Relationship between 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

Objective: To determine the presence of anti-SARS-CoV-2 antibodies in colostrum and mature milk in women who had SARS-CoV-2 infection during pregnancy or at delivery; to investigate the correlation between anti-SARS-CoV-2 antibodies in milk with antibody in maternal blood, severity of infection and time-interval from active illness; and to evaluate immunoglobulin evolution from colostrum to mature milk. Design: prospective cohort-study Setting: six hospitals in Spain and Hong-Kong. Sample: pregnant women with confirmed SARS-CoV-2 infection during pregnancy or at delivery. Methods: Colostrum and mature milk were collected by manual expression with strict contact precautions. Colostrum samples were tested with rRT-PCR-SARS-CoV-2 and both, maternal milk and serum were tested against SARS-CoV-2 specific immunoglobulin M, A and G reactive to receptor binding domain of SARS-CoV-2 spike protein-1. Results: All rRT-PCR-SARS-CoV-2 tested negative. IgA and IgG were present in 111/135 (82.2%) and 2/135 (1.5%) colostrum samples and 27/81 (33.3%) and 0/81 mature milk samples, respectively. Concentrations of immunoglobulins were not associated with the timing of infection but women with SARS-CoV-2 pneumonia had higher levels of IgA and IgG in colostrum than those who were asymptomatic or had mild symptoms. Conclusion: No SARS-CoV-2 virus was found in human milk, however, high levels of antibodies were found in colostrum, specially IgA, irrespective of the time of infection. All women should be encouraged to breastfeed, undertaking strict contact precautions when there is active disease. Funding: Spanish Government grant (Instituto de Salud Carlos III: COV20/00188). Synlab Diagnostics' Globales (Madrid, Spain). Perkin Elmer.

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(*Shortened running title* **:** Human milk anti-SARS-CoV2 antibodies)

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Abstract:

Objective : to determine the presence of anti-SARS-CoV-2 antibodies in colostrum and mature milk in women who had SARS-CoV-2 infection during pregnancy or at delivery; to investigate the correlation between anti-SARS-CoV-2 antibodies in milk with antibody in maternal blood, severity of infection and time-interval from active illness; and to evaluate immunoglobulin evolution from colostrum to mature milk.

Design : prospective cohort-study

Setting : six hospitals in Spain and Hong-Kong.

Sample : pregnant women with confirmed SARS-CoV-2 infection during pregnancy or at delivery.

Methods : Colostrum and mature milk were collected by manual expression with strict contact precautions. Colostrum samples were tested with rRT-PCR-SARS-CoV-2 and both, maternal milk and serum were tested

against SARS-CoV-2 specific immunoglobulin M, A and G reactive to receptor binding domain of SARS-CoV-2 spike protein-1.

Results : All rRT-PCR-SARS-CoV-2 tested negative. IgA and IgG were present in 111/135 (82.2%) and 2/135 (1.5%) colostrum samples and 27/81 (33.3%) and 0/81 mature milk samples, respectively. Concentrations of immunoglobulins were not associated with the timing of infection but women with SARS-CoV-2 pneumonia had higher levels of IgA and IgG in colostrum than those who were asymptomatic or had mild symptoms.

Conclusion: No SARS-CoV-2 virus was found in human milk, however, high levels of antibodies were found in colostrum, specially IgA, irrespective of the time of infection. All women should be encouraged to breastfeed, undertaking strict contact precautions when there is active disease.

Funding : Spanish Government grant (Instituto de Salud Carlos III: COV20/00188). Synlab Diagnostics' Globales (Madrid, Spain). Perkin Elmer.

Keywords: Human Breast Milk, Colostrum, SARS-CoV-2, COVID-19, anti-SARS-CoV-2 specific antibodies.

Tweetable abstract: Colostrum (n = 135) and mature milk (n = 81) were collected from pregnant women who had SARS-CoV-2 infection during pregnancy or at delivery. All colostrum samples tested negative by rRT-PCR-SARS-CoV-2. However, IgA and IgG were present in 111 (82.2%) and 2 (1.5%) colostrum samples and 27 (33.3%) and 0 mature milk samples, respectively. Concentrations of immunoglobulins were not associated with the timing of infection but women with SARS-CoV-2 pneumonia had higher levels of IgA and IgG in colostrum than those who were asymptomatic or had mild symptoms. All women should be encouraged to breastfeed, undertaking strict contact precautions when there is active disease.

