

# Viral, Nutritional, and Bacterial Safety of Flash-Heated and Pretoria-Pasteurized Breast Milk to Prevent Mother-to-Child Transmission of HIV in Resource-Poor Countries

## A Pilot Study

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**Background:** Heat-treated breast milk of HIV-positive mothers has potential to reduce vertical transmission. This study compared the impact of flash-heating (FH) and Pretoria pasteurization (PP) on HIV, nutrients, and antimicrobial properties in human milk.

**Methods:** Milk samples were spiked with  $1 \times 10^8$  copies/mL of clade C HIV-1 and treated with FH and PP. We measured HIV reverse transcriptase (RT) activity before and after heating ( $n = 5$ ). Heat impact on vitamins A, B<sub>6</sub>, B<sub>12</sub>, and C; folate, riboflavin, thiamin, and antimicrobial proteins (lactoferrin and lysozyme) was assessed. Storage safety was evaluated by spiking with *Escherichia coli* or *Staphylococcus aureus*.

**Results:** Both methods inactivated  $\geq 3$  logs of HIV-1. FH resulted in undetectable RT activity. Neither method caused significant decrease in any vitamin, although reductions in vitamins C and E were noted. Heat decreased immunoreactive lactoferrin ( $P < 0.05$ ) but not the proportions of lactoferrin and lysozyme surviving digestion. FH seems to retain more antibacterial activity. Both treatments eliminated spiked bacteria.

**Conclusions:** FH may be superior to PP in eliminating all viral activity; both methods retained nutrients and destroyed bacterial

contamination. Heat-treated breast milk merits further study as a safe and practical infant feeding option for HIV-positive mothers in developing countries.

**Key Words:** HIV, breast milk, mother-to-child transmission, pasteurization, heat treatment, perinatal transmission

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In developed countries, obstetric intervention, antiretroviral (ARV) drugs, and avoidance of breast-feeding have drastically reduced mother-to-child transmission (MTCT) of HIV, but these options are not available to most women in the developing world. One third to one half of pediatric HIV infections in sub-Saharan Africa are attributed to breast-feeding.<sup>1</sup> Complete avoidance of breast-feeding for HIV-positive mothers in resource-poor communities may not be possible because of cost, lack of safe water, unsanitary conditions, and sociocultural factors. In these settings, formula feeding has been associated with a 14- to 25-fold increase in diarrhea-associated infant mortality<sup>2–6</sup> because of contamination and lack of the protective factors intrinsic to breast milk. Thus, many HIV-positive mothers and health care workers face a desperate dilemma: risk HIV transmission through breast-feeding versus increased morbidity and mortality with infant formula or other breast milk substitutes.

Although the prevailing form of breast-feeding worldwide is mixed breast-feeding (supplementing with non-breast milk liquids and/or solids),<sup>7</sup> several recent studies suggest that this may increase the risk of HIV transmission up to 4-fold compared with exclusive breast-feeding (EBF) to 6 months.<sup>8–10</sup> Increased risk among mixed feeders is hypothesized to be attributable to an increased risk of maternal mastitis, causing an increase in milk viral load, and to the introduction of antigens, which may compromise the infant's gut integrity.<sup>11,12</sup> Currently, the World Health Organization (WHO) recommends that HIV-positive mothers in developing countries practice EBF for the first months and then wean unless alternative feeding options are acceptable, feasible, affordable, safe, and sustainable.<sup>13–15</sup> One WHO-endorsed alternative, manually expressed heat-treated

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breast milk, has not yet been fully investigated.<sup>16,17</sup> The WHO lists (1) direct boiling, shown to cause significant nutritional damage, and (2) pasteurization, such as Holder pasteurization (62.5°C for 30 minutes),<sup>16</sup> which has been reported to inactivate HIV, although retaining most of breast milk's protective elements,<sup>18-21</sup> but requires temperature gauges and timing devices unavailable in most at-risk communities.<sup>22</sup> This pilot study investigated and compared the virologic, nutritional, and antimicrobial safety of flash-heating (FH) and Pretoria pasteurization (PP), 2 recently developed heat treatments that a mother in a developing country could implement over a fire or in her kitchen.<sup>23,24</sup>

