Bacterial Safety of Flash-heated and Unheated Expressed Breastmilk during Storage

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Summary

Heat-treated breastmilk is one infant-feeding option recommended by the WHO to reduce mother-to-child transmission of HIV in developing countries. Flash-heat, a simple pasteurization method that a mother could perform in her home, has been shown to inactivate cell-free HIV-1. Since heating may affect the naturally occurring antimicrobial properties found in breastmilk, storing heated breastmilk may present a safety issue in resource-poor settings due to lack of refrigeration and potential contamination. To address this, we investigated the ability of flash-heat to eliminate bacteria and to prevent growth over time compared with unheated breastmilk. We collected breastmilk samples from 38 HIV positive mothers in South Africa and aliquoted them to flash-heated and unheated controls. Samples were stored at room temperature for 0, 2, 6 and 8 h and then plated and incubated for 24 h at 37°C in CO_2. We performed total colony counts and identified *Escherichia coli, Staphylocuccus aureus* and Group A and Group B streptococci. Unheated samples had a significantly higher number of samples positive for bacterial growth at each time point ($p < 0.0001$), as well as mean colony-forming units (CFU)/ml in those samples that were positive at each time point ($p < 0.0001$). In addition, unheated samples had a significantly higher rate of bacterial propagation over time than flash-heated samples when comparing log values of CFU/ml across 0–8 h ($p < 0.005$). No pathogenic growth was observed in the flash-heated samples, while the unheated samples showed growth of *E. coli* ($n = 1$) and *S. aureus* ($n = 6$). Our data suggest that storage of flash-heated breastmilk is safe at room temperature for up to 8 h.

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Introduction

Mother-to-child transmission (MTCT) is responsible for ~90% of the 725 000 HIV infections that occur each year among the children of the world, of which 90% are in sub-Saharan Africa [1]. Breastfeeding for extended periods is widespread and is responsible for one-third to one-half of paediatric HIV infections in sub-Saharan Africa. Even the low-cost, two-dose nevirapine prophylaxis does not substantially decrease the transmission from prolonged breast-feeding [2, 3]. For HIV positive mothers in developing countries, complete avoidance of breastfeeding may not be a safe option due to cost, lack of safe water, unsanitary conditions and socio-cultural factors. In addition, formula-fed infants who lack the immune protection conferred by breastmilk experience increased rates of morbidity and mortality due to diarrhoeal, respiratory and other infections [4–8].

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In light of this, the current World Health Organization (WHO) guidelines stipulate that HIV positive women avoid breastfeeding when replacement feeding options are acceptable, feasible, affordable, safe and sustainable. If these conditions are not in place, the WHO recommends that mothers exclusively breastfeed for the first months of life, then abruptly wean [9, 10]. Modifications to breastmilk are also a recommended alternative. Use of manually expressed, heat-treated breastmilk is one such modification recommended by WHO, UNICEF and UNAIDS [9, 10]. We previously reported that flash-heat, a simple in-home pasteurization method for mothers in developing countries, is capable of inactivating cell-free HIV in HIV-spiked breastmilk samples, while retaining the milk's nutritional value [11]. In addition, our ongoing research suggests that flash-heat can also destroy HIV in naturally infected breastmilk from HIV positive mothers [12]. However, safe storage of manually expressed and heated breastmilk is of concern in countries that lack refrigeration as bacterial contamination could result in infant morbidity, such as diarrhoeal illness.

Previous studies have shown a wide range of bacterial levels in donated expressed breastmilk (EBM), from no growth to 10^6 colony-forming units (CFU)/ml [13–15]. This variation may be due to the mode of breastmilk collection and storage, and differences in personal hygiene practices [14]. Breastmilk obtained by manual expression has been reported to have less risk of contamination than milk obtained with breast pumps, although manual expressing at home resulted in higher bacterial contamination than that performed in a hospital [15–17]. Commercial heat treatment methods, such as Holder Pasteurization (62.5°C for 30 min), are used by human milk banks to eliminate potential pathogens in donated EBM [18]. However, appropriate low-technology methods are needed for use in resource-poor countries. Jeffery *et al.* [19] reported that one simple method, Pretoria Pasteurization, eliminated clinically significant bacteria in 93% of the EBM samples tested. Similarly, we previously reported pilot results that the flash-heat method eliminated spiked *Escherichia coli and Staphylococcus aureus* in breastmilk from healthy mothers in the United States [11]. The objectives of this study were to determine if flash-heat could eliminate naturally occurring bacteria in EBM and to assess if flash-heated EBM could be safely stored at room temperature for up to 8 h.

