Effects Of Storage Process On The Bacterial Growth-Inhibiting Activity Of Expressed Human Breast Milk On Common Neonatal Pathogens, *Staphylococcus aureus*, *Escherichia coli* And *Klebsiella pneumoniae*

First Prize Winner of the Research Paper Contest at the 18th PIDSP Annual Convention

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KEYWORDS
Breast milk, breast milk storage, *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae*

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ABSTRACT

Storage of human breast milk is unavoidable especially among working mothers who need to report back to work soon after delivery. The aim of this study was to describe the effects of storage process on the bacterial growth-inhibiting activity of expressed human breast milk on common neonatal pathogens.

Methods: This was an experimental study performed at the Ospital ng Makati on the breast milk of mothers who were without infection prior to delivery, did not take any medications, and spontaneously delivered their babies full term. Fifteen mothers expressed breast milk within the first seven days up to one month, postpartum. Each milk sample was stored and tested with the following time series: immediately after expression; thawed after being frozen for 24; and thawed after being frozen for 72 hours. Each of the broth medium containing Staphylococcus *aureus, Escherichia coli* or *Klebsiella pneumonia* was added to 1 ml of expressed breast milk. Tenfold dilutions were made. Each dilution was plated at zero and four hours and colony-forming units (CFU) were counted after 24 hours incubation. Same procedure was performed on a negative control.

Results: The number of CFU for the three tested organisms (had lower nominal counts with the breast milk solution when compared to control solutions both at zero-hour and four-hour test samples. Breast milk exhibited bacteriostatic properties against most pathogens except for *Escherichia coli* after being frozen for 24 hours. For the breast milk that was frozen for 72 hours, most tests did not show significant lower counts when compared to control solutions, except still for *Staphylococcus aureus* and *Escherichia coli* both at 0 hour.

Conclusion: Breast milk has bacterial inhibiting property against common neonatal pathogens. Bacteriostatic property of breast milk started to diminish after being frozen for 72 hours, but still exerted its inhibiting property.

INTRODUCTION

Human breast milk provides infants with defensive factors against many illnesses.[1] According to numerous reports, infants who are breastfed appear to be less susceptible to certain infections than bottle-fed infants. There are many agents found in human milk that are important in imparting protection to infants; these agents include lysozymes, lactoperoxidase, lactoferrin, interferon, complement components, leukocytes and immunoglobulins. These protective properties are particularly beneficial during the first few months of an infant's life, when an effective immune response against foreign organisms cannot be mounted.[2]

Storage of human breast milk for limited periods of time is unavoidable in neonatal units and in many households, especially among working mothers who need to report back to work soon after delivery. Many mothers who are certain of the importance of prolonged exclusive or complementary breastfeeding, often express and store human milk for use during the period of separation.[3]

OBJECTIVES

The aim of this study was to describe the effect of storage processes on the bacterial growth-inhibiting activity of expressed human breast milk on common neonatal pathogens, *Staphylococcus aureus, Escherichia coli and Klebsiella pneumoniae.*

MATERIALS AND METHODS

Study Design

This was an experimental study which was conducted in a tertiary government hospital on November 2010.

Procedure

Breast milk was collected from mothers in the neonatal ward, who were clinically healthy, thus, with no evidence of infection prior to delivery, had no chronic illnesses, were free from intake of any medications for at least one week, except multivitamins and iron supplements, and had spontaneously delivered full term babies. Mothers who were only seen within one week postpartum were included in the study.

An informed consent was obtained from these mothers and baseline data on the age, parity, gender of the baby, maturity index, birth weight and postpartum day were recorded in a data collection form. The researcher assisted the mothers on the proper use of the manual breast pump for expression. Prior to the collection of the milk sample, the mothers were instructed to take a bath in the morning, to practice proper hand washing with soap and water for 15 minutes and to wear sterile gloves. Using a manual breast pump, 15 mothers expressed 5-to-10 ml of breast milk within the first seven days to one month postpartum. The collected samples of breast milk were placed in sterile specimen bottles.

Gram stain was performed on each milk sample to rule out the presence of contamination. Samples that yielded any organism on gram stain were not included for further inhibitory testing. Every milk sample of a mother was divided into three aliquots.

Fifteen milk samples were tested immediately. The other 15 samples were stored in a freezer set at -8°C for 24 hours, while the remaining 15 samples were stored in a freezer for 72 hours. The samples that were frozen were thawed at room temperature before testing.

Three common pathogens in the nursery were obtained from the bacteriology section with their corresponding media namely: *Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae*. Each bacteria was collected from the streaked Petri dish which was diluted in 1 mL of tyrpticase soy broth and turbidity set at 0.5 McFarland standard. Using a calibrated pipette, 0.02 mL from each of the broth medium was added to 1 mL of expressed breast milk to make a concentration of 2×10^5 cfu per mL. Sterile water was used as a negative control and the same procedures were performed as breast milk.

Tenfold dilutions were made. Using a calibrated pipette, 0.02 mL from each of the broth medium with breast milk and the negative control were plated to a plate count agar. Plates were incubated for 24 hours at 37°C.

