Pasteurized Donor Human Milk Maintains Microbiological Purity for 4 Days at 4°C

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Abstract
Background: Most protective components in human milk are stable during prolonged storage at 4°C; however, pasteurization reduces some microbial activities responsible for suppressing microbial growth and protecting against infection. Donor milk used by neonatal intensive care units (NICUs) is frozen pasteurized donor human milk (PDHM) defrosted and stored at 4°C. Current Human Milk Banking Association of North America (HMBANA) Best Practice guidelines recommend that milk be discarded 24 hours after being thawed, but experimental data on the duration of microbiological purity in thawed PDHM are sparse.
Objective: This study evaluates microbiological purity of thawed PDHM during prolonged storage at 4°C.
Methods: A total of 42 independent, randomly selected PDHM samples were thawed at 4°C. As is typical in NICUs, each bottle was opened at 3-hour intervals and 3 mL was withdrawn with a sterile syringe and transferred into a sterile tube. The 3 mL samples removed at 0, 24, 48, 72, and 96 hours, and 9 days were tested for the presence of any microorganisms by a clinical laboratory that routinely screens PDHM for microbes.
Results: No evidence of microbial growth was observed in cultured samples taken at 0 to 9 days after thawing of the milk samples.
Conclusion: There was no evidence of microbes in PDHM as dispensed by HMBANA milk banks when defrosted and stored at 4°C for up to 9 days. Extended storage of PDHM in the NICU could reduce waste of donor milk, thereby increasing availability of human milk to vulnerable neonatal patients.

Keywords: breastfeeding, donor milk, human milk, milk banking, NICU, pasteurization

Well Established
Pasteurized donor human milk (PDHM) from nonprofit Human Milk Banking Association of North America milk banks is widely utilized in North American neonatal intensive care units. After slow thawing, unused PDHM in an aliquot is discarded after 24 hours of refrigerated storage at 4°C.

Newly Expressed
Thawed PDHM stored at 4°C in a refrigerator does not grow bacteria over the course of 9 days with simulated bedside preparation. The utilization of PDHM for greater than 24 hours after thawing would conserve PDHM.

Background
For premature and critically ill infants hospitalized in a neonatal intensive care unit (NICU), human milk provided by their biological mother is the optimum feeding choice, yielding the best possible outcomes. However, when the biological mother is unavailable or unable to provide sufficient milk, pasteurized donor human milk (PDHM) is the best alternative feeding option.¹²

Human Milk Banking Association of North America (HMBANA) milk banks typically provide PDHM to NICUs in bottles normally containing 50 mL or 100 mL (~1.5 or 3 oz). Before PDHM is dispensed, it is pasteurized and then must test negative for the presence of any bacteria by 48-hour culture assays after a representative sample is inoculated on blood agar and incubated at 35°C.³

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Preterm infant feedings may be as small as a few milliters. In the NICU, one 100 mL aliquot of milk could provide feedings for multiple infants for multiple days in the unit. Nurses or feeding preparation technicians in the NICU prepare feedings from the aliquot of PDHBM provided by milk banks using clean technique, either pouring out a portion of thawed milk or by aspirating a volume with a sterile syringe after thorough mixing of the completely thawed PDHBM. Currently, any remaining PDHBM thawed and stored in a refrigerator is discarded within 24 hours of being defrosted and stored at 4°C.

The data guiding storage of thawed PDHBM in the refrigerator is sparse. The current HMBANA Best Practice guidelines recommend that milk be discarded 24 hours after it is thawed and stored in a 4°C refrigerator. However, this current policy was based on expert opinion and formulated at a time when no direct evidence was available and was deliberately conservative. Therefore, the current investigation is to identify if and when bacteria begin to grow in thawed, refrigerated PDHBM when handled under standard NICU conditions. The sampling methods used in this study were designed to mimic common techniques used in the preparation of PDHBM feedings in the NICU.

**Methods**

**Sample Selection**

All donors to the Mothers’ Milk Bank of North Texas provide written consent to the use of their donated milk for research. The use of de-identified human milk samples for research was granted exempt status by the Institutional Review Board of Boston College (14.024.01e), and those performing the microbiologic analyses were blind to the identity of the milk. Each pasteurization batch was pooled from 2 to 5 individual donors prior to pasteurization, had tested negative by bacterial culture post pasteurization, and had been approved for dispensing. One 100 mL PDHBM bottle was randomly selected from each of 42 batches. The samples were thawed in a refrigerator at 4°C. The first hour after the samples were completely thawed was designated hour one of the study.