## METHODS

### Sample Collection

Healthy lactating women, 2 to 12 months postpartum and not receiving antibiotics or corticosteroids, were recruited in northern California between August 2003 and May 2004. Institutional review board approval and written informed consent were obtained. After cleansing each breast with soap and water to reduce contamination from normal skin flora, each woman donated 80 to 120 mL of breast milk using a pump or hand expression. Samples were transferred to sterile containers and transported on ice for laboratory assays within 2 hours of expression.

### Heat Treatments

Two methods of heat treatment were performed using a single-burner butane stove to imitate the intense heat of a fire. PP has been described elsewhere.<sup>23,25,26</sup> Briefly, 450 mL of water was brought to a boil in a 1:1 Hart 1 quart aluminum pan and then removed from the heat. A 50-mL sample of breast milk in a 16-oz glass peanut butter jar with a lid was placed in the water bath for 20 minutes; it was then removed from the water bath, uncovered, and allowed to cool to 37.0°C. The FH method was modified from methods described elsewhere.<sup>24</sup> Using the same equipment, a 50-mL sample of breast milk in an uncovered glass peanut butter jar was placed in 450 mL of water in an aluminum pan. The water and milk were heated together over a flame until the water reached 100°C and was at a rolling boil. The breast milk was immediately removed from the water and allowed to cool to 37.0°C. Temperature data were collected at 15-second intervals using thermometer probes (Cole-Palmer Digi-Sense DualLogR Thermocouple Thermometers).

### HIV Assays

Five fresh whole breast milk samples and phosphate-buffered saline (PBS) were inoculated with  $1 \times 10^8$  copies/mL of cell-free clade C HIV and aliquoted to (1) unheated HIV-spiked milk, (2) FH HIV-spiked milk, (3) PP HIV-spiked milk, (4) unheated HIV-spiked PBS, (5) FH HIV-spiked PBS, (6) PP HIV-spiked PBS, and (7) unheated milk not spiked with HIV.

Samples were assayed for HIV using 3 techniques: (1) TaqMan real-time RNA polymerase chain reaction (PCR); TaqMan PCR), (2) reverse transcriptase (RT) enzymatic activity assay, and (3) semiquantitative cultures. We compared these assay methods because it was necessary to discriminate between live and inactivated HIV. Because TaqMan PCR

assays detect only viral nucleic acid and do not distinguish between viable and nonviable HIV, we required an additional assay to assess the viability of HIV. Because previous studies have shown the RT assay to correlate well with RNA quantification and because functional RT is required for cellular infection, we chose quantitative measurement of RT activity as a quantitative marker for the presence of viable HIV.<sup>27</sup>

RNA was extracted using the Viral RNA Kit (Qiagen, Valencia, CA). The TaqMan PCR assay methods have been described elsewhere.<sup>28</sup> TaqMan oligonucleotides were HIVB-579f: ACATCAAGCAGCCATGCAAAT, HIVB-682r: TCTG GCCTGGTGCAATAGG, and HIVB-612p: CTATCCCATTC TGCAGCTTCCTCATTGATG. The ExaVir Quantitative HIV-Reverse Transcriptase Load Kit (Cavidi, Uppsala, Sweden) was used according to the manufacturer's instructions.

