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ARTICLES

Cytomegalovirus (CMV) Inactivation in Breast Milk: Reassessment of Pasteurization and Freeze-Thawing

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ABSTRACT

Breast-feeding mothers frequently transmit cytomegalovirus (CMV) to preterm infants of very low birth weight. Current recommendations for prevention of virus transmission are based on data published 20 y ago in the context of human milk banking. Two recent clinical trials examined storage of breast milk at −20°C to reduce virus transmission. However, in both studies, CMV transmission occurred. Using sensitive tools like quantitative PCR, CMV pp67 late mRNA assay, and a high-speed, centrifugation-based microculture assay for quantification of CMV infectivity, we reassessed the virological and biochemical characteristics of freeze-storing breast milk at −20°C, compared it with traditional Holder pasteurization (30 min at 62.5°C), and a new short-term pasteurization (5 s at 72°C) based on the generation of a milk film. Both heat treatment procedures were able to destroy viral infectivity and pp67 RNA completely. Preliminary results showed short-term heat inactivation below 72°C was less harmful in reducing the activity of marker enzymes than Holder pasteurization. Freezing breast milk preserved the biochemical and immunologic quality of the milk; however, late viral RNA and viral infectivity was also preserved. Compared with viral DNA, CMV-RNA more directly reflects infectious CMV in human milk samples. Further studies are necessary to evaluate short-term heat treatment below 72°C as an effective tool for prevention of CMV transmission. **(*Pediatr Res* 56: 529–535, 2004)**

Abbreviations

AP, alkaline phosphatase
CMV, cytomegalovirus
HFF, human foreskin fibroblasts
IE, immediate early
NASBA, nucleic acid sequence–based amplification
p.p., post partum
sIgA, secretory IgA
VLBW, very low birth weight

Breast-feeding in premature infants is beneficial (1). However, breast-feeding of a high-risk group of preterm infants (birth weight <1000 g, gestational age at birth <30 wk) may be associated with symptomatic CMV infection (2,3). If CMV transmission from breast milk to preterm infants is clinically important, then prevention of virus transmission will be critical (4).

Recent recommendations for virus inactivation in human milk by freezing, which destroys the virus (5,6), are still based on three studies that evaluated freezing for viral decontamination of stored milk in the context of human milk banking 20 y ago (7–9). Only two studies analyzed the effect of storage at −20°C using naturally infected milk specimens (8,9). Storage of milk for 3 d at −20°C reduced the viral titre by more than 99% (9), and storage of naturally infected milk specimens for 7 d or longer at −20°C eliminated infectivity (8). On the other hand, CMV survived when the milk sample was frozen at

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−15°C for 10 d in the presence of low lipase levels (7). Interestingly, the persistence of viral infectivity or the enhanced CMV recovery after overnight storage at 4°C was discussed in the context of cell-free or cell-associated virus (8). Recent analysis revealed that cell-free virus is present in nearly every seropositive milk (2).

In contrast, Holder pasteurization (30 min at 62.5°C) markedly diminished pathogenic bacteria and also completely destroyed CMV infectivity in human milk (7–9). However, heating of spiked milk at 56°C for 30 min was not able to reliably eliminate viral infectivity (7,8). Using a modified plate heat exchanger designed for treating large volumes of cow milk, at least 5 s of heating at 72°C was necessary to eliminate CMV infectivity. This treatment supposedly did not destroy the nutritional and immunologic qualities of human milk (10).

Recently, routine freezing of breast milk at −20°C was recommended to prevent transmission of CMV from breast milk of seropositive mothers to uninfected preterm infants (11). This study was also based on previous data of CMV cryoinactivation (8,9). Using new and very sensitive tools for quantitative detection of CMV in human milk, we reassessed and compared the effectiveness of Holder pasteurization and freezing for CMV inactivation with our new short-term heating procedure based on the generation of a milk film. Biochemical analysis revealed the influence of all three inactivation procedures on the nutritional and immunologic milk quality. Short-term heat inactivation using temperatures below 72°C seemed to be superior to Holder pasteurization, whereas cryoinactivation reduced viral infectivity very insufficiently—especially during physiologic peak level of viral load.

