MATERIALS & METHODS
MATERIAL AND METHODS

Newborn Nursery:

This study was conducted in the Department of Pediatrics, Wanless Hospital, Miraj and Civil Hospital Sangli, during the period January 1990 to April 1993. The Wanless Hospital has a neonatal intensive care unit with a managing capacity of 12-15 babies at a time and an average of 50 admissions every month. The neonatal intensive care unit is well equipped with a ventilator, ECG monitor, apnea monitors, pulse oximeter, oxygen monitor and other facilities to handle neonatal emergencies.

72 cases of suspected nosocomial infection were studied. The study group consists of 72 neonates, 40 full term, 32 premature which included 45 male and 27 females with clinical signs and symptoms suggestive of nosocomial infection and 78 age matched healthy controls. The healthy controls had no complications at the time of birth or during the hospital stay. There was no evidence of infection by clinically and by laboratory investigations.

The newborn in this study group had clinical manifestations suggestive of nosocomial infections such as "not doing
well", poor feeding, hypothermia abdominal distension, vomiting, diarrhoea, apnea, Jaundice, irritability, lethargy and high pitched cry etc.

Detailed clinical examination of the babies was done. Gestational age of the babies was determined by enquiring into the first day of mother’s LMP and subsequently confirmed by clinical criteria.

Clinical information collected for each neonate included birth weight, sex, gestational age, postnatal age, maternal illness or complications (fever — 37.5°C within 24 hrs. of delivery, use of intrapartum antibiotics etc.), intrapartum complications (time interval between rupture of membrane and delivery, meconium stained amniotic fluid, Apgar score) and nature of delivery. In those babies delivered in outside hospitals, the history about antenatal, natal and postnatal period was obtained.

Both nostrils, throat, umbilicus and rectum of all suspected newborns were swabbed on day 0, 1 and 3 by sterile, dry, cotton wool swabs. On day 0, swabs were taken in delivery room or in operation theatre. Premature babies were kept in the neonatal intensive care unit and hence on day 1 and 3 swabs were taken when the babies were in the neonatal
intensive care unit. While normal full-term newborns were kept with their mother’s in the PNC ward.

The method used for resuscitation when it was required were,

a) Suction b) endotracheal intubation. c) resuscitation with sodium bicarbonate, diluted with 10 % dextrose given through umbilical vein.

Cord care was taken by keeping the cord clean and dry only. Daily bath was not taken given to pre-mature babies; while full term babies bathed daily. Warm water was used to remove blood and varnish.

All newborns were observed daily for clinical features of any bacterial disease. In case of suspected bacterial disease, samples were collected for isolation and identification of pathogens and for their antibiotic susceptibility. All specimens were stored in refrigerator before being sent to Microbiology Department.

All the specimens were streaked directly on blood agar and Mac Conkey’s agar plates by standard plating method. Innoculated plates were incubated aerobically at 37°C for 18-24 hours.
The swabs were then smeared for Gram's staining and examined microscopically for presence of Gram positive and Gram negative organisms.

The inoculated plates after a period of incubation, were examined for the presence of bacterial colonies. Organisms were identified by colonial appearance, staining, morphology and by standard biochemical reactions. Tube coagulase method was used for detection of coagulase negative and coagulase positive Staphylococci.

Antibiotic susceptibility was performed by the method of Bouer et. al. (1966).

**BLOOD CULTURE:**

3 ml blood was collected under all aseptic precautions from femoral vein preferably prior to antibiotic therapy.

1 ml of blood was collected in 10 ml of tryptose phosphate broth and 1 ml in 10 ml of bile broth [Bhokoo et. al. (1974)] by inserting the sterile needle through the rubber washer and incubated at 37°C.

First subcultures were made from the tryptose phosphate
broth and bile broth on blood agar and MacConkey's agar medium on 2nd day and again after 5-7 days. 2nd subcultures were done if the 1st subcultures appeared negative. [Alex Sonnenwirth, Leonard Jarete (1980); Koshi (1981)].

