0.16–5.63	0.842	0.51	0.20–1.44	0.180	1.43	0.07–10.25	0.752

Antibody evolution from colostrum to mature milk

In mature milk samples, IgG was positive in 2/62 (3·23%) (two positivizations in women with active disease at birth and no negativizations); IgA was positive in 17/62 (27·42%) (32 negativizations from colostrum; p-value for the difference < 0.001); and IgM was positive in 0/51 (6 of 51 were positive in colostrum).

Discussion

Main findings

The study has demonstrated that, firstly, all human breast milk tested for rRT-PCR SARS-CoV-2 are negative; secondly, antibodies against SARS-CoV-2 present in the colostrum do not seem to vary significantly in relation to the time when the infection has occurred during pregnancy or in relation to their presence in the maternal blood; and thirdly, IgA is the predominant immunoglobulin found in human breast milk and its concentrations are significantly lower in the mature milk compared to the colostrum.

Study strengths and limitations

To our knowledge, this is the largest series of breast milk samples from women with SARS-CoV-2 infection during pregnancy or at the time of delivery. We also collected paired colostrum and mature milk samples and studied the serological status of the mother at the time of milk sampling, which allowed us to study immunoglobulin concordance between the colostrum, mature milk and maternal blood. Additionally, the protocol for collection, handling, and storage of samples was defined early and implemented in all centers(9).

However, the main limitations relate to the technical difficulties that reduced the sample further, which may have prevented us to recognize other possible association or significant finding.

Interpretation

It is well known that breastfeeding protects babies against gastrointestinal and respiratory infections(26–29). IgA represents around 90% of all immunoglobulins present in human milk and its concentration is higher in the colostrum, decreasing during the first year of lactation(9). Due to its low degradation and absorption rate in the infant's gastrointestinal system, IgA is the most important immunoglobulin in human milk, protecting the infant against infections at mucosa level. Recently, it has been demonstrated that anti-SARS-CoV-2 antibodies in breast milk neutralize the virus *in-vitro*(16,30,31). Therefore, anti-SARS-CoV-2 IgA in human breast milk could protect the infant against the SARS-CoV-2 infection at a local level, similar to what happens with other viral infections(32).

In our study, most of the colostrum samples tested positive for IgA, irrespective of the time of SARS-CoV-2 infection. When evaluating longitudinal changes in the colostrum and the mature milk, a significant reduction in IgA positivity was found. This is similar to what happens with other viral infections(9). Importantly, IgA was even present in the colostrum from mothers with a negative serological status at the time of delivery, contrary to what happened with IgG, which was more likely to be present when IgG in serum was also present. A possible explanation for this could be related to the fact that IgA is secreted from Peyer patches, while IgG is mostly filtered from the maternal plasma(33). Peyer patches belong to the Gastrointestinal Antigen Linfoid Tissue (GALT) system and they represent maternal immunological memory(34,35). This system is responsible for secreting antibodies against common infections prevalent in maternal living area(36). IgM is also secreted by this system, but at much lower concentrations.

In this study 29 samples from women with active disease at the time of delivery were tested by rRT-PCR-SARS-CoV-2 and all of them were negative. Evidence suggesting the presence of SARS-CoV-2 in the breast milk is conflicting(1–4,6–8,13) and it is possible that cross-contamination was responsible for the

positive results(16). Goad et al investigated the presence of cell-specific expression of angiotensin-converting enzyme 2 (ACE2), proteases TMPRSS2, and cathepsins CTSB and CTSL in breast epithelium and they did not find co-expression of ACE2/TMPRSS2 or ACE2/CTSB/L, which is important for the entry of the virus into the cell. Therefore, they concluded that there was no risk of vertical transmission of SARS-CoV-2 in neonates through breastfeeding(37).

Clinical implications

This study confirms that SARS-CoV-2 is not detected in breast milk, even when active infection is present at the time of delivery and therefore, the possibility of vertical transmission while breastfeeding is extremely low. Furthermore, since antibodies are found in the colostrum irrespective of the time of infection, all women should be encouraged to breastfeed their infants, undertaking contact precautions when there is active disease. Nevertheless, since IgA concentrations drop significantly from the colostrum to mature milk, we could speculate that they might be even lower beyond six weeks postpartum, so public health measures should still be maintained in order to reduce the risk of the babies in acquiring the infection.

Conclusions

Our study has provided further evidence that breastfeeding is safe during maternal SARS-CoV-2 infection as the virus has not been detected in human breast milk. In addition, breastfeeding should be encouraged in every woman with active or past SARS-CoV-2 infection as the anti-SARS-CoV-2 antibodies are present in both the colostrum and mature milk, irrespective of the time when the infection has occurred during the pregnancy.

Abbreviations

rRT-PCR: real-time reverse-transcriptase-polymerase-chain-reaction

LMP: Last Menstrual Period

BMI: Body mass index

IgM: Immunoglobulin M

IgA: Immunoglobulin A

IgG: Immunoglobulin G

RBD: Receptor binding domain

protS1: SARS-CoV-2 spike protein 1

ELISA: Enzyme-Linked Immunosorbent Assay

IQR: Interquartile range

Rho: Spearman correlation coefficient

CI: Confidence interval

GALT: Gastrointestinal Antigen Linfoide Tissue

ACE2: Angiotensin-converting enzyme 2

Declarations

Ethics approval and consent to participate

The study was approved by each one of the Local Research Ethics Committees at the participant centers. All women gave written informed consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interest.

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