

## SHORT REPORT

## Cytomegalovirus remains viable in naturally infected breast milk despite being frozen for 10 days

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Arch Dis Child Fetal Neonatal Ed 2005;90:F529–F530. doi: 10.1136/adc.2004.067769

Cytomegalovirus culture positive breast milk was obtained from four mothers of very premature babies. The milk was stored at 0–5°C in a domestic refrigerator for 48 hours or frozen for different durations at –20°C. Cytomegalovirus survived in breast milk despite being frozen for 10 days at –20°C.

Cytomegalovirus (CMV) re-activation and shedding in breast milk is common during the postnatal period, and the virus is often transmitted to the baby.<sup>1</sup> Although CMV infection usually presents no threat to term infants, its clinical consequence in very premature babies is still uncertain.<sup>2</sup> The efficacy of freezing naturally infected breast milk to eliminate CMV is also uncertain.<sup>3</sup> This study aimed to investigate the freezing duration required to eliminate viable CMV from breast milk and whether baseline CMV viral load affects the effectiveness of freezing.

## METHODS

## Subjects and milk storage

CMV culture positive breast milk was obtained from four mothers of very premature babies at the Royal Women's Hospital, Melbourne, Australia after informed consent. The milk was divided into multiple 3 ml aliquots for culture before or after storage at 0–5°C in a domestic refrigerator for 48 hours or freezing for different durations at –20°C.

## CMV culture

After thawing at 37°C in an incubator, milk samples were defatted and separated into cellular and aqueous (whey) fractions by centrifugation. CMV culture was undertaken on each fraction separately using the highly sensitive rapid enhanced tissue culture immunofluorescence (RETCIF) method.<sup>4</sup> Briefly, fractions were diluted, vortexed, sonicated, and centrifuged. Supernatant (200–300 µl) from the cellular and aqueous fractions was inoculated separately into tissue culture plates. After further centrifugation, supernatant from each well was replaced by viral maintenance medium. After incubation at 37°C for two days, the cultures were tested for the presence of CMV by direct immunofluorescence using monoclonal antibodies. Negative samples were tested again after 10 days incubation.

## Quantification of baseline CMV viral load

A 200 µl aliquot of stored (–70°C) milk from baseline was thawed and centrifuged. DNA from the cellular and supernatant (whey) fractions was prepared using an automated MagNA Pure LC (Roche Diagnostics, Indianapolis, Indiana, USA) with the associated DNA Isolation Kit I protocol. Quantification was by real time polymerase chain reaction using a Lightcycler (Roche Diagnostics). Primers (gBfor and

gBrev) targeted to a 254 bp region on the glycoprotein B gene were used with hybridization probes labelled with fluorescein or LC-Red 640 for a nested region within the amplification area. CMV was quantified by comparison of amplification crossing points from five 10-fold serial dilutions of a plasmid standard containing the primer spanning region of the glycoprotein B gene to the samples. The presence of adequate amplifiable DNA in each sample was assessed by separately amplifying a 260 bp product of the human β globin gene using GH20 and PC04 primers.

## RESULTS

Table 1 details the results. Initial CMV viral load in the seven samples tested varied between 1.8 and 110.1 copies/µl. CMV was cultured from all the samples after freezing for 24 hours and in five of seven (72%) after freezing for 48 hours. In the three samples tested after longer freezing, viable CMV was still detected after 10 days. CMV was undetectable after freezing for 20 days in the two samples tested. Baseline viral load of CMV was lower in samples in which CMV was undetectable after freezing for 48 hours, than those that tested positive. After refrigeration for 48 hours, CMV was undetectable in three of the seven (43%) samples.

## DISCUSSION

Concerns about CMV transmission to very premature babies have led to the suggestion that breast milk from CMV seropositive mothers should be routinely frozen at –20°C.<sup>3</sup> Our study is the first to show the persistence of viable CMV in naturally infected breast milk after being frozen for 10 days at –20°C. This finding is consistent with the recently published study by Hamprecht *et al*<sup>5</sup> that showed infectivity of breast milk artificially spiked with CMV despite 10 days of freezing at –20°C. Of note, in our study, CMV persistence was found in samples of variable initial viral load, including as few as 10.9 copies/µl, a factor of at least 50 times lower than the concentrations tested in Hamprecht's spiking experiments.

The RETCIF culture method for CMV is highly sensitive.<sup>4</sup> The relation between detection of CMV using a sensitive culture technique and infectivity of breast milk remains unknown, and the clinical significance of our findings is therefore uncertain. Only further prospective clinical studies can determine the importance of CMV transmission from breast milk to very premature babies and whether this has a significant impact on babies' clinical status and outcome. In the meantime, our study shows that routine freezing may not eliminate CMV from breast milk from mothers of very premature babies.

**Abbreviations:** CMV, cytomegalovirus; RETCIF, rapid enhanced tissue culture immunofluorescence

**Table 1** Results of cytomegalovirus (CMV) testing of frozen and refrigerated breast milk

Mother		Baby		Milk		Refrig	Duration of freezing						
No	Age (years)	Parity	Gest (weeks)	BW (g)	Day	VL	48 h	24 h	48 h	5 days	7 days	10 days	20 days
1	19	1	25	754	8	1.8	--	++	--				
					21	7.1	++	++	--				
					31	10.1	++	++	++				
					43	13.5	++	++	++				
2	24	1	25	604	71	11.0	++	++	++	++	++		
3	37	3	28	890	47	110.1	--	++	++	++	++	++	--
4	25	1	26	665	19	10.9	--	++	+-	++	+-	++	--

Gest, Gestation of baby; BW, birth weight of baby; Day, day after delivery milk collected; VL, initial CMV viral load (copies/ $\mu$ l); Refrig, duration of refrigeration. ++, CMV detected by culture in whey and cells; +-, CMV detected by culture in whey only; --, CMV not detected by culture in whey or cells.

### What is already known on this topic

- CMV re-activation and shedding in breast milk is common during the postnatal period and the virus is often transmitted to babies
- The clinical consequence of CMV infection by this route in very premature babies is uncertain

### What this study adds

- Freezing breast milk from mothers of very premature babies for up to 10 days does not eliminate viable CMV
- This study underlines the importance of further prospective clinical studies to determine whether CMV transmission from breast milk to very premature babies has a significant impact on clinical status and outcome

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Competing interests: none declared

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Accepted 11 February 2005

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