**Serum Immunoglobulin Quantitation:**

Quantitative estimation of immunoglobulins was carried out in cord sera of all premature and full term newborns and in their mother's sera and also in 78 healthy neonates as control group.

Two ml of blood from the umbilical cord of newborns was collected and 3 ml of blood was collected by venipuncture, from mother's within the first 24 hours after delivery. Sera were separated and preserved by addition of one drop of sodium azide and stored at 20°C until processed.

Quantitative estimation of immunoglobulins were performed by using plates from Hoechst India Limited.

**C- Reactive Proteins : (CRP)**

1 ml of blood sample was collected from formal vein in a plain bulb. The CRP was done by latex slide agglutination
method using Ortho - CRP - slide agglutination kit.

Epidemiological study was taken up in later part.

Swabs moistened in nutrient broth were taken from anterior nares to study carrier rates in the hospital staff, nurses, surgeons, ward attendents and other para medical staff. Who were coming in contact with the patients.

A total of 175 samples were taken from different sites i.e. nose, throat and hands of hospital staff.

Simultaneously bacetriological study of hospital environment was done as follows :

Total 290 Samples were collected.

The blood agar and MacConkey’s agar plates were exposed to ward environment for 1 hour and colony counts were made after 18-24 hours incubation at 37°C. This was routinely done at interval of 15 days for dressing rooms, wards and varandah. The samples from ward floor, bath tub, walls, windows, tables incubators, cots, i.v. sets, water samples and mattresses were taken at the same time.
Individual organisms were isolated and identified as per standard procedures - [Cruickshank (1975)] and antibiotic susceptibility of the isolated organisms were carried out.

MATERIAL AND METHODS

BURNS:

The study was conducted over a period extending from January 1990 to April 1993. Patients admitted to the old burn ward, Wanless Hospital, Miraj were examined and samples were collected.

A total of 325 cases of different percentage of burns were studied for isolation of microbes. The study was continued for a period of three weeks in each case, whenever necessary it was extended further.

The first sample of blood culture and wound cultures were taken immediately after admission of the patient in the hospital.

Second set of samples were taken after one week. The samples from wounds were taken roughly from the site from
where the first samples were taken.

Third set of samples were taken in third week, the same procedure was followed.

The samples of blood of culture were taken in MacCortney bottles containing 50 ml of bile broth and 50 ml of tryptose phosphate broth. Ten ml of blood was collected from anticubiotal vein and 5 ml of blood was transferred to each blood culture medium. Blood samples were processed as routine. The bile broth and tryptose phosphate broth were incubated aerobically at 37°C for 48 hours.

The media were prepared as follows:

**Tryptose Phosphate broth**: (DIFCO manuel 1977)

- **Tryptose**  -  20 gm
- **Dextrose**  -  2 gm
- **Disodium Phosphate**  -  2.5 gm
- **Sodium Chloride**  -  5 gm
- **Distilled water**  -  1000 ml

pH is adjusted to 7.3 and autoclaved at 10 lbs for 10 minutes.
Bile Broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab Lemco</td>
<td>3.5 ml</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 gm</td>
</tr>
<tr>
<td>Sodium taurocholate</td>
<td>5 gm</td>
</tr>
<tr>
<td>Dextrose (Glucose)</td>
<td>10 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH is adjusted to 7.6 and bile salt is dissolved and autoclaved at 108°C for 15 minutes, cooled and dispensed in autoclaved bulbs with aseptic precautions and bottles were sealed.

First subcultures were made from tryptose phosphate broth and bile broth on blood agar and on MacConkey medium on 2nd day and again after 5-7 days. 2nd subcultures were done, if the first subcultures appeared negative. [Alex Sonnenwirth, Leonard Jarete (1980); Koshi (1981)].

Serum Immunoglobulin Quantitation:

Total 47 adult burn patients were selected irrespective of sex and with burns ranging from 15 per cent to 93 per cent.

