APPENDIX-I

A. Clinical profile

Sample no.:	Patients on antibiogram:
Name:	Type of specimen:
Address:	Method of collection:
Sex / Age:	Time of collection:
Ward / Bed no.:	Date:

Short clinical history:

B. Collection of clinical specimens

1. Urine

i. Before the provision of container, each patient was provided with proper instruction for urine specimen collection.
ii. Five to 10 ml of CCMSU was requested in clean, sterile leak proof container.
iii. The specimen is transferred in laboratory as soon as possible.

2. Blood

i. Using a tourniquet, a suitable vein was located in the arm.
ii. Wearing gloves, the venipuncture site was thoroughly disinfected by using 70% ethanol and allowed to dry.
iii. Using a sterile syringe and needle, about 2-10 ml blood (10ml for adults and 2-5ml for children). Was withdrawn from the patients and dispensed to the sterile screw capped culture bottle containing 50ml BHI).

3. Pus

i. While collecting pus sample from abscesses, wound or other sites aseptic technique was followed so that the specimen not be contaminated by the skin microflora or by other external means.
ii. Pus from the abscess was collected at the time the abscess was incised or after it has been ruptured naturally.
iii. Five ml of pus was aspirated or collected from a drain tube and transported to a leak proof sterile container.
iv. If it was not available needle capped syringe itself was transported.
v. A sterile cotton swab was used to collect a sample from the infected site in the case of scanty or un-discharged pus.

4. Sputum:

i. Sputum collection was supervised by professional personnel familiar with the methods to achieve clinical correlations.

ii. For examination first morning specimen was preferred. Specimen was collected in a sterile, disposable, impermeable container with a screw cap or tightly fitting cap.

5. Fluid sample:

i. Fluid samples were collected by aspiration technique with a needle and syringe by an experienced and authorized physician.

ii. Aspiration site was located and the area was disinfected by 70% ethanol and allowed to dry.

iii. Using a sterile syringe and a needle about 1-5 ml of fluid was aspirated and transferred to 20ml BHI broth and transported to laboratory immediately.

C. List of equipments and materiales used during the study

1. Materiales

i.	Plastic containers	vi.	Pipettes	xii.	Inoculating loop
ii.	Plastic applicator sticks	vii.	Forceps	xiii.	Inoculating wire
		viii.	Droppers	xiv.	Petri plates
iii.	Glass slides	ix.	Glass rods	xv.	Cotton swab
iv.	Beakers	x.	Sticker	**xvi.**	Burner
v.	Test tubes	xi.	Filter paper		

2. Equipments:

Microscope:	Olympus, Japan
Refrigerator:	Toshiba, Japan
Incubator:	Vortex, India
Hot air oven:	Scout, USA
Water bath:	Grant, OLS 200, England
Safety cabinet	
Centrifuge	

3. Microbiological media (Hi-Media)

i.	Nutrient Agar	iv.	Blood Agar
ii.	Nutrient broth	v.	Muller Hinton Agar
iii.	Mac Conkey Agar		

4. Chemical reagents:

i. Catalase reagent (3% H_2O_2)

ii. Oxidase reagent (1% Tetramethyl p-phenylene diamine dihydrochloride)

iii. Plama

5. Antibiotics Discs

I. Hi-Media

amoxicillin (AMX, 10mg)

amikacin (AK, 30mcg)

ampicillin (AMP, 10mcg)

chloramphenicol (C, 30mcg)

ciprofloxacin (CIP, 30mcg)

cotrimoxazole (COT, 25mcg)

levofloxacin (LE, 5mcg)

nitrofurantoin (NIT, 30)

norfloxacin (NX, 10mcg)

oxacillin (OX, 1mcg)

vancomycin (VA, 30mcg)

cephalexin (CN, 30mcg)

cefotaxime (CTX, 10mcg)

erythromycin (E, 5mcg)

cloxacillin (30mg)

II. MAST and OXOID

cloxacillin (CLX, 1mcg, 5mcg, 30mg)

APPENDIX-II

A. Composition and preparation of different types of culture media (Hi-Media)

(Note: All compositions are given in grams per litre and at 25^{o}C temperature.)

