APPENDIX-I

CLINICAL AND MICROBIOLOGICAL PROFILE OF PATIENT

Clinical Profile:

Name: … … … … … … … … … Lab No: …

Age / Sex: … … … … … … Date: …

Address: … … … … … … … … … …

Brief Clinical History:

Patient on antibiotics: Yes No

If Yes, Antibiotic(s) taken: 1) … … … … … … … … … 2) … … …

Duration of treatment: … … … …… … … …… … … …

Microbiological Profile:

Day 1 (… … / … … / … … … …)

Specimen: … … … … … … … … …

Time of sample collection: … … … … … … … … …

Mode of Collection: … … … … … … … … … Receiving time at the laboratory: … … …

Direct Microscopic observation (if necessary):

1) … … … … … … … … …

Incubation: 1) Aerobic 2) Anaerobic 3) Microaerophilic

Incubation temperature: … … … … … … … … … Incubation time: … … … … … … … … … …

Culture on: 1) … … … … 2) … … … … … 3) … … … … …

Day 2 (… … / … … / … … … …)

Reading of Culture Plates:

Media Used	Features	Shape	Size	Color	Texture	Opacity	Consistency

Gram staining results: … … … … … … … … … … … … … … … … …

Catalase: … … … … … … … … … … Oxidase: … … … …

Coagulase: … … … … … … … … … … Others: … … … … …

Provisional identification of organism: … … … … … … … … … … …

Inoculation on: 1) … … … … … … 2) … … … … … … 3) … … … …

Day 3 (… … / … … / … … … …)

Biochemical tests

TSI: … … … … … … … … … … SIM: … … … … … …

Citrate: … … … … … … … … … … Urea: … … … … … …

Others: … … … … … … … … … …

Organism identified as: … … … … … … … … … … … … … … …

Antibiotic sensitivity test method: Modified Kirby-Bauer method

Antibiotics used	Zone of inhibition (mm)	Interpretation

Day 4 (... .../ ... .../ ... ...)

Screening of the **m**ultidrug resistant isolates for ESBL **p**roduction

Screening agents used	Zone of inhibition (mm)	Interpretation
Cefotaxime		
Ceftazidime		

Day 5 (... .../ ... .../ ... ...)

ESBL **c**onfirmatory **t**est

Combination discs used	Increase in size of zone of inhibition (mm)	Interpretation
Cefotaxime-clavulanate		
Ceftazidime-clavulanate		

Confirmatory test for MBL production

MBL: Positive Negative

APPENDIX-II

LIST OF MATERIALS

1. Equipments

Autoclave Incubator

Hot air oven Compound Microscope

Refrigerator Deep Freeze

Weighing machine Water Bath

Gas burners Glasswares

Inoculating wire and loops

2. Microbiological media (HIMEDIA^{TM})

Blood Agar	Hugh and Leifson Media
Choocolate Agar	Sulphur Indole Motility Media
Mac conkey agar	MRVP Broth
Mueller Hinton Agar	Triple Sugar Iron Agar
Mueller Hinton broth	Simmon's Citrate agar

3. Chemicals and reagents

Catalase reagent (3% H_2O_2)

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

Kovac's reagent

Barritt's reagent (40% KOH, 5% α-naphthol in a ratio of 1:3)

Barium Chloride

Sulphuric acid

Ethylene Diamine Tetra Acetate (EDTA)

Glycerol

Gram's reagent

4. Antibiotic discs

Amikacin (30µg)	Cefotaxime (30µg),
Ceftazidime (30µg)	Cotrimoxazole (25µg)
Ciprofloxacin (5µg)	Chloramphenicol (30µg),
Imipenem (10µg)	Norfloxacin (10µg)
Nitrofurantoin (300µg)	Nalidixic acid (30µg)
Ofloxacin (5µg)	Cefalexin (30µg)

5. Discs for ESBL confirmation

MASTDISCS^{TM} ID

Extended Spectrum β-lactamase (ESBL) Detection Discs (D52C)

APPENDIX-III

BACTERIOLOGICAL MEDIA

1. Blood agar (BA)

Blood agar base (infusion agar) + 5-10% sheep blood

Ingredients	gm/liter
Beef heart infusion	500.0
Tryptose	10.0
Sodium Chloride	5.0
Agar	15.0
Final pH (at 25^0C) 7.3±0.2	

Preparation: About 42.50 grams of the blood agar base medium was suspended in 1000 ml distilled water and sterilized by autoclaving at 121^0C (15lbs pressure) for 15 minutes. After cooling to 40-50^0C, 7% sterile defibrinated sheep blood was added aseptically and mixed well before pouring.