The feeding preparation simulation protocol in this study was conducted in a milk bank workroom that does not qualify as a clean room. The refrigerator in which samples were stored was opened 27 times per day on average. To simulate removal of “feedings” from each bottle, staff members wore fresh, clean gloves and used a new sterile syringe for removal of each aliquot. The countertop was disinfected between every sample withdrawal to avoid cross-contamination. Each bottle was opened at 3-hour intervals and 3 mL was withdrawn with a sterile syringe and transferred into a sterile tube. The 3 mL samples removed at 0, 24, 48, 72, and 96 hours and 9 days were tested for the presence of any microorganisms by a clinical laboratory that routinely screens PDHBM for microbes.

**Positive Controls**

To control for any fluctuation in technique at the milk bank, hospital laboratory, or transportation between the 2 that may have accounted for negative findings in the experiment, the results of testing milk samples from the routine operation of the bank over the period of the study were collated as a reference group. These were the sentinel samples used by the milk to validate that each pasteurized batch of milk was safe to dispense; they are run for every batch of pasteurized milk by every HMBANA milk bank and were independent of the experimental milks in this study. Because all samples in the study were negative for bacteria at all time points, it was imperative to ascertain that the system for detecting bacteria was functioning at its proper level of sensitivity. The number of positive sentinel bottles for the period of this study was consistent with prior and subsequent rates, indicating that all aspects of the routine bacterial testing were functioning normally. Therefore, differences between the experimental group and the concurrent baseline controls for bacterial contamination could be attributed to an actual lack of bacteria in the milk stored in the refrigerator rather than to a random transient fluctuation in the ability to detect bacteria.

**Quantitative Analysis**

The representative 3 mL PDHBM samples in sterile lab tubes were sent to the medical laboratory that is routinely used to test post-pasteurization microbial purity using the HMBANA Standard Operating Procedure for Culturing Pasteurized Donor Human Milk. A 100 μL aliquot of each PDHBM sample was spread onto a plate of sheep blood agar. After aerobic incubation at 35°C for 48 hours, colony counts were determined with a Quebec Colony Counter (Reichert, Inc, Depew, New York, USA). Results are reported as colony-forming units per 100 μL (CFU/100 μL).

**Statistical Analysis**

The proportion of pasteurized milk samples that displayed microbial growth was compared between those samples of milk stored at 4°C and comparison controls. The controls were those donor milk samples that, after processing and pasteurization, were routinely tested at the same microbiological testing laboratory by the same procedures during the time period of the study. This comparison ascertained that all results were obtained by fully functional assays running concurrently with the identical reagents by the same personnel in the same laboratory and that any differences between groups was attributable to actual lack of bacteria in refrigerated human milk rather than random systematic depression in the ability to detect microbes by the assay. The significance of differences was calculated using Pearson’s chi-square test with Yates’ continuity correction for small sample sizes, with confirmation of results through Fisher’s exact test. All
statistical analysis was conducted using R version 3.1.0 (R Foundation for Statistical Computing). A P value < .05 (95% confidence interval) was used as the threshold of statistical significance.

Results

Each of the 42 samples were randomly selected from batches of pasteurized milk whose sentinel sample had been found to be free of bacteria when tested just subsequent to pasteurization. Consistent with this, at time 0 they all tested negative for the presence of any bacterial growth. Furthermore, all of these 42 samples remained free of detectable bacteria for the duration of the 9-day storage at 4°C. The protocol for periodic removal of 3 mL aliquots, as if for feeding, simulated typical NICU feeding protocols for PDHM, with the exception that the storage was extended from 1 day to 4. When these were tested by the standard protocol for testing for potential microbial contamination, all 210 cultures collected over 96 hours were negative for any microbial growth. Surprisingly, even when the remaining milk from the 42 samples was cultured after 9 days of storage at 4°C, they all still remained negative for microbial growth. During the time period of the study, an additional 117 pasteurized donor milk samples were tested for microbial growth as part of routine milk screening procedures; 5 of these samples tested positive for the presence of Bacillus growth. The NICU-simulated PDHM feeding samples collected at 0 to 4 days after pasteurization and stored in the refrigerator exhibited a statistically significant absence of microbial growth relative to the overall rate of positive microbial growth tests in pasteurized milk samples processed in the same batches using the same reagents, and personnel in the same facility, during the time period of the study (0/210 vs 5/117; P = .01; χ² test) or P = .005 (Fisher’s exact test). Thus, the difference between groups was attributable to treatment rather than random systematic depression in the ability to detect microbes by the assay, even when excluding data from the originally unplanned 9-day time point to enhance statistical rigor.