Total 47 patients admitted to the burn unit of the Wanless Hospital, Miraj were examined and blood samples were
collecting during first two weeks of post burn period.

25 healthy adults - (Staff and donors), irrespective of sex were studied as control group for humoral immunity alone.

Serum Separation:

Collection of blood:

5 ml of blood in plain bulb was collected with aseptic precautions. Serum was separated and for half ml of serum 1 drop of sodium azide was added as preservative. Sera were preserved in deep freeze till processing.

Quantitative estimation of immunoglobulins were performed on immunodiffusion plates by single radial immunodiffusion technique. Plates from Hoechst India Limited were used and instructions given in the leaflet were strictly followed.

Quantitative estimation of C₃ levels were performed on NOR-partigen immunodiffusion plates from Behring.

C-reactive proteins were done by latex slide agglutination method using Ortho-CRP-slide agglutination kit.
The wound swabs were collected with the help of three swabs moistened in nutrient broth. One of the swab was used for aerobic culture and other was used for anaerobic culture in R.C.M. specimen from wound was also collected for fungal isolation.

Robertson's cooked meat medium was used for anaerobic culture for Clostridium tetani.

The aerobic culture was done on MacConkey's medium and blood agar plates. Aerobic culture was incubated for 18-24 hours at 37°C and anaerobic incubation was done for 5 days.

For aerobic organisms colonies were followed using standard procedures, - [Cruickshank, (1965)].

The final identification of organisms was made by extending the biochemical reactions, agglutination etc. One swab was inoculated on Sabouraud's medium for isolation of fungi.

A total of 330 samples were collected from burn ward staff and 757 samples were collected from burn ward environment. Simultaneously bacteriological study of hospital environment was done as follows.
The blood agar and MacConkey's agar plates were exposed to ward environment for 1 hour and colony count were made after 18-24 hours incubation at 37°C. This was routinely done at interval of 15 days for dressing rooms, wards and varandah. The samples from ward floor, bath tub, walls, windows, tables, cots, i.v. sets, bed-pans, water and mattresses were taken at the same time. Individual organisms were isolated and identified as per standard procedure, - (Cruikshank, 1975); and antibiotic sensitivity of the isolated organisms were carried out.

The isolated organisms were tested for various antibiotics by disc diffusion technique as recommended by [Bauer, et al, (1966)].

The following antibiotic discs of different strength were used.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>10 u</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 mcg</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30 mcg</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30 mcg</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 mcg</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 mcg</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 mcg</td>
</tr>
<tr>
<td>Polymyxin-B</td>
<td>300 u</td>
</tr>
</tbody>
</table>
For testing antibiotic sensitivity, 3 to 4 hours nutrient broth culture at 37°C of test strains as well as NCTC strains were used. The test strain was inoculated on nutrient agar plate. After the inoculum got dried up, the antibiotic discs were placed firmly on the medium. The plates were then incubated at 37°C overnight and result were read next day.

From time to time disc potencies were checked by using Oxford Staphylococcus NCTC 6571, Esch. coli NCTC 10418 and Pseudomonas aeruginosa NCTC 10662 by method recommended by Stokes, (1975).

Following tests were carried out for Staphylococcus aureus:

**CATALASE TEST**

This test demonstrates the presence of the enzyme catalase which catalyses the release of oxygen from hydrogen
peroxide. It helps to differentiate the Staphylococci micrococci group from other Gram positive cocci.

About 2 ml of hydrogen peroxide was taken in a test tube and a few colonies of Staphylococci picked up with a clean sterile glass rod and inserted into the solution. The production of effervescence almost immediately was taken as positive test.

The Staphylococci were then differentiated from Micrococci by their anaerobic growth and fermentation of glucose.

Coagulase Test:

The reagent used was human plasma. The suitability of the plasma was tested in slide and tube tests with standard known positive and negative strains as control.

