Bacteriological Safety of Human Milk Storage

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ABSTRACT

Human milk is the optimal food for infants because it fulfills their nutritional requirements and strengthens the bond between mother and child. However, because mothers have increasing difficulty continuing to breastfeed because of the demands of modern life, expressed milk is often stored for later use. This study examined the effects of different storage methods on bacterial counts and species in expressed human milk. Samples of human milk from 20 healthy lactating mothers were stored frozen for 4 weeks, refrigerated for 24 or 48 hours, or left at room temperature for 3 or 6 hours. The samples were then analyzed for bacterial counts and species and compared with freshly collected samples. The bacterial counts of frozen and refrigerated samples of human milk were significantly lower than those in fresh samples, whereas those of samples left at room temperature were significantly higher. However, bacterial counts were not zero, even after refrigeration or freezing, which suggests that bacterial counts depend on the number of bacteria present when the milk was collected. The bacterial species present did not change, regardless of the storage method, which suggests species composition also depends on the species present at the time of collection.

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Key words : human milk, bacteria, bacterial species, storage, breastfeeding, milk banking

Introduction

Human milk is the optimal food for neonates because it fulfills their nutritional requirements and strengthens the bond between mother and child[1-3]. Furthermore, as neonatal immune function is poor, particularly with regard to infection control and antibody production, human milk can be regarded as a complement to the neonatal immune system. Human milk prevents infection through the binding of its immunoglobulins to specific pathogens, such as viruses and bacteria.

Human milk contains particularly high levels of immunoglobulins, which are most often secretory immunoglobulin A[1], 2 to 4 days after delivery. The secretory immunoglobulin A in breast milk combines with many bacteria, toxins, viruses, and parasites, thereby acting to control infection[4]. Furthermore, human milk possesses a variety of other immunological components, such as lactoferrin, lactoperoxidase, lysozyme, and oligosaccharides.

The protective effects of breastfeeding have also been reported in a number of conditions, including diarrhea, meningitis, sepsis, respiratory diseases, otitis media, and necrotizing enterocolitis[1,5]. Therefore, the protection against infection provided by breastfeeding is important.

However, despite the benefits of human milk, some women are unable to breastfeed because of flat or inverted nipples, and breastfeeding may not be possible after admission to the neonatal intensive care unit.   Therefore, expressed milk is increasingly being stored, most often after being frozen, for later use.

In 2010, the Academy of Breastfeeding Medicine published a protocol to make expressed milk safe for consumption by neonates[6], and various bacteriological studies have been performed[7-10].   However, because of procedural differences, these studies cannot readily be compared, and the safety of expressed milk remains uncertain.   In Japanese maternity wards, awareness of the bacteriological safety of expressed milk remains poor, and the storage periods vary from facility to facility.

The present study examined the effects of different storage methods on bacterial counts and species in human milk obtained from 20 healthy lactating mothers.

Materials and Methods

1. Samples

The use of human milk samples in the present study was approved by the human milk outpatient clinic of The Jikei University Daisan Hospital, in Tokyo, from November 2010 through January 2011.   The contents of human milk differ depending on the birth term, i.e., full-term or preterm[11-13], and on the lactation period[2], i.e., less than 2 months after birth or more than 2 months after birth.   Therefore, this study enrolled puerperal women with normal, full-term pregnancies, at 2 to 6 weeks after birth, and with healthy human-fed neonates.   Mothers with mastitis, fever (38.5°C or more), or other infections were excluded.   Twenty-four puerperal women were seen in the human milk outpatient clinic, but 4 women who did not meet the criteria were excluded.   Therefore, samples of human milk were provided by 20 healthy puerperal women.

Each participant was briefed on the study goals, risks, and inclusion and exclusion criteria and gave written, informed consent before participation.   They also completed a comprehensive questionnaire.   Two formula milks (Sukoyaka : Bean stalk Snow Co., Ltd. ; Hohoemi : Meiji Co., Ltd.) were used as controls in this study.

