Microbiological Assessment of fresh expressed breast milk on room temperature at Dr Soetomo Hospital Neonatal Unit

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MICROBIOLOGICAL ASSESSMENT OF FRESH EXPRESSED BREAST MILK ON ROOM TEMPERATURE AT DR. SOETOMO HOSPITAL NEONATAL UNIT

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ABSTRACT

Storing EBM at room temperature in several hours before consuming, frequently found in Indonesia. Based on Academy of Breastfeeding Medicine guidelines EBM can last for 6 to 8 hours in room temperature (25°C or 77°F). However, currently there hasn't been study in tropical country especially Indonesia for the guidelines. This study aimed to assess microbiological quality of EBM on room temperature, including bacterial growth and major bacterial found on EBM for health care and society recommendations. An observational study of 30 expressed breast milk samples provided by 30 healthy women with term baby below 6 month old. The samples were kept sterile and laid at plates for 0 hours, 2 hours, 4 hours and 6 hours in room temperature (26°-32° C) and used drop plate technique on several culture media. Data was analyzed by Chi-square and paired sample T-test. Thirty of unheated fresh EBM from 30 lactating mothers were stored at room temperature, examined for the degree of bacterial contamination at 0 hour, 2 hours, 4 hours, and 6 hours. All the EBM samples were contaminated at 2 hour. Bacterial species identified was Coagulase-negative Staphylococcus (CNS), Escherichia coli, Klebsiella pneumoniae and Streptococcus faecalis, range of growth 10^9 cfu/ml-63 x 10^9 cfu/mm3 after 6 hour of storage. The EBM exposed at room temperature (30-36°C) for more than two hour reduce the quality and do not recommended to be given to the infants.

Keywords: *Expressed breast milk; room temperature; bacterial growth; human milk; breast milk*

ABSTRAK

Menyimpan EBM pada suhu kamar dalam beberapa jam sebelum dikonsumsi, sering ditemukan di Indonesia. Berdasarkan pedoman Academy of Breastfeeding Medicine, EBM dapat bertahan selama 6 hingga 8 jam dalam suhu kamar (25°C atau 77°F). Namun, saat ini belum ada studi di negara tropis terutama Indonesia untuk pedoman. Penelitian ini bertujuan untuk menilai kualitas mikrobiologis EBM pada suhu kamar, termasuk pertumbuhan bakteri dan bakteri utama yang ditemukan pada EBM untuk perawatan kesehatan dan rekomendasi masyarakat. Sebuah penelitian observasional terhadap 30 sampel ASI yang diekspresikan diberikan oleh 30 wanita sehat dengan bayi cukup bulan di bawah 6 bulan. Sampel disimpan steril dan diletakkan di piring selama 0 jam, 2 jam, 4 jam dan 6 jam dalam suhu kamar (26°-32°C) dan menggunakan teknik drop plate pada beberapa media kultur. Data dianalisis dengan uji Chi-square dan paired sample T-test. Tiga puluh EBM segar yang tidak dipanaskan dari 30 ibu menyusui disimpan pada suhu kamar, diperiksa tingkat kontaminasi bakteri pada 0 jam, 2 jam, 4 jam, dan 6 jam. Semua sampel EBM terkontaminasi pada 2 jam. Spesies bakteri yang diidentifikasi adalah Coagulase-negative Staphylococcus (CNS), Escherichia coli, Klebsiella pneumoniae dan Streptococcus faecalis, kisaran pertumbuhan 10^9 cfu/ml-63 x 10^9 cfu/mm3 setelah penyimpanan 6 jam. EBM yang terpapar pada suhu kamar (30-36 °C) selama lebih dari dua jam mengurangi kualitas dan tidak direkomendasikan untuk diberikan kepada bayi.

Kata kunci: *ASI; suhu kamar; pertumbuhan bakteri; susu manusia; ASI*

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INTRODUCTION

Breast milk plays a protective roles against a wide range of infectious and other diseases, especially in developing countries, because of anti-microbial and other immunological substances such as imunoglobulins, lysozymes, lactoperoxidase, lactoferrin, complement components and active leucocytes (Lorico et al 2012).