Semiquantitative culture was performed using established techniques.<sup>29</sup> Briefly,  $5 \times 10^4$  PM1 cells in 100  $\mu$ L of RPMI 1640/10% fetal bovine serum (FBS) were placed into a 96-well plate in 6 groups of 4 wells each (quadruplicate). One hundred microliters of serial 10-fold dilutions of the milk or PBS sample diluted  $10^{-1}$  to  $10^{-6}$  in RPMI 1640/10% FBS were added to the appropriate well. Cultures were incubated at 37°C in 5% CO<sub>2</sub>. To remove residual virus, on day 1, cultures were washed 3 times by removing tissue culture medium, adding fresh medium, respinning the cells, and centrifuging at 400 g for 10 minutes. Cultures were fed every 3 days beginning at day 1 by removing 150  $\mu$ L of culture supernatant and replacing it with an equal volume of medium. Samples were taken for p24 assays at days 1 and 3 (background residual p24 from inoculum) and at days 7, 14, 21, and 28.

### Nutrient Assays

Vitamin and protein analyses were performed on unspiked, fresh, whole breast milk samples (120 mL) collected from mothers as described previously. Sample sizes varied per nutrient assay (Table 1). Each milk sample was aliquoted to (1) unheated, (2) FH, or (3) PP and was frozen at  $-20^\circ\text{C}$  to await analysis.

For the vitamin A analysis, the sample was mixed with ethanolic potassium hydroxide-containing pyrogallol and heated for 1 hour at 60°C. Retinol was extracted into hexane using o-ethyl-retinal-oxime as an internal standard and was analyzed by reverse-phase high-performance liquid chromatography (HPLC) as previously described.<sup>30</sup>

For the ascorbic acid analysis, an aliquot of sample was mixed with an equal volume of metaphosphoric acid. After centrifugation, vitamin C was measured by HPLC.

Thiamin diphosphate was measured by HPLC after derivatization with alkaline ferricyanide in a slightly modified version as described by Lynch et al<sup>31</sup> and Baines.<sup>32</sup>

After precipitation with metaphosphoric acid, riboflavin was measured by reverse-phase HPLC.

After protein precipitation with 10% metaphosphoric acid, plasma pyridoxal-5 phosphate (vitamin B<sub>6</sub>) was measured using HPLC with on-line derivatization with sodium bisulfite and fluorometric detection.

Folate and vitamin B<sub>12</sub> were measured using automated competitive chemiluminescent immunoassays on the Bayer Advia: Centaur instrument.

**TABLE 1.** Nutrient Assay Results in Heated and Unheated Breast Milk

	Mean (SD)		
	FH	PP	No Heat
Vitamin A (µg/dL) (n = 5)	41.4 (9.77)	44.0 (9.83)	40.2 (8.56)
Vitamin B <sub>12</sub> (pg/mL) (n = 6)	862.8 (667.0)	862.3 (651.1)	724.5 (488.0)
Vitamin C (mg/dL) (n = 5)	4.2 (2.5)	3.6 (2.2)	5.3 (1.6)
Vitamin E (mg/L) (n = 9)	2.7 (2.0)	2.4 (1.3)	3.0 (1.2)
Pyridoxal-5-phosphate (µg/L) (n = 5)	29.0 (17.2)	28.0 (17.4)	28.8 (17.3)
Folate (ng/mL) (n = 5)	18.7* (3.8)	19.0* (1.5)	13.3 (1.2)
Riboflavin (µg/L) (n = 5)	728.0 (1142.9)	704.4 (1094.5)	667.2 (955.0)
Thiamin (nmol/L) (n = 4)	18.0 (9.1)	17.8 (6.8)	12.3 (4.6)
Lactoferrin concentration (ELISA, mg/mL) (n = 10)	0.214* (0.272)	0.826* (0.246)	1.341 (0.154)
Lactoferrin digestion (% digested) (n = 9)	84.0 (10.1)	80.3 (8.8)	87.7 (12.5)
Lysozyme digestion (% digested) (n = 9)	70.6 (21.0)	75.8 (14.9)	89.0 (14.1)

\*P < 0.05 compared with unheated control.