Tube Method: (Guillespie, 1943)

A 1:10 dilution of human plasma in saline (0.85 percent NaCl) was made 1 ml of dilute plasma was taken in a small tube. 0.1 ml of an 18-24 hours broth culture was added into the tube. The tubes were incubated at 37°c and were examined for coagulation at 1, 3, 6 hours. Negative tubes
were left at room temperature overnight and re-examined.

The conversion of the plasma into a soft or stiff gel best seen by tilting the tube to the horizontal position, - indicates positive results.

Since the coagulum may be liquify sometime after it has been formed; it is necessary to examine the tubes at each of the time prescribed above.

A known positive and negative strains and a tube of uninoculated plasma was also set up as a controls.

VOGES PROSKAUER TEST :

This test depends on the production of acetylmethyl carbinol from pyruvic acid as an intermediate state in its conversion to 2:3 butylene glycol. In presence of alkali and atmospheric oxygen the small amount of acetylmethyl carbinol present in the medium is oxidised to diacetyl which reacted with the peptone of the broth to give a red colour.
Medium:

Glucose phosphate peptone water:

- Peptone 5 gm
- Dipotassium hydrogen phosphate $K_2HPO_4$ 5 gm
- Glucose 10% solution 50 ml

Dissolve the peptone and phosphate, adjust the pH to 7.6, filter, dispense in 5 ml amounts and sterilized at 121°C for 15 minutes. Sterilize the glucose solution by filtration and add 0.25 ml to each tube (final concentration 0.5 percent).

METHOD: (BARRITT)

Inoculate the liquid medium with colonies, incubate at 37°C for 48 hours. Add 1 ml of 40 percent potassium hydroxide and 3 ml of 5 percent solution of alpha-napthol in absolute alcohol. Aerate by shaking at intervals to ensure maximum aeration.

A positive reaction is indicated by the development of a pink colour in 2-5 minutes, becoming to Crimson in 30 minutes.
Isolated Staphylococcus aureus were sent to Moulana Azad Medical College, New Delhi for phage typing.

The strains of Pseudomonas aeruginosa isolated were identified by the following criteria:

1. Gram negative, motile bacilli.
2. Oxidase test positive
3. Non-fermentation of sugars, except glucose with acid production.
4. Utilization of citrate.

Then the following test were applied for final identification of the organism.

1. Pigment Production:

The pigment production was observed on nutrient agar. The isolates which fail to produce pigment on nutrient agar plate, then the Wahba and Darrells (1965) modified Sierra medium was used to induce pigment production.

The pyocynin production was observed after 24 hours incubation of cultures as a deep greenish blue colour, which is diffused into the medium.

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OXIDASE TEST (Kovaes, 1956) :

This test depends on the presence in bacteria of certain oxidase that will catalyse the transport of electrons between electron donors in the bacteria and a redox dye—tetramethyl-p-phenylene-diamine. The dye is reduced to a deep purple colour.

DRY FILTER PAPER METHOD :

Strips of Whatman's No. 1 filter paper are soaked in a freshly prepared 1 percent solution of tetramethyl-p-phenylene-diamine dihydrochloride. After draining for about 30 seconds the strips are freez-dried and stored in dark bottle.

For use, the strip is moistened with distilled water. The colony was picked up from culture plate and rubbed on moistened paper strip with the help of glass rod.

A positive reaction is indicated by an intense deep-purple colour appearing within 10 seconds.
NITRATE REDUCTION:

This test depends upon the capability of organisms of reducing either nitrate or nitrite (or both) to gaseous nitrogen.

The Nitrate/Nitrite broth was prepared as:

- Heart infusion broth: 25 gm
- Potassium nitrate: 2 gm
- Distilled water: 1000 ml

pH 7.0

Nitrate broth with Durham's tube were prepared. The broth was inoculated and incubated at 37°C for overnight. Gas formation in the Durham's tube indicates nitrate reduction.
CITRATE UTILIZATION TEST (SIMMONS)

Principle:

This is a test for the ability of an organism to utilize citrate as the sole carbon and energy source for growth and an ammonium salt as the sole source of nitrogen.