The milk samples and the negative control with 0.02 mL of bacteria were made to stand at room temperature for four hours. After four hours, ten-fold dilutions were made and plated to a plate count agar.

The same procedure was repeated after the breast milk was frozen at 24 hours and 72 hours. Bactericidal and bacteriostatic activities were analyzed according to the number of folds of decrease from the initial inoculum.

Definition of Terms

Bactericidal – if the four-hour colony count reveals a fourfold or greater decline from the initial inoculum.

Bacteriostatic – if the four-hour colony count reveals less than a fourfold decline

Non-Inhibitory – if the colony counts at four hours demonstrated a greater fourfold increase than in the inoculum

Analysis of Data

Data was encoded and tallied in SPSS version 14 for windows. Descriptive statistics was generated for all variables, while nominal data frequencies and percentages were computed. For numerical data, mean ±SD with 95% confidence intervals, the median and the range were generated. The following statistics was done to compare the different variables under study: Paired *t*-test for comparative analysis of mean CFU from 0 hour to 4 hours; and 2 sample *t*-tests for the comparative analysis between test and control samples. Significant P-value was set at <5%.

RESULTS

Fifteen samples of expressed breast milk were collected from 15 mothers. Prior to inhibitory tests, gram staining proved that all milk samples were free of bacterial contamination. Demographic profiles of mothers and babies were tallied and are shown **in Table 1.**

Table 1. Demographic Profiles of Mothers and Babies

	Descriptive Statistics
Age	Mean = 28.53 (95% CI = 25.75 – 31.32) Range = (19-38 yrs)
Gravida	Mean = 3.47 (95% CI = 1.97 – 4.96) Median = 2.0 Range = 1-11
Parity	Mean = 3.40 (95% CI = 2.01 – 4.79) Median = 2.0 Range = 1-10
Gender of baby Male Female	 9 (60%) 6 (40%)
Maturity(weeks AOG)	Mean = 37.93 (95% CI = 37.40 – 38.47)
Birth weight (grams)	Mean = 3.04 (95% CI = 2.86 – 3.21)
Days postpartum	Mean = 5.33 (95% CI = 4.59 – 6.08)

The age range of mothers who were enrolled in this experimental study was 19-to-38 years old with mean age of 28.5 years and mostly multiparous. Majority of the babies were male, full term (mean gestational age= 38 weeks), and weighed within the range of 2.5 kilograms to 3.9 kilograms. Range of postpartum days was noted to be within three-to-seven days.

On the first aliquot plated immediately after expression, the CFU for all breast milk samples initially and after four hours remained significantly lower than control.

The comparison in the mean differences of CFUs from zero-to-four hours between test and control samples did not show any significance for all test organisms.

Table 3 shows the 2nd aliquot which was frozen for 24 hours. Most tests still exhibited

significantly lower CFU against control but the test plate with *Staphylococcus aureus* at four hours was not significantly lower.

Table 2. Mean Bacterial Colony Counts Mean difference at 0 and 4 hours of breast milk plated immediately after expression against control.

	No. of Hours	BREAST MILK (CFUx10^2) N=15	CONTROL (CFUx10^2) N=15	Mean Difference	P value
S. aureus	0	126. 13	451.87	325.73	0.0001 (S)
	4	571.20	892.93	321.73	0.0001 (S)
	Mean Differ	445.1	441.1	- 4.0	0.954 (NS)
E. coli	0	153.67	336.33	182.67	0.0001 (S)
	4	474.93	711.27	236.33	0.0001 (S)
	Mean differ	321.3	374.9	53.67	0.137 (NS)
K. pneumoniae	0	180.13	429.07	248.93	0.0001 (S)
	4	528.13	779.00	250.87	0.0001 (S)
	Mean differ	348.0	349.9	1.93	0.976 (NS)

Table 3. Mean Bacterial Colony Counts at 0 and 4 hours of breast milk plated after being frozen for 24 hours against control.

	No. of Hours	BREAST MILK (CFUx10^2) N=15	CONTROL (CFUx10^2) N=15	Mean Difference	P value
S. aureus	0	114.73	337.60	222.87	0.0001 (S)
	4	516.33	667.93	151.60	0.057 (NS)
	Mean differ	401.6	330.3	- 71.27	0.325 (NS)
E. coli	0	117.00	224.13	107.13	0.0001 (S)
	4	654.27	772.00	117.73	0.038 (S)
	Mean differ	537.3	547.9	10.60	0.863 (NS)
K. pneumoniae	0	146.87	286.40	139.53	0.0001 (S)
	4	498.73	677.73	179.00	0.018 (S)
	Mean differ	351.9	391.3	39.47	0.543 (NS)

The comparison in the mean differences of CFUs from zero-to-four hours between test and control samples after 24 hours of freezing did not show any significance for all test organisms.

The breast milk frozen for 72 hours was not significantly lower against control, except for *Staphylococcus aureus* and *Escherichia coli* both at zero hour.