Lactoferrin was quantified before and after treatment by a sandwich enzyme-linked immunosorbent assay (ELISA).<sup>33,34</sup> In vitro digestion was performed as previously described<sup>34,35</sup> with minor modifications. Milk samples were adjusted to pH 3.8 with 1 M of HCl, and porcine pepsin (Sigma, St. Louis, MO) was added to make a pepsin/protein ratio of 0.08. The mixtures were incubated for 30 minutes at 37°C with shaking at 200 rpm. After incubation, pH was adjusted to 7.0 with 1 M of sodium bicarbonate. Porcine pancreatin (Sigma, 8 × United States Pharmacopoeia) was then added to make a pancreatin/initial protein ratio of 0.016. After 1 hour of incubation at 37°C with shaking at 200 rpm, enzymes were inactivated by heating (85°C for 3 minutes). Undigested proteins were separated from peptides and low-molecular-weight compounds by rapid gel filtration using PD-10 columns. Total protein and undigested protein were analyzed by the Lowry method, which is known to correlate well with micro-Kjeldahl analysis,<sup>36</sup> and digested protein by difference. Proteins remaining after in vitro digestion were identified by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blotting with specific antibodies. Samples were applied for SDS-PAGE under reducing conditions, and the gels were stained with Coomassie blue. For Western blotting, an SDS-PAGE gel was electroblotted to a 0.45-µm nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). The blot was blocked with 5% nonfat dry milk in PBS, pH 7.4, for at least 2 hours, followed by 3 washes with PBS, pH 7.4, for 10 minutes each. Membranes were incubated with 5% bovine serum albumin and 0.05% Tween-20 in PBS for 1 hour to block nonspecific binding. Membranes were washed 3 times and then incubated with horseradish peroxidase–conjugated antibodies against lactoferrin and lysozyme for 1 hour at room temperature. After another wash, immunologically active proteins were detected by enhanced chemoluminescence Western blot detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

**Microbiology Assays**

To determine the effect of heat treatments on the natural antimicrobial properties of fresh whole breast milk, 1 sample

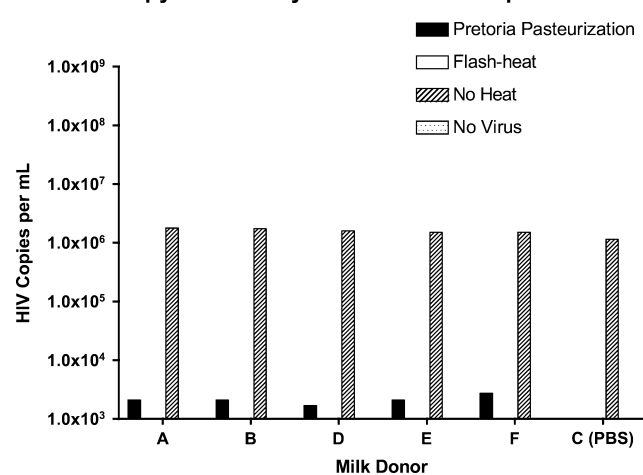
each was aliquoted to (1) unheated milk, (2) FH milk, (3) PP milk, and (4) a saline control and was inoculated with 1 × 10<sup>6</sup> colony-forming units (CFUs)/mL of *Staphylococcus aureus* or *Escherichia coli*. Samples were incubated at 25°C for 12 hours, and quantitative subcultures were then performed using salt blood agar (SBA) (Fig. 1). To determine if storing before heating or heating before storing breast milk was safer, 1100 mL of fresh whole breast milk pooled from 10 mothers was spiked with 1 × 10<sup>6</sup> *E coli* or *S aureus* and then aliquoted to (1) FH, (2) PP, or (3) unheated. Samples 1 and 2 were further aliquoted to (1) FH or PP immediately and then stored at 25°C and subcultured on SBA at 0, 4, 8, and 12 hours or (2) stored for 0, 4, 8, or 12 hours at 25°C and then FH or PP and subcultured on SBA. Unheated, unspiked breast milk served as a control.