Introduction:

On March 11 2020 the COVID-19 pandemic emergency was declared by the World Health organization (WHO)[1]. Since then, all obstetric 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 SARS-CoV-2 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[2–5] while others have not[6–8], but 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 SARS-CoV-2 active or past infection, 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 of newborn 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 concentrations of anti-SARS-CoV-2 antibodies correlate with the milk's ability to 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 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 (Abbott Realtime SARS CoV-2 Amplification reagent kit. Abbott molecular Inc. Des Plaines, IL USA)[17] , during pregnancy or at the time of delivery, who consented to participate in the study. In probable 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].

Breast milk samples were collected from six recruiting units, Hospital Universitario de Torrejon (Madrid, Spain), Hospital Universitario Vall d'Hebron (Barcelona, Spain), Hospital Universitario Clinico San Cecilio (Granada, Spain), the Chinese University of Hong Kong COVID-19 collaborative network *(* Hong Kong SAR China*),* Hospital Clinico Universitario Virgen de la Arrixaca (Murcia, Spain) and Hospital Universitario Principe de Asturias (Madrid, Spain) from March 2020 to March 2021. The study was approved by each one of the Local Research Ethics Committees at the participant centers. All women gave written informed consent.

Each participant had one sample of colostrum (between the day of delivery until day 4 postpartum), and one sample of mature milk (from day 7 postpartum until 6 weeks 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.

Clinical data, including maternal age, body mass index (BMI) at the beginning of pregnancy, date of last menstrual period (LMP) and COVID-19 severity, was recorded for every participant, pseudo-anonymized and entered into a common secured 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]. The time interval in days between maternal diagnosis of COVID-19 and delivery was calculated. Gestational age was estimated by first trimester sonographic assessment of fetal crown-rump length[20] or confirmed by LMP.

Virological 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 Diagnosticos Globales Laboratory (Alcobendas, Madrid, Spain) on monthly basis from Spanish sites and in a single batch 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]. Both techniques used CE marked and validated kits.

IgA and IgG were considered positive, indeterminate and negative when results were >1*1, 0*8-1*1 and <0*8, respectively; IgM was considered positive, indeterminate and negative when results were >1*1, 0*9-1*1 and <0*9, respectively. IgM could not be analyzed in all the samples send to the laboratory due to insufficient sample (82 colostrum samples and 12 mature milk samples).

Colostrum samples from mothers with active illness at the time of collection were tested for SARS-CoV-2 by rRT-PCR. 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^{TM} 2019-nCov Assay kitv2 Thermofisher (s,ORF1ab and N targets). For Hong Kong samples, viral RNA was extracted using RNeasy(r) 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. Samples that contained organic or inorganic contaminants that interfered in the amplification of the PCR were considered inhibited.

Statistical analysis

Descriptive data was expressed as median and interquartile range (IQR) and in proportions (absolute and relative frequencies). Spearman correlation coefficient (Rho) was calculated as a measure of the degree of the relationship between each immunoglobulin type measured in colostrum and maternal serum, and between each immunoglobulin type measured in mature milk and maternal serum. Linear regression analysis was performed to assess the relationship between maternal symptoms and immunoglobulin levels. Coefficients, 95% confidence interval (CI) and p-value were reported. To graphically represent the relationship between antibody levels and time lapse to disease, we adjusted a locally weighted scatterplot smoothing (LOWESS) regression which, essentially, joins together the curves generated by several regressions derived from different segments of the timeframe[23].

Lastly, to see how each immunoglobulin type evolved from the colostrum to the mature milk, paired t-test was used. This analysis was performed separately for women with active disease and those who had recovered from SARS-CoV-2 infection. In all cases mean of the differences, 95% CI and p-value were reported. Level of significance was set up at 0*05.

The statistical software R version 4*0*3 (Vienna, Austria) was used for all data analyses[24].

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Results

Overall, 177 women were recruited, and breast milk samples were collected for analysis from the six recruiting units. Maternal, pregnancy and disease characteristics are shown in Table 1. 29 (16*38%) women had active SARS-CoV-2 infection at the time of delivery. 28 (15*82%), 55 (31*07%) and 65 (36*74%) women acquired the infection in the first trimester (<14 weeks), second trimester (14-28^{+6}) and third trimester (>28^{+6}) of pregnancy, respectively.

148 samples of colostrum were collected between the day of delivery until day 4 postpartum. In 13 cases there was insufficient sample for analysis and therefore colostrum from 135 women was included in the final analysis. Of these, serological status of the women at the time of colostrum collection was available in 124 cases (Table 2). In 83 women milk samples were collected after day 7 postpartum (mature milk). In two

samples there was insufficient mature milk for analysis and therefore 81 samples were finally included. Of these, serological status of the women at the time of mature milk collection was available in 66 (Table 2; Figure 1).