METHODS

Milk samples. For inactivation, sequential 60–80 mL samples of freshly expressed milk from each of two seropositive and two seronegative mothers from our study (2) and two healthy volunteers of term infants (one mother seronegative, one mother seropositive transmitter) were used for 10 freezing experiments with storage at −20°C for up to 10 d (18 h, −20°C, $n = 2$; 4 d, −20°C, $n = 5$; 10 d, −20°C, $n = 3$), 4 Holder pasteurization experiments, and 14 short-term pasteurizations with heating for 10 s ($n = 6$) and 5 s at 72°C ($n = 8$), respectively. Another 120 mL of seronegative milk were used for a spiking experiment comparing Holder pasteurization with short-term heat inactivation below 72°C. Informed consent for breast milk donation was obtained from all lactating mothers. This study was approved by the local ethical committee.

Virus spiking experiments. Raw milk samples from seronegative mothers were spiked with an inoculum of cell-free culture supernatant (3000 *g*) of CMV laboratory strain AD 169 using 10^4–10^5 $TCID_{50}$ (tissue culture infective dose 50%) per milliliter raw milk.

Preparation of milk whey and milk fat. Naturally infected milk from seropositive CMV reactivating mothers and spiked milk from seronegative mothers was separated according to previously published protocols (12,13).

Quantitative detection of viral DNA in milk. Viral DNA load (copies/mL) was determined by quantitative PCR (COBAS Amplicor CMV Monitor Test; Roche Molecular Biochemicals, Mannheim, Germany) using milk whey from naturally infected milk and spiked milk after virus inoculation (13).

Quantitative detection of viral infectivity in milk. CMV present in the milk whey fraction was concentrated by centrifugation of 2× 1 mL whey for 1 h at 50,000 *g*. Resuspended pellets were inoculated on microcultures of HFF for detection of virus infectivity after 18 h or 5 d incubation at 37°C and 5% CO_2. Long-term incubation allowed detection of CMV plaque formation, which is associated with fully replication-competent virus. Read-out was the number of specific CMV immediate early antigen-(IEA) stained nuclei (18 h) or plaques (5 d) per milliliter whey (virus load) (13).

Detection of CMV pp67 late mRNA in milk. Viral late pp67 mRNA was detected in naturally infected milk whey using the NASBA technique (NucliSens CMV, Organon Teknika, Boxtel, The Netherlands) (14). Therefore, immediately after preparation of milk whey, 100 μL were added to 900 μL of NASBA lysis buffer [4.7 M guanidinium thiocyanate, 46 mM Tris, pH 6.4, 20 mM EDTA, 1.2% (wt/vol) Triton X-100], and the mixture was stored at −70°C until further processing (13).

Short-term heat inactivation. This procedure for processing individual milk samples is technically based on the generation of a thin milk film by rotating at least 20 mL of raw milk in special heating and cooling devices. The heating and cooling process was performed automatically in the following sequence: heat-up time was approximately 90 s, followed by a ramp temperature of 72°C (or 62–65°C) for 5 s (or less) and cooling for 30 s at 5°C to reach 30°C. The machine (Klaus Lauf, Tübingen, Germany) and the procedure are presented in detail elsewhere (International Patent Application WO 00/74494).

Holder pasteurization. Each 20 mL sample of naturally infected milk and spiked milk specimens was incubated for 30 min at 62.5°C in a water bath using the LABU-Muttermilchpasteur (Labor Buchrucker, Ottensheim, Austria).

Cryoinactivation. Each 20 mL sample of milk was frozen in a household freezer at −20°C and stored for 18 h, 4 d, and 10 d, respectively. Thereafter, the milk whey fraction was prepared for further analysis.

Biochemical analysis. Total protein concentration and activity of AP and lipase was determined by standard procedures (Roche Molecular Biochemicals) using a Hitachi 917 Automatic Analyzer. Folic acid and vitamin B12 concentrations were determined by chemiluminescence immunoassays using an Advia Centaur Analyzer (Bayer, Leverkusen, Germany). Levels of sIgA and lysozyme were quantified by radial immunodiffusion (The Binding Site, Heidelberg, Germany) and evaluated using a plate reader.