**RESULTS**

**Heat Treatments**

Figure 2 shows typical temperature curves of the FH and PP. FH methods typically reached temperatures higher than

**HIV Copy Number by Reverse Transcriptase Activity**



**FIGURE 1.** Comparison of RT activity in FH, PP, unheated, and unspiked milk and PBS samples.

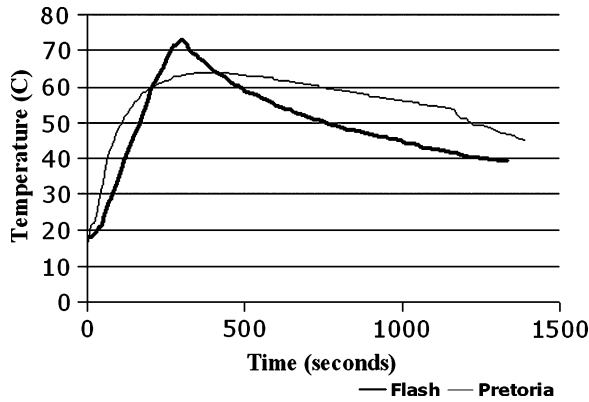


FIGURE 2. Temperature curves of FH and PP.

56.0°C for 6 minutes 15 seconds and peaked at 72.9°C. PP methods typically reached temperatures greater than 56.0°C for 13 minutes 52 seconds and peaked at 63.8°C.

### HIV Assays

TaqMan PCR assay showed no decrease in the amount of cell-free viral RNA when comparing unheated controls (8.00 log, SD = 0.030) with samples heated using FH (7.95 log, SD = 0.032) or PP (7.99 log, SD = 0.038). Both heating methods showed inactivation of  $\geq 3$  logs of HIV-1 as detected by enzymatic activity of RT, however. Figure 1 shows no evidence of RT in all 5 samples of FH milk, whereas a small amount of residual RT activity was observed in 4 of 5 samples that underwent PP. Similarly, 0 of 5 FH samples were positive for virus on coculture compared with 1 of 5 PP samples and 3 of 5 unheated controls.

### Nutrient Assays

Table 1 shows that both treatments significantly reduced the concentration of lactoferrin (FH by 84% [SD = 20] and PP by 38% [SD = 18]), although reductions in the more biologically active measures of digestibility of lactoferrin (FH by 4% [SD = 12] and PP by 8% [SD = 10]) and lysozyme (FH by 21% [SD = 24] and PP by 15% [SD = 18]) were not statistically significant. Reductions in vitamin C (FH by 21% [SD = 47] and PP by 32% [SD = 42]) and vitamin E (FH by 10% [SD = 67] and PP by 20% [SD = 43]) were also observed, although these differences were not significant. Both heat treatments were associated with increases in concentrations of the B vitamins and significantly so for folate.

### Microbiology Assays

At 12 hours after inoculation, the unheated breast milk sample showed greater inhibition of *E coli* and *S aureus* when compared with FH and PP samples as detected by quantitative subcultures (Fig. 3). FH inhibited *S aureus* and *E coli* growth by factors of 2.2 and 2.9, respectively, when compared with PP. In the samples spiked with bacteria before heating, FH and PP successfully eliminated the spiked *S aureus* or *E coli*, with  $< 100$  CFUs in all but 1 sample with 200 CFUs and maintained bacterial inhibition regardless of whether heating was done before or after the storage period of 0, 4, 8, and 12 hours (data not shown).

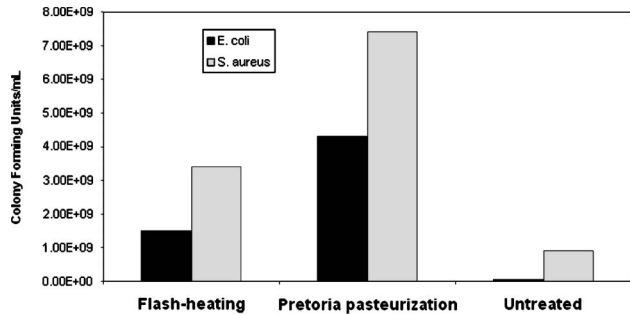


FIGURE 3. Antimicrobial effect of heated breast milk; comparison of bacterial counts in heated and control milk samples 12 hours after inoculation with *E coli* or *S aureus*.