Materials and Methods

HIV positive breastfeeding mothers, not currently receiving antiretrovirals or antibiotics, were recruited during postnatal clinic visits at an informal settlement in Durban, South Africa between October and December 2004. Approximately 80% of the mothers in this community of 120 000 are unemployed. Within the settlement, 50% of the homes have no running water, electricity or sanitation. Thirty-eight mothers agreed to participate in this study. Following the washing of their hands with soap and water, each of them manually expressed 75–150 ml of breastmilk into a sterile glass jar. Breastmilk samples were covered and stored immediately in an ice water bath, then transported within 2 h to the laboratory where the same sterile glass jar was used for flash-heating. Fifty millilitres of each EBM sample were aliquoted to be flash-heated and the remaining volume was aliquoted to be used as an unheated control.

The flash-heat method has been described in detail elsewhere [11]. Briefly, 50 ml of EBM in an uncovered sterile 16 oz commercial glass food jar was placed in 450 ml of water in a 1 : 1 Hart brand 1 quart aluminium pan. Water and milk were heated together over a single burner butane stove, used to imitate the intense heat of a fire, until the water reached 100°C and was at a rolling boil. The breastmilk was immediately removed from the water bath and allowed to cool to 37.0°C. Temperature data were collected at 15 s intervals using thermometer probes (Cole-Palmer Digi-Sense® DuaLogR® Thermocouple Thermometers). Flash-heat typically reached temperatures above 56.0°C for 6 min and 15 sec, and peaked at 72.9°C.

Flash-heated and unheated samples were stored at 2–8°C overnight to be processed for microbiology assays the next morning, ~18–24 h after collection. At this time, both flash-heated and unheated aliquots were placed at room temperature (~23°C) and allowed to stand, in capped vials, for up to 8 h. For both the flash-heated and unheated aliquots, at 0, 2, 4, 6 and 8 h, 100 µl of undiluted EBM and 100 µl of a 1 : 100 dilution of EBM were plated with sterile streaking loops on cysteine lactose electrolyte deficient (CLED) medium, colistine nalidixic acid blood agar (CNA) and mannitol salt agar (MSA). Plates were incubated for 24 h at 37°C in CO_2. The dilution with a number of colonies between 20 and 200 at time zero, or baseline, was used to determine the number of CFU/ml at each subsequent time point irrespective of the number of colonies at the subsequent time. CFU/ml were determined using 33/38 and 5/38 undiluted and 1 : 100 diluted samples, respectively. Growth on the CLED agar was used to determine the total count, while growth of *E. coli, S. aureus* and β-haemolytic streptococci was quantified on CLED, MSA and CNA, respectively. If >200 colonies were observed, this was considered too numerous to count (TNTC). For all cases where colony counts yielded values below the set minimum (20) and above the set maximum (200) number of colonies at the dilution used, we substituted a proxy value. We calculated the geometric mean between the

CFU/ml obtained from the highest number of colonies countable before designating TNTC, 2000 CFU/ml (>200 colonies in 100 µl aliquot), and the CFU/ml value obtained from the count just below the minimum acceptable for 1 : 100 dilutions, which would be 19 000 CFU/ml (<20 colonies in 100 µl aliquot of 1 : 100 dilution). The geometric mean of these observed values at baseline, 2000 CFU/ml and 19 000 CFU/ml, was calculated to be 6166 CFU/ml (log value = 3.79 CFU/ml). This value was then used for all time points with TNTC values.

All statistical analyses were performed using Stata, version 8.0, Stata Corporation, College Station, Texas.

This study was approved by the Committees for the Protection of Human Subjects at the University of California campuses at Berkeley and Davis and the University of KwaZulu-Natal.