2. Data collection and stored

All samples of human milk were obtained by midwives who wore sterilized gloves after washing their hands with soap and water.   Nipples and the surrounding tissue were cleaned with sterilized water and sterile cotton (no antiseptic) to prevent contamination.   Ten-millimeter samples of human milk were collected in sterile tubes and kept at 4°C until they were delivered to the laboratory approximately 5 minutes later.   Formula milks were made with once-boiled hot water cooled to 70°C, in accordance with the manufacturers' instructions.

The samples were divided into 3 aliquots, which were 1) stored in a freezer at -20°C for 4 weeks, thawed with running water and stored in a refrigerator (5°C) for 24 hours ; 2) stored in a refrigerator (5°C) for 24 or 48 hours ; or 3) kept at room temperature (approximately 20°C) for 3 or 6 hours.   And the control group assumed the sample just after the collection.   Of the 20 samples of frozen human milk, only 13 could be analyzed because 7 samples were in a poor state of preservation owing to freezer malfunction.   The samples were then subjected to bacteriological analysis.

Table 1 shows the characteristics of the women enrolled in the study, including age, parity, gestational age of the neonate at birth, time of recruitment after birth, and feeding method.   All women had given birth at 37 to 41

Table 1.   Participants by age of mother, birth history, gestational week, recruitment day after birth, and method of feeding

	$n=20$
Maternal age	
mean (SD)	32.8±4.5
min, max	20, 40
Birth history	
primipara	14
multipara	6
Gestational week	
mean (SD)	39±1.4
min, max	37, 41
Recruitment day after birth	
mean (SD)	22.7±6.8
min, max	15, 35
Method of feeding	
breastfeeding only	13
mixed feeding	7
bottle feeding only	0

weeks' gestation and had decided to breastfeed.

3. Data Analysis

All samples were cultured on NHM-II blood agar medium (Kyokuto Pharmaceutical, Tokyo, Japan) and were aerobically incubated at 35℃ for 24 hours.   Microbiological data, recorded in terms of colony-forming units (CFU) per milliliter, were transformed to logarithmic values before statistical analysis.   Values for bacterial counts are given as means ± SD, and bacterial species were identified by a clinical laboratory technician.   Bacterial counts were compared among fresh, frozen, refrigerated, and room-temperature samples.   The comparison of the samples went in fresh sample and 6 pairs of each storage method (fresh samples and 1. freezer for 4 weeks, 2. refrigerator 24 hours after the freezing for 4 weeks, 3. refrigerator for 24 hours, 4. refrigerator for 48 hours, 5. room temperature for 3 hours, 6. room temperature for 6 hours) to clarify what kind of changes a number of bacteria included by a storage method.   We examined even 3 pairs (freezer : for 4 weeks VS and then refrigerator for 24 hours, refrigerator : for 24 hours VS 48 hours, room temperature : for 3 hours VS 6 hours) of the time lag on each storage condition to clarify a change by the storage time at the same time.   After the Shapiro-Wilk test had been performed, the Wilcoxon signed-rank test was used to compare bacterial counts in all samples.   Statistical significance was examined with the Holm adjustment to solve the problem of multiple comparisons.   Analyses were performed with a significance level of $p < 0.05$, and collected data were analyzed with the statistical software package SPSS (version 16).

4. Ethical considerations

The Committees for the Protection of Human Subjects at The Jikei University Daisan Hospital and The Jikei University approved all study protocols.   This research was supported by the Research Fund of the School of Nursing, The Jikei University, project number 22-056.   The authors had no conflicts of interest.

RESULTS

1. Bacterial counts and species in fresh, frozen, refrigerated, and room-temperature samples of formula milk

No bacterial growth was observed in most samples of formula milk, although one sample kept at room temperature for 3 hours showed a single colony (*Bacillus* sp.). However, formula milk samples did not show additional bacterial counts after 6 hours at room temperature.

