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Antimicrobial and Antiviral Effect of High-Temperature Short-Time (HTST) Pasteurization Applied to Human Milk

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ABSTRACT

In the United States, concerns over the transmission of infectious diseases have led to donor human milk generally being subjected to pasteurization prior to distribution and use. The standard method used by North American milk banks is Holder pasteurization (63°C for 30 minutes). The authors undertook an experiment to validate the effects of a high-temperature short-time (HTST) pasteurization process (72°C for 16 seconds) on the bioburden of human milk. It was concluded that HTST is effective in the elimination of bacteria as well as of certain important pathogenic viruses.

INTRODUCTION

DONOR HUMAN MILK has been used in the United States and elsewhere for many years. By common consensus, milk banking guidelines all indicate the need for pasteurization of donor milk prior to use.[1,2] The method of pasteurization used almost uniformly in milk banking is the Holder method (63°C for 30 minutes). The commercial dairy industry, by contrast, having to process much larger volumes than the typical milk bank, has used HTST methodology (usually 72°C for 15 seconds) for many years. For a variety of operational reasons (the possibility of continuous processing and preservation of vitamins, enzymes, and immunoglobulins), the authors thought that an HTST method would be well suited to a large-scale donor milk processing operation. Prior to introducing such a system, it was thought to be appropriate to validate its ability to reduce both bacteriologic and, particularly, viral bioburden. An experimental model was set up to perform that evaluation.

MATERIALS AND METHODS

Viral spiking experiment

Selection of viruses and cells. Three lipid-enveloped (LE) and two non-LE (NLE) viruses were selected for validation. LE viruses: human immunodeficiency virus (HIV, strain HTLV-

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IIIB; National Cancer Institute, Bethesda, MD) was selected as a relevant blood-borne virus, bovine viral diarrhea virus (BVDV, strain NADL; VR-534; ATCC, Rockville, MD) was selected as a model virus for hepatitis C virus (HCV), and pseudorabies virus (PRV), strain Aujeszki Bartha K61 (Duphar, Weesp, The Netherlands) was selected as a general model virus for LE DNA viruses, such as hepatitis B virus. NLE viruses: hepatitis A virus (HAV, strain HM175/18F; Organon, Boxtel, The Netherlands) was selected as a relevant blood-borne virus, and porcine parvovirus (PPV, strain NADL-2; VR-742; ATCC) as a specific model virus for human parvovirus B19.

BVDV was cultured on (Madin and Darby) bovine kidney cells (MDBK) (CCL-22; ATCC) and titrated on EBTr cells (ID-Lelystad, Lelystad, The Netherlands). HIV was cultured on H9 cells (National Cancer Institute) and titrated on MT2 cells (Wellcome, Beckenham, Kent, United Kingdom). HAV was cultured and titrated on BSC-1 cells (Organon). PPV was cultured and titrated on swine testis (ST) cells (CRL-1746; ATCC). PRV was cultured and titrated on PD5 swine kidney cells (Duphar).

Test for cytotoxicity. A cytotoxicity assay was performed to determine whether there was a direct cytotoxic effect of the (diluted) starting material on the appropriate cell line and if so, the lowest dilution in culture medium that did not cause cytotoxic effects was determined. Prior to the initiation of the cytotoxicity assay, cells were suspended in 4.0 mL of culture medium, transferred into 25-cm^2 tissue-culture flasks, and incubated for 1 day at 37°C. Subsequently, threefold serial dilutions of the test sample were prepared in culture medium and tested in 0.5 mL volumes on cells in duplicate. Unexposed cells were used as control cultures. Subsequently, all cell cultures were incubated at 37°C for the period required for the respective virus systems. Cytotoxicity is expressed as the lowest dilution of the test sample that did not cause any cytotoxic effects.

Test for stop and storage conditions and interference. The test for stop and storage conditions and interference was performed for three purposes. First, the appropriate dilution of starting material in culture medium that prevented direct virus inactivation was determined (stop condition). Second, the appropriate dilution of starting material in culture medium that did not cause inhibition of virus infection on the appropriate cells was determined (interference). Third, the diluted starting material was frozen and stored, and the virus titer was determined to show that there was no loss of virus infectivity after storage (storage condition).

