

MICROBIOLOGICAL AND BIOCHEMICAL ASSESSMENT OF THE SURFACE AREA OF BREAST NIPPLES AND BREAST MILK OF LACTATING WOMEN IN CALABAR, SOUTHEASTERN NIGERIA

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Abstract. Microbiological and biochemical analyses of 59 breast nipple swab samples and 15 manually expressed breast milk samples of lactating mothers aged 15 to 40 years, was carried out using standard procedures. The incidence of bacterial species in swab samples was *Staphylococcus aureus* (63.8%), *Streptococcus* sp (25.5%), *Escherichia coli* (6.4%) and *Klebsiella* sp (4.26%), indicating the poor sanitary status of the lactating mothers. *S. aureus* was recovered from only 1 (6.7%) of the milk samples, indicating that breast milk is relatively sterile. The nutritional values for the breast milk were 22.5 mg/ml (protein), 0.3 mg/ml (calcium), 3.5 mg/ml (sugar) and 300 µg/ml (vitamin A) in age group 15-20 years, and 16.4 mg/ml (protein), 0.16 mg/ml (calcium), 1.8 mg/ml (sugar) and 100 µg/ml (vitamin A) in the age group 36-40 years. In conclusion, the nutritive and antimicrobial properties of breast milk decrease with increasing age of lactating mothers. The need for public health enlightenment of lactating mothers regarding hygiene, and the provision of oral vitamin A supplement to infants, is discussed.

INTRODUCTION

The nutritional and immunological advantages of human breast milk make it desirable and ideal for newborn babies and infants (Whitehead, 1983; Deodhar and Joshi, 1991). Human breast milk is naturally balanced and constitutes an important source of carbohydrates, proteins, lipids, calcium, water and vitamins for the growing infant (Belavady, 1978; Boediman *et al*, 1979; Jansson *et al*, 1981; Pitkin, 1985). Useful vitamins, such as riboflavin, pyridoxin, biotin, niacin and panthothenic acid have been reported to occur in human milk (Nancy, 1867; Pitkin, 1985; Kadiri and Obembe, 1999). Other valuable components of human milk include milk cells, lysozymes and micronutrients, such as sodium (Na) and iron (Fe) as well as lactoferrin, albumin, immunoglobulins (Ig), such as IgG and IgA, and active leukocytes (Boediman *et al*, 1979; Deodhar and Joshi, 1991). These unique components of human breast milk enhance resis-

tance to infection (Deodhar and Joshi, 1991; Kadiri and Obembe, 1999; May, 1999). Vitamin A, monolaurin and the protein lactoferrin, have been shown to be very effective antimicrobial factors against the growth of cytomegalovirus (CMV), which infects infants from human milk (Clark and May, 2000).

Colostrum, a yellowish, sticky and transparent fluid, which has a high level of albumin, IgA, lactoferrin and leukocytes, is secreted from the breast during the first five days after parturition. This is followed by the formation of transitional milk, which is secreted six to ten days after parturition, before the formation of true milk (Harvy, 1985). This is the period when chemical and immunological changes occur to transform the milk to "matured milk". Transitional milk has a higher concentration of phosphorus than colostrum and "matured" milk.

Microbial contamination of human milk and associated infant infection are rare, especially as human milk is known to contain some antimicrobial factors, which protect infants against various infections. However, the high nutritive value of human milk makes it susceptible to con-

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tamination by microorganisms. Besides, HIV whose risk of infection from mothers to infants through breast feeding has not yet been clarified (Deodhar and Joshi, 1991), some contaminants, such as cytomegalovirus, may be transferred to infants from seropositive mothers, without adverse effects to the infants (May, 1999). May (1999) reports that in spite of the rarity of microbial contamination of human milk, various bacterial contaminants present in expressed human milk have caused infections.

This study was undertaken with a view to determining the status of the surface area of the breast nipple and the breast milk of lactating women of various age brackets with respect to bacterial contamination, biochemical composition and nutritive values.

MATERIALS AND METHODS

Collection of samples

Specimens were collected from healthy lactating mothers age 15 to 40 years from 5 randomly selected private clinics in Calabar, South-eastern Nigeria. The babies of the lactating mothers ranged from one day to three months old.