1. Nutrient Agar (NA)

Peptone	5.0g
Sodium Chloride	5.0g
Beef Extract	1.5g
Agar	1.5g
Final P^{H}	7.4=0.2

2.8 gms of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^{o}C for 15 minutes.

2. Nutrient broth (NA)

Peptone	5.0g
Sdium chloride	5.0g
Beef Extract	1.5g
Yeast Extract	1.5g
Final P^{H}	7.4=0.2

1.3 gms of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^{o}C for 15 minutes.

3. Mac Conkey Agar (MA)

Pancreatic digest of gelatin	17.0g
Peptone	3.0g
Lactose	10.0g
Sodium Chloride	5.0g
Bile salt	1.5g
Agar	13.5g
Neutral red	0.03g
Final P^{H}	6.9-7.3

5.5gms of media was dissolved in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^{o}C for 15 minutes.

4. Blood Agar (BA)

Composition of Blood Agar base	15.0g
Proteose peptone	2.5g
Liver digest	5.0g
Yeast Extract	5.0g
Sodium Chloride Agar	15.0g
Final P^H	7.4

Blood agar base medium was prepared and autoclaved at 121^{o}C for 10 minutes. It is then cooled down to 48^{o}C and blood (7-10%) is added aseptically and mixed thoroughly. About 18-20 ml. of the media was then poured on Petri-plates. If bubbles appear in the poured plates, a flame is passed over the bubbled before the media sets.

5. Muller Hinton Agar

Beef infusion Broth	300.0g
Casein Acid Hydrolysate	17.0g
Starch	1.0g
Agar	17.0g
Final P^H	7.0=0.2

3.8 gms of media was suspended in 100 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at 121^{o}C for 15 minutes. It was then poured while at 45-48^{o}C into sterile petriplates in 25 ml quantity.

6. Peptone water

Peptone	10g
NaCl	5g
Final P^H	7.2=0.2

15 gm was dissolved in 1000 ml distilled water and was sterilized by autoclaving at 15 lbs pressure at 121^o C for 15 minutes.

B. Composition and preparation of different types of biochemical media

1. Hugh and Leifson Media (O/F media)

Peptone	2.0g
Sodium chloride	5.0g
Dipotassium hydrogen phosphate	0.3g
Bromothymol Blue	3ml
Agar	3g
Distilled water	1 litre
Final P^H	7.1

The P^H is adjusted to 7.1 before adding the bromothymol blue and the medium is autoclaved in a flask at 121^o C for 15 minutes. The carbohydrate to be added is sterilized separately and added to give a final concentration of 1%. The medium is then tubed to a depth of about 4 cm.

C. Composition and preparation of different reagents

1. Gram staining reagents

I. Crystal violet Gram stain

Crystal violet	20g
Ammonium oxalate	9g
Ethanol or methanol, absolute	95ml
Distilled water	1 litre

Preparation:

Crystal violet is weighed and transferred to a clean bottle and absolute ethanol is added and mixed until dye is completely dissolved.

Ammonium oxalate is weighed and dissolved in about 200 ml of distilled water. Then it was added to the stain and total volume is made 1 litre by adding distilled water and mixed well.

II. Iodine Solution

Potassium iodide	1.5g
Iodine	1.0g
Distilled water	150ml

Preparation:

Potassium iodide is weighed and transferred to a clean bottle

30-40 ml of distilled water is added to Potassium iodide and mixed until it is fully dissolved.

Iodine is weighed and added to potassium iodide solution and mixed well.

Final volume is made 150ml by adding distilled water and mixed well.

III. Acetone-alcohol decolorizer

Acetone	500ml
Ethanol (absolute)	475ml
Distilled water	25ml

To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then 500 ml acetone was added and mixed well.