Mothers are not always disposed to breastfeed their babies adequately because of several reasons such as working or schooling, consequences on poor breastfeeding on their babies. This situation forces mothers to expressed breast milk (EBM) that has been advocated as an effective way of encouraging and maintaining lactation when the mother is separated from the baby for a while.

Milk is a good medium for the growth of many microorganisms, thereby making it a possible means of transmission of microbial infections when it is not properly collected, processed and stored (Obiajuru et al 2017). Bacterial growth at suboptimal storage temperatures (temperature 15-38ºC) is often found to increase in developing countries as well as in workplace situations in industrialized countries.

Like living tissues and other fluids, breast milk is very sensitive to temperature changes, and some nutrient content and bioactive content may be affected by storage (Bransburg-Zabary et al 2015). Storing EBM in room temperature in several hours before consuming frequently found in Indonesia. Academy of Breast-feeding Medicine guidelines states that EBM can last for 6 to 8 hours in room temperature (25ºC or 77ºF), but currently there hasn't been study of microbiological assessment of EBM on room temperature in tropical country, especially Indonesia for the guidelines.

This study was undertaken to determine microbiological quality of EBM on room temperature, including bacterial growth and major bacterial found on EBM for health care and society recommendations.

MATERIALS AND METHODS

This study was conducted among a group of healthy lactating woman at Dr. Soetomo Hospital Surabaya Indonesia. Samples of EBM were collected from 30 lactating women who willingly donated their EBM. Inclusion criteria for participating in this study was that the mothers must have lactating for less than 6 month, term birth, and have not been placed on antibiotics for up to six weeks before sample collection. The sample collected using electric breast pump swing model on Maret-May 2017. A structured questionnaire was distributed to the subjects to elicit information on their practices concerning expressed and stored breasr milk.

Five ml of EBM was taken using a sterile syringe and transferred to a sterile breast milk plastic bag, and brought to the microbiology section and considered to be a 0 hour. The samples were stored at open plates for 2 hours, 4 hours and 6 hours in room temperature (30°-32°C) and used drop plate technique on several culture media. The samples were analyzed for types of bacteria and bacterial load at 0, 2, 4, and 6 hour with drop plate technique using agar medium.

Culture media used this study include Nutrient Agar, Blood Agar and MacConkey`s Agar, sterilized using autoclave at 121ºC for 15 minutes. 1 ml of each breast milk sample was serially diluted (10-1, 10-2, 10-3 and 10-4). The dilution was properly shaken and a dilution of (10-2) was inoculated on sterile media for colony counting using Nutrient agar (NA). Blood agar was used to identify Gram-positive and Gram-negative bacteria. Mac Conkey agar was used to differentiate between lactos and non-lactose fermenting organisms. Cultures were grown at 35-37ºC for 24 hours as per laboratory protocol. After 24 hours, a sterile wire loop was used to pick isolates from the plate and was streaked on freshly prepared blood agar and MacConkey agar and then incubated for 4 hours at 37ºC in order to get pure culture. After incubation all colonies form a section of incubated plates were examined microscopically for Gram reaction, cell morphology and motility. Biochemical analysis included catalase, oxidase and urease activities as well as indole tes and patterns of sugar utilization. The isolates were identified based on the results obtained from biochemical characterization and microscopic examinations. Data of bacterial counts was analyzed by Chi-square and paired T-test using SPSS version 16.0. The value of $p<0.05$ was considered as statistically significant.

RESULTS

Table 1 shows the socio-demographic characteristics of the lactating mother (n=30). More than half subjects (56.67%) of the subjects were between age range of 20-6 years. Half mother had basic education suggested by Indonesian goverment (elementary until senior high shool/ 9 years of education), while half of the rest got education only elementary and junior high school. 66.67% of the lactating mother were full ttime housewives, the rest were working mother (goverment and private employer). More than half the income (60%) with income between 1-5 million rupiah, there is (30%) with income <1 million rupiah and the rest with income >5 million rupiah.

Practices of the mother on storage of EBM revealed that most mother store it on refrigerator (66.67%), while the rest stored it at room temperature (33.33%). 70% of the mother using bottle feeding for EBM to feed their babies, and 30% using spoon and cup to feed their baby by EBM.