Results

Thirty-eight EBM samples were flash-heated and compared with unheated controls for bacterial growth over 8 h at room temperature. At baseline, immediately after heating, 16% (6/38) of the flash-heated samples showed some bacterial growth, compared with 100% (38/38) of the unheated samples. No growth to very-limited growth (<99 CFU/ml) was observed overall time points for the majority of the flash-heated samples (89–92%) compared with unheated controls (3–5%) ($p < 0.0001$), while substantial growth (>1000 CFU/ml) was observed in very few flash-heated samples (0–3%) compared with the majority of unheated controls (61–66%) ($p < 0.0001$). Similarly, the majority of unheated samples (61%) had $>1 \times 10^3$ CFU/ml starting at baseline and continued up to 8 h. Eleven percent (4/38) of the unheated samples, including one 1 : 100 dilution, at baseline and 42% (16/38), including two 1 : 100 dilutions, at 8 h had unreadable plates, and were considered TNTC. Additionally, after 8 h incubation, zero bacterial growth was observed in the majority, 84% (32/38), of the flash-heated samples, compared with 0% (0/38) of the unheated samples. These differences between flash-heated and unheated samples were found to be statistically significant when comparing the number of samples positive for bacterial growth at each time point (Table 1) as well as the mean log values of CFU/ml at each time point (Fig. 1, Table 2).

We observed a decline in CFU/ml among breastmilk samples positive for bacterial growth in at least one time point over the 8 h in 83% (5/6) of flash-heated samples and 82% (31/38) of the unheated samples, although this decrease was not statistically significant. Unheated samples had significantly greater bacterial propagation over time than flash-heated samples when comparing log values of CFU/ml across 0–8 h ($p < 0.005$, Wilcoxon signed-rank test).

TABLE 1
Comparison of number of flash-heated and unheated samples with bacterial growth at each time point

Time point	Flash-heated (n=38)	Unheated (n=38)
0	6	38
2	11	38
4	6	37
6	5	38
8	5	38

$p < 0.0001$, paired Student's t-test.

None of the flash-heated samples were considered TNTC at any time point. Among unheated samples, four were considered TNTC at baseline, five at 2 h, seven at 4 h, thirteen at 6 h, and sixteen at 8 h. These samples were assigned the imputed value of 6166 CFU/ml (log value = 3.79 CFU/ml).

Among the flash-heated samples, 0/38 showed pathogenic growth at any time point (Table 3, Fig. 2). Among the unheated samples, 20 CFU/ml of *E. coli* were observed in one sample at 6 h and *S. aureus* was observed at $\leq 1 \times 10^3$ CFU/ml in 8% (3/38) of samples. Similar to the total bacterial growth described above, we observed a decline in CFU/ml in at least one time point for pathogens in 100% (7/7) of unheated samples, although this decrease also was not statistically significant. Neither the flash-heated nor the unheated samples had Group A or B streptococcus growth at any time point.

Discussion

Flash-heat was successful in completely eliminating bacteria in the majority of samples, and prevented substantial growth for up to 8 h when stored at room temperature. We observed significantly less bacterial growth in the flash-heated samples compared with unheated ones at each time point. Although the majority of unheated samples had $>1 \times 10^3$ CFU/ml starting at baseline through 8 h, unfortunately, because of the dilutions used, we were not able to ascertain the upper limits of growth for those considered TNTC. The interpolated value we used was derived only from time point 0. This suggests that by 8 h, our samples of unpasteurized EBM stored at room temperature (23°C) had substantial bacterial growth. Current recommendations by the Human Milk Bank Association of North America state that storage of EBM at room temperature is safe for up to 6–8 h. Based on our results and previous findings [20], however, we would urge that further research is needed to evaluate the safe

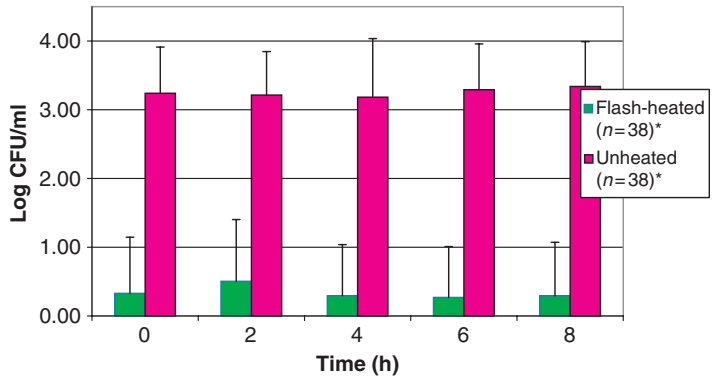

FIG. 1. Mean log comparison of non-pathogenic growth in flash-heated or unheated positive breastmilk samples at 0–8 h storage ($p < 0.0001$). Samples labelled TNTC were set to 6166 CFU/ml (log value = 3.79), which may underestimate the actual bacterial growth in these samples. (No flash-heated samples were considered TNTC at any time point. Among unheated samples, 4,5,7,13 and 16 samples were considered TNTC at 0,2,4,6 and 8 h, respectively.)

