Chapter III

MATERIALS AND METHODS

“The art of discovery consists in seeing what everyone else has seen and then thinking what nobody else has thought”

A. SZENT GYÖRGYI

Nobel Prize for Physiology or Medicine, 1937
3.0. MATERIALS AND METHODS

The experimental procedure adopted in the study “Evaluating Beneficial Effect of Breast Milk through Biochemical, Microbiological and Molecular Methods” is described in the following sequence.

3.1. SELECTION OF SUBJECTS:

Forty lactating women in the age group ranging between 20-35 years and forty years who came to Kovai Medical centre and Hospital, Coimbatore, Tamilnadu and Government hospital, kodaikanal, KHMS hospital in kodaikanal for regular checkup were selected for this study. Twenty non-lactating women with corresponding age group were chosen as controls. Forty cow milk samples and ten coliform mastitis samples were collected from clinical ward, Government Veterinary hospital, kodaikanal, and surrounding areas of kodaikanal.

3.2. COLLECTION OF SAMPLES:

3.2.1. Swab 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.

3.2.2. Collection of Serum and Plasma samples:

10 ml of whole blood was collected from the lactating women and a non lactating woman blood was collected in tubes previously coated with 1% EDTA and was subsequently centrifuged
at 2,500rpm for 15 min for separation of plasma. Blood taken in separate tube was allowed to clot for 20 min and was subsequently centrifuged for 15 minutes at 2,500rpm, for serum separation. The serum and plasma samples were collected and refrigerated (15°) until biochemical analysis.

3.2.3. Colostrum samples;

Approximately 5ml of colostrum was collected from a single breast within 24 hours of delivery from each subject into sterile storage vials and stored at -20°C and were analyzed within two weeks.

3.2.4. Milk Samples:

Milk samples (approximately 5ml) were collected randomly, at the mother’s convenience, by hand expression into clean polypropylene tubes (5ml, disposable products) and frozen immediately in either the hospitals or the mother’s home, freezer and then transported on ice to the laboratory to be stored at -20°C for analysis. Where milk was collected specifically for milk culture gloves worn for the expression of the samples. The nipple and areola areas of each breast were cleaned with sterile water and allowed to dry. The first 5ml (approximate) were collected into a clean sample tube for biochemical analyses and a further, mid stream sample was then expressed into a sterile polypropylene tube (5ml). The mid stream sample was transported on ice and stored at -20°C, for no longer than 6 hours, until plated for culture.

HUMAN BREASTMILK IN VARIOUS STAGES
3.2.5. Cow milk samples

20 Milk samples were collected from mastitis affected cows. The samples were inoculated in Triptose soya broth. After an incubation period of 4-6 hours, the samples were streaked on differential media namely Mac Conkey agar, Eosine Methylene blue agar. In Mac Conkey agar, out of 20 samples 15 samples produced pink colour colonies. In Eosine Methylene blue agar out of 15 samples 10 sample produced colonies with metallic sheen. Further identification of *E. coli* isolates was done by biochemical and sugar fermentation test using Enterbacteriaceae rapid kit. Simultaneously antibiotic sensitive test was also done to identify the sensitivity pattern of the *E. coli* isolates. PCR was done for the detection of virulence genes. The coliform mastitis further confirmed by 16s rRNA sequencing.

3.3. BIOCHEMICAL ANALYSIS IN SERUM AND PLASMA SAMPLES

- **Determination of Total Protein** Biuret method (Harold Varley, 1988).
- **Determination of Serum Albumin** Biuret Method (King et al., 1956).
- **Determination of Vitamin A** (Raghuramulu et al., 1983).
- **Determination of Vitamin B1**
  - The Vitamin B12 Assay was designed to quantitative patient sample between 60pg/ml and 1500pg/ml.
- **Determination of Iron** Dipyridyl method (Ramsay, 1958).
- **Determination of Calcium** O-Cresolphthalein Complexone method (Gitelman, 1967)
- **Determination of IgA**
  - The Determination of human IgA. Is based on the specific reaction between antigen and corresponding antibody. IgA in the serum was determined by Radial Immuno Diffusion test as described by Fahey and Mckelvey (1965) and Mancini et al., (1965).
- **Determination of Prolactin (PRL)**
Determination of prolactin is based on the electro chemiluminescence Immuno Assay (ECLICA) by using Elecsys by using Elecsys 1010 immunoassay analyzer (Smith et. al., 1996).