Specimens were collected from the breast nipple using a sterile cotton swab stick previously moistened with physiological saline (0.85% NaCl, ANALAR). The samples were immediately transported to the laboratory in an ice box at 4°C for microbiological analysis within 2-3 hours. After thoroughly cleaning the lactating mothers' nipples with 70% alcohol, 20 ml of the breast milk were expressed and collected in sterile sampling bottles (4 oz or 114 ml) and immediately frozen prior to microbiological and biochemical analyses.

Microbiological analysis of breast nipple samples

Samples were inoculated on triptone soya agar (Oxoid, England), a general purpose agar that can support the growth of both aerobes and anaerobes when supplemented with 1% (w/v) cysteine hydrochloride (BDH chemicals, UK) (APHA, 1998) and McConkey agar (Oxoid, England) for the isolation of coli- aerogenes-like enteric organisms (Itah and Ben, 2004), and in-

cubated at 37°C for 24 hours. Primary isolates were repeatedly subcultured on fresh media using streak plating techniques to obtain pure cultures.

The purified isolates were stored in agar slants at 4°C and later characterized and identified using John L's bacterial identification scheme (Lindquist, 1999) adopted from Cowan (1974). In this scheme, some chemoheterotrophic bacterial genera are sorted out based on various primary tests, such as Gram staining and shape of bacteria along with the results of glucose fermentation tests, catalase and oxidase reactions, and other tests, such as a motility test, possession of endospores, aerobic and anaerobic growth.

The coagulase test was performed on staphylococcal isolates and those which were catalase-positive were cultured on mannitol salt agar to confirm *Staphylococcus aureus*, based on their growth pattern (Itah and Ben, 2004). Suspected *Escherichia* isolates were differentiated from *Klebsiella* by inoculating the isolates in lactose peptone water and incubating at 44°C. Gas production confirmed *E. coli*, which was further completely confirmed by Methyl Red Voges Proskauer (MRVP) test, since *Enterobacter aerogenes* has many characteristics in common with *E. coli*.

Microbiological analysis of human milk samples

Ten-fold serial dilutions ranging from 10^{-1} to 10^{-6} were prepared using sterile distilled water (Atlas and Bartha, 1992). One milliliter aliquots of sample dilutions of 10^{-3} - 10^{-6} were each seeded into sterile disposable Petri dishes (90 mm diameter) in triplicates and the total viable bacterial count was determined by pour plate technique (APHA, 1998) using tryptone soya agar. The cultures were incubated at 37°C for 48 hours after which bacterial colony counting was carried out. Isolates were repeatedly subcultured to obtain pure cultures which were stored in agar slants for later characterization and identification of the organisms based on John L's scheme described earlier in this paper. The growth pattern of catalase-positive staphylococcal isolates on mannitol salt agar (Oxoid, England) was used to confirm *S. aureus* (indicated by colonies surrounded by bright yellow

zones).

Physio-chemical and biochemical analysis of human milk

Determination of calcium and pH value of breast milk. Using the method of IITA (1979), 1 ml of the milk sample was pipetted into a beaker and diluted to 100 ml with sterile water, followed by 2 drops of methyl red and dropwise addition of ammonium hydroxide until a brownish-orange color was obtained. Next, 2 drops of dilute hydrochloric acid were added and the solution was diluted with 50 ml water. After boiling the solution, 10 ml of hot 4.2% ammonium oxalate solution was added with stirring until a precipitate was formed. The precipitate was filtered, washed with 40% ammonium hydroxide solution and dissolved in a mixture of 125 ml water and 5 ml 98% sulphuric acid, followed by heating to 70°C using a water bath and titrated against 0.05N sodium permanganate solution.

The pH was determined by dipping the electrode of a pH meter in 10 ml of the human milk sample and recording the pH.

Determination of protein and sugar contents of the milk samples. Protein was determined using Folin Ciocalteau reagent (Lowry *et al*, 1951). This involved adding 3 ml of solution A to 1 ml of the milk sample, followed by the addition of 1 ml Folin Ciocalteau reagent. After 30 minutes, the optical density of the resulting solution was read at 570 nm against a blank that had 1 ml milk sample replaced with 1 ml distilled water. Solution A consisted of 50 ml 2% Na₂CO₃ in 0.1M NaOH mixed with 1 ml of a mixture of 10% CuSO₄.5H₂O and 2% sodium tartarate.

