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Microbiological and biochemical assessment of the surface area of breast nipples of lactating women in India

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ABSTRACT

Microbiological and biochemical analyses of 40 breast nipple swab samples and was carried out using standard procedures. The incidence of bacterial species in swab samples was *Staphylococcus aureus*, P*antoea agglomerans,*, indicating the poor sanitary status of the lactating mothers *S. aureus and* P*antoea agglomerans,* was confirmed through PCR and 16srna sequencing and the database submitted in genbank. The need for public health enlightenment of lactating mothers regarding hygiene, and the provision of oral vitamin A supplement to infants. Swab samples of the breast nipples of lactating women for all age groups studied revealed the presence of varying amounts of chemo heterotrophic bacteria, including some enteric bacteria, which are of sanitary significance of the 40 swab samples from breast nipples of lactating women in the various age groups, *Staphylococcus aureus (KF525236)* had the highest incidence and *Pantoema agglomerans (KF477281)* also occurred, indicating the sanitary status of some of the lactating women. In this study, only 1 of the 15 breast nipple swab samples analyzed microbiologically showed evidence of bacterial contamination, which could have arisen as a contaminant from improper cleaning of the nipples with 70% alcohol before collection

of the swab sample. 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.

Keywords: PCR-Polymeric chain reaction, Vit-A-Vitamin A

1. INTRODUCTION

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. To study the microbiological and biochemical analyses of 40 breast nipple swab samples and 40 manually expressed breast milk samples of lactating mothers aged 15 to 35 years samples are analyzed. To study the incidence of bacterial species in swab samples, breast milk and cow milk samples are further confirmed by PCR 16srna sequencing analysis. 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, immunoglobulin (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 resistance 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).

Not all the properties of breast milk are understood, but its nutrient content is relatively stable. Breast milk is made from nutrients in the mother's bloodstream and bodily stores. Breast milk has just the right amount of fat, sugar, water, and protein that is needed for a baby's growth and development. Because breastfeeding uses an average of 500 calories a day, it helps the mother lose weight after giving birth. The composition of breast milk changes depending on how long the baby nurses at each session, as well as on the age of the child (Chung M, Raman G, et al., 2007). 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 contamination 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.

2. MATERIALS AND METHODS

Collection of samples

Specimens were collected from healthy lactating mothers’ age 20 to 35 years from 5 randomly selected private clinics in kodaikanal in Tamilnadu. 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 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, (APHA, 1998) and Mac Conkey agar, for the

isolation of coli- aerogenes-like enteric organisms (Itah and Ben, 2004), and incubated at 37°C for 24 hours. Primary isolates were repeatedly sub cultured on fresh media using streak plating techniques to obtain pure cultures.

Gram Staining

Gram staining is used for identification of Gram positive and Gram negative organism. E.coli is a gram negative rod. Shaped bacterium. In the present study the 10 isolates were subjected to gram staining all the isolates were found to be gram negative rods. This work is supported by previous work done to identify micro organism (Cruickshank et al., 1994) where they identified Ecoli as gram negative rods.

Inoculation into Broth

In the laboratory the milk sample was inoculated into tryptose soy broth under sterile condition. After inoculation, the test tube was incubated at 37oC for overnight (Cruickshank et al., 1975).

Inoculation into Tryptose Soy Agar

A loopful of culture from the tryptose soy broth was streaked onto tryptose soy agar plates. After inoculation the plates were incubated at 37oC overnight (Cruickshank et al., 1975).

Inoculation into Agar Plates

Well isolated colonies from the TSA plates were streaked onto the following agar plates,

-Mac Conkey Agar

-Eosin Methylene Blue Agar (Cruickshank et al., 1975).

Biochemical Tests

Indole Test

A loop full of culture from 24h growth medium was inoculated in Tryptone medium and incubated at 37° C for 24 h. After incubation 0.5ml of Kovac's reagent was added to it and observed for the ring formation (Cruickshank et al., 1975).

Methyl Red Test

Sterile MR broth was inoculated with the isolate and incubated at 37° C for 24-48 hours after incubation, methyl red solution was added and shaken well (Cruickshank et al., 1975).