Table 1. Socio-demographic characteristics of lactating women (n=30)

Maternal data	Subjects	Percentage %
Age		
<20 years	4	13.33
20-30 years	17	56.67
> 30 years	9	30
Weight (kg)		
< 50 kg	2	6,67
50-70 kg	19	63.33
> 70 kg	9	30
Baby Birth Weight		
<2500g	19	63.33
>2500 g	11	36.67
House wife	20	66.67
Working	10	33.33
Educational Status		
Elementary school	9	30
Junior High school	6	20
Senior High School	8	26.67
College	7	23.33
Income		
<1,000,000 (Rupiahs)	9	30
1,000,000-5,000,0000 (Rupiahs)	18	60
>5,000,0000 (Rupiahs)	3	10
Stored EBM		
Refrigerator (Freezer)	20	66.67
Room temperature	10	33.33
Feeding Methods		
Bottle	21	70
Cup and spoon	9	30
Frequency of expressed breast milk		
Routine	5	16.67
Not routine	12	40
Never	13	43.33

Table 2. Percentage of positive and negative samples of EBM found in 0, 2, 4 and 6 hour after exposed at room temperature

Microorganism	0 hour	%	2 hour	%	4 hour	%	6 hour	%
Positive contaminated	2	0.67 %	16	76.67 %	23	83.33 %	30	100 %
Negative contaminated	28	93.33 %	14	23.33 %	7	16.67 %	-	-
Total	30	100 %	30	100 %	30	100 %	30	100 %

Bacteriological test of the samples (Table 2) showed that at 0 hour there are 2 samples are positive contaminated by bacteria. The amount of contaminated samples increase by the time at 2 hour ($2x10^2$-$1.2x10^5$; averange $1,09x10^4$ cfu/ml) and 4 hour ($2x10^{6}$-$1.4x10^8$; averange value $3.2x10^8$ cfu/ml). At 6 hour all samples are contaminated (10^8-$60x10^9$ averange value $26.2x10^9$ cfu/ml).

At 2 hour of exposure, 16 samples are positive contaminated by the bacteria, but only four samples (of contaminated samples) had bacterial count $>10^4$ cfu/ml (see Table 3).

Table 3. Laboratory assessment of bacteria found in EBM

No	0 hour	2 hour	Bacteria counts cfu/ml	4 hour	Bacteria counts cfu/ml	6 hour	Bacteria counts cfu/ml	Coagulase-negative *Staphylococcus*	*E. coli*	*Klebsiella pneumonia*	*Streptococcus faecalis*
1	-	+	$2x10^2$	+	$8x10^6$	+	$38x10^9$	+	-	+	-
2	-	-	-	+	$12x10^6$	+	$23x10^9$	+	-	+	-
3	-	+	$2x10^3$	+	$14x10^7$	+	$22x10^9$	+	+	-	-
4	+	+	$9x10^3$	+	$19x10^6$	+	$4.5x10^9$	+	+	-	-
5	-	+	$7x10^2$	+	$21x10^5$	+	$2.9x10^9$	+	-	+	-
6	-	-	-	-	-	+	$40x10^9$	+	-	+	-
7	-	+	$2x10^3$	+	$7x10^6$	+	$1.7x10^9$	+	+	-	-
8	-	+	$3x10^4$	+	$11x10^7$	+	$25x10^9$	+	+	-	-
9	-	-	-	-	-	+	$31x10^9$	+	+	-	+
10	+	+	$12x10^4$	+	$24x10^6$	+	$60x10^9$	+	-	-	+
11	-	+	$11x10^4$	+	$23x10^6$	+	$58x10^9$	+	-	+	-
12	-	-	-	-	-	+	$0.8x10^9$	+	-	+	-
13	-	+	$3x10^3$	+	$13x10^5$	+	$28x10^9$	+	+	+	-
14	-	+	$2x10^2$	+	$11x10^5$	+	$0.1x10^9$	+	+	-	-
15	-	-	-	-	-	+	$37x10^8$	+	-	-	-
16	-	+	$5x10^2$	+	$11x10^6$	+	$25x10^9$	+	-	+	-
17	-	+	$4x10^4$	+	$14x10^6$	+	$31x10^9$	+	-	+	-
18	-	-	-	+	$13x10^5$	+	$2.5x10^9$	+	-	+	-
19	-	-	-	+	$20x10^6$	+	$3.1x10^9$	+	-	-	+
20	-	-	-	+	$15x10^5$	+	$34x10^9$	+	-	+	+
21	-	-	-	-	-	+	$29x10^9$	+	-	+	-
22	-	-	-	+	$16x10^6$	+	$43x10^9$	+	-	+	-
23	-	-	-	-	-	+	$37x10^9$	+	-	+	-
24	-	-	-	+	$18x10^5$	+	$27x10^9$	+	-	+	-
25	-	+	$4x10^3$	+	$11x10^5$	+	$31x10^9$	+	-	+	-
26	-	+	$2x10^3$	+	$7x10^6$	+	$0.2x10^9$	+	-	-	-
27	-	-	-	+	$24x10^6$	+	$51x10^9$	+	-	-	+
28	-	-	-	-	-	+	$37x10^9$	+	-	-	-
29	-	+	$6x10^3$	+	$32x10^7$	+	$63x10^9$	+	-	-	-
30	-	+	$3x10^2$	+	$9x10^5$	+	$1x10^8$	+	-	-	-
Mean			2.06×10^4		$3.2x10^8$		2.6×10^{10}				