MEDIA:

Sodium Chloride 5.0 g
Magnesium Sulphate 0.2 g
Ammonium dihydrogen phosphate 1.0 g
Potassium dihydrogen phosphate 1.0 g
Sodium Citrate 5.0 g
Agar 20.0 g
Bromothymol blue 40 ml
(0.2 percent)
Distilled water 1000 ml

The pH should be 6.8. The medium is dispensed in the tubes and sterilized by autoclaving at 121°C for 15 minutes.
Method:

A light inoculum from a colony of growth of the test organism is streaked to the surface of the agar slant and incubated at 35°C for 24 hours.

Interpretation:

Positive: Blue colour and streak of growth.
Negative: Original green colour and no growth.

Oxidation metabolism of Glucose:

Oxidative fermentative (O-F) Test (Hugh and Leifson)

Principle:

The O-F medium of Hugh and Leifson differs from Carbohydrate fermentation media as follows:

1. The concentration of protein (peptones) has been decreased from 1% to 0.2%.

2. The concentration of carbohydrate is increased from 0.5% to 1.0%.
3. The concentration of agar is decreased to 0.25 from 1.5 making it semisolid in consistency.

The lower protein to carbohydrate ratio reduces the formation of alkaline amines that can neutralize the small quantities of weak acids that may form from oxidative metabolism. The relatively larger amount of carbohydrate serves to increase the amount of acid that can potentially be formed. The semisolid consistency of the agar permits acids that form on the surface of the agar to permeate throughout the medium, making interpretation of the pH shift of the indicator easier to visualise.

**MEDIA:**

O-F Medium of Hugh and Leifson

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>2.00 gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10.00 gm</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>0.03 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>2.50 gm</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>0.30 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The pH is adjusted to 7.1 before adding the bromothymol blue and the medium is autoclaved at 121°C for 30 minutes. The
carbohydrates sterilized separately and added to give a final concentration of 1 percent. The medium is tubed to a depth of about 4 cm.

METHOD:

Duplicate tubes of medium are inoculated by stabbing the medium almost to the bottom of the tube. One tube is covered by layer of sterile liquid paraffin to a height of 3 cms, and both are incubated at for 48 hours.

Oxidizing organisms - i.e. Pseudomonas - produce an acid reaction only in the open tube.

GELATIN LIQUIFICATION:

Principle:

Gelatinases are proteolytic enzymes capable of breaking down gelatin and other proteins into peptides and amino acids. Those bacteria that secrete gelatinase can be detected by observing the liquification of culture media or substrates containing gelatin following inoculation of the test organism and incubation for the appropriate period of time.
**MEDIA:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5 gm</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>3 gm</td>
</tr>
<tr>
<td>Gelatin</td>
<td>120 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Final pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

**PROCEDURE:**

Gelatin tubes must be kept in the refrigerator until just prior to use. With an inoculating wire containing a heavy inoculum from an 18 to 24 hours culture of the organisms to be tested stab the medium to a depth of 1/2 to 1 inch. Set up an uninoculated control tube. Incubate at 35°C for 24 hours to 14 days.

**Interpretation:**

Tubes were checked daily for 2 weeks. At the end of each 24 hour the tubes were placed in the refrigerator for 2 hours and examined for liquification.
ARGININE DIHYDROLASE TEST

Principle:

This test is based on the ability of some bacteria to decarboxylate an amino acid to the corresponding amine with the liberation of carbon dioxide. The production of these decarboxylases is induced by low pH and as a result of their action, the pH rises to neutrality or above.