Voges Proskauer Test

VP broth was inoculated with cultures and tubes were incubated at 37° C for 24-48 hours. Baritt's reagent was added in all test tubes and the result was observed (Cruickshank et al., 1975).

Citrate Utilization

The test culture was inoculated into Simmons citrate medium and inoculated at 37° C for 24 hours and observed for colour change (Cruickshank et al., 1975).

Eijkman Test

1. Durham's tube was placed inside the test tubes containing Mac conkey broth. Care should be taken that there should be no air bubbles.
2. Inoculate the isolate into the sterilized test tubes.
3. Incubated for 24 – 48 hours at 44° C Formation of air bubbles inside the Durhams tube indicated the positive results (Cruickshank et al., 1975).

Sugar Fermentation Test

The cultures were incubated in the respective sugar medium (glucose, sucrose. Lactose, maltose, mannitol, mannose, raffinos, xylose, sorbitiol, trehalose, fructose) and incubated at 37 C over night. After incubation, Andrads indicator was added to the culture tubes. Red colour was formed as a result of the production of acid and gas confirms the positive reaction (Cruickshank et al., 1975).

The Protocol of Sequencing Service

1.Sequencing Kit: ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits
2.Sequencer: ABI PRISM® 3730XL Analyzer (96 capillary type) (Over 20)
3.PCR machine: MJ Research PTC-225 Peltier Thermal Cycler
4.Sequencing protocol

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using [Universial or what you selected] primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Primer information

	Primer Name	Type	Type2	Sequence (5 to 3)
1	518F	Universal	Forward	CCAgCAgCCgCggTAATACg
2	800R	Universal	Reverse	TACCAgggTATCTAATCC
3	27F	Universal	Forward	AgAgTTTgATCMTGGCTCAg
4	1492R	Universal	Reverse	TACggYTACCTTgTTACgACTT

Note:
Primer 1 & 2 for Sequencing Reference.
Primer 3 & 4 for PCR Amplification

Analysis Procedure

1. Preparation of template DNA

It is important to use a pure cultivated bacterium for identification. Colonies are picked up with a sterilized toothpick, and suspended in 0.5 ㎖ of sterilizes saline in a 1.5 ㎖ centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet is suspended in 0.5 ㎖ of InstaGene Matrix (Bio-Rad, USA). Incubated 56℃ for 30 min and then heated 100℃ for 10 min. After heating, supernatant can be use for PCR.

2. PCR

Add 1 ㎕ of template DNA in 20 ㎕ of PCR reaction solution. Use 27F/1492R primers for bacteria, and then perform 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec. DNA fragments are amplified about 1,400 bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

3. Purification of PCR products

Remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore).

4. Sequencing

The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers as described (Primer Name File). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

3. RESULTS AND DISCUSSION

Swab samples of the breast nipples of lactating women for all age groups studied revealed the presence of varying amounts of chemo heterotrophic bacteria, including some enteric bacteria, which are of sanitary significance (Tables 2, 3 & 4). Of the 40 swab samples from breast nipples of lactating women in the various age groups, *Bacillus* had the highest incidence 30 (63.8%) followed by *Staphylococcus aureus* 12 (25.5%). and *Enterococcus* Species 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 entropathogenic *Escherichia coli*, which is a well known agent in infantile and

traveller's diarrhea (Itah and Ben, 2004). The high incidence of *Staphylococcus aureus (KF525236)*, 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. *Pantoea agglomerans (KF477281)* is a Gram-negative bacterium that belongs to the family Enterobacteriacea. Formerly called *Enterobacter agglomerans*, this bacterium is known to be an opportunistic pathogen in the immunocompromised, causing wound, blood, and urinary-tract infections (Plate 1 & 2). It is commonly isolated from plant surfaces, seeds, fruit (e.g. mandarin oranges), and animal or human feces. It is an opportunistic pathogen in the immunocompromised, causing wound infections, bacterium, and urinary tract infections. This species is currently listed as a Biosafety level 2 (BL2) organism due to clinical reports as an opportunistic human pathogen. Septic arthritis or synovitis is usually common, clinical manifestations caused by *P. agglomerans (KF477281)* and often correlated with a predisposing factor i.e. immunodeficiency (diabetes mellitus, malignancies, extremes of age) or use of central catheter.