Statistical analysis. Continuous variables were expressed as a percentage of control. All five treatment methods were compared with respect to virus load by the nonparametric Kruskal-Wallis test. Four of the treatment methods were compared with respect to the biochemical variables by an ANOVA of the log-transformed data. The results were back-transformed for easier interpretation. Relative frequencies were compared using Fisher's exact test.

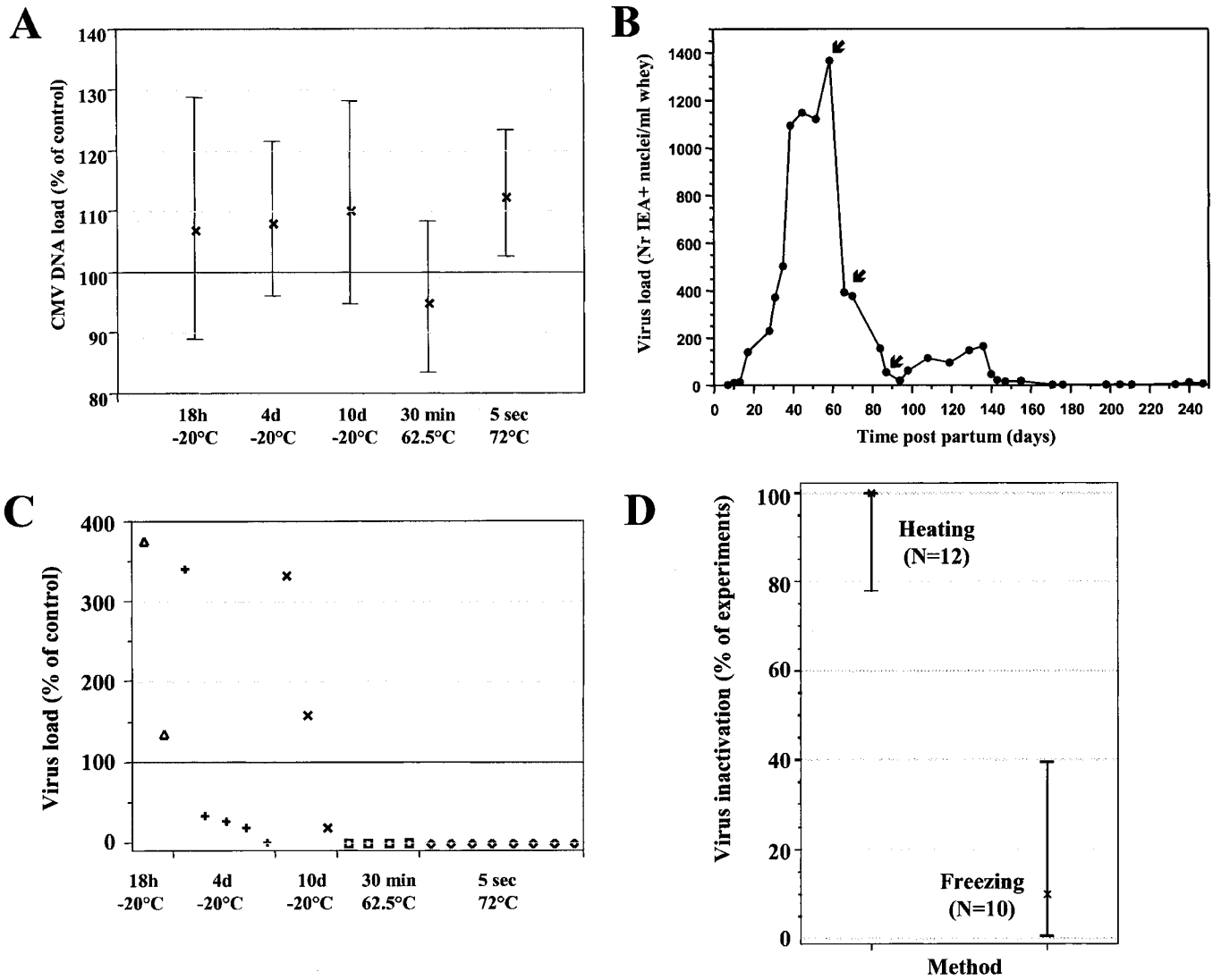

Figure 1. Virological parameters of different procedures for CMV inactivation in breast milk. (*A*) Stability of viral DNA after freezing or heating. (*B*) Unimodal kinetics of CMV viral load during lactation of a maternal transmitter (see Table 1, mother C) of a term infant. The *arrows* indicate d 59, 66, and 87 p.p.; d 61: onset of viruria of the infant. (*C*) Virus load (infectivity) expressed as a percentage of the control for the five inactivation methods (Kruskal-Wallis test, $p = 0.002$). Number of experiments using spiked milk (AD) / naturally infected milk (wt): 18 h, −20°C, 2 AD; 4 d, −20°C, 2 AD / 3 wt; 10 d, −20°C, 3 AD; 30 min, 62.5°C, 3 AD / 1 wt; 5 s, 72°C, 4 AD / 4 wt. (*D*) Virus elimination by heating *vs* cryoinactivation (observed percentages together with exact 95% confidence intervals; $p < 0.0001$).

RESULTS

Viral DNA load is stable after freeze-thawing and pasteurization. Initially, the influence of different inactivation procedures on viral DNA load in milk whey was studied. Neither freeze-thawing experiments storing milk for 18 h to 10 d at −20°C nor Holder or short-term pasteurization reduced the viral DNA load significantly (Fig. 1*A*).

Determination of virus load in milk whey did not result in cytotoxicity in the microculture assay. Spiking experiments revealed a discrepancy between high viral DNA levels and low infectivity of the inoculated milk containing freeze-thawed culture supernatants of cell-free CMV laboratory strain AD 196 (Table 1: mother A). Interestingly, the viral DNA load (data not shown) and the virus load in naturally infected milk illustrated an unimodal course, reaching very high levels in some maternal transmitters (Fig. 1*B*).