Every one of the 42 samples of PDHM tested free of bacteria at the outset, and when thawed slowly and stored at 4°C with “feeding” aliquots removed every 3 hours with a sterile syringe, mimicking typical use in the NICU, every milk bottle remained free of bacteria not only for 96 hours (4 days), but even for 9 days.

Discussion

Expressed human milk often contains bacteria, frequently resembling the skin flora. In addition, maternal infections, especially those of the breast, can result in microorganisms entering the milk. Expressed human milk may also be contaminated with environmental pathogens. In a study of over 1000 samples of either pooled or individual-donor raw milk, both non-pathogenic and pathogenic bacteria were found in freshly expressed and stored breast milk. The researchers identified group B Streptococcus, Staphylococcus aureus, coagulase-negative Staphylococcus, alpha-Streptococcus, Enterococcus, and Bacillus. In human milk purchased through private Internet sites, frozen milk shipped directly to the researcher contained aerobic bacteria, coliforms, Staphylococcus species, and Streptococcus species. Specifically identified organisms included Klebsiella, Escherichia coli, Staphylococcus epidermidis, Viridans streptococci, Staphylococcus aureus, and Salmonella, along with small numbers of additional species.

In addition to potential pathogens, human milk also contains a moderate level of benign bacteria. These are often viewed as normal and beneficial in the development of a healthy immune system. Moreover, human milk innately contains systems that suppress bacteria. Human milk has maximal bactericidal activity when it is freshly expressed, while freezing or refrigeration of human milk reduces bactericidal activity in a time-dependent manner. Freshly pumped human milk changes minimally in overall microbiological integrity (bacterial growth, cell count, and components) during storage at room temperature for several hours or with refrigerator storage over several days. Slutzah et al found that fresh (never frozen) human milk stored in the NICU and stored at 4°C with over 70 refrigerator openings was not compromised with regard to microbiological purity over a time course of 96 hours.

Our study had an average of 27 door openings per day, or ~ 100 openings over 96 hours, which slightly exceeds the estimate of typical NICU refrigerator traffic of Slutzah et al., and thus of exposure to bacteria through this route. A major target population of PDHM is premature infants, whose mothers may initially not be able to produce adequate milk for the immediate needs of the infant and whose high risk allows them to display the greatest benefit from a donor milk supply. The mucosal immune system of the premature infant gut is immature and not fully functional, which creates concern regarding even the low levels of bacteria present in many human milk samples, despite the potent bactericidal activity of freshly expressed human milk. Thus, donor milk is pasteurized to eliminate all detectable bacteria. This heat treatment also reduces the bactericidal properties of breast milk. Although donor milk may not provide the same level of anti-microbial protection as milk provided by the mother directly, there are no bacteria in pasteurized PDHM. Therefore, the data on fresh (never frozen) milk microbiological purity during storage at 4°C may not be relevant to storage of PDHM under the same conditions.

There had been no evidence on which to base storage recommendations for PDHM that has been frozen and thawed. The recommended limit of refrigerator storage time of thawed PDHM was based on recommendations at that time for unpasteurized, raw milk with the rationale that the bactericidal activity of previously frozen milk is compromised, potentially allowing rapid growth of microorganisms. Thus,
expert opinion of both HMBANA and the Academy of Nutrition and Dietetics was that unpasteurized frozen and thawed (without warming), breast milk be stored in the refrigerator no longer than 24 hours.