3.6. THE FOLLOWING BIOCHEMICAL PARAMETERS WERE ANALYZED IN EACH SAMPLE OF COLOSTRUM:

- Total proteins measured by Lowry’s method
- Triglycerides were estimated by GPOPAP method
- Cholesterol by enzymatic method of Richmond
- Lactose measured by Folin –Wu method
- Glucose by Hexokinase method
- Calcium was estimated by Arsenazo III colorimetric method
- IgA was estimated by Immuno-turbidimetric assay
- Determination of calcium and pH value of breast milk. Using the method of IITA (1979),
- Protein was determined using Folin Ciocalteau reagent (Lowry et al, 1951).
- Sugar content of human breast milk, the phenol-sulphuric acid method of Dubois et al (1956)

3.7. ISOLATION, CHARACTERIZATION, DETERMINATION OF PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA FROM HUMAN MILK

3.7.1. Isolation of Lactic Acid Bacteria from Human Breast Milk

The isolation material was human milk obtained from 40 healthy mother volunteers in KMCH hospital, Coimbatore and Kodaikanal government hospital and KHMS Hospital. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were taken to the procedure for isolation. Pour plate technique was used to isolate the organisms. Samples were used directly and also diluted to 10-1, 10-2 and 10-3 using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into MRS(M an, Rogosa and Sharpe) agar (pH 6.2 and pH5.5), TPY (Trypticase Phytone Yeast) agar (pH 6.5) and MRS-cystein agar (pH 5.5). The plates were incubated at 37 °C for 3 days under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact). The using of these mediums aimed to isolation and enumeration of lactobacilli, streptococci and enterococci. After incubation, individual colonies were selected and transferred into sterile broth mediums. The
following step is purifying the selected colonies with streak plate technique. The isolates were examined according to their colony morphology, catalase reaction and gram reaction. Gram positive and catalase negative cocci and bacilli colonies were taken to the glycerol stocks as lactic acid bacteria.

3.7.2. Gram Staining

The gram reaction of the isolates was determined by light microscopy after gram staining. LABS are known to be gram positive. It means that they give blue-purple color by gram staining. Cultures were grown in appropriate mediums at 37 °C for 24 h under anaerobic conditions. Cells from fresh cultures were used for gram staining. After incubation cultures were transferred aseptically into 1.5 ml eppendorf tubes and centrifuged for 5 min at 6000 rpm. Then, supernatant was removed and cells were resuspended in sterile 23 water. Gram staining procedure was applied. Then, under light microscopy gram Positives and purified isolates were determined.

3.7.3. Catalase Test

Catalase is an enzyme produced by many microorganisms that breaks down the hydrogen peroxide into water and oxygen and causes gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.

\[2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2\]

Catalase test was performed to isolates in order to see their catalase reactions. For this purpose, two methods can be applied. Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 h 3% hydrogen peroxide solution was dropped onto randomly chosen colony. Also fresh liquid cultures were used for catalase test by dropping 3% hydrogen peroxide solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were chosen. Since, LAB is known as catalase negative.

3.7.4. Long Term Preservation of Isolates

Gram positive and catalase negative isolates were preserved in MRS broth Medium containing 20% (v/v) glycerol as frozen stocks at -80 °C. The glycerol stocks of samples were prepared by mixing 0.5 ml of active cultures and 0.5 ml MRS medium including 40% sterile glycerol.

3.7.5. Probiotic Properties of Isolates

For the determination of probiotic properties of isolates these major selection criteria were choused: resistance to low pH, tolerance against bile salt and the Antimicrobial activity.
3.7.6. Resistance to Low pH

Resistance to pH 3 is often used in vitro assays to determine the resistance to stomach pH. Because the foods are staying during 3 hrs, this time limit was taken into account (Prasad, et al. 1998). For this purpose, active cultures (incubated for 16-18 h) were used. Cells were harvested by centrifugation for 10 min at 5000 rpm and 4 °C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2). Then cell pellets were resuspended in PBS (pH 3) and incubated at 37 °C. Viable microorganisms were enumerated at the 0., 1., 2, and 3. Hours with pour plate techniques. Appropriate dilutions were done and plates were incubated at 37 °C under anaerobic conditions for 48 h. Also growth was monitored at OD620 (Thermo Multiskan EX).