2. Bacterial counts in fresh, frozen, refrigerated, and room-temperature samples of human milk

Of the 20 samples, inoculation of the samples onto NHM-II blood agar plates led to bacterial growth.

Bacterial counts in samples of fresh human milk were 3.9×10^2 to 3.2×10^4 CFU/mL (mean, 7.2×10^3 CFU/mL). Bacterial counts did not exceed 5×10^4 CFU/mL in any sample but did vary substantially.   In samples of fresh human milk, bacterial counts were 10^3 to 10^4 CFU/mL (10^2 CFU/mL in 3 samples, 10^3 CFU/mL in 10 samples, and 10^4 CFU/mL in 7 samples).   The counts in samples of frozen human milk were 3.2×10^2 to 1.5×10^4 CFU/mL (mean, 5.3×10^3 CFU/mL).   The counts in samples of thawed frozen human milk were 1.8×10^2 to 1.5×10^4 CFU/mL (mean, 4.2×10^3 CFU/mL).   The counts in samples of refrigerated human milk were 1.5×10^2 to 1.3×10^4 CFU/mL (mean, 5.0×10^3) at 24 hours and 5.0×10 to 1.0×10^4 CFU/mL (mean, 2.8×10^3 CFU/mL) at 48 hours.   In samples kept at room temperature for 3 and 6 hours, counts were 4.8×10^2 to 3.5×10^4 CFU/mL (mean, 9.0×10^3 CFU/mL) and 4.6×10^2 to 3.5×10^4 CFU/mL (mean, 1.0×10^4 CFU/mL), respectively. The samples frozen for 4 weeks, thawed, and refrigerated for 24 hours showed significantly fewer viable bacteria than did fresh samples.   The samples refrigerated for 48 hours showed significantly fewer viable bacteria than did samples refrigerated for 24 hours.   Samples kept at room temperature for 3 hours showed significantly more viable bacteria than did fresh samples (Fig. 1).

The bacterial counts in frozen samples (freezer for 4 weeks and refrigerator for 24 hours after freezer for 4 weeks) of human milk decreased from 10^3 to 10^2 CFU/mL or from 10^4 to 10^3 CFU/mL.

3. Bacterial species in fresh, frozen, refrigerated, and room-temperature samples of human milk

Each sample of fresh human milk contained 1 to 5 bacterial species.   Coagulase-negative staphylococci (CNS) were most prevalent and were detected in all samples.   In descending order of prevalence, bacterial species were classified as α-*Streptococcus* (10 samples), *Staphylococcus aure-*
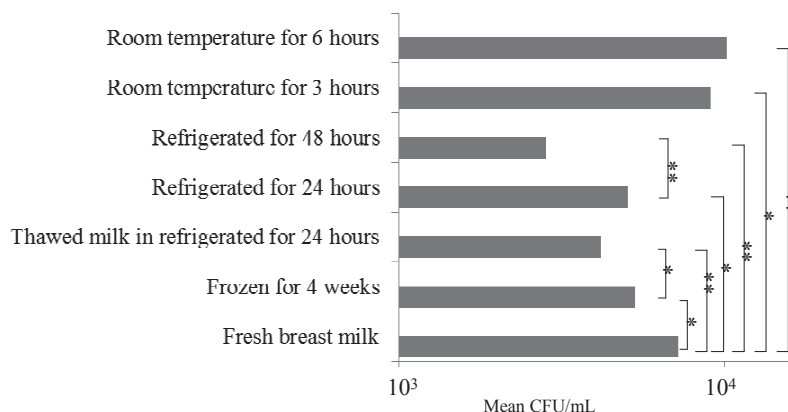
Fig. 1. Mean bacterial counts (CFU/mL) in fresh, frozen, refrigerated, and room-temperature samples of human milk. Multiple comparisons of samples stored at different temperatures were performed. * : ($p<0.05$), ** : ($p<0.01$).

us, *Streptococcus* species (3 samples), *Escherichia coli* (2 samples), *Klebsiella pneumoniae*, and Gram-positive rods (1 sample).