The protein standard curve was prepared from various concentrations of casein in 0.1M NaOH (Lowry *et al*, 1951).

To determine the sugar content of human breast milk, the phenol-sulphuric acid method of Dubois *et al* (1956) was adopted. This involved adding 1 ml of 5% phenol and 5 ml concentrated sulphuric acid to 1 ml of the human milk sample and the optical density (OD) read against a blank at 490 nm using a DR/3000 HACH spectrophotometer. The blank contained 1 ml of distilled water and the standard curve was prepared using various concentrations of

Table 1
Genera of bacteria isolated from the surface breast nipple.

Gram stain (young culture) and shape	Aerobic growth	Anaerobic growth	Endospores	Motility	Catalase reaction	Oxidase reaction	Glucose fermentation to acid or to acid +gas	Organism (Genus)
Gram +ve coccus (clusters)	+	+	-	-	+	-	+	^a Staphylococcus
Gram +ve coccus (chains)	+	+	-	-	-	-	+	Streptococcus
Gram +ve bacillus	+	+	-	-	+	-	+	Klebsiella
Gram -ve bacillus	+	+	-	+	+	-	+	^b Escherichia

+ = Positive result; - = negative result
^a = *Staphylococcus aureus* confirmed; ^b = *Escherichai coli* confirmed.

glucose (Dubois *et al*, 1956).

Estimation of vitamin A. To be able to determine antimicrobial factors in the breast milk one of the easily estimated factors, vitamin A, was estimated in colostrum, transitional milk and “matured” milk.

The method of Bessey *et al* (1946) was adopted in the determination of vitamin A. This involved adding 1 ml of absolute ethanol followed by 4 ml of n-hexane to 0.2 ml of the milk sample in a 30 ml capacity test tube. The vitamin A in the milk was extracted by shaking the mixture with the aid of a votex mixer for 5 minutes, after which the mixture was centrifuged for 10 minutes at 2,000 rpm. About 3 ml of the hexane layer was transferred to a 10 ml silica cuvette and the absorbance (A₁) read at 430 nm using a DR/3000 HACH spectrophotometer. The cuvette was then exposed to the long wavelength of ultra violet light (6 inches away from a block ray long wavelength violet lamp) for 1 hour before reading the absorbance (A₂) was read again at 430 nm. Vitamin A was calculated by multiplying the difference between A₁ and A₂ by the concentration of a standard (200 mg/deciliter) divided by absorbance of the standard (0.280 nm) and expressing the result in mg/100 ml of milk.

RESULTS

Isolates recovered from breast nipple swab and milk samples

The results of the microbiological analysis of 59 breast nipple swab samples collected from lactating women between the ages of 15 and 40 are shown in Table 1. This shows the presence of the bacterial genera: *Staphylococcus*, *Streptococcus*, *Klebsiella* and *Escherichia*. *Staphylococcus* and *Escherichia* were confirmed to be *Staphylococcus aureus* and *Escherichia coli*, respectively.

The incidence of the bacterial species isolated from the nipples of the lactating mothers is represented in Table 2. A total of 47 isolates were recovered from 59 samples examined. Thirty (63.8%) were *Staphylococcus aureus*, while 12 (25.5%), 2 (4.3%) and 3 (6.4%) were *Streptococcus* species, *Klebsiella* species and