IV. Counterstain solution

Safranine	10gm
Distilled water	1 lit

In a piece of clean paper, 10 gm of safranine was weighed and transferred to a clean bottle. Then 1 lit. distilled water was added to the bottle and mixed well until safranine dissolves completely.

IV. Catalase reagent (To make 100 ml)

Hydrogen peroxide solution	3ml
Distilled water	97ml

Preparation:

To 97 ml distilled water, 3 ml of hydrogen peroxide solution was added and mixed well.

V. Oxidase reagent (To make 100 ml)

Tetramethyl P-Phenylene diamine dihydrochloride (TPD)	0.1gm
Distilled water	10ml

Preparation:

This reagent was made by dissolving 0.1 gm TPD in 10 ml distilled water. To that solution strips of Whatman's no. 1 filter paper were soaked and drained for 30 secs. Then these stripes were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

APPENDIX-III

A. Colony morphology of staphylococci in different culture media

Blood agar

i. Large 2-4mm circular, smooth and glistening surface, entire edge, soft butyrous consistency and opaque pigmented appearance.

ii. The pigmentation is golden, with yellow, orange and cream buff or white.

iii. Some strains are haemolytic when grown aerobically.

MaCconkey agar

i. Small pin head size about 0.1-0.5mm, pink or orange due to lactose fermentation

B. Procedure of different biochemical tests

1. Gram's strain: (Mackie and McCartney Vol.2., 14th edition).

Isolated colony selected for staining:

1. Smear was made from pure culture by emulsifying a colony in normal saline and heat fixed.
2. Smear flooded with crystal violet for 1 mint.
3. Wash with water
4. Add Gram's Iodine for 1minute.
5. Wash with water.
6. Decolourise with absolute alcohol for 10-15secs.
7. Wash with water
8. Flood with saffranin for 1minute.
9. Wash with water, blot dry and examine under oil immersion objective of the microscope.

2. Catalase test

i. A small amount of isolated colony from pure culture was transferred to the surface of clean dry glass slide.

ii. A drop of 3% H_20_2 was placed onto the inoculum.

iii. The evolution of oxygen bubbles was recorded immediately.

iv. The slide was then discarded into a disinfectant.

3. Oxidase test

i. A piece of filter paper was placed in a clean petridish and 2-3 drops of freshly prepared oxidase reagent was added.

ii. Using a glass rod, a colony of test organism was smeared on the filter paper.

iii. It was observed for the development of blue purple colour within a few seconds

Observation – Spherical Gram positive cocci in group and pairs.

Test	***Staphylococcus***	***Micrococcus***
Catalase	+	+
Oxidase	-	+
O/F	Fermentative	Oxidative

4. Coagulase test

I. Slide test (to detect bound coagulase)

i. A drop of physiological saline was placed on end of a slide and colony of test organism was emulsified in each of the drops to make two thick suspensions.

ii. A drop of plasma was added to one of the suspensions and mixed gently. It was looked for clumping of the organism within 10 seconds. But no plasma was added to second suspension. This is used to differentiate any granular appearance of the organism from the coagulase clumping.

II. Tube test (to detect free coagulase)

i. The plasma was diluted 1 in 10 physiological saline (mixing 0.2 ml of plasma with 1.8 ml of saline)

ii. 3 tubes were taken and labeled as:
T = test organism (18-24 hour broth culture),
P = Positive control (*S. aureus* broth culture),
N = Negative control (sterile broth).

iii. 0.5 ml of diluted plasma was pipetted into each tube.

iv. About 5-5 drops each of test organism, *S. aureus* culture, and sterile broth was added to the tubes labeled 'T', 'P' and 'N' respectively.

v. After mixing gently, 3 tubes were incubated at 37^{o}C. It was examined for clotting after 1 hour. If no clotting occurs tubes were examined at 30 minutes intervals for up to 6 hours.