IgA and IgG were present in 111/135 (82*23%) and 2/135 (1*48%) of the colostrum samples, respectively. Due insufficient sample, IgM could only be analyzed in 67 colostrum samples, and it was present in 14 (26*42%) cases. While IgG was negative in all mature milk samples, IgA was present in 27/81 (33*33%). IgM could only be analyzed in 59 mature milk samples and all tested negative (Table 2).

70 colostrum samples were tested against rRT-PCR SARS-CoV2. In 8 samples there was insufficient sample for analysis and therefore 61 were finally tested. 2 were inhibited and 59 tested negative.

Breast milk antibody correlation with maternal serological status

Among the 111 cases with positive IgA in the colostrum, maternal blood at the time of colostrum collection tested negative for IgM, IgG and IgA in five cases (Table 4). There was a significant correlation between IgM (rho=0*49; p=0*001), IgA (rho=0*3; p=0*008) and IgG (rho=0*27; p=0*018) measured in the colostrum and maternal serum (Figure 2). There was also a significant correlation between IgA (rho=0*49; p=0*002) measured in the mature milk and maternal serum but there was no association for IgM rho=0*31; p=0*056) and IgG (rho=-0*17; p=0*216) (Figure 3).

Breast milk antibody correlation with severity of SARS-CoV-2 infection

Women with pneumonia had significantly higher IgA (2*370, IQR 0*29-4*44; p=0*03) and IgG (0*19, IQR 0*07-0*31; p=0*001) in the colostrum than those who were asymptomatic. IgG was also higher in women with pneumonia than in women with mild symptoms (-0*19, IQR 0*08-0*31; p=0*001) (Figures 4a and 4b). No other significant correlations were identified.

Breast milk antibody correlation with time interval from active infection

No correlation was found between IgM, IgA or IgG in the colostrum or mature milk and the time interval from acute infection of SARS-CoV-2. However, higher rates of IgA and IgM positivity were noted in the colostrum when SARS-CoV-2 infection occurred during the second and third trimesters of pregnancy although these differences were not statistically different (Table 3).

Antibody evolution from colostrum to mature milk

Paired colostrum and mature milk samples were analyzed for IgA and IgG in 47 women. IgA concentration in the colostrum was higher than in the mature milk (2*42, 95% CI 1*46 to 3*37; p<0*001) while IgG concentration was not significantly different between the two types of milk (0*016, 95% CI -0*0178 to 0*050; p=0*341). Paired colostrum and mature milk samples were analyzed for IgM in 20 women. No significant differences were found between them (1*043, 95% CI -0*016 to 2*102, p=0*053).

12 paired samples corresponded to women with active COVID-19 at the time of delivery. Only IgA concentration was significantly higher in the colostrum than in the mature milk (3*07, 95% CI 1*23 to 4*92; p<0*005). 35 paired samples corresponded to women who had recovered from the disease at the time of delivery. Only IgA concentration was significantly higher in the colostrum than in mature milk (2*19, 95% CI 1*04 to 3*35; p<0*001).

Active SARS-CoV-2 infection at the time of delivery

29 pregnant women had SARS-CoV-2 infection at the time of delivery. Two colostrum samples had insufficient volume for the r-RT-PCR SARS-CoV-2 analysis, and one was inhibited. 26 samples were tested, and all were negative.

Five colostrum samples had insufficient volume for serological analysis and, therefore, 24 colostrum samples were analyzed. IgA and IgG were positive in 18 (75%) and 1 (4*16%) cases, respectively. Due insufficient sample, IgM could only be analyzed in 10 samples, and it was positive in 3 (30%) cases (Table 3). No

correlation was found in IgM (rho=0*71; p=0*088), IgA (rho=0*46; p=0*153) and IgG (rho=0*33; p=0*322) between the colostrum and the maternal blood.

Discussion:

Main findings

The study has demonstrated that, firstly, all 59 human breast milk tested negative for rRT-PCR SARS-CoV-2; secondly, antibody levels against SARS-CoV-2 present in the colostrum and mature milk do not seem to vary significantly in relation to the time when infection has occurred during pregnancy; 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; and fourthly, women with SARS-CoV-2 pneumonia have higher levels of both IgG and IgA in the colostrum than those who are asymptomatic or with mild symptoms.