One to 4 bacterial species were present in each sample of frozen human milk. The most prevalent species was CNS, which were detected in all samples. In descending order of prevalence, other bacterial species were α-*Streptococcus* (5 samples), *E. coli* (3 samples), *Baccillus* sp., *S. aureus* (2 samples), and *K. pneumoniae* (1 sample). One to 2 bacterial species were present in each sample of human milk frozen, thawed, and refrigerated for 24 hours. CNS were detected in all samples. In descending order of prevalence, bacterial species were classified as α-*Streptococcus* (3 samples), *Baccillus* sp., *S. aureus*, and *K. pneumoniae* (1 sample).

Each sample of human milk refrigerated for 24 hours contained 1 to 4 bacterial species, with CNS being the most prevalent. In descending order of prevalence, bacterial species were classified as α-*Streptococcus* (5 samples), *S. aureus*, *E. coli* (3 samples), *Streptococcus* sp. (2 samples), *K. pneumoniae*, Gram-positive rods, and *Baccillus* sp. (1 sample).

One to 4 bacterial species were present in each sample of human milk refrigerated for 48 hours. The number of bacterial species in the 48-hour sample was lower than that in the 24-hour sample in the milk from 1 subject. However-er, the number of bacterial species did not differ between 24 and 48 hours of refrigeration in the milk from the remaining 12 subjects.

The number of bacterial species in room-temperature samples did not differ from that in fresh samples in the milk

from 11 subjects. In the milk from the remaining 2 subjects, *E. coli* and *Baccillus* sp. were detected after 3 hours at room temperature. In all detected *E. coli*, pathogenicity was ruled out by the confirmation of the O antigen.

DISCUSSION

The present study has shown that human milk refrigerated for 48 hours have lower bacterial counts than does fresh, frozen, or room-temperature milk. The lower bacterial counts in refrigerated milk is probably related to the fact that among the many immune factors present in human milk, macrophages and neutrophils decrease at temperatures of 0°C to −4°C, and lymphocytes decrease at −20°C[14]. Human milk has various immune components[5], and the results of the present study confirm that bacteria do not proliferate greatly in human milk at room temperature or under refrigeration, as these components function effectively. At the same time, bacterial counts decreased significantly under both refrigeration and freezing. However, bacterial counts varied greatly between the samples of human milk in the present study, ranging from 3.9×10^2 to 3.2×10^4 CFU/mL at the time of expression, a 100-fold difference. In other words, the rate of decline in bacterial counts due to storage method likely did not exceed individual differences between subjects.

In Japan, the "Ministerial Ordinance Concerning the Ingredient Standards for Milk and Dairy Products" issued by the Ministry of Health, Labour and Welfare[15] calls for negative coliform bacteria and fewer than 5×10^4 CFU/mL of viable bacteria in formula milk. If we were to apply this

standard to human milk, bacterial counts after 6 hours at room temperature did not exceed this maximum value, although the counts will vary depending on temperature and environment.   The average temperature during the autumn–winter period, when this study was performed, in the Kanto region of Japan is 10°C, with room temperature being around 25°C.   Thus, bacterial counts may exceed 5.0×10^4 CFU/mL in areas with high room temperature during the summer, and when more than 1×10^4 CFU/mL bacteria are mixed in human milk when it was expressed.   In these cases, administering stored milk to neonates may need to be prohibited.