A virus stock was thawed and diluted in culture medium to $\approx 10^{5.3}$ tissue culture infectious dose 50% ($TCID_{50}$) per mL (virus inoculum). To test the efficacy of the stop condition, the selected dilution of a test sample was prepared in culture medium, and 9.5 mL of this dilution was spiked with 0.5 mL of virus and then incubated for 30 minutes at room temperature. After incubation, the infectivity of the virus inoculum and the test sample were measured directly to determine the efficacy of the stop condition and lack of interference. The virus inoculum was titrated with the standard $TCID_{50}$ assay (dilution in culture medium), whereas the test sample was titrated in a modified $TCID_{50}$ assay (dilution in prediluted test sample to test interfering effects). To determine whether the selected dilution of the test sample also provided an effective storage condition, samples of the virus inoculum and spiked-and-incubated test sample were frozen and stored for at least 7 days, and subsequently tested for infectivity. If the selected dilution of test sample provides an effective stop or storage condition, and does not cause interference, no significant loss of infectivity is expected (clearance factor < 1 $\log_{10}$).

Virus assays. Infectivity was measured in validated $TCID_{50}$ assays and bulk culture tests. For $TCID_{50}$ assays, threefold serial dilutions of samples were prepared in culture media and 50 μL (or 0.5 mL for HIV) volumes were tested in eight replicates. To detect small amounts of virus, up to 60 mL of prediluted sample was tested in duplicate bulk culture tests using 25-, 80-, and 175-cm^2 flasks. BVDV, PPV, and PRV cultures were inspected microscopically for cytopathic effects at 6, 7, and 5 days postinfection

(dpi), respectively. Supernatants of HAV cultures were harvested 14 dpi and subsequently tested in an HAV enzyme-linked immunosorbent assay (ELISA). HIV cultures were inspected microscopically twice a week for the formation of syncytia until 21 dpi. Virus titers were calculated by the Spearman-Kärber method, and expressed as $TCID_{50}$/mL. If all cultures were negative, the titer ($TCID_{50}$/mL) was considered to be less than 1 ÷ total test volume (mL). Reduction factors (RF) were calculated by the following formula: RF = $\log_{10}$ (total amount of virus spiked as derived from the reference sample ÷ total amount of virus recovered from the treated sample). Clearance factors (CF) were calculated by the formula: CF = $\log_{10}$ (total amount of virus spiked as derived from the virus stock sample ÷ total amount of virus recovered from the treated sample).

Downscaling. A bench scale HTST pasteurizer was set up to perform the experiment. Milk was pumped through a stainless steel tube submerged in a water bath heated so that the fluid in the tube attained a temperature of 72°C. The flow rate through the tubing was adjusted so that the milk was maintained at that temperature for varying times up to 16 seconds. Between runs, the setup was rinsed with 50 mL of a 0.1 N solution of NaOH and then with 100 mL of PBS and 100 mL of distilled water. If the pH was still above 7, an additional rinse with PBS was performed.

A volume of 78 mL of milk was placed in a reaction container with a magnetic stirrer and was cooled to 4 ± 2°C. At that time 2 mL of virus inoculum (2.5% v/v) was added to the milk and mixed for 1 minute. A sample was then taken and diluted with an appropriate amount of culture medium. This sample was tested for the presence of infectious virus immediately and the rest of the material was frozen and stored for back-up purposes.

The remaining spiked milk was then pumped through the test apparatus and subsequently collected in a tube placed in melting ice to eliminate further heat exposure. Once the spiked milk was collected, a portion was tested for the presence of infectious virus immediately and the rest was frozen and stored for back-up purposes.

Bacterial spiking experiment

Selection of bacteria.

1. *Escherichia coli* CIVO.B.0505 (human feces)
2. *Staphylococcus aureus* NCCB70054/CIVO.B. 1245
3. *Streptococcus agalactiae* CIVO.B.0062 (mastitis cow)

Although *bacillus cereus* is a potential pathogen commonly found in milk, it was not included in this experiment as it is known that the d50 for this organism is well in excess of the time and temperature used in this process.

The bacteria were cultured for 24 hours at 37°C, subsequently centrifuged for 10 minutes at 400 rpm, and then 1 mL of the sediment was resuspended in 999 mL of human milk to achieve a level between 10^6 and 10^8 cells/mL of each test strain. The human milk, which had been stored frozen at –20°C before spiking, was allowed to thaw overnight at 5°C and after spiking was kept at 4°C until it was run through the pasteurizer.

