RESEARCH

Holder pasteurization of donated human milk is effective in inactivating SARS-CoV-2

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ABSTRACT

BACKGROUND: Provision of pasteurized donor human milk, as a bridge to mother's own milk, is the standard of care for very low-birth-weight infants in hospital. The aim of this research was to confirm that Holder pasteurization (62.5°C for 30 min) would be sufficient to inactivate severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in donated human milk samples.

METHODS: We spiked frozen milk samples from 10 donors to the Rogers Hixon Ontario Human Milk Bank with SARS-CoV-2 to achieve a final concentration of 1×10^7 TCID$_{50}$/mL (50% of the tissue culture infectivity dose per mL). We pasteurized samples using the Holder method or held them at room temperature for 30 minutes and plated serial dilutions on Vero E6 cells for 5 days. We included comparative controls in the study using milk samples from the same donors without addition of virus (pasteurized and unpasteurized) as well as replicates of Vero E6 cells directly inoculated with SARS-CoV-2. We reported cytopathic effects as TCID$_{50}$/mL.

RESULTS: We detected no cytopathic activity in any of the SARS-CoV-2–spiked milk samples that had been pasteurized using the Holder method. In the SARS-CoV-2–spiked milk samples that were not pasteurized but were kept at room temperature for 30 minutes, we observed a reduction in infectious viral titre of about 1 log.

INTERPRETATION: Pasteurization of human milk by the Holder method (62.5°C for 30 min) inactivates SARS-CoV-2. Thus, in the event that donated human milk contains SARS-CoV-2 by transmission through the mammary gland or by contamination, this method of pasteurization renders milk safe for consumption and handling by care providers.

Mother's milk is the optimal source of nutrition for infants and contains a myriad of bioactive and immunomodulatory factors, including cytokines, lactoferrin, oligosaccharides and secretory immunoglobulins, which help orchestrate immune system development and provide first-line defence against respiratory tract and gastrointestinal tract infection.[1–5] For vulnerable infants, such as very low-birth-weight (born < 1500 g) infants, use of mother's milk is associated with a shorter hospital stay and reduces their risk of sepsis and necrotizing enterocolitis, a severe bowel emergency.[6–9] It is the standard of care in Canada to provide very low-birth-weight infants in hospital with pasteurized donor human milk until their mother's supply is established.[10]

Past global epidemics, such as HIV/AIDS, have had devastating effects on donor human milk banking because of perceived risks. In the 1980s, with the knowledge that HIV could be transmitted into human milk, 22 of the 23 Canadian donor human milk banks closed.[11] Several viruses, in addition to HIV, can be transmitted through human milk, including hepatitis, cytomegalovirus and human T-cell lymphotropic virus type 1.[12] Some viruses may be secreted into milk by paracellular passage as tight junctions open in response to maternal illness and inflammation.[3] Other routes of transmission include contamination from respiratory droplets, skin, breast pumps and milk containers. Milk banks affiliated with the Human Milk Banking Association of North America (HMBANA) and the European Milk Bank Association (EMBA) pasteurize milk using the Holder method (62.5°C for 30 min) before dispensing for use; the Holder method is effective in inactivating the aforementioned viruses.[13,14]

Very little is known of the prevalence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in human milk, or its infectivity; however, the virus has been detected in human milk by reverse transcription polymerase chain reaction (RT-PCR) testing.[15–18] Mothers donating milk are verbally screened for symptoms associated with coronavirus disease 2019 (COVID-19) at HMBANA-affiliated milk banks, but direct assessment for SARS-CoV-2 by nasopharyngeal swabs and RT-PCR testing is not performed. Although there is no direct evidence showing that Holder pasteurization inactivates SARS-CoV-2 in human milk, this virus is known to be heat sensitive.[19] The aim of this research was to confirm that Holder pasteurization would be sufficient to inactivate SARS-CoV-2 in donated human milk samples.