2. MacConkey Agar (MA)

(Without sodium taurocholate, without salt and crystal violet)

Ingredients	gm/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Sodium chloride	5.0
Neutral Red	0.04
Agar	20.0
Final pH (at 25^0C) 7.4±0.2	

Preparation: As directed by manufacturer company, 55 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

3. Mueller Hinton Agar (MHA)

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Acid Hyrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C) 7.4±0.2	

Prepartion: As directed by manufacturer Company, 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve. 10 ml was distributed in test tubes and sterilized by boiling in water bath for 10 minutes.

4. **Nutrient Agar (NA)**

Ingredients	gm/litre
Peptone	10.0
Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25^0C) 7.4±0.2	

Preparation: About 37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes.

5. **Nutrient Broth (NB)**

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Final pH (at 25^0C) 7.4±0.2	

Preparation: About 13 grams of the medium was dissolved in 1000 ml distilled water and autoclaved at 121^0C for 15 minutes.

6. Tryptic Soy broth+ 20% Glycerol

Ingredients	gm/litre
Pancreatic Digest of Casein	15.0
Enzymatic Digest of Soybean Meal	5.0
Sodium Chloride	5.0
Glycerol	200ml

Preparation: About 40 gram of the media was suspended in 1 litre of distilled water containing 200ml glycerol and mixed thoroughly. It was boiled completely and autoclaved at 121^0C for 15 minutes.

Biochemical Test Media

1. MR-VP Medium

Ingredients	gm/litre
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^0C) 6.9±0.2	

Preparation: As directed by manufacturing company, 17 grams was dissolved in 1000 ml distilled water. 3 ml of medium was distributed in each test tube and autoclaved at 121^0C for 15 minutes.

2. **Hugh and Leifson's Medium**

Ingredients	gm/litre
Tryptone	2.0
Sodium Chloride	5.0
Dipotassium Phosphate	0.3
Bromothymol Blue	0.08
Agar	2.0
Final pH (at 25^0C) 6.8±0.2	

Preparation: About 9.4 grams of the medium was rehydrated in 1000 ml cold distilled water and then heated to boiling to dissolve completely. The medium was distributed in 100 ml amounts and sterilized in the autoclave for 15 minutes at 15 lbs pressure (121^0C). To 100 ml sterile medium aseptically added 10ml of sterile Dextrose and mixed thoroughly and dispensed in 5 ml quantities into sterile culture tubes.

3. Sulphide Indole Motility (SIM) medium

Ingredients	gm/litre
Beef Extract	3.0
Peptone	30.0
Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0
Final pH (at 25^0C) 7.3±0.2	

Preparation: As directed by manufacturing company, 36 grams of the medium was suspended in 1000 ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized.

4. Simmon Citrate Agar

Ingredients	gm/litre
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25^0C) 6.8±0.2	

Preparation: As directed by the manufacturing company, 24.2 grams of the medium was dissolved in 1000ml distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

5. **Triple Sugar Iron (TSI) Agar**

Ingredients	gm/litre
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25^0C) 7.4±0.2	

Preparation: About 65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of thickness.

Staining and Test Reagents

1. Gram's Stain reagents:

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml

Distilled Water (D/W) to make 1 litre

(b) Gram's Iodine solution

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

2. Normal saline

Sodium Cholride	0.85 g
Distilled Water	100 ml

3. Test Reagents

For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Preparation: To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

For Oxidase Test

Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1gm
Distilled Water	100 ml

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

For Indole Test

Kovac's Indole Reagent

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Hydrochloric acid	10 ml

Preparation: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

For Methyl Red Test

Methyl Red Solution

Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Preparation: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

For Voges-Proskauer Test (Barritt's Reagent)

Solution A

α-Napthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Preparation: To 25 ml D/W, 5 g of α-Napthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Preparation: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

100mM EDTA solution for MBL detection

1.86 gm of EDTA (Disodium salt, MW 372.24) was dissolved properly in 50 ml of sterilized distilled water and the reagent was kept aseptically.