Freeze-thawing and storing for various times at −20°C. CMV infectivity was eliminated in only 1 out of 10 [95% confidence interval (CI): 0.25–44.5%] cryoinactivation experiments (Fig. 1*C*). In the case of naturally infected milk (Table 1: mother C, d 87 p.p.; Fig. 1*B*), initial (control) viral infectivity was low compared with peak level and viral RNA could not be amplified after cryoinactivation. In contrast, in 4 out of 10 cryoinactivation experiments, virus load was incompletely destroyed and in the residual 5 cases virus load was even enhanced up to 3.8-fold in relation to the control level (Fig. 1*C*). Interestingly, infectious virus was also detectable in milk fat of naturally infected milk during maximum of viral load (Table 1, mother C: d 59 p.p.). In this case, cryoinactivation resulted also in an enhanced infectivity in the creamy top layer. In Figure 2, viral plaque formation after freeze-storing of spiked milk for 10 d at −20°C is shown. Although viral

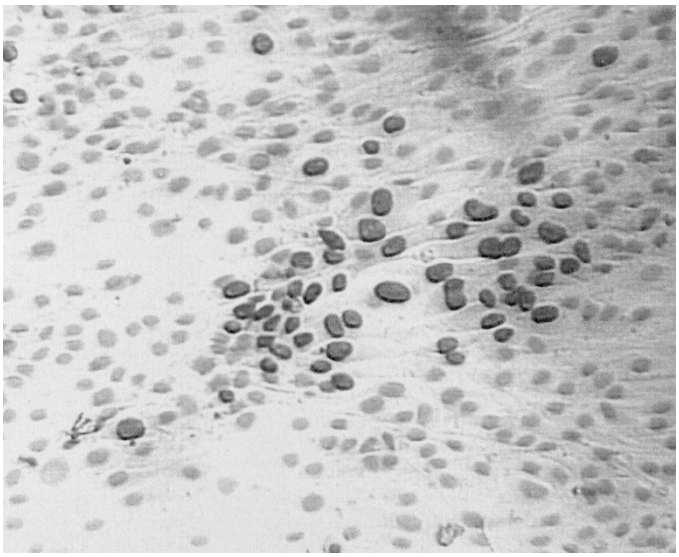

Figure 2. Freezing is insufficient for inactivation of CMV infectivity: detection of plaque-forming virus after freeze-storing of spiked milk. Native seronegative milk was spiked with 10^5 $TCID_{50}$/mL CMV laboratory strain AD196 and stored for 10 d at −20°C. After thawing, milk whey was prepared and inoculated on microculture-monolayers of HFF. After incubation for 5 d, CMV IEA staining was performed (13). Intranuclear staining of CMV IEA (*brown*) revealed replication competent virus with plaque formation. Control: spiked milk without freezing, 150 plaques/microculture; freeze-thawed milk: 29 plaques/microculture. Counterstaining of noninfected fibroblast nuclei (*blue*) was performed using Mayer's hemalum/hematoxylin solution (13).