The pasteurization process was performed in a manner analogous to that described for the viral studies. Pump speed was adjusted to allow for testing of samples after varying periods of exposure to heat. Between runs, the setup was rinsed with 50 mL of a 0.1 N solution of NaOH, followed by a rinse with 100 mL of 0.01 M PBS at pH 7.5, 100 mL of demineralized water and 100 mL of 96% alcohol.

After pasteurization, a dilution series in Peptone Physiological Salt Solution (1 g/L casein peptone + 8.5 g/L NaCl in 1000 mL distilled water was prepared for both pasteurized and unpasteurized samples. Of each dilution, 1 mL was spread onto two Plate Count Agar (PCA; Oxoid CM325) plates for enumeration of the total viable aerobic count and onto six Nutrient Agar plates (NA; Oxoid CM3) for enumeration of *E. coli*, *S. aureus*, and *S. agalactiae*. The Nutrient Agar plates were incubated at room temperature for 4 hours to allow for resuscitation of the heat damaged cells. Each plate was then covered with 15 mL of selective medium. For enumeration of *E. coli*, two NA plates were covered with Violet Red Bile Glucose Agar (VRBG; Oxoid CM485). For enumeration of *S. aureus*, two NA plates were covered with Baird Parker Agar (BP; Oxoid CM 275

with 50 mL/L of egg yolk tellurite emulsion; Oxoid SR54). For enumeration of *S. agalactiae*, two NA plates were covered with New Granada medium (proteose peptone no. 3; Difco, 25 g; soluble starch, 20 g; morpholinepropanesulfonic acid [MOPS], 11 g; Na_2HPO_4, 8.5 g; glucose, 2.5 g; sodium pyruvate, 1 g; $MgSO_4$, 0.2 g; methotrexate sodium salt, 6 mg; crystal violet, 0.2 mg; cholistine sulfate, 5 mg; metronidazole, 10 mg; agar 10 g; and water, 1000 mL).[4,5]

PCA, VRBGA, and BPA were incubated aerobically for 24 to 48 hours at 37°C, after which the number of specific colonies was counted: on PCA the total number of colonies; on VRBG the red colonies in red agar; on BPA the gray-black colonies with a clear halo. New Granada medium was incubated anaerobically (85% N, 10% H, 5% CO_2) at 37°C, after which the number of specific, orange-red *S. agalactiae* colonies was counted.

RESULTS

Virus inactivation

HTST pasteurization was tested on 3 LE and 2 NLE viruses. All virus studies were performed in duplicate and the RF values obtained for each virus at each time point are presented in Figure 1. The RF values of the duplicate runs for each virus were very similar. Results for individual viruses are discussed in the following.

BVDV. The total amount of spiked virus was $10^{7.74}$ $TCID_{50}$ and $10^{7.80}$ $TCID_{50}$ and the total amount of virus recovered from the unheated spiked material was $10^{7.61}$ $TCID_{50}$ and $10^{8.03}$ $TCID_{50}$, indicating that there was no direct effect of the breast milk on the amount of infectious virus. Already after exposure to the pasteurization process for 4 or 8 seconds, no infectious virus was detectable, resulting in an RF value of >3.55 $\log_{10}$ and >3.97 $\log_{10}$. Because for time points 12 and 16 seconds bulk cultures were tested also, the RF values in these cases were >5.42 $\log_{10}$ and >5.84 $\log_{10}$.

HAV. The total amount of spiked virus was $10^{7.39}$ $TCID_{50}$ and $10^{7.27}$ $TCID_{50}$ and the total amount of virus recovered from the unheated spiked material was $10^{7.55}$ $TCID_{50}$ and $10^{7.67}$ $TCID_{50}$, indicating that there was no direct effect of the breast milk on the amount of infec-