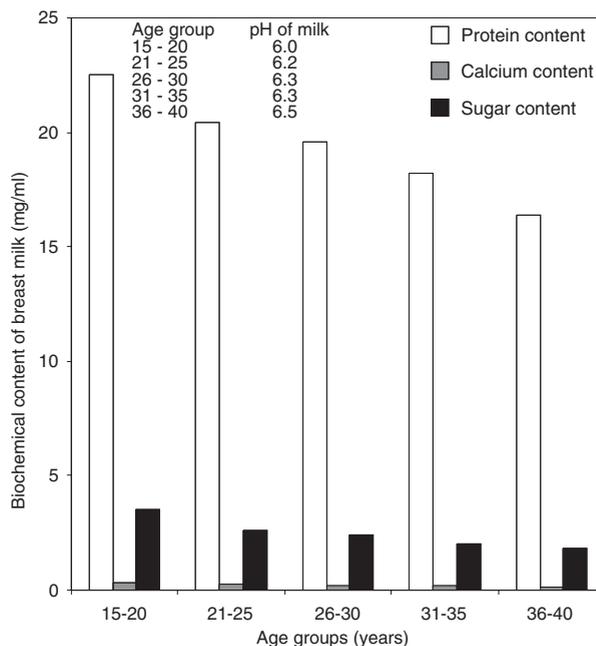


Fig 1—Levels of biochemical substances in the breast milk of lactating mothers.

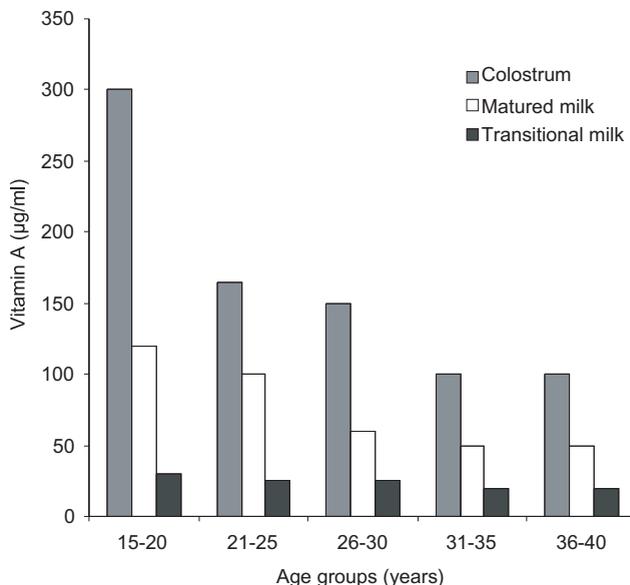


Fig 2—Vitamin A levels in the breast milk of lactating mothers.

Escherichia coli, respectively.

The mean total bacterial counts of breast milk samples are represented in Table 3 which shows the presence of bacteria in one of the two

Table 2
Incidence of bacterial species isolated from the nipples of lactating women.

Organism	No. of samples analyzed	Age groups (Years)					Total	% bacteria isolated in 59 samples
		15-20	21-25	26-30	31-35	36-40		
<i>S. aureus</i>		6 (66.7)	5 (55.6)	8 (61.5)	4 (66.7)	7 (70.0)	30	63.8
<i>Streptococcus</i> sp		2 (22.7)	3 (33.3)	5 (38.5)	2 (33.3)	0 (0)	12	25.5
<i>Klebsiella</i> sp		0 (0)	0 (0)	0 (0)	0 (0)	2 (20.0)	2	4.3
<i>E. coli</i>		1 (11.1)	1 (11.1)	0 (0)	0 (0)	1 (10.0)	3	6.4
Total		9	9	13	6	10	47	100

Numbers in parenthesis represent incidence

Table 3
Mean total bacterial counts for breast milk in lactating women.

Age groups of lactating women	No. of samples treated in each age group	Mean total bacterial count (cfu/ml x 10 ⁴)	Identified bacterial species
15-20	4	0	Nil
21-25	5	0	Nil
26-30	2	0	Nil
31-35	2	1.0±0.01	<i>Staphylococcus aureus</i>
36-40	2	0	Nil
Total	15	1.0±0.01	Nil

Nil = No bacterial growth

samples collected from lactating mothers of the age group 31-35 years. This shows that 1 (6.7%) of the 15 samples analyzed showed the presence of bacteria. Samples from the rest of the age groups were relatively sterile.

Biochemical contents of breast milk of lactating women

The results of the biochemical analysis of breast milk of lactating women are presented in Figs 1 and 2. Protein levels decreased progressively from 22.5 mg/ml (age group 15-20 years) to 16.4 mg/ml (age group 36-40 years). Sugar levels also decreased from 3.5 mg/ml (age group 15-20 years) to 1.8 mg/ml (age group 36-40 years). There were no major differences in the levels of calcium among the age groups. The pH of the breast milk increased with increasing age.