infectivity was reduced by about 80%, 29 plaques were still detectable per microculture. This demonstrates impressively that high viral loads in milk are reduced very insufficiently by freeze-storing for 10 d.

Long-term (Holder) and short-term heat treatment. In contrast to freeze-thawing, heat treatment of human milk was able to kill CMV in each stage of lactation (Fig. 1*C*, Table 1). Infectivity was completely destroyed in six initial experiments using 72°C for 10 s (data not shown), therefore all subsequent experiments with short-term pasteurization were performed by heating at 72°C for 5 s. Holder ($n = 4$) and short-term pasteurization ($n = 8$) were powerful tools to kill CMV in each of the 12 heat inactivation experiments (100%; 95% CI: 73.5–100%). CMV infectivity and CMV-RNA were completely eliminated. Comparison of virus elimination by freeze-thawing and heating revealed highly significant differences ($p < 0.0001$) (Fig. 1*D*).

Biochemistry. Biochemical analysis of milk whey after the various inactivation procedures did not reveal any significant alteration of log (sample/control) values for total protein, folic acid, and vitamin B12 (data not shown). Freezing for 4 d and 10 d at −20°C did not alter the activity of the marker enzymes AP and lipase or the levels of sIgA (Fig. 3, *A, B,* and *D*). Lysozyme levels were slightly reduced (Fig. 3*C*). In contrast, both heat-inactivation procedures completely destroyed the activity of AP and lipase (Fig. 3, *A* and *B*; Table 1). Holder pasteurization and short-term heat inactivation reduced lysozyme levels to 65% and 80% of the control, respectively (Fig. 3*C*). Both heat-inactivation procedures reduced levels of sIGA to 80% of the control (Fig. 3*D*).

Short-term inactivation below 72°C. Preliminary data on the influence of our short-term heating procedure using ramp temperatures below 72°C derived from an additional spiking experiment (Table 2). All ramp temperatures resulted in successful virus killing. However, Holder pasteurization, like short-term heating for 2 s at 72°C, destroyed the activity of AP and lipase nearly completely. In contrast, short-term inactivation for 2 s at 62°C was able to preserve up to 57% of AP activity and 10% of lipase activity (Table 2).

DISCUSSION

Current recommendations on freezing (5,6) as well as a recent clinical study outlining routine freezing of breast milk at −20°C to prevent CMV transmission (11) are based on former data using conventional cell culture of unseparated milk samples (8,9). Our study reassessed the effectiveness of CMV inactivation procedures in human breast milk using very sensitive tools. Additionally, a new short-term heat inactivation method based on the generation of a milk film for rapid temperature exchange was presented.

Cell-free CMV is shed according to unimodal and self-limited kinetics into milk during lactation (13) and the presence of cell-free viral DNA in milk whey is the gold standard for detection of CMV reactivation during lactation (12,13). However, using quantitative PCR in combination with milk fractionation, we could demonstrate the stability of CMV DNA against freeze-thawing and short- and long-term pasteurization. Therefore, DNA PCR is not able to evaluate the success of CMV inactivation methods, inasmuch as even high viral DNA loads reflect only the presence of replication-incompetent virus particles in milk whey (13). The observed discrepancy between high viral DNA load and low viral infectivity of spiked milk specimens may be explained by the heterogenous composition of cell-free culture supernatants as published by Topilko and Michelson (15). Their ultrastructural analysis of cell-free CMV culture supernatants revealed that only 38% of the particles in the extracellular virus stock inoculum consisted of complete virions (15). Therefore, only the combination of the high-speed, centrifugation-based microculture assay in conjunction with the NASBA technique enabled us to accurately determine the success of any virus inactivation step by sensitive detection of CMV infectivity and late CMV mRNA (13).