3.7.7. Tolerance against Bile

Because the mean intestinal bile concentration is believed to be 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 h (Prasad, et al. 1998). The experiment was applied at this concentration of bile for 4 h. MRS medium containing 0.3% bile (Oxoid) was inoculated with active cultures (incubated for 16-18 h). During the incubation for 4 h, viable colonies were enumerated for every hour with pour plate technique and also growth was monitored at OD620 (Thermo Multiskan EX).

3.7.8. Antimicrobial Activity

For the antimicrobial activity test, spot on lawn method was used. After 18 h incubation active cultures were spotted on the surface of MRS agar plates (2 strains for each plate). Then MRS plates were incubated to grow cultures for 24 h at 37 °C under anaerobic conditions. The next step is preparing the indicator microorganisms (Table 2.1). Overnight indicator pathogens inoculated (1%) to soft agar containing 0.7% agar and this inoculated agar were overlaid on MRS plates. These plates were incubated according to the appropriate conditions for indicator microorganisms. At the end of the incubation, inhibition zone diameters (surrounding the spotted isolates) were measured. Lots of researches were observed to give the results. Accordingly; isolates, which gave an inhibition zone bigger than 1 mm, were determined to have antimicrobial activity.

3.7.9. Indicator microorganism Incubation conditions

Bacillus cerus 37 °C in Nutrient broth medium
Escherichia coli 37 °C in TSB medium
Staphylococcus aureus 37 °C in Nutrient broth medium
3.7.10. Physiological and Biochemical Characterization
Biochemical tests were run according to methods offered by Bulut, 2003.

3.7.11. Gas Production from Glucose
In order to determine the homonfermentative and heterofermentative characterization of isolates, CO2 production from glucose test was applied. Citrate lacking MRS broths and inverted Durham tubes were prepared and inoculated with 1% overnight fresh cultures. Then the test tubes were incubated at 37 °C for 5 days. Gas occurrence in Durham tubes was observed during 5 days which is the evidence for CO2 production from glucose.

3.7.12. Growth at Different Temperatures
Temperature test media, MRS containing bromocresol purple indicator, was prepared and transferred into tubes as 5 ml. Then fifty µl of overnight cultures inoculated to tubes and incubated for 7 days at 10 °C, 15 °C, 45 °C. During these incubation time cells growth at any temperatures was observed by the change of the cultures, from purple to yellow.

3.7.13. Growth at Different NaCl Concentrations
Isolates were tested for their tolerance against different NaCl concentrations. For this purpose 4% and 6.5% NaCl concentrations were selected. Test mediums containing bromocresol purple indicator were prepared according to the appropriate concentrations and transferred into tubes in 5 ml. these tubes were inoculated with 1% overnight cultures and then incubated at 37 °C for 7 days. The change of the color from purple to yellow was proofed the cell growth.

3.7.14. Arginine Hydrolysis Test
Arginine MRS medium and Nessler’s reagent were used in order to see ammonia production from arginine. MRS containing 0.3% L-arginine hydrochloride was transferred into tubes as 5 ml and inoculated with 1% overnight cultures. Tubes were incubated at 37 °C for 24 h. After incubation, 100 µl of cultures transferred onto a white background. The same amount of Nessler’s reagent was pipetted on the cultures. The change in the color was observed. Bright orange color indicated a positive reaction while yellow indicated the negative reaction. A negative control, which did not contain arginine, was also used as negative control.