Plate 1
Genera of bacteria isolated from the surface

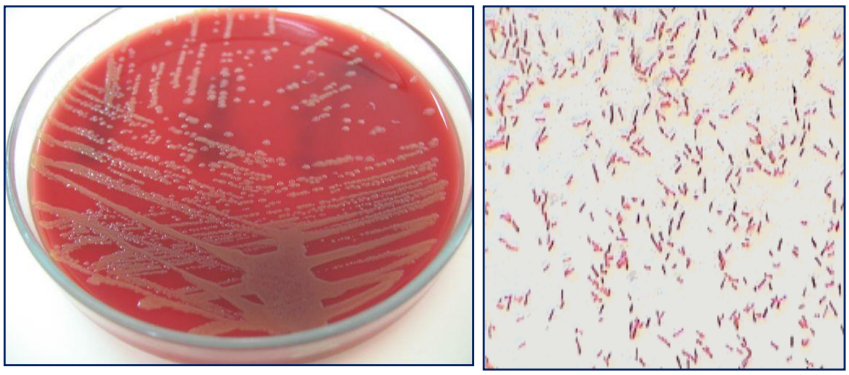

Plate 2
Pantoea agglomerans (KF477281) Isolated from the Surface Breast Nipple of Lactating Women and Gram Staining

Table 2
Incidence of bacterial species isolated from the breast nipple swab of lactating women

organism	No of samples analyzed	Age groups (years)			Total (45)	% bacteria isolated in 40 samples
		15 -20	20-25	25-35		
Bacillus		6(66.7)	8(61.5)	7(70.0)	21	63.8
S.aureus		2(22.7)	3(33.3)	5(55.6)	10	25.5
Enterococcus Species		1(11.1)	1(11.1)	3(30.0)	5	10.8
Total		9	12	15	36	100

Table 3
Biochemical Test in Breast Nipple Swab Samples of Lactating Women

Gram stain (young culture) and shape	Aerobic growth	Anerobic growth	Endo spores	motility	Catalase reaction	Oxidase reaction	Glucose fermentation to acid or acid to gas	Organism (Genus)
Gram +ve	+	+	-	-	+	-	+	**S.aureus**
Gram-ve	+	+	-	+	+		+	**Pantoea agglomerans**

+=Positive result, - =negative result

Table 4
Genera of bacteria identification of the isolate from the surface breast nipple swab samples of Lactating women in 16srna sequencing

S.No	Identified Isolates	Genbank Accession No
1.	***Staphylococcus aureus***	**KF525236**
2.	***Pantoea agglomerans***	***KF477281***

4. CONCLUSION

Swab samples of the breast nipples of lactating women for all age groups studied revealed the presence of varying amounts of chemo heterotrophic bacteria, including some enteric bacteria, which are of sanitary significance of the 40 swab samples from breast nipples of lactating women in the various age groups, *Staphylococcus aureus (KF525236)* had the highest incidence and *Pantoema agglomerans (KF477281)* also occurred, indicating the sanitary status of some of the lactating women. In this study, only 1 of the 15 breast nipple swab samples analyzed microbiologically showed evidence of bacterial contamination, which could have arisen as a contaminant from improper cleaning of the nipples with 70% alcohol before collection of the swab sample. 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.

SUMMARY OF RESEARCH

The *Staphylococcus aureus* had the highest incidence and *Pantoema agglomerans* also occurred, indicating the sanitary status of some of the lactating women. In this study, only 1 of the 15 breast nipple swab samples analyzed microbiologically showed evidence of bacterial contamination, which could have arisen as a contaminant from improper cleaning of the nipples with 70% alcohol before collection of the swab sample.

DISCLOSURE STATEMENT

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