Table 4. Mean Bacterial Colony Counts at 0 and 4 hours of breast milk plated after being frozen for 72 hours against control

	No. of Hours	BREAST MILK (CFUx10^2) N=15	CONTROL (CFUx10^2) N=15	Mean Difference	P value
S. aureus	0	575.13	713.60	138.47	0.0001 (S)
	4	1235.93	1305.13	69.20	0.570 (NS)
	Mean differ	660.8	591.5	- 69.27	0.578 (NS)
E. coli	0	545.93	662.07	116.13	0.024 (S)
	4	1261.53	1411.93	150.40	0.136 (NS)
	Mean differ	715.6	749.9	34.27	0.755 (NS)
K. pneumoniae	0	697.80	802.00	104.20	0.309 (NS)
	4	1241.47	1359.67	118.20	0.327 (NS)
	Mean differ	543.7	557.7	14.00	0.903 (NS)

Table 5. Mean degree of change at 0 – 4 hours in Colony Forming Units in breast milk immediately after expression and after being frozen for 24 and 72 hours

	Immediately after expression	Thawed after frozen for 24 hours	Thawed after frozen for 72 hours
S. aureus	3.62 (Bacteriostatic)	3.43 (Bacteriostatic)	1.17 (Bacteriostatic)
Escherichia coli	2.26 (Bacteriostatic)	5.46 (Non-inhibitory)	1.43 (Bacteriostatic)
Klebsiella pneumoniae	2.04 (Bacteriostatic)	2.54 (Bacteriostatic)	0.91 (Bacteriostatic)

The comparison in the mean differences of CFUs from 0 to 4 hours between test and control samples after 72 hours of freezing did not show any significance for all test organisms.

Breast milk exhibited bacteriostatic properties against most pathogens except for *Escherichia coli* after being frozen for 24 hours to which it was non-inhibitory (5.46 x increase in CFU). It is also noted that, in general, the bacteriostatic activity of breast milk for all three bacteria rapidly decreased with 72 hours of freezing.

DISCUSSION

It has been established that human milk is uniquely superior for infant feeding, in addition to its unique immunological, growth and developmental benefits.[7] When it is not possible to breastfeed an infant in the postnatal period, expressed breast milk, fresh or frozen, may provide both nutritional and immunological benefits.[8, 9]

This study showed that breast milk expressed from mothers has bacteriostatic properties against *Staphylococcus aureus, Escherichia coli*, and *Klebsiella pneumoniae*. However, the significant difference between test and control samples differed between the three aliquots as experimented on a time series (immediately after expression, and those frozen after 24 and 72 hours). These data correspond with the previous local studies[11, 12] done on breast milk.

Results of a study performed by Ogundele[3], revealed that bactericidal activities of refrigerated human milk diminishes rapidly. The ability of milk fat globule membrane to adhere to suspended bacteria was gradually lost in frozen milk samples, while it was greatly enhanced during the first few days in refrigerated samples, before declining sharply.[3] Human milk possesses bactericidal activity that remains stable during the first 48 hours of refrigerated storage, but it is significantly reduced beyond 72 hours.[4] The study of Igumbor[5] showed that although freezing temperature (0-4°C) seemed safest for breast milk storage, short-term storage in a freezer is not recommended due to the hazards of the thawing process. This is due to the growth of organisms detected after storage which were both pathogenic and non-pathogenic although with low counts.[5]

Many antimicrobial components have proven to be present in breast milk and one of these very important components is Lactoferrin. One of Lactoferrin's important antibacterial properties is related to its high-binding affinity to iron which can deprive the bacteria of this essential nutrient for their growth.[12]

It was noted in this study that the bacterial-inhibiting property of breast milk started to diminish around 72 hours. A study done by Ezz, El Din, et al. showed that breast milk samples stored in the refrigerator showed significant decrease in Vitamins A, C and E.[6] The authors verified that the mechanism of Vitamin C loss during storage at the freezer is due to lactoperoxidase activity. There were small reductions in the fat, protein and lactose content of breast milk after refrigeration for 24 hours but these were not statistically significant. All values after storage are still within the international reference ranges.

The results match with those of Friend, et al., who reported that frozen storage tended to increase lipase activity. Milk became progressively more acidic during storage.[3] The ability of milk fat globule membrane to adhere to suspended bacteria will be gradually lost in frozen milk samples. Lopez, et al., reported that the inhibitory property of breast milk may persist up to 48 hours but significantly decrease after refrigeration for 72 hours.[15]

CONCLUSIONS

This study supports that breast milk has bacterial inhibiting property among common neonatal pathogens. The number of CFU for the three tested organisms (*Staphylococcus aureus, Escherichia coli, and Klebsiella pneumoniae*) had lower nominal counts with the breast milk solution when compared to control solutions both at 0 hour and 4 hour test samples. Breast milk exhibited bacteriostatic properties against most pathogens except for *Escherichia coli* after being frozen for 24 to 72 hours.

The bacteriostatic property of breast milk started to diminish around 72 hours, but still exerted its inhibiting property. The comparison for the degree of mean differences between test and control samples had all been insignificant for all aliquots and test organisms.

RECOMMENDATIONS

The researcher recommends that further studies be pursued in order to determine the specific antimicrobial components of breast milk that were altered during storage processes, which in turn compromises its inhibitory property.

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