5. Oxidative-fermentative (O/F) test

i. Using a sterile straight wire, the test organism was inoculated to the bottom of two tubes of sterile O/F media.

ii. The inoculated medium in one of the tubes was covered with a 10mm deep layer of sterile paraffin oil.

iii. The tubes were incubated at 37^{o}C for 24 hours and then examined for carbohydrate utilization.

C. Antibiotic disc used and procedure of susceptibility test

1. Antibiotic disc used

Antibiotics used	symbol	Disc Content (mcg)	Diameter of Zone of inhibition (mm)		
			Resistant	Intermediate	Sensitive
Amikacin	AK	30	14	15-16	17
Amoxicillin	AMX	10	19	-	20
Ampicillin	AMP	10	28	-	29
Cloxacillin	CLX	1 5 30	18 18 30	- - -	24 30 40
Chloramphenicol	C	30	12	13-17	18
Cefotaxime	CTX	30	14	15-22	23
Cephalexin	CN	30			
Cotrimoxazole	COT	25	10	11-15	16
Ciprofloxacin	CIP	5	15	16-20	21
Levofloxacin	LE	5	13	14-16	17
Nitrofurantoin	NIT	30	14	15-16	17
Norfloxacin	NX	10	12	13-16	17
Oxacillin	OX	1	10	11-12	13
Erythromycin	E	15	13	14-22	23
Vancomycin	VA	30	14	15-16	17

2. Procedure of sensitivity test (Kirby-Bauer's Disc Diffusion Method)

In vitro susceptibility of the pure bacterial species to fifteen different antibiotics was determined using Kirby- Bauer disk diffusion technique using Muller-Hinton agar and antibiotic discs as described by the National Committee for Clinical Laboratory Standards (CLSI, 2006). One ml of each bacterial isolates prepared directly from an overnight agar plates adjusted to 0.5 McFarland Standard was inoculated using sterile swab into each of the Petri-dishes containing Mueller-Hinton agar and were allowed to stand for 30 minutes for pre-diffusion of the inoculated organisms.

Antibiotic discs were seeded into the petri dishes containing Mueller-Hinton agar (MHA) for each bacterial isolates. The AST of the isolates towards various antimicrobial discs was done by modified Kirby-Bauer M2-A9 disc diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI) using MHA as follows:

i. MHA was prepared and sterilized as instructed by the manufacturer.

ii. The pH of the medium was adjusted to 7.2-7.4 and the depth of the medium at 4mm (about 25 ml per plate) was maintained in petri dish.

iii. Using a sterile wire loop, a single isolated colony whose susceptibility pattern is to be determined was touched and inoculated into MHB tube and was incubated at 37oC for 2-4 hrs.

iv. After incubation, the turbidity of the suspension was matched with the McFarland standard tube number 0.5 (which is equivalent to 10 to power 4 organisms).

v. Using a sterile swab, an MHA plate was inoculated with the matched suspension using a carpet culture technique.

vi. The plate was then allowed to stand for 20-30 minutes for the pre-diffusion of the inoculated organisms.

vii. Using clean and sterile forceps, the above mentioned antibiotic discs (6 mm) were placed on the MHA. The discs were placed at the considerable distance apart from each other on a 90 mm Petri-dish. Then the plate was incubated at 37oC for 24 hrs.

viii. After incubation, the plates were observed for zone of inhibition and the diameters of inhibition zones were measured in millimeters (mm). The measurement was interpreted as sensitive and resistant according to the manufacture's standard zone size interpretative manual of CLSI (2006).

ix. The percentage resistance was calculated using the formula *PR=a/b×100, where 'PR'* was percentage resistance, '*a*' was the number of resistant isolates and '*b*' was the number of isolates tested with the antibiotic. The percentage sensitivity was calculated using the formula *PS=c/d×100, where 'PS'* was percentage sensitivity, '*c*' was the number of sensitive isolates and '*d*' was the number of isolates tested with the antibiotic.