TABLE 2
Comparison of mean log values of CFU/ml for flash-heated and unheated samples at each time point

Time point	Flash-heated		Unheated	
	Mean log (S.D)	Median	Mean log (S.D)	Median
0	0.328 (0.817)	0	3.239 (0.672)	3.251
2	0.504 (0.898)	0	3.213 (0.632)	3.111
4	0.294 (0.745)	0	3.182 (0.853)	3.127
6	0.269 (0.741)	0	3.292 (0.667)	3.161
8	0.292 (0.779)	0	3.339 (0.653)	3.159

$p < 0.0001$, paired Student *t*-Test.
Samples labelled TNTC were set to 6166 CFU/ml (log value = 3.79), which may underestimate the actual bacterial growth in these samples. (No flash-heated samples were considered TNTC at any time point. Among unheated samples, 4,5,7,13 and 16 samples were considered TNTC at 0,2,4,6 and 8 h, respectively.)

TABLE 3
Flash-heated and unheated breastmilk samples with non-pathogen and pathogen growths at any time point over 0–8 h

	Flash-heated (*n*=38) No. of samples positive (%)	Unheated (*n*=38) No. of samples positive (%)
Pathogens		
E. coli	0 (0)	1 (2.6)
S. aureus	0 (0)	6 (15.8)
Group B strep	0 (0)	0 (0)
Non-pathogens	13 (34.2)	38 (100)

duration for storing EBM at room temperature—for consumption of either raw or pasteurized EBM.

We observed decreases in bacterial growth at several time points in some flash-heated and unheated breastmilk samples. Although these decreases were not statistically significant, previous studies have suggested similar decreases and fluctuations in bacterial growth over time in breastmilk due to its naturally occurring antimicrobial activity [21–24]. We find it interesting that data from several of our samples agree with these previous findings that an initial increase in bacterial growth was followed by a decrease and then subsequent increase again. This fluctuation in bacterial growth suggests a delay in anti microbial activity and is hypothesized to be due to possible activation and involvement of complement and to a progressive increase in free fatty acids by milk lipases in stored milk, which are known to have cytotoxic effects on pathogenic organisms [25–28].

Breastmilk is not a sterile bodily fluid and can play an important role in promoting the infant immune response if the bacterial concentrations are at acceptable levels. Common bacteria found in donated EBM include non-pathogens such as *Staphylococcus epidermidis*, α-haemolytic streptococcus, *Bacillaceae* species, as well as pathogens such as *S. aureus* and *E. coli*. The criteria for safe donor milk, as specified by the Human Milk Banking Association of North America, are

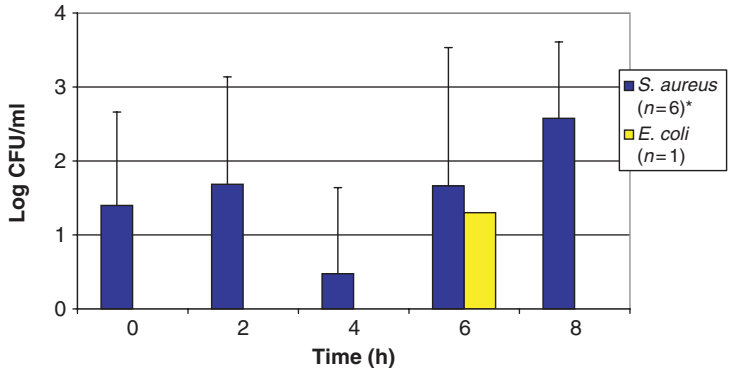

FIG. 2. Mean log of pathogenic growth in unheated breastmilk samples positive for *S. aureus* ($n = 6$) and *E. coli* ($n = 1$) at 0–8 h storage. Samples labelled TNTC were set to 6166 CFU/ml (log value = 3.79), which may underestimate the actual bacterial growth in these samples. (No flash-heated samples were considered TNTC at any time point. Among unheated samples 4,5,7,13 and 16 samples were considered TNTC at 0,2,4,6 and 8 h, respectively.)

counts of $<1 \times 10^5$ CFU/ml for non-pathogens, $<1 \times 10^3$ CFU/ml of *S. aureus* and no *E. coli* in pre-pasteurized samples and no growth of any species in post-pasteurized samples after 48 h stored at 4°C [29].