Table 4. Distribution of microbial analysis of the samples

No	Bacteria Species	Total samples	%
1	Coagulase-negative *Staphylococcus*	30	62.50 %
2	*Klebsiella pneumoniae*	11	22.92 %
3	*Escherichia coli*	4	3.33 %
4	*Streptococcus faecalis*	3	6.25 %

Bacterial identification of the samples as presented in Table 4 showed that the predominant microorganisms isolated were coagulase-negative Staphylococcus (62.50 %), *Escherichia coli* (13.33 %), *Klebsiella pneumoniae* (36.67 %) and *Streptococcus faecalis* (10%). The most common species of contaminant is coagulase-negative Staphylococcus, presence in all samples.

Coagulase-negative Staphylococcus is a group Gram positive, catalase positive and grow well in blood agar, representing the majority of normal skin flora. *Escherichia coli* is Gram negative, indole positive and coagulase negative, while *Streptococcus faecalis* is Gram positive, motile, and coagulase negative, catalase negative and ferments glucose. *Klebsiella pneumoniae* is Gram negative, motile, and coagulase negative.

Statistical analysis showed that there were strong correlation between 2 hours storing with 4 hours storing ($p=0.001$); total colony at 4 hour and total colony at 6 hour ($p=0.047$); and 2 hour and 6 hour ($p=0.02$). There were also significant difference between bacterial colony and the duration itself ($p=0.026$).

DISCUSSION

There are several information about microbiological safe preparation of EBM considered acceptable: less than 10^4 cfu/ml of mesophilic bacteria, with the total count of enterobacteria lower than 10 cfu/ml (Asquith & Harrod 1979); and no presence of pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Enterobacter sakazakii*, (ß-hemolytic) *Streptococcus pyrogenes*, species of Pseudomonas, Proteus and Salmonella (Serra et al 2013).

The findings achieve in the study showed that after 2 hours exposed at open room temperature, 76.67% of EBM samples have been contaminated (the colony count is $>10^4$ cfu/ml) and the presence of several pathogens (*Escherichia coli*, *Streptococcus faecalis* and *Klebsiella pneumoniae*) suggested that the EBM samples are not safety to be given to the infants. The study conducted by Karimi et al reveal that 85% samples collecting from Neonatal Intensive Care Unit, Yazd Iran were infected by bacteria, and the dominant genus was Klebsiella (Karimi et al 2013).

Study has shown that the best method of storing expressed breast milk is in refrigerator at 4ºC temperature, especially in hot climate (Marin et al 2009, Martinez-Costa et al 2007). Other studies (Tully 2000, Zinn 2000) have been carried out in optimum conditions of storage noted that EBM could last for up to 3-8 days in the refrigerator. Indonesia is a tropical country with two seasons all year (summer and rainy season) with extreme temperatures between the two seasons (30-37ºC in summer and 27-28ºC rainy).