MEDIA:

Peptone - 5 g
Meat Extract - 5 g
Glucose - 0.5 g
Pyridoxal - 5 mg
Bromocresol Purple - 5 ml
(1:500 solution)

Cresolred
(1:500 solution) - 2.5 ml
Distilled water - 1000 ml

Dissolve the solids in water and adjust the pH to 6.0 before the addition of the indicators. This is the basal medium and to it is added the amino acid whose decarboxylation is...
to be tested. Divide the basal medium into two portions and treat as follows:

1. Add 1 percent L-arginine hydrochloride
2. No addition (control)

Readjust the pH to 6.0 if necessary. Distribute 1 ml Quantities in the small tubes add sterile liquid paraffin to provide a layer about 5 mm thick above the medium. Autoclave at 121°C for 15 min.

Procedure:

The medium was inoculated lightly through the paraffin layer with a straingth wire and incubated at 37°C for overnight. Violet colours indicates positive test. The control should remain yellow.

Growth at 42°C and 4°C

The test strains were inoculated on to nutrient agar plates. One plate was incubated at 42°C and another at 4°C by keeping it in the refrigerator. Next day growth was observed at 42°C and no growth at 4°C.

Preservation of Ps. aeruginosa:
The strains identified as Pseudomonas aeruginosa were maintained on semisolid (0.5 percent) nutrient agar. These were stock cultures and were stored in refrigerator.

In all strains of Pseudomonas aeruginosa were used for 'pyocine typing' study. These strains were called as test strain. All these strains were subjected to pyocine typing and antibiotic sensitivity tests.

Pyocine typing:

Pyocine typing was done according to the method of Shriniwas (1974); which is a "Scrape and Streak" method. A set of 10 indicator strains were obtained from Prof. Shriniwas, N.P.C., Department of Microbiology, All India Institute of Medical Sciences, New Delhi.

The indicator strains were Whaba's eight indicator strains namely M₈, B₁₀, S₁₇, B₂₇ B₃₉, A₅₂, 8/39, 10/55. Indicator strains 9 and 10, AIIMS 785/76 and AIIMS 790/76 were isolated at All India Institute of Medical Sciences, New Delhi.
(A) MATERIALS:

The media include

1: Tryptone Glucose Agar (RICHER MEDIUM)
2: Nutrient Agar
3: Glucose broth

Tryptone Glucose Agar - (RICHER MEDIUM)

1: Tryptone - 5 gm

2: Beef extract - 3 gms

3: Glucose - 1 gm

4: Agar - 30 gms

5: Distilled water - 1000 ml

Dissolve the ingredients and adjust the pH 7.2. Sterilize
the medium by auto claving at 121°C for 15 minutes; add 0.05
% or 0.08 % CaCl₂ pour into suitable petriplates to
thickness of 8 mm.
B] METHOD FOR TYPING:

The test strains to be typed were inoculated first on nutrient agar plate. After overnight incubation of the plate a single colony of the test strain was picked up and inoculated into glucose broth and incubated at 37°C for 4 hours. The four hour growth of the test strain was streaked as 2 cm wide band in the middle of tryptone glucose agar with a sterile cotton swab. This plate was incubated at 32°C for 16 to 20 hours. The resulting growth at the end of incubation period was scrapped off using sterile glass slides and cotton swabs. The microscopic growth was killed by exposing it for 1-2 hours to chloroform placed in the lid of eliminated from plate by exposing into air for a few minutes.

Indicator strains:

Isolated colonies of 10 indicator strains were inoculated into glucose broth and incubated for 4 hours at 37°C. These indicator strains were streaked in parallel lines at right angles to the site of inoculation of test strains using sterile glass rods. Plates were then incubated at 32°C overnight. Any pyocine produced by the test strains diffuses into the medium during the first period of
incubation and then exert their inhibitory action on strains during the second incubation. The pyocine types of the test strains were recognised from the pattern of inhibition produced on the indicator strains.

The results were read as follows:

1 - Complete inhibition of growth of indicator strain

2 - No inhibition of indicator strain.

The inhibition patterns were recorded and labelled as typable and untypable. Random strains were again studied by the same method for its reproducibility and reliability.