We present the first longitudinal course of quantitative virolactia of a breast-feeding mother of a term infant. Like mothers of preterm infants, the dynamics of CMV reactivation during lactation also illustrates unimodal kinetics (13). The success of freeze-thawing strongly depends on the level of cell-free virolactia at the time of inactivation, as shown in three independent freeze-storing experiments during peak level (d 59 p.p.) and decreasing viral loads (d 66 and 87 p.p) of a lactating maternal transmitter. Freezing is not sufficient to kill CMV during maximum virolactia. Moreover, even in the phase of decreasing viral load, freezing is not able to completely destroy viral infectivity. Only at the beginning and the end of viral reactivation during lactation does freezing result in complete inactivation of low CMV loads. The clinical relevance of this important issue resulting in a symptomatic CMV infection

Table 1. *Synopsis of virological and biochemical raw data after CMV inactivation of artificially and naturally infected milk*

			Virological characteristics‡			Biochemical characteristics‡			
Virus strain	Maternal status†	Virus inactivation	Infectivity (IEA+/mL)	Viral transcripts (late pp67 mRNA)	DNA load (copies/mL)	AP (U/L)	Lipase (U/L)	Lysozyme (mg/L)	sIgA (mg/L)
AD 169*	A, 19, IgG neg	Control	18	Not done	559,000	43	2224	196	3680
	Nontransmitter	5 s 72°C	0	Not done	663,000	0	6	96	2880
		30 min 62.5°C	0	Not done	750,000	1	3	68	3200
		10 d −20°C	60	Not done	615,000	43	2115	174	3370
Wild type	B, 31, IgG pos	Control	23	Positive	4,360	15	1605	392	1130
	Transmitter	5 s 72°C	0	Negative	4,560	1	9	340	900
Wild type	C, 59, IgG pos	Control	1366 (19)§	Positive	56,300	21	1894	420	780
	Transmitter	5 s 72°C	0 (0)	Negative	58,000	3	10	368	660
		4 d −20°C	373 (31)	Positive	62,100	23	1894	368	760
Wild type	C, 66, IgG pos	Control	392 (5)	Positive	49,000	25	1855	543	940
	Transmitter	5 s 72°C	0 (0)§	Negative	62,700	2	19	415	865
		4 d −20°C	70 (0)	Positive	51,900	26	1857	430	1000
Wild type	C, 87, IgG pos	Control	55 (0)	Positive	21,300	19	1705	1370	1010
	Transmitter	5 s 72°C	0 (0)	Negative	23,500	3	57	1160	730
		30 min 62.5°C	0 (0)	Negative	17,200	1	12	1090	740
		4 d −20°C	0 (0)	Negative	25,900	20	1690	1200	910

* In case of seronegative mother: spiking experiment with 10^5 tissue culture infective dose 50% of CMV laboratory strain AD 169 per mL of raw milk ($TCID_{50}$/mL).

† Individual mothers (A–C), milk donation on day post partum (p.p.), anti-CMV serostatus, and retrospective characterisation as maternal transmitter or nontransmitter.

‡ Data derived from milk whey.

§ Milk fat analysis (data in parentheses).

of a preterm infant exclusively fed with frozen milk could be demonstrated (16). As reported recently, initial freeze-thawing of milk may reduce the transmission rate to preterm infants (17), but it is important to consider that the CMV reactivation during lactation is a unimodal process with a peak load of DNA-lactia (13,17) and virolactia (13), which is associated with the initial detection of virus shedding into the urine of the preterm infants (13). Even during maximum viral load, preservation of milk at −20°C for an undefined time (17) may be a risk for VLBW infants under high risk to acquire a symptomatic CMV infection. In 50% of the experiments using freeze-thawing of spiked milk samples, we observed enhanced viral load in milk whey prepared after freeze-thawing, as shown also in one case for the milk fat fraction of a naturally infected milk. An explanation could be the different distribution of laboratory strain AD169 and wild-type virus between the aqueous milk whey and the fat fraction after freeze-thawing. Even after storing naturally infected milk for 1 y at −20°C followed by three freeze-thawing cycles in total, we were still able to isolate infectious virus from milk whey (16). Up until now, a threshold level of CMV DNA lactia and virolactia resulting in transmission of CMV by breast-feeding cannot be given. In fact, our results indicate that even quantitative CMV DNA lactia cannot be taken as a reliable indicator of viable infectious virus in milk or milk whey after the different inactivation procedures, because DNA levels do not alter significantly. Therefore, the presence of viral pp67 late mRNA detected by NASBA in cell-free milk whey after freeze-thawing is an important molecular approach to confirm the results of quantitative virolactia by microculture. Furthermore, quantification of viral pp67 mRNA by NASBA (14,18) in naturally infected milk may provide a more suitable alternative that requires further analysis.