Appendix-IV

MICROBIOLOGICAL PROCEDURES

A. **Gram-staining Procedure**

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

B. Catalase test

During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. Catalase enzyme breaks down hydrogen peroxide into water and oxygen. The enzyme catalase is

present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* sp.

A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g., Blood Agar) or if an iron wire loop is used.

C. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test.

A piece of filter paper was soaked with few drops of oxidase reagent. Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

D. Oxidation-Fermentation test

This test is done to determine the oxidative or fermentative metabolism of carbohydrate resulting in production of various organic acids as end product. Some bacteria are capable of metabolizing carbohydrates (as exhibited by acid production) only under aerobic conditions, while others produce acid both aerobically and anaerobically. Most medical bacteria are facultative anaerobes.

The test organism was stabbed into the bottom of two sets of tubes with Hugh and Leifson's media, bromothymol blue being the pH indicator. The inoculated medium in one of the tubes was covered with a 10 mm deep layer of sterile paraffin oil. The tubes were then incubated at 37°C for 24 hours. After incubation the tubes were examined for carbohydrate utilization as shown by acid production.

Fermentative organism utilizes the carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium from green to yellow.

Oxidative organisms, however, are able to use the carbohydrate only in the open tube.

E. Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indoleacetic acid.

A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 0.5 ml of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

F. Methyl Red test

This test is performed to test the ability of an organism to produce sufficient acid from the fermentation of glucose to give a red color with the indicator methyl red (denotes changes in degree of acidity by color reactions over a pH range of 4.4-6.0).

A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity.

G. Voges Proskauer (VP) test

This test is employed to detect the production of acetyl methyl carbinol (a neutral end product) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates.

A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red colour.

H. Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

I. Motility test

The motility media used for motility test are semisolid, making motility interpretations macroscopic. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

J. Triple Sugar Iron (TSI) Agar

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium).

The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

APPENDIX-V

ANTIBIOTIC SUSCEPTIBILITY TESTING

Disc diffusion methods for antibiotic susceptibility testing

A. **Preparation of 0.5 Mc Farland standard**

Add 0.5 ml of 0.048M $BaCl_2$ (1.17% w/v BaCl2.2H2O) to 99.5 ml of 0.18M H_2SO_4 (1% v/v) with constant stirring.

B. **Preparation of inoculum**

By touching 2-3 morphologically similar colonies with sterile loop, inoculate into MHB or NB and incubate at 37^0C until turbidity matches with that of 0.5 Mc Farland Standard. Direct colony suspension method can also be used.

C. Inoculation of agar plates

The agar plates, canister of discs are brought to room teperature before use. It should be made sure that the agar surface doesn't have any moisture, if so should be dried by keeping it in incubator. Using a sterile swab, a plate of Mueller-Hinton agar is inoculated with the bacterial suspension using carpet culture technique. The plate is left for about 5 minutes to let the agar surface dry. Using sterile forceps, appropriate antimicrobial discs (6 mm diameter) is placed, evenly distributed on the inoculated plates, not more than 6 discs are placed on a 90 mm diameter Petri plate. Within 30 minutes of applying the discs, the plates are incubated at 35^0C for 16-18 hrs. After overnight incubation, the plates are examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition in mm is measured and results interpreted accordingly.