Methods

Study design

The Rogers Hixon Ontario Human Milk Bank in Toronto, Canada, is a provincial milk bank that follows guidelines established by HMBANA, whereby donors are screened by health and lifestyle interview and serology, and counselled about safe procedures for expression, handling and storage of human milk. Once donors have collected a minimum volume of milk at home, the milk is shipped frozen to the milk bank by express priority courier. We chose 1 container of frozen human milk (approximately 150 mL) at random from shipments received from each of 10 donors. The number of samples included align with previous investigations of viral inactivation in human bodily fluids, where it is common to pool samples before spiking with virus.[20–22] We specifically avoided pooling in this study because of the known variability in human milk composition. After all identifiers had been removed from milk containers, they were transported frozen to the Combined Containment Level 3 Unit at the University of Toronto, where we completed all experiments.

We thawed the milk samples on ice, homogenized them and individually spiked two 840 µL aliquots of milk from each woman with 160 µL of SARS-CoV-2 SB2 passage 3 (titre = 6.29×10^7 50% of the tissue culture infectivity dose [$TCID_{50}$] per mL) to achieve a concentration of 1×10^7 $TCID_{50}$ in each 1 mL of milk-containing solution.[23] One spiked milk sample from each mother was allowed to sit at room temperature for 30 minutes (unpasteurized milk). We pasteurized the second spiked milk sample from each mother in a water bath by warming the milk to 62.5°C, holding for 30 minutes and then cooling in ice to mimic HMBANA and EMBA guidelines.[13,14] We processed individual aliquots of milk in hard plastic microfuge tubes of composition similar to containers normally used during Holder pasteurization at the milk bank. Not unexpectedly, given the complex carbohydrate, lipid and immune factor content of human milk, we found undiluted human milk to be cytotoxic to Vero E6 cells, even without SARS-CoV-2. Hence, we diluted all samples 1:100 in serum-free Dulbecco's Modified Eagle's Medium (DMEM), the medium used to maintain cultures of Vero E6 cells, before conducting the experimental procedures described below. Although the dilution of the treated samples does result in a 1-log decrease in sensitivity of viable virus detection in the subsequent titration assay, the high input titre of SARS-CoV-2 used to spike the milk samples balances the dilution factor such that a 6-log reduction in viable virus would still be quantifiable.

We used spiked milk samples to determine viral titres in samples from all treatment conditions, as previously described.[23,24] Briefly, we prepared 6 serial 10-fold dilutions of each SARS-CoV-2 milk solution (inoculum) and applied 50 µL of each to monolayers of Vero E6 cells with DMEM (0.2×10^6 cells/mL) in flat-bottom 96-well plates. We incubated the plates at 37°C and 5% CO_2 for 1 hour, with gentle shaking every 15 minutes to promote uniform distribution of the inoculum across the wells. After 1 hour, we removed the inoculum and then reconstituted the plate with 200 µL of DMEM with 2% fetal bovine serum, and allowed it to progress for 5 days. Positive controls consisted of

undiluted milk-free samples of 160 µL of viral stock solution and 840 DMEM (SARS-CoV-2 alone). Negative controls consisted of unpasteurized and pasteurized human milk with no viral inoculum (mock infection).

Virologic analysis

We observed cytopathic effects at 5 days after infection and reported them as $TCID_{50}$/mL. We calculated the viral titres using the Spearman–Karber method.[25,26] At 5 days after infection, we passaged supernatants onto fresh Vero E6 monolayers in flat-bottom 96-well plates and refreshed the original monolayers with 200 µL of DMEM with 2% fetal bovine serum. We monitored these for 14 days for any signs of emergence of breakthrough cytopathic effects not evident in the initial 5 days of culture. The limit of detection for the $TCID_{50}$ assay was 20 $TCID_{50}$/mL.

Ethics approval

Donors to the Rogers Hixon Ontario Human Milk Bank provide written informed consent that their milk may be used for quality control and research purposes. We obtained human research ethics approval from Sinai Health and the University of Toronto.

Results

Cytopathic effects were identical at 3 and 5 days after infection and are presented in Table 1. We detected no cytopathic activity in any of the SARS-CoV-2–spiked milk samples that had been pasteurized using the Holder method (62.5°C for 30 min), even after the passaging of inoculum and subsequent observation for 14 days. Of note, in the positive control samples (SARS-CoV-2

Table 1: Stability of SARS-CoV-2 in donated human milk with and without Holder pasteurization ($TCID_{50}$/mL)*