3.7.14. Carbohydrate Fermentations
Isolates were characterized according to their fermentation profiles of ability to ferment 17 different carbohydrates. All reactions were performed by using 96-well microtitre plates. Active cells and sugar solutions were prepared separately. For preparation of active cells; isolates were activated in 10 ml MRS medium and incubated at 37 °C for 24 h. Then, they were centrifuged 10 min at 10000 rpm. Pellets were washed twice and resuspended in MRS without glucose and containing pH indicator bromecresol purple. Each sugar solutions were prepared at a final concentration of 10% (w/v), only salicin was prepared at concentration of 5%. Then the solutions were filter sterilized with filters (0.22 µm pore diameter). After preparation steps the procedure was applied. Forty µl of sugar solutions were pipetted into each well and 160 µl of suspended cells were added onto the sugar solutions. Thus, 2% final sugar concentration was obtained. All the reactions were performed twice. Also positive and negative controls were used to indicate any contamination. 160 µl of suspended cells + 40 µl of glucose solution were used as positive control while 200 µl of suspended cells was used as negative one. After overnight incubation at 37°C, the turbidity and the color change from purple to yellow was recorded as positive fermentation results compared with the positive and negative controls. Also results were compared with the absorbance of samples read at 620 nm in an automated microplate reader (Thermo Multiskan EX).

3.8. MOLECULAR AND BACTERIOLOGICAL EXAMINATION OF COW MILK IN COLIFORM MASTITIS

- **Tryptose soy broth** (Appendix 1).
- **Tryptose soy agar** (Appendix 2).
- **Mae Conkey agar** (Appendix 3).
- **Eosine Methylene Blue agar** (Appendix 4).

3.8.1. GRAM STAINING REAGENTS:

1. Methyl violet (hucker’s ammonium oxalate crystal violet) (Appendix 5)
2. Gram’s iodine (Appendix 6)
3. Ethanol 95%
4. Saffranin 1% (Cruickshank et al., 1975).

3.8.2. BIOCHEMICAL TEST (Cruickshank et al., 1975).

1. Tryptone water (tryptone broth) (Appendix 7).
2. Kovac’s reagent (Appendix 8)
3. Methyl Red Reagent (Appendix 9)
4. Voges Proskauer Reagent (Appendix 10)
   - VP Reagent – a
   - VP Reagent – b

3.8.3. Citrate Test (Appendix 11) (Cruickshank et al., 1975)

3.8.4. Eijkman’s Test (Appendix 12) (Cruickshank et al., 1975)

3.8.9. SUGAR FERMENTATION TEST (Cruickshank et al., 1975)
   - Sugar disc
   - Culture
   - Andrade’s reagent

3.8.10. Peptone Water (Appendix 13) (Cruickshank et al., 1975)

3.9. ANTIBIOGRAM TEST (Cruickshank et al., 1975)
   - Antibiotic disc
   - Trypsoe Soy Agar
   - Culture

3.10. 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 37°C for overnight. (Cruickshank et al., 1975).

3.11. 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 37°C overnight. (Cruickshank et al., 1975).

Antibiotic Disc with Standard Cone.

<table>
<thead>
<tr>
<th>S. No</th>
<th>ANTIBIOTICS</th>
<th>STRENGTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gentamycin (G)</td>
<td>10mcg</td>
</tr>
<tr>
<td>2</td>
<td>Amoxyclov (Ac)</td>
<td>30mcg</td>
</tr>
<tr>
<td>3</td>
<td>Erythromycin (E)</td>
<td>15mcg</td>
</tr>
<tr>
<td>4</td>
<td>Trimethoprim (Tr)</td>
<td>30mcg</td>
</tr>
</tbody>
</table>
5 Ciprofloxacin (cf) 5mcg
6 Norfloxacin (Nx) 10mcg
7 Ampicillin (A) 10mcg
8 Azithromicin (AZ) 30mcg
9 Enrofloxacin 10mcg

3.12. INOCULATION INTO AGAR PLATES:
-Well isolated colonies from the TSA plates were streaked onto the following agar plate -Mac Conkey Agar
- Eosin Methylene Blue Agar. (Cruickshank et al., 1975).

3.13. GRAM STAINING:
A thin smear was made the Tryptose Soya Agar plate on a clean glass slide and subjected for Gram staining
1. A smear was made on a clean slide from the overnight culture, air dried and heat fixed.
2. Crystal violet [primary stain] was added on the smear and kept for 1 minute and washed with tap water.
3. Gram’s iodine [mordant] was then added, kept for 1 minute and washed with tap water.
4. Few drops of ethanol [decolorized] was added and washed with tap water.
5. Saffranin [counter stain] was added and kept for 30 seconds and washed with tap water.
6. The slide was blot dried and observed under oil immersion objectives. (Cruickshank et al., 1975).