In the present study numerous bacterial strains were detected in expressed human milk.   First, CNS were detected in all samples.   A possible reason for this finding is that among the indigenous bacteria, CNS show particularly strong viability at optimal conditions, and nutrient–rich human milk is a favorable environment.   Unlike CNS, however, the indigenous bacterium α–*Streptococcus* was not found in many samples.   This is probably because α–*Streptococcus* requires carbon dioxide to survive, and human milk is thus a harsh environment for this strain.   Furthermore, α–*Streptococcus* is an indigenous oral bacterium, which suggests the possibility that oral bacteria from the neonate become attached to the nipple during suckling.   In addition, although α–*Streptococcus* was present in almost all samples stored at room temperature, it was absent from about half of the samples that were refrigerated or frozen.   This finding suggests that the immune factors in human milk act against α–*Streptococcus* during refrigeration or freezing.   Both *S. aureus* and Gram–positive rods are widely distributed indigenous bacteria in Japan.   The detection rate of *S. aureus* in the present study was 23%, which is similar to the rate of *S. aureus* in community–acquired infections in Japan (20% to 30%).

The intestinal bacteria *E. coli* and *K. pneumoniae* are not normally found adhering to the nipple, but in the present study, *E. coli* was detected in 23% of samples and *K. pneumoniae* in 7.7%.   In previous studies, the detection rates were 4.5%[16] and 6.5%[17] for *E. coli* and 6.1% for *Klebsiella*[17].   These findings suggest that it is not uncommon for intestinal bacteria to adhere to the neonate via the mother's amniotic fluid at the time of birth.   In the present study the 3 samples of human milk in which *E. coli* and *K. pneumoniae* were detected had been collected from the mother around 20 days after birth.   This finding suggests that the expressed human milk was contaminated with intestinal bacteria that adhered to the nipple via the action of suckling or the mother's fingers.   The possibility should be recognized that intestinal bacteria, such as *E. coli,* are present.

In the present study, 1 to 5 strains of bacteria were detected in each sample of human milk, but the strains differed greatly among individual samples because the strains present depended on the indigenous bacterial flora of the mother after birth.   Human milk contains many immune components, and the number of bacterial strains decreases when expressed human milk is stored, because of the action of these components[1].   Nonetheless, bacterial strains were not eliminated through any of the storage methods. Bacterial counts did decrease somewhat under refrigeration. But the number of CNS decreased, but did not in all samples regardless of the storage method.   We suggests that the effects of immune substances in human milk do not include sterilization.

The results show that largely sterile formula milk can be prepared in accordance with the prescribed method. The results also show that formula milk can remain sterile under any of the storage methods.   Thus, although formula milk has the same nutritional composition as human milk, the likelihood of bacterial contamination is lower when formula milk is prepared and stored under sterile conditions.

The final endpoint of a bacteriological study of human milk is that a neonate does not cause an infection of high pathogenicity bacterium.   However, the endpoints of the present study were the quantity and species of bacteria. Studies of infection symptoms should be considered.

Because the period of safe refrigeration of human milk in current guidelines ranges from 2 to 8 days[18], both healthcare workers and mothers might be confused.   We analyzed bacterial search of the human milk using a manual milking method that many mothers performed with a Japanese home household refrigerator.   Refrigerated human milk remains safe, in bacteriological terms, for at least 2 days.   In addition, human milk can be safely stored in a general Japanese household freezer for at least 1 month.   In expressed milk, indigenous bacteria continued to be present after storage, and pathogenic species continued to exist slightly.   Human milk has considerable advantages, such as passing on favorable indigenous bacte-

rial flora from the mother to the neonate, and guidance in expressing milk is an important nursing technique for promoting breastfeeding and ensuring its continuation. The bacteria present in expressed human milk depend on the bacterial count at the time of expression, regardless of the method of storage. It is, therefore, essential when giving guidance on milk expression to teach breastfeeding mothers that their hands should be washed thoroughly with soap and running water or, if they are not extremely dirty, should be cleaned as appropriate with quick-drying alcohol. Mothers should also be given careful guidance on techniques to minimize bacteria adhering to containers for expressed milk. If the container is not sterilized it should be washed thoroughly, or if a plastic bag is used, it should be a strong bag that can be hermetically sealed. The container should be able to be stored immediately after expression.

Authors have no conflict of interest.

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