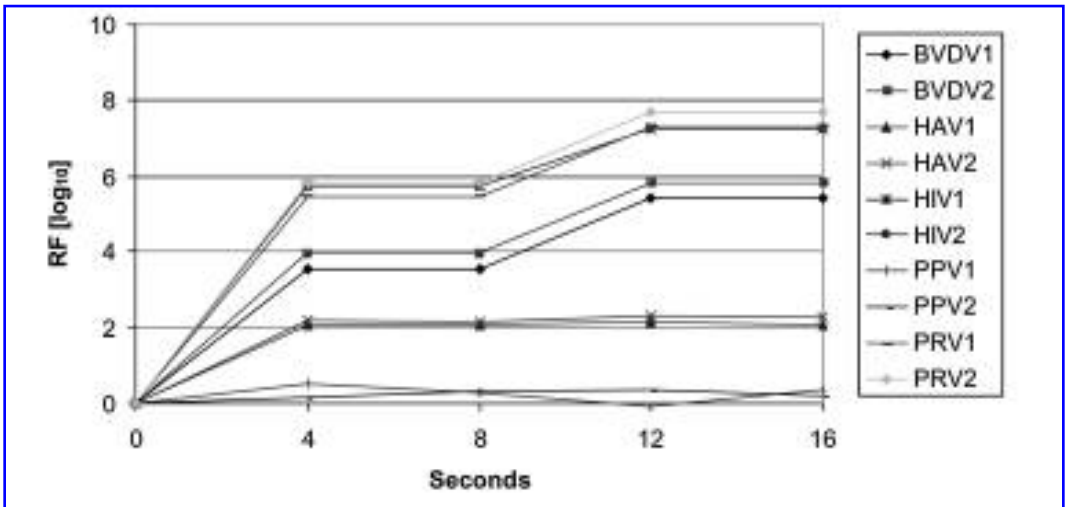

FIG. 1. Virus reduction.

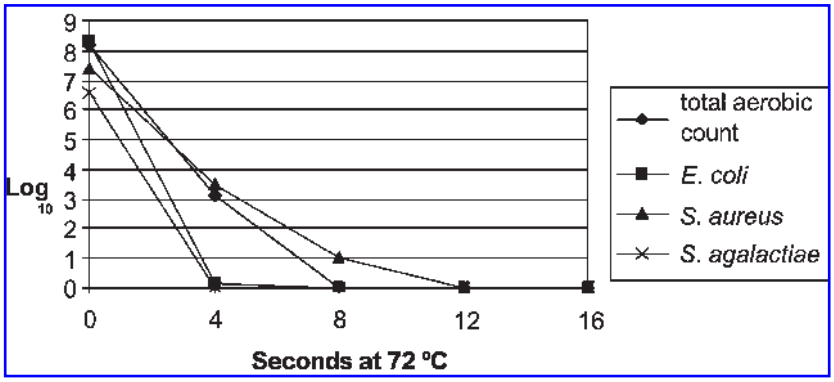

FIG. 2. Inactivation of pathogenic bacteria during HTST bacterial count pasteurization at 72°C.

tious virus. Already after exposure for 4 seconds a RF value of approximately 2 $\log_{10}$ was observed. However, this RF value remained stable at approximately 2 $\log_{10}$ for the later time points and for all time points tested infectious virus was still detectable.

HIV. The total amount of spiked virus was $10^{8.35}$ $TCID_{50}$ and the total amount of virus recovered from the unheated spiked material was $10^{8.52}$ $TCID_{50}$, indicating that there was no direct effect of the breast milk on the amount of infectious virus. Already after exposure to the pasteurization process for 4 or 8 seconds, no infectious virus was detectable, resulting in a RF value of >5.77 $\log_{10}$. Because for time points 12 and 16 seconds bulk cultures were tested also, the RF value in these cases was >7.27 $\log_{10}$.

PPV. The total amount of spiked virus was $10^{8.58}$ $TCID_{50}$ and the total amount of virus recovered from the unheated spiked material was $10^{8.39}$ $TCID_{50}$, indicating that there was no direct effect of the breast milk on the amount of infectious virus. Exposure to the pasteurization process for 4, 8, 12, and 16 seconds did not result in inactivation of infectious virus. For all time points tested the RF values were 0.50 $\log_{10}$ or less.

PRV. The total amount of spiked virus was $10^{8.64}$ $TCID_{50}$ and the total amount of virus recovered from the unheated spiked material was $10^{8.57}$ $TCID_{50}$ and $10^{8.93}$ $TCID_{50}$, indicating that there was no direct effect of the breast milk on the amount of infectious virus. Already after exposure to the pasteurization process for 4 or 8 seconds, no infectious virus was detectable, resulting in an RF value of >5.46 $\log_{10}$ and >5.82 $\log_{10}$. Because for time points 12 and 16 seconds bulk cultures were tested also, the RF values in these cases were >7.32 $\log_{10}$ and >7.68 $\log_{10}$.