3.14. BIOCHEMICAL TESTS
3.14.1. 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).

3.14.2. 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).

3.14.3. 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).

3.14.4. CITRATE UTILISATION
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).

3.14.5. EIJKMAN TEST

1. Durhams 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).

3.15. 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).

3.16. ANTIBIOGRAM TEST

The 8 h bacterial culture was spread on Trypbose soy agar using a sterile swab. The plates were dried and then the antibiotic disc were placed by means of a sterile forceps and incubated at 37°C overnight. Clear zone around the antibiotic disc indicated that the organism is sensitive. The diameter of each zone of inhibition was measured, recorded and interpreted according to the zone size interpretative charts (Cruickshank et al., 1975).

3.17. THE PROTOCOL OF SEQUENCING SERVICE

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 [Universal 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).

Table-2

**Primer Information**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Type</th>
<th>Type2</th>
<th>Sequence (5 to 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 518F</td>
<td>Universal</td>
<td>Forward</td>
<td>CCAgCAgCCgCggTAATACg</td>
</tr>
<tr>
<td>2 800R</td>
<td>Universal</td>
<td>Reverse</td>
<td>TACCAgggTATCTAATCC</td>
</tr>
<tr>
<td>3 27F</td>
<td>Universal</td>
<td>Forward</td>
<td>AgAgTTTgATCMTGGCTCAg</td>
</tr>
<tr>
<td>4 1492R</td>
<td>Universal</td>
<td>Reverse</td>
<td>TACggYTACCTTgTTACgACTT</td>
</tr>
</tbody>
</table>

Note:

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

**3.17.1. 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 ml of sterilizes saline in a 1.5 ml centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet is suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA). Incubated 56°C for 30 min and then heated 100°C for 10 min. After heating, supernatant can be use for PCR.

2. **PCR**

   Add 1 μl of template DNA in 20 μl of PCR reaction solution. Use 27F/1492R primers for bacteria, and then perform 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C 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).

3.18. STATISTICAL ANALYSIS

The level of significance in the variations of above biochemical parameters in blood in lactating women were determined by performing students ‘t’ test. It t value is 1.960 and above but below 2.576, then P<0.05 significant at 5% level. If t value is 2.576 and above, then P<0.01-1% level.

RESULTS AND DISCUSSION

Chapter IV
Biochemical Changes of Lactating Women Compare to Non Lactating Women in Serum and Plasma Samples
RESULTS AND DISCUSSION

Chapter IV

4.0. Biochemical Changes of Lactating Women Compare To Non Lactating Women in Serum and Plasma Samples

4.1. Introduction:

Lactation that is the formation of milk is possibly initiated in two ways; first, the act of suckling evokes, by a nervous reflex, the secretion of prolactin, growth hormone and ACTH by the anterior pituitary. These are all essential to lactation. Secondly, delivery of the placenta causes a sudden fall in concentration of progesterone in the blood, and this allows the release of prolactin.

The expulsion of milk from the alveoli is also controlled reflexly. Suckling causes the secretion of oxytocin by the neurohypophysis, and this stimulates the myoepithelial cells of the alveoli to contract. It is probable that thyroxine plays a part in the maintenance of an optimum yield of milk.

The first secretion of the breasts, following birth, is not milk but colostrum. This has a yellow color and contains cells from the alveoli. It is rich in the protein globulin, but low in fat. It is believed to be a means of passing antibodies, particularly IgA, from mothers to baby.

The aim of this study is to quantify the biochemical parameters in lactating women blood samples compare to non lactating women. Hence the biochemical parameters Serum Total
protein, Serum Albumin, Serum Vitamin – A, Serum Vitamin – C, Serum Vitamin – B12, Serum Iron, Serum Calcium, Serum IgA, Serum Prolactin are estimated.

Breast feeding helps the uterus shrink back its normal size. There is evidence that longer-term breastfeeding may help to reduce. Postnatal weight women who have breast fed may have a reduced risk of premenopausal breast cancer, ovarian cancer and hip fractures due to osteoporosis. Recent evidence from the World Health Organization has shown that breastfeeding is more than 98% effective as a birth control method if the mother is a full time breast feeder and feeding on demand day and night.