## DISCUSSION

This preliminary work suggests that FH and PP can denature HIV, although retaining most of the nutritional as well as some of the antimicrobial properties of breast milk. Detecting cell-free HIV in heat-treated breast milk was a challenge, however. TaqMan PCR assays were not useful in differentiating active virus versus inactive viral fragments. Although coculture is generally considered the “gold standard” for determining infectivity, it is difficult to demonstrate total inactivity because of the inherent insensitivity of cocultures, particularly in breast milk, as previously reported.<sup>37</sup> The RT assay was an effective assay in determining inactivity of cell-free HIV-1 in breast milk, however. Although both heating methods inactivated 3 logs or more of HIV-1, the FH method more effectively eliminated RT activity (and infectivity as measured by the less reliable coculture) than PP.

Recent data suggest that cell-associated HIV may play a more important role in transmission of HIV via breastfeeding than does cell-free virus,<sup>38</sup> which was studied here. We hypothesize that the cell-associated HIV provirus should become inactive on cell death, which is likely to occur when the temperature is higher than 45°C for a few minutes and that any remaining HIV should have thermal inactivation properties similar to cell-free virus. Thus, we are confident that the FH method would inactivate cell-free and cell-associated HIV in breast milk.

The nutritional results were encouraging. The substantial decreases in the overall amount of lactoferrin observed with FH and PP may be attributable to partial denaturation of the lactoferrin, resulting in less recognition by the antibody used in the assay. It is likely that such denaturation does not impair the biologic activity of lactoferrin.<sup>34,39,40</sup> Treatment effects on the *in vitro* digestibility (the relative proportion that survives digestion) were much more modest, again suggesting that biologic activity may persist in the infant’s gastrointestinal tract. Additional research is needed to investigate the impact of these heating methods on the bioactivity of these proteins as well as on other important anti-infective components in breast milk, particularly secretory immunoglobulin A.

No significant decreases were observed in the vitamins assayed because of heat treatment, although power was limited because of the small sample size. Postheating increases in amounts of vitamins A and B<sub>12</sub>, folate, riboflavin, and thiamin

may be attributable to release of the vitamins from binding proteins in the milk; similar findings have been reported elsewhere.<sup>41</sup>

Because many mothers in developing countries do not have access to refrigeration, safe storage of expressed breast milk is of particular concern.<sup>42-45</sup> In samples spiked with *E coli* and *S aureus* after heating, bacterial growth was greater than in unheated controls, suggesting that heat inhibited the natural antimicrobial components in human milk that reduce the ability of pathogens to multiply.<sup>42-45</sup> This effect was less marked with FH than with PP. In samples spiked before heating, both methods eliminated inoculated as well as naturally occurring bacteria, regardless of whether the milk was heated or stored first. This finding confirms the findings of a previous study showing PP to be capable of killing bacteria in expressed breast milk, allowing it to be safely stored without refrigeration for up to 12 hours.<sup>25</sup> This suggests that the safest approach in the field may be to use the same container to store the expressed breast milk and then to heat-treat the milk just before use, because heat would destroy not only the HIV but any potential contamination.<sup>25</sup>