Bacterial inactivation

HTST pasteurization was tested on three pathogenic microorganisms that can be present in expressed milk because of contamination from the breast (mastitis) or fecal contamination via the hands. The bacterial studies were performed in duplicate. The results of the studies are presented in Figure 2.

Total aerobic count

An initial inoculum of 1.85×10^8 CFU was recovered from unheated milk. After 8 seconds at 72°C, no viable bacteria could be detected anymore. The total count at time 0 reflects the inoculum of *E. coli*, whereas the total count at 4 seconds reflects the number of surviving *S. aureus* cells.

Escherichia coli. An initial inoculum of 2.1×10^8 CFU of *E. coli* was recovered from unheated milk. By 4 seconds no viable *E. coli* could be cultured, resulting in >32 $\log_{10}$ reductions after 16 seconds at 72°C.

Staphylococcus aureus. An initial inoculum of 2.5×10^7 was recovered from unheated milk. After 4 seconds still 3×10^3 CFU were detected, after 8 seconds 20 CFU and there was no growth detected by 12 seconds. The inactivation rate (^{72}D-value) for *S. aureus* was 1.08 $\log_{10}$

reduction per second, resulting in 15 $\log_{10}$ reductions after 16 seconds at 72°C.

S. agalactiae. An inoculum of 3.8×10^6 of *S. agalactiae* was recovered from unheated milk, and once again by 4 seconds no bacteria could be cultured from the treated milk, resulting in >26 $\log_{10}$ reductions after 16 seconds at 72°C.

DISCUSSION

Human milk is the optimum food for the human infant. The American Academy of Pediatrics goes so far as to say that even in the neonatal intensive care unit (NICU), breast milk is the optimal food and in the event a mother cannot provide breast milk, donor milk may be an adequate substitute.[3] For a variety of reasons, donor milk has not been widely used in NICUs in the United States. One of those reasons is concern about the transmission of disease via breast milk. This seems to be the case even though all donors are screened and all donor milk is pasteurized using the holder method of pasteurization.

In an effort to make specialty milk formulations for use in the NICU as safe as possible to address these concerns, the authors undertook a study to validate the antimicrobial activity in human milk after a high-temperature short-time (HTST) pasteurization process. Although pasteurization is well known to be effective against bacteria in cow's milk, activity against pathogenic bacteria in human milk was studied, as this process has not previously, to the authors' knowledge, been applied to human milk. In addition, the effectiveness of the process against viruses was also determined, because the dairy and juice industries are not concerned with the transmission of viruses by their products, whereas this is obviously of concern with fluids of human origin.

As the data indicate, the HTST process is highly effective against HIV and marker viruses for HBV and HCV. The present experiments clearly show that all viruses were very rapidly destroyed by this pasteurization method. These viruses are all lipid enveloped and of pathogenic potential in humans. The ability to eliminate these viruses, as well as other viruses for which they serve as markers (e.g. CMV, EBV) should be reassuring to those who must recommend the use of these products.

Studies on PPV, a non-lipid enveloped virus, showed no effect of HTST on viral load.; however, the authors observed a reproducible inactivation of approximately 2 $\log_{10}$ for HAV. This is a limited inactivation, but it is a small contribution to inactivation of a non-lipid enveloped virus that has been described in milk. Also this might be considered as a model for other NLE viruses and can contribute to virus safety for these viruses. Of note, a Medline search on Parvovirus B19 and human milk yielded no reports, which indicates a lack of importance of this route in transmission of this virus.

The HTST process quite easily killed the highest bacterial load of well-known human pathogens that could be produced in the laboratory, as was to be expected, based on long-term experience in the dairy and food industries. From the inactivation data $\log_{10}$ reduction rates of 15 to >32 could be calculated for the different test strains.

The present data document a strong effect of the authors' HTST process on bacteria and lipid-enveloped viruses, as well as a small, yet reproducible effect on at least one non-lipid enveloped virus. That, together with data obtained in a comparative experiment looking, among other things, at levels of IGA and other proteins thought to play a role in immune defense of the infant, indicating that HTST preserves more of the important milk protein than Holder pasteurization (manuscript in preparation) leads the authors to the conclusion that it may be the method of choice for the treatment of human milk. The major drawback of HTST is that it is extremely expensive, prohibitively so if one cannot benefit from economies of scale. In that case the existing Holder methodology has shown itself to be sufficiently robust over several decades of experience. However, when the appropriate scale can be achieved, the use of HTST may indeed be preferable.

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