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 from many women and, for many of them, their serological status at the time of milk sampling was also recorded, which allowed us to study correlations between colostrum, mature milk and maternal serological status. Additionally, the protocol for collection, handling and storage of samples[9] was early defined and implemented in all centers.

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

Interpretation

It is well known that breastfeeding protects babies against gastrointestinal and respiratory infections[25–28]. 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. Therefore, anti-SARS-CoV-2 IgA in human breast milk could protect the infant against the infection at a local level, similarly to what happens with other viral infections[28]. Recently, it has been demonstrated that anti-SARS-CoV-2 antibodies in breast milk neutralize the virus*in-vitro* [16,30,31].

In our study, most of the colostrum samples tested positive for IgA, irrespective of the time of SARS-CoV-2 infection, and it was the only immunoglobulin present in mature milk. In contrast, IgG was present in less than 2% of the colostrum samples and in none of the mature milk samples. When evaluating longitudinal changes in the concentrations of immunoglobulins both, in the colostrum and the mature milk, a significant reduction in IgA levels was found, similarly 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 maternal levels of IgG in serum were higher. A possible explanation for this could be related to the fact that IgA is secreted from Peyer patches, while IgG is mostly filtered from maternal plasma[32]. Peyer patches belong to the Gastrointestinal Antigen Linfoide Tissue (GALT) system and they represent maternal immunological memory[33,34]. This system is responsible of secreting antibodies against common infections prevalent in maternal living area[35]. IgM is also secreted by this system, but at much lower concentrations.

According to the severity of the disease, we have found higher concentrations of colostrum immunoglobulins in women with severe symptoms (pneumonia) as compared to those with mild or no symptoms. A higher immunological response has also been previously demonstrated in non-pregnant population[36].

In this study 26 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[2–8,13] and it is possible that crossed-contamination was responsible of 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 supports the idea that SARS-CoV-2 is not detected in breast milk even when active infection is present at the time of delivery and therefore, possibility of vertical transmission while breastfeeding is extremely low. Furthermore, since antibodies are found in the colostrum irrespectively of the time of infection, all women should be encouraged to breast feed their infants, undertaking strict contact precautions when there is active disease. Nevertheless, since IgA drops significantly from the colostrum to mature milk, we could speculate that they might lower even further afterwards, so public health measures should still be maintained.

Conclusions:

No SARS-CoV-2 virus was found in human breast milk and anti-SARS-CoV-2 antibodies were present in both the colostrum and mature milk, irrespective of the time when the infection occurred during the pregnancy. IgA was the predominant immunoglobulin found in human breast milk and its concentrations were significantly lower in the mature milk than in the colostrum. Women who had SARS-CoV-2 pneumonia during pregnancy or at delivery had higher levels of IgG and IgA in the colostrum than those who were asymptomatic or with mild symptoms.

AUTHOR CONTRIBUTIONS

Conceptualization: IFB, LCP, MMG

Data curation: IFB, NR, JCS, BS, OOH, BWL, JLD, DSN, SV, LM, AS, RPT, VR, BS, MMG, LCP.

Formal analysis: IFB, NR, VR, MMG

Investigation: IFB, NR, JCS, BS, OOH, BWL, JLD, DSN, SV, LM, AS, RPT, VR, BS, MMG, LCP.

Methodology: IFB, MMG, LCP

Project administration: MMG, LCP

Supervision: MMG, LCP

Validation: IFB, NR, JCS, BS, OOH, BWL, JLD, DSN, SV, LM, AS, RPT, VR, BS, MMG, LCP.

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Writing – review & editing: IFB, NR, JCS, BS, OOH, BWL, JLD, DSN, SV, LM, AS, RPT, VR, BS, MMG, LCP.

DECLARATION OF INTEREST STATEMENT

The authors declare no competing interest.

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TABLES

Table 1. Maternal, pregnancy and disease characteristics.

Table 2. Human breast milk and maternal blood serology.

Table 3. Presence of immunoglobulins in human colostrum according to the trimester of the acute infection of SARS-CoV-2.

Table 4. Correlation between antibodies present in breast milk and maternal blood.

Figure 1. Samples flow chart

Figure 2. Correlation between immunoglobulin levels in colostrum and maternal blood. IgM, IgA and IgG are represented in figures 2a, 2b and 2c, respectively.

Figure 3. Correlation between immunoglobulin levels in mature breast milk and maternal blood. IgM, IgA and IgG are represented in figures 3a, 3b and 3c, respectively.

Figure 4. Correlation between immunoglobulin levels in colostrum and severity of COVID-19 disease. IgA and IgG are represented in figures 4a and 4b, respectively.

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