The levels of vitamin A in colostrum, matured milk and transitional milk are presented in Fig 2. Colostrum appeared to contain the high-

est level (300 µg/ml) in the age group 15-20 years, and declined to 100 µg/ml in the age groups 31-35 and 36-40 years. This pattern of decline was also applicable to matured milk, which had the highest level (120 µg/ml) in the age group 15-20 years. Transitional milk did not appear to exhibit a progressive pattern of decline, although the older age groups still showed lower levels of vitamin A. In general, older lactating women appeared to have lower levels of protein, calcium, sugar and vitamin A than younger women.

DISCUSSION

Swab samples of the breast nipples of lactating women for all age groups studied revealed the presence of varying amounts of chemoheterotrophic bacteria, including some enteric bacteria, which are of sanitary significance (Tables 1 and 2). Of the 59 swab samples from

breast nipples of lactating women in the various age groups, *Staphylococcus aureus* had the highest incidence 30 (63.8%) followed by *Streptococcus* species 12 (25.5%). *Klebsiella* species 2 (4.3%) and *E. coli* 3 (6.4%) also occurred, indicating the sanitary status of some of the lactating women. The ubiquity of enteric bacteria, such as *E. coli*, *Klebsiella*, *Citrobacteria* and *Proteus*, and the likelihood of their being shed from the body, clothing, etc, has been reported (Itah and Ben, 2004). Therefore, babies of lactating women with such a poor sanitary status are at risk of being infected with enteropathogenic *Escherichia coli*, which is a well known agent in infantile and traveller's diarrhea (Itah and Ben, 2004). The high incidence of *Staphylococcus aureus*, which, of course, forms part of the normal microbial flora of the skin, upper respiratory tract and intestinal tract (Cheesbrough, 1991; Deodhar and Joshi, 1991), further reveals the unsanitary condition of the breast nipples of some lactating women. This calls for public health enlightenment of lactating women at antenatal through to the post-natal period, aimed at protecting newborn babies and infants from infection obtained from breast nipples and milk.

The microbiological quality of the breast milk (Table 3) indicates that breast milk is relatively sterile and thus safe for consumption by babies. In this study, only 1 (6.7%) of the 15 milk samples analyzed microbiologically showed evidence of bacterial contamination, which could have arisen as a contaminant (May, 1999) from improper cleaning of the nipples with 70% alcohol before expression of the milk sample. The sterility of human milk and its being the optimal source of nutrition for infants has earlier been reported (American Academy of Pediatrics Committee, 1978). Kadiri and Obembe (1999) reported the absence of bacterial growth in cultured human milk. May (1999) reported microbial contaminants in human milk are rare, as are infant infections from milk, although some contaminants, such as cytomegalovirus (CMV) may be transferred to infants from seropositive mothers without adverse effects on the infants.

Protein, calcium, sugar and vitamin A levels in the breast milk were observed to decline with increasing age (Figs 1 and 2), indicating that

the older a lactating woman, the less the nutritive quality of her breast milk. This implies that babies from older lactating women are less likely to have healthy bodies and strong bones than babies from younger lactating women, especially as a decline in calcium level can adversely affect bone formation (Pitkin, 1985). The pH of the breast milk was observed to increase with age. Protein is known to be the principal substance present in casein which contains essential acids that are important substrates for protein synthesis in babies. Thus, an increase in pH level with age can result in a proportional decrease in the protein level of the human breast milk. Similar results of biochemical studies of human breast milk of lactating women in western Nigeria have been reported (Kadiri and Obembe, 1999).

Variation in the vitamin A content of colostrum, matured milk and transitional milk exhibited the same pattern as protein, calcium and sugar content. Colostrum had the highest level of vitamin A (300 µg/ml) in age group 15-20, which declined in matured milk (120 µg/ml), with a minimum of 30 µg/ml in transitional milk. The same scenario was observed in other age groups. However, vitamin A level generally decreased with age (Fig 2). Vitamin A is one of the most effective antimicrobial factors in human breast milk that protects infants against microbial infectious agents, particularly cytomegalovirus (May, 1999; Clark and May, 2000). This calls for a doubling of effort by the World Health Organization (WHO) to provide oral vitamin A supplement to infants, especially those born by women in their later years.

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