4.1.1. Materials and Methods:

4.1.1. Collection of Serum and Plasma samples:

10 ml of whole blood was collected from the lactating women and a non lactating woman blood was collected in tubes previously coated with 1% EDTA and was subsequently centrifuged at 2,500rpm for 15 min for separation of plasma. Blood taken in separate tube was allowed to clot for 20 min and was subsequently centrifuged for 15 minutes at 2,500rpm, for serum separation. The serum and plasma samples were collected and refrigerated (15°) until biochemical analysis.

4.1.2. Biochemical Analysis in Serum and Plasma samples

- **Determination of Total Protein** Biuret method (Harold Varley, 1988).
- **Determination of Serum Albumin** Biuret Method (King etal., 1956).
- **Determination of Vitamin A** (Raghuramulu et al., 1983).
- **Determination of Vitamin B1**
- The Vitamin B12 Assay was designed to quantitative patient sample between 60pg/ml and 1500pg/ml.
- **Determination of Iron** Dipyridyl method (Ramsay, 1958).
- **Determination of Calcium** O-Cresolphthalein Complexone method (Gitelman, 1967)
- **Determination of IgA**
The Determination of human IgA is based on the specific reaction between antigen and corresponding antibody. IgA in the serum was determined by Radial Immuno Diffusion test as described by Fahey and Mckelvey (1965) and Mancini et al., (1965).

- **Determination of Prolactin (PRL)**

Determination of prolactin is based on the electro chemiluminescence Immuno Assay (ECLICA) by using Elecsys by using Elecsys 1010 immunoassay analyzer (Smith et. al., 1996).

### 4.1.3. Results and Discussion

Forty lactating women in the age group ranging between 20-35 years and forty years who came to Kovai Medical centre and Hospital, Coimbatore, Tamilnadu and Government hospital, kodaikanal, KHMS hospital in kodaikanal for regular checkup were selected for this study. Twenty non-lactating women with corresponding age group were chosen as controls.

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Total Protein g/dl, Albumin g/dl, Vitamin A Mg/dl, Vitamin C Ug/dl, Vitamin B12 pg/dl, Serum Iron ug/dl, Serum IgA mg/dl, Prolactin ug/dl, Calcium mg/dl in lactating women compare to non lactating women</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S.No</th>
<th>Serum Samples</th>
<th>Non Lactating Women (A) Mean ±SD</th>
<th>Lactating Women (B) Mean ±SD</th>
<th>non lactating compared to lactating groups</th>
<th>T value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total Protein</td>
<td>6.63 ± 0.046</td>
<td>6.22 ± 0.034</td>
<td>A vs. B</td>
<td>17.492 **</td>
</tr>
<tr>
<td>2</td>
<td>Albumin</td>
<td>4.42 ± 0.040</td>
<td>3.92 ± 0.029</td>
<td>A vs. B</td>
<td>40.272 **</td>
</tr>
<tr>
<td>3</td>
<td>Vitamin A</td>
<td>45 ± 0.816</td>
<td>112.50 ± 0.192</td>
<td>A vs. B</td>
<td>61.307 **</td>
</tr>
<tr>
<td>4</td>
<td>Vitamin C</td>
<td>0.88 ± 0.816</td>
<td>1.30 ± 0.192</td>
<td>A vs. B</td>
<td>23.69 **</td>
</tr>
<tr>
<td>5</td>
<td>Vitamin</td>
<td>547 ± 1.633</td>
<td>121 ± 0.816</td>
<td>A vs. B</td>
<td>3.988 ns</td>
</tr>
</tbody>
</table>
It was observed that; Mean total protein level was decreased in lactating women when compared with non lactating women. The decreased in the mean total protein in lactating women was statistically significant at 1% level (P<0.01) level when compared with non lactating women. The mean serum albumin in serum of lactating women and non lactating women group are presented in Table I. It was evident from the data serum.