Both heat treatments were adapted from established heating methods. PP is similar to the low temperature, long time (LTLT) method, such as Holder pasteurization at 62.5°C for 30 minutes. Although it is widely held that LTLT-treated milk maintains most of its immunologic components and macronutrients, previous research has demonstrated a reduction in lactoferrin, vitamins, and immunoglobulins as well as inactivation of a major lipase with Holder pasteurization.<sup>46-49</sup> FH is similar to the high temperature, short time (HTST) heat treatment method used commercially, typically heating to 72°C for 15 seconds. Studies have shown that the HTST method effectively killed bacteria and cytomegalovirus, with no decrease in vitamins, lactoferrin, total IgA concentrations, or secretory IgA activity; thus, it is viewed as the preferable heating technique.<sup>41,50,51</sup> Our findings are in agreement with previous studies that showed the  $D_{69^\circ\text{C}}$  of HIV, the time required to reduce the titer or concentration of HIV by 10-fold at 69°C, in culture media to be <1 second; thus, 15 seconds at this temperature would result in a reduction of viral titer by >15 logs.<sup>18</sup> Our FH temperatures typically reached greater than 72°C, whereas PP peaked near 62°C, explaining the residual HIV activity after PP and the total viral destruction achieved with FH.

The small number of breast milk samples from healthy women in the United States limits application of these findings to the field. These safety data require verification in a large enough number of naturally infected breast milk samples from HIV-positive mothers from developing countries to ensure statistical power and relevance to the mothers and infants in need of this option. We only examined a single possible scenario, 50 mL of breast milk heated in a 450-mL water bath over a butane stove, but a variety of volumes, containers, and heat sources may influence milk temperatures, and thus effectiveness of heat treatment in the home setting. The findings of Jeffery and Mercer<sup>23</sup> that there was little temperature variation in PP because of varying volumes is reassuring,<sup>23</sup> but additional work is needed to evaluate variations in milk and water volumes, jar or pan size or shape, heat source, and even

altitude on the effectiveness of heat treatments before transferring the method to the field. Finally, this study did not address acceptability and feasibility issues because of the stigma that may arise for a mother who is not breast-feeding or who is expressing and heat-treating her milk. Our previous qualitative study in Zimbabwe, however, found that mothers and other stake holders in infant feeding concluded that heat-treated breast milk, if proven safe and nutritious, could be a more sustainable and low-cost alternative for mothers than infant formula,<sup>22</sup> but more research on attitudes, barriers, and feasibility in different settings is required.

ARVs may offer protection during breast-feeding,<sup>52-56</sup> and extended regimens are currently under investigation; however, they are unlikely to be widely implemented in the near future.<sup>57</sup> Because breast-feeding is a key route of MTCT in developing countries, even in the presence of ARVs, practical infant feeding alternatives are urgently needed for HIV-positive mothers in these settings.<sup>58</sup> Manually expressed heat-treated breast milk, which is then fed to the infant using a cup or spoon without the need for breast pumps or bottles, could serve as an affordable mother-controlled alternative in settings in which safe formula is not possible. It may also serve as a low-tech pasteurization process for milk banks in resource-poor areas. Enhanced counseling and support, including method demonstrations, may be necessary for mothers and communities to implement this option successfully.<sup>59</sup>

Heat treatment may be most feasible on the introduction of complementary foods, because milk production would be well established after months of EBF. This may also be the time when heating breast milk is most efficacious in preventing MTCT of HIV while still providing continued immune protection, because mixed feeding carries a greater risk than does EBF.<sup>8,9</sup> It may, however, also be feasible that heat-treated breast milk could be partially or totally substituted for feeding infants at the breast at any age.<sup>4,9,60</sup> Results from a study of Kenyan mothers suggest that even a partial reduction in exposure to HIV-infected breast milk could translate into a meaningful reduction in the risk of HIV infection for infants.<sup>61,62</sup> This might be accomplished by providing the infant with 1 or more feedings of heat-treated milk per day.

In summary, our findings suggest that these 2 simple heating methods, which do not require technical equipment, may be capable of HIV denaturation while retaining many of the protective elements of breast milk. FH may be more reliable than PP for complete viral destruction and may have less impact on nutritional and antimicrobial properties. Heat treatment of breast milk could play an important role in the promotion of safer breast-feeding practices to reduce MTCT of HIV in developing countries.

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