Sample	Unpasteurized (room temperature for 30 min)	Pasteurized (62.5°C for 30 min)
A	2.0×10^5	Undetected
B	6.3×10^4	Undetected
C	6.3×10^5	Undetected
D	6.3×10^6	Undetected
E	6.3×10^5	Undetected
F	2.0×10^5	Undetected
G	6.3×10^5	Undetected
H	6.3×10^5	Undetected
I	6.3×10^5	Undetected
J	6.3×10^5	Undetected
SARS-CoV-2 alone (positive control)	6.3×10^6	6.3×10^3
Mock infection (negative control)	Undetected	Undetected

Note: SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2, $TCID_{50}$ = 50% of the tissue culture infectivity dose.
*$TCID_{50}$/mL calculations defined by duplicate dilution series of indicated samples.

alone), this heat treatment did not completely inactivate the virus. In the SARS-CoV-2–spiked milk samples that were not pasteurized but were kept at room temperature for 30 minutes, we observed a reduction of approximately 1 log in comparison with virus spiked into DMEM alone, although we observed variability between milk donors (range of reduction 2 log to none). The mean ± standard deviation $TCID_{50}$/mL was $1.05 \times 10^6 \pm 1.86 \times 10^6$.

Interpretation

Very few milk samples from women positive for COVID-19 have been tested for SARS-CoV-2. Of the few available cases reported in the literature, there are now at least 3 reports of the presence of SARS-CoV-2 nucleic acid in human milk, although none of these have measured the viability of the virus in these samples. The World Health Organization recommends that human donor milk be fed to low-birth-weight infants when there is an insufficient volume of mother's milk.[27] Human milk banking is growing rapidly internationally, with more than 650 milk banks globally that rely on the Holder method to ensure the safety of donor milk.[28]

Although this technique is assumed to result in inactivation of SARS-CoV-2, it is important to confirm this in a human milk matrix, for the safety of milk bank staff, caregivers and recipients of human donor milk. In this study, pasteurization of human milk spiked with SARS-CoV-2 using the Holder method (62.5°C for 30 min) resulted in complete viral inactivation, as measured by $TCID_{50}$/mL. The high viral titre used to spike samples in these experiments enabled us to confirm a 10^6 reduction. The impact of pasteurization on coronaviruses in a human milk matrix has not previously been reported in the literature.[29]

The results are in keeping with evidence of coronavirus inactivation in other matrices, including culture media and plasma using a variety of pasteurization protocols. The virus causing severe acute respiratory syndrome, SARS-CoV, has been shown to be completely inactivated with temperatures as low as 56°C for 20 minutes, as well as at higher temperatures, such as 70°C for 5 minutes.[30–33] The virus causing Middle East respiratory syndrome was shown to be inactivated at 56°C for 60 minutes.[34,35] A recent report by Chin and colleagues showed SARS-CoV-2 in virus transport media to be completely inactivated at 56°C for 30 minutes or 70°C for 5 minutes.[19] In the present investigation, we did not see complete inactivation of SARS-CoV-2 in media that did not contain human milk (positive control) after pasteurization at 62.5°C for 30 minutes, which differs from the report of Chin and colleagues. This finding suggested to us that the biological matrix in which the virus resides needs to be considered when assessing effective inactivation conditions.

Interestingly, we observed some reduction in the cytopathic effects of SARS-CoV-2 in milk samples that were not heat treated but held at room temperature for 30 minutes. This is very likely a result of the multitude of immune components found in human milk — including secretory 1gA antibodies, lactoferrin, lactadherin, mucins from milk fat globules and oligosaccharides — that have significant antiviral activity.[1,2] Notably, Hamilton Spence and colleagues reported the same finding for human milk samples inoculated with Ebola virus and held at room temperature for 30 minutes.[36]

Limitations

We studied only 10 samples of milk. However, our ability to study more was limited because of the complexity of ensuring safety using the SARS-CoV-2 virus, and this sample size is larger than has been used in similar studies of other viruses.[20–22]

Conclusion

Pasteurization of human milk by the Holder method (62.5°C for 30 min) inactivates SARS-CoV-2. In the event that a woman who has COVID-19 donates human milk that contains SARS-CoV-2, whether by transmission through the mammary gland or by contamination through respiratory droplets, skin, breast pumps and milk containers, this method of pasteurization renders milk safe for consumption. Furthermore, previously frozen, thawed human milk appears to contain sufficient antiviral activity to partially reduce the infectivity of SARS-CoV-2 in human milk.

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