The microbiologic assay to assess PDHM for contamination involves inoculation of a blood agar plate with an aliquot of pasteurized milk, incubation at 35°C for 48 hours, and only if no evidence of microbial growth occurs can it be dispensed. Therefore, if handled similarly to the testing inoculum, PDHM should respond similarly, and remain sterile for at least 48 hours, providing the rationale for testing thawed PDHM stored for 48 hours in the refrigerator. Reevaluation of these guidelines is also warranted by preliminary data from other laboratories, which indicate that PDHM remains sterile for several days when thawed in the refrigerator and handled carefully, and PDHM remains free of bacteria when stored refrigerated for 1 week. However, these studies did not include a simulation of the type of frequent opening of containers and the removal of contents that occurs in a NICU when PDHM is accessed for multiple feeds, limiting the applicability of these data to the evidence base on limitations of storage of PDHM in the NICU. At the most technical end of the spectrum, some NICU feedings are prepared by technicians in laboratory apparel in specially constructed clean feeding preparation laboratories with separate ventilation systems, high-frequency particle air-forced filtration systems, or under laminar flow hoods. At the lowest technical end of the spectrum, some NICU feedings are prepared by nurses or technicians at the bedside without special consideration to attire or to location. Our study simulates PDHM handling methods representative of the less technical feeding preparation methods used in NICUs to account for environmental factors and human error that might contribute to microbial contamination.

PDHM is a valuable resource whose supply is limited by a variety of biological, social, and economic factors. The demand from NICUs for PDHM from HMBANA milk banks has increased over the past decade.

The current standard protocol is that 24 hours after PDHM is thawed, any remaining is discarded. This protocol was formulated before evidence for the persistence of microbiological purity in PDHM for 48 hours under conditions of repeated withdrawal that emulates NICU practice was available. To our surprise, PDHM may indeed be free from microbes for even longer, 96 hours under the conditions of NICU storage and for even 9 days of refrigeration. Were the current protocol replaced with one that reflects these new data, the prolonged storage life in the refrigerator would allow NICUs to extend the utility of PDHM, reducing health care expenditures, but most importantly, conserving PDHM to serve more infants who are at high risk for morbidity and mortality.

The unexpected total absence of bacteria in PDHM after defrosting and being stored at 4°C for 9 days elicited several questions. First, was the bacterial assay accurate for the period of time that the study was performed? This was addressed by reviewing the records of other milk assays sent to the same laboratory unrelated to this study over the same period of time and finding that they had the normal amount of bacteria as would be expected. Thus, the assay was functionally normal and detected bacteria when they were present. Second, the question was asked whether the 210 bacteria free milk samples in this study could have occurred by chance. This was addressed through statistical comparison of the bacterial incidence of this study (0 positive samples of 210 total) with the measured bacterial incidence in an unrelated population of PDHM samples (5 positive samples of 117) measured in the same laboratory over the same period of time. Both chi-square analysis and Fisher's exact ratios concurred that this difference had less than 1 chance in 100 of occurring by chance, and hence the conclusion that there is a total lack of bacterial growth in refrigerated defrosted PDHM samples should be accepted as valid. Finally, the question of a biological mechanism to account for the bacterial growth in PDHM would reinforce the credence of the study results. Human milk is unique in its high content of antibacterial bioactive components, many, but not all, of which survive pasteurization. Thus, the observation that raw human milk is strongly antibacterial gives precedent to the strong antibacterial activity of PDHM that could underlie the lack of bacterial growth observed in this study. In aggregate, these data strongly support reevaluation of the policy to discard PDHM within 24 hours of its being defrosted and stored at 4°C.

Limitations

This study searched for bacterial growth as a function of the duration of PDHM storage at 4°C and found none in 42 samples stored for 96 hours under conditions that simulated storage in the NICU and for 9 days of refrigeration. Although our data strongly weighed against a 24-hour expiration for thawed PDHM, they cannot indicate a definitive time point at which bacterial growth may be expected or an actual expiration date for storage of refrigerated PDHM or freshly expressed milk. Moreover, there are criteria other than bacterial growth that may limit the duration of PDHM utility, hence 9-day-old milk may not be suitable for premature infants for reasons independent of microbiological purity. Studies on any changes of other parameters in human milk during prolonged storage may be warranted.

Conclusion

The utilization of PDHM for premature and vulnerable infants can be lifesaving, providing unique nutrients and immune modulators for this vulnerable population. Securing adequate PDHM requires coordinated efforts of health care professionals, researchers, and donors to advocate for the lifesaving potential of donating breast milk. Furthermore, HMBANA milk bank standards of practice and recipien
NICU protocols should be periodically reviewed and revised as new data are added to the evidence base, to maximize the supply of PDIM and use it efficiently and effectively. The results of this study demonstrate that HMBANA milk bank PDIM shows no evidence of microbiologic contamination well beyond 48 hours after cold thawing. Extending the acceptable storage time of PDIM to 48 hours in the NICU would extend the availability of this limited resource to serve a greater population of infants.

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