Ighogboja et al (1996) state that many mothers will not give EBM because they think EBM is not safe given to babies after more than 8 hours of storage. Further study that EBM would very easily be contaminated with bacteria, toxic, and can become diluted and acid after storage. Research of the time difference in the length of storage can cause many changes the nutrient contents (Ighogboja et al 1996).

Freshly collected breast milk is rarely sterile and normally contains bacteria originated from the maternal skin and nipple duct micro-flora, but it also contain potential pathogens (Ogundele 2000). Gransden et al (1986) reported that the breast pump was an important sources of bacterial contamination of EBM. The study conducted by Boo et al (2001) confirmed that manual expression (using hands) had lower risk of contamination than expressed breast milk using breast pump.

It is well known that differences in geographic locations and in the time and methods of milk collection affect the results of analysis of breast milk microbiota (Li et al 2017). Fitzstevens et al (2017) suggested that two genera, Streptococcus and Staphylococcus, may be universally predominant in human milk, regardless of differences in geographic location or analytical methods.

There are four species isolated: Staphylococcus coagulase, *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus faecalis*. Mense et al (2013) observed the bacterial contamination of mecanically extracted breast milk in 50 mother including species of Staphylococcus coagulase, *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus faecalis*. The other species were *Staphylococcus aureus*, Alpha-hemolytic streptococci and dermal bacteria.

Coagulase-negative Staphylococcus (CNS) was a predomintants species, presents in all samples, because this species is a dominant organism in normal skin flora and most commonly isolated from blood cultures (Cunha et al 2004). Delgado et al (2009) proposed that disruption in the normal bacterial flora balance in breast milk may lead to CNS overgrowth and mastitis.

It is believed that about 8% of nosoclominal infections occuring in patients of neonatal intensive care units is caused by *Klebsiella pneumoniae* (KLP) (Donowitz et al 1981). Dorota et al's (2017) study observing newborns with recurrent infections found out that the source of infection was the contaminated breast milk with *Klebsiella pneumoniae*.

Escherichia coli presents in the samples indicated that contamination by coliform microorganisms may originate from the environment, means the low level of hygiene and sanitary practices (Serafini et al 2003). Among the enterobacterias, coliforms have been singled out as particularly important bacteriological control of HMBs, since their presence may indicate faecal contamination, even if it originates from an indirect sources (Serafini et al 2003). Other study found *Escherichia coli* in 8.5% of the 59 sample of breast milk (Szollosy et al 1974) and 2% of 44 samples (Eidelman & Szigagyi 1979).

Breast milk has high agglutinating titres to a variety of *E. coli* serotype (Gindrat et al 2000). Lorico et al (2012) observed the bacterial growth inhibiting activity of EBM on Eschericia coli, *Klebsiella pneumoniae* and *Staphylococcus aureus* and stated that breast milk exhibit bacteriostatic properties againts most pathogens except for *Escherichia coli* after being frozen for 24 hours.

Table 5. Treatment of EBM depending on bacterial count, modified according to Henker (1987)

Group	Bacterial count (cfu/ml)	Bacterial differentiation	Consequence
I	< 1,000 (10^3)	n/a	No pasteurization
II	<10,000 (10^4)	n/a	Pasteurization for baby weight <1,500 g; no pasteurization for baby weight >1,500 g
IIIa	<100,000 (10^5)	<10,000/ml potential pathogenic bacteria	Pasteurization
IIIb	<100,000 (10^5)	>10,000/ml potential pathogenic bacteria	Discarding
IV	>100,000 (10^5)	n/a	Discarding

CFU: colony-forming units.

It is recommended to reduce the amount of bacteria and pathogens in breast milk according to Henker (1987) (table 5) before giving the contaminated EBM to feed the infants.

CONCLUSION

Evaluation results that the EBM exposed at room temperature (30-36°C) for more than two hour reduce the quality because bacterial counts > 10^4 cfu/ml and the presence of pathogens *E. coli*, *Streptococcus faecalis* and *Klebsiella pneumoniae* were found.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7