The certain protein fractions in human milk may aid in generating helper cell responses and in performing other immune regulatory functions. Albumin level was decreased in the
lactating women. The decrease in serum albumin level was statistically significant at 1% level when compared to non lactating women. Mal nutrition was characterized by lower weight to height indices and serum concentrations of total protein, albumin, IgG, IgA. The mean Vitamin-A level was increased in lactating women when compared with non lactating women. The increase in the mean Vitamin-A in lactating women was statistically significant at 1% level (P<0.01) level when compared with non lactating women. Hence Vitamin-A is important predictive marker for lactating.

PLATE 1
SINGLE RADIAL IMMUNO DIFFUSION FOR IMMUNOGLOBULIN A

Along with ensuring adequate vitamin A levels in breast milk for the infant, improving the vitamin A status of lactating women may benefit maternal health directly. Recent studies in human subjects indicate that an adequate dietary intake of retinol and carotene helps to prevent
breast and cervical cancer. Retinol derived from the circulating RBP-retinol complex is transferred from blood to milk. Most of it is re-esterified in the mammary glands and occurs as retinyl esters in milk whereas most of the vitamin A activity in mature human breast milk is in the form of retinol (retinyl esters), some is provided by carotene. β-carotene is stored in the mammary glands during pregnancy and is rapidly secreted into milk during the first few days of lactation. Thus, carotene provides almost 20% of the retinol equivalents during the first day, but this drops to less than 5% by the end of the first week. Unlike retinol, carotene is a very effective antioxidant and thus provides the infant a defense against oxygen toxicity. This may be particularly important during the first several days of life, as the infant adjusts to its new oxygen-rich environment.

The vitamin A content of human milk is significantly affected by maternal nutrition during pregnancy and lactation. The fat content of the milk, time after birth (postpartum age), gestational age at birth, parity of the mother, and individual variation also have an influence. The use of oral contraceptives is reported to affect the amount of retinol in human milk. Thus, it is important to consider these factors when evaluating milk vitamin A levels.

The mean Vitamin-C level was increased in lactating women when compared non lactating women. The increase in the mean Vitamin-C in lactating women was statistically significant at 1% level (P<0.01) level when compared with non lactating women. Season variations in Vitamin-A, Vitamin-C, riboflavin and folate intakes and status of lactating women. The nutrients in human milk most likely to be present in lower than normal concentrations in response to chronically low maternal intakes are the Vitamins. Especially vitamins B6, B12, and D those maintained at the expanse of maternal stores or tissues include the macro nutrients most minerals and folate. The mean serum Vitamin B12 in serum of the lactating women and non lactating women group. There were no significant variations in the mean Vitamin B12 in the lactating women.

The decrease in the mean iron in lactating women was statistically significant at 1% level (P<0.01) level when compared with non lactating women. In lactating women, the daily loss in milk about 0.3mg. Together with the basal iron losses of 0.8mg the total iron requirement during the lactation period amount to 1.1mg/day.

The decrease in the mean calcium in lactating women and non lactating women was statistically significant at 1% (P<0.01) level when compared with non lactating women. In
lactating rural women who have marginal nutrition and consume a high fiber diet, negative calcium balance may be expected.

The mean serum IgA in serum of the lactating women and non lactating women group. There was no significant variation in the mean IgA in the lactating women. The lower levels of IgG but similar levels of IgA in colostrums from well nourished and poorly nourished women. The increase in the mean prolactin in lactating women was statistically significant at 1% (P<0.01) level when compared with non lactating women. Hence prolactin is an important marker for lactating women.

**Conclusion**

This study was done in lactating women who were healthy. It was observed that their results showed an increase in the level of Vitamin-A, Vitamin-C, a decrease in the Iron, Calcium and Total Protein, Albumin, Vitamin-B12 and normal level of IgA. The present study has clearly shown that the biochemical changes in lactating women though there is an increase in Vitamin-A, Vitamin- C and prolactin and decrease in Iron, Calcium, and Total Protein, Albumin, Vitamin B12 and IgA levels were found to be normal in lactating women. There is abundant evidence that women are able to produce milk with adequate content of protein, fat, carbohydrate, and most minerals even when their supply of nutrients is limited. The nutrients in human milk most likely to be present in lower than normal concentrations in response to chronically low maternal intakes are the vitamins, especially vitamins B6, B12, A, and D.