Freezing did not alter the activity of AP and lipase or the levels of sIgA. As reported previously, storage of milk by deep-freezing at −20°C for 3 mo produced no appreciable loss of lysozyme and IgA (19). Taken together, cryoinactivation preserved the nutritional quality of human milk, but unfortunately also CMV infectivity. In contrast, Holder pasteurization and short-term heat inactivation for 5 s at 72°C destroyed the activity of the marker enzymes AP and lipase completely.

Compared with freezing only, heat treatment may eliminate viral infectivity in milk in each stage of lactation, reflecting the unimodal kinetics of CMV reactivation during lactation. Feeding of freeze-stored milk from a seropositive mother with an unknown virus load to a VLBW preterm infant under risk may be associated with CMV transmission and acquisition of a symptomatic CMV infection. Only screening for (quantitative) DNA-lactia, RNA-lactia, or virolactia may predict mothers under risk to transmit the virus.

Preliminary data from short-term heat inactivation using ramp temperatures below 72°C revealed that CMV infectivity was eliminated very efficiently, preserving up to 56% of the activity of the AP. However, further clinical trials are necessary to characterize the superiority of our short-term heat inactivation method based on the generation of a thin milk film using a ramp temperature below 72°C compared with traditional Holder pasteurization. Thus, the lowest optimal temperature and time for complete destruction of viral infectivity and for simultaneous conservation of the nutritional and immunologic milk quality still remains to be defined. In contrast to freeze-storing, Holder pasteurization and short-term heat treatment need special equipment. Long-term pasteurization is commercially available, whereas our short-term heat procedure has been performed with a prototype. Therefore, further efforts are necessary to develop commercially available technical