In resource-poor settings where infants may continually be exposed to potential pathogens, it is important that the immuno-protective elements of breastmilk remain after heating. Examples of important vertically transferred anti-infective components include oligosaccharides, leukocytes, secretory IgA, lactoferrin and lysozyme, which are protective against enteric pathogens. This biochemical protection manifests itself in a dose-responsive inverse correlation between lower morbidity and mortality rates and milk volume consumption and duration of breastfeeding among breastfed infants. Oligosaccharides are simple sugars that bind bacteria and form complexes that are then safely excreted in the infant's urine [30, 31]. Leukocytes, including neutrophils, macrophages and lymphocytes, actively respond to the presence of enteric pathogens [32]. Secretory IgA, which is the primary immunoglobulin in human milk, is an important immune factor for epithelial surfaces [33–36]. Lactoferrin, in addition to its antiviral, antioxidant, anti-inflammatory, immune-modulating and anticancer activities, causes breastmilk to become bacteriostatic for some bacteria, including *E. coli, Stretococcus mutans*, and *Vibrio cholerae* [37–44]. Human lysozyme kills most Gram-positive bacteria by damaging their surface peptidoglycan and is also active against Gram-negative organisms. Other studies have found that storage of breastmilk in refrigeration or deep freeze has been associated with increased anti microbial properties, thought to be due to an increase in levels of free fatty acids [21, 28, 45]. Moreover, the presence of non-pathogenic bacteria in EBM are thought to inhibit pathogenic growth [46].

Low-temperature, long-time (LTLT) heat treatment methods, such as Holder Pasteurization at 62.5°C for 30 min, are reported to maintain the majority of such immunological components and macronutrients of breastmilk, although research shows a substantial reduction in lactoferrin, vitamins and immunoglobulins [47–50]. Thus, the flash-heat method was designed to imitate high-temperature, short-time (HTST) heat treatments used commercially, which typically heat to 72°C for 15 s. HTST methods are considered to be superior since they can kill bacteria and cytomegalovirus, with no decrease in vitamins, lactoferrin, total IgA concentrations or secretory IgA activity [51–53].

This study had several limitations. Since samples were refrigerated overnight prior to processing, this delay may have allowed lipolysis of fatty acids resulting in enhanced antimicrobial ability of both flash-heated and unheated samples. Additionally, in order to have quantifiable data, samples identified as TNTC were set as 6166 CFU/ml, based on the geometric mean between the observable baseline values of 2000 and 19 000 CFU/ml. This may underestimate our values after time zero since potential bacterial growth after the baseline reading of these samples was not captured. In light of this, although we found the difference to be significant, the actual magnitude of this difference between bacterial growth in flash-heated vs unheated samples may have been greater than that presented here.

The purpose of this study was to determine if flash-heat was capable of eliminating naturally

occurring bacteria in EBM and in preventing contamination. We acknowledge that our study design does not allow us to accurately assess the antimicrobial activity remaining in flash-heat breastmilk since additional contaminants were not introduced post-heating to specifically test this. It may be safest to flash-heat the breastmilk immediately after expressing and prior to storage to avoid potential replication of pathogens over time, such as *S. aureus* whose toxin may remain post-heat [54]. Further research is needed in this area.

In summary, this study suggests that flash-heat is capable of eliminating pathogenic and non-pathogenic bacteria and that an 8 h storage period outside the refrigerator does not result in a significant increase of bacteria. This is an important finding since HIV is not the only microbe of concern that must be eliminated in EBM for safe infant consumption. Flash-heat is a simple EBM pasteurization method that could be a safe infant-feeding option for mothers in need of breastmilk modifications, such as HIV positive mothers in developing countries where resources such as refrigeration are lacking.

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