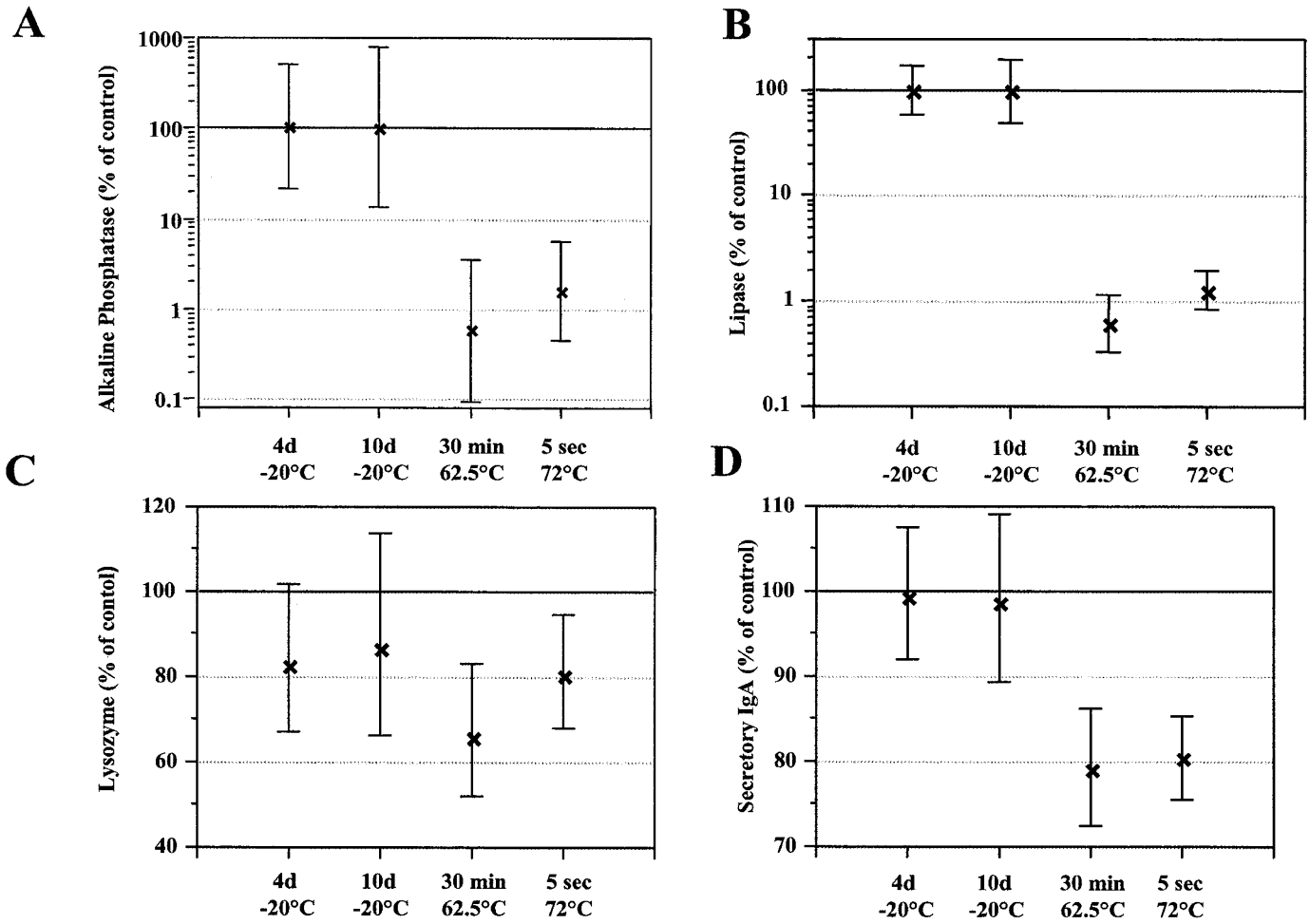

Figure 3. Biochemical parameters of different procedures for CMV inactivation in breast milk. *A–D* show the geometric mean values together with their 95% confidence limits of four biochemical parameters expressed as percentage of control for four inactivation methods. (*A, B*) Stability of marker enzyme activities of AP and lipase by freeze-storing, complete loss of marker enzyme activities by Holder pasteurization and short-term pasteurization. (*C*) Moderate loss of lysozyme levels (about 20%) by freezing and short-term pasteurization. (*D*) No loss of sIgA levels by freezing, moderate loss of sIgA by Holder and short-term pasteurization (about 20%).

Table 2. *Short-term heating is less harmful to the activity of marker enzymes in milk than Holder pasteurization*

	Mother		Virological characteristics*		Enzyme activity			
Virus strain	Lactation days p.p.	Virus inactivation	Infectivity (IEA+/mL)	DNA load (copies/mL)	AP (U/L)	% activity	Lipase (U/L)	% activity
AD 169†	D, 139	Control	1260	92,500	23	100	59	100
	CMV-IgG negative	2 s 72°C	0	105,000	1	4.4	4	6.8
		2 s 65°C	0	112,000	3	13.0	7	11.9
		2 s 63°C	0	86,600	12	52.2	6	10.2
		2 s 62°C	0	90,600	13	56.5	6	10.2
		30 min 62.5°C	0	108,000	1	4.4	3	5.1

* Data from milk whey.

† Spiking experiment with 10^5 tissue culture infective dose 50% of CMV laboratory strain AD 169 per milliliter of raw milk ($TCID_{50}$/mL).

equipment for an optimized short-term heat inactivation of CMV in human milk, which overcomes the known biochemical disadvantages of Holder pasteurization resulting in prevention of CMV transmission to VLBW infants by breast-feeding. The actual debate on the importance of prevention strategies for symptomatic CMV primary infections of preterm infants under risk (4,11,17) resulted recently in a consensus paper of the Austrian Society of Pediatrics that recommended Holder pasteurization of every seropositive milk up to the end of gestational wk 34 of the preterm infant (20).

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