D. Quality Control

QC strains Escherichia coli ATCC 2592

- Monitoring Accuracy
- Running AST for QC strains side by side with pathogenic bacteria
- Monitoring the expiry date of antibiotic discs and MHA
- Comparing zone size with CLSI QC tables

1**. Inhibitor potentiated disc diffusion (IPDD) test/ combined disc assay for ESBL confirmation using MASTDISCS^{TM} ID extended spectrum β-lactamase(ESBL) detection discs (D67C)**

ESBL production was confirmed among the suspected bacterial strain according to the gudelines of CLSI (Clinical and Laboratory Standard Institute) for phenotypic confirmatory testing. According to these guidelines, when confirming ESBL production among the suspects using Combined Disk (CD) assay, an increase in zone size of ≥5 mm from either of the combination disk i.e. clavulanate containing disk indicates the presence of ESBL in the test organism.

The suspected organism was inoculated into Mueller hinton broth and incubated at 37^0C until the turbidity matched 0.5 Mc Farland atandard.Using a sterile cotton swab the test orgaism was carpet cultured on a MHA plate.

With the help of sterile forcep, the ESBL detection discs were placed onto the inoculated medium ensuring that they are evenly spaced.

The plate was incubated at 35-37^0C for 18-24 hours and the results interpreted.

Interpretation of results

Compare the zone of inhibition for the ceftazidime, cefotaxime and cefpodoxime discs to that of the ceftazidime, cefotaxime and cefpodoxime plus clavulanic acid combination discs. An increase in zone diameter of ≥5 mm in the presence of clavulanic acid from any or all of the discs indicates the presence of ESBL in the test organism.

Quality control

Check for sign of deterioration. Quality control mst be performed with at least one organism to demonstrate positive reaction and at least one to demonstrate negative reaction.

Positive Control: *Escherichia coli* NCTC 13351
Negative Control: *Escherichia coli* ATCC 25922

2. EDTA combined disk -Confirmation of MBL production using Imipenem and EDTA Disk Synergy Test (EDST)

Currently there are no Clinical and Laboratory Standard Institute (CLSI) criteria for the phenotypic detection of MBL production. MBL detection thus depend on the ability of the chelating agent like EDTA and thiol based compounds which remove the zinc from active site of the enzyme thus inhibiting its activity. In this study, we used a comparatively simple method for the detection of MBL production based on the bacterial genera to be tested for MBL production.

For *Pseudomonas aeruginosa*

A suspension of the test organism equivalent in density to a Mc Farland 0.5 opacity standard was prepared using a pure and fresh culture. Using a sterile swab, test organism was carpet cultured on Mueller Hinton Agar. With the help of sterile forcep two ceftazidime (30µg) disks were placed at 4-5 cm distance apart. A blank disc (Mast BD0680) was placed near one ceftazidime disk with center to center distance of 20mm. To the blank disc 5µl of 100mM EDTA solution was added aseptically. The plate was incubated at 35-37^{0}C for 18-24 hours.

Inetrpretation of result

A synergy between two disks is indicative of MBL production.

For Enterobacteriaceae and others:

A suspension of the test organism equivalent in density to a Mc Farland 0.5 opacity standard was prepared using a pure and fresh culture. Using a sterile swab, test organism was carpet cultured on Mueller Hinton Agar. With the help of sterile forcep two Imipenem (10µg) disks were placed at 4-5 cm distance apart. To one Imipenem disk 10µl of 100mM EDTA solution was added aseptically. The plate was incubated at 35-37^{0}C for 18-24 hours.

Inetrpretation of result

The plate is observed for the increase in inhibition zone of the Imipenem-EDTA disk as compared to Imipenem Disk alone. The increase in zone size of ≥5mm is positive for MBL production.

APPENDIX VI

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF ANTIBIOTICS (CIPROFLOXACIN)

Principle

Minimum inhibitory concentration are considered the gold standard for determining the susceptibility of organisms to antimicrobials and therefore used to judge the performance of all other methods of susceptibility testing. MICs are widely used to give a definitive answer when borderline result is obtained by other methods of testing or when disc diffusion methods are not appropriate and are important in the evaluation of antibiotics breakpoints.

Materials

Media, equipment and reagent for determination of MIC were as described in appendix I.

Quality control

A. QC strains

1. Escherichia coli ATCC 25922

B. Monitoring accuracy

- Test QC strains by following routine procedure, and record results. Record lot number and expiration date of antibiotic powder.
- Compare to expected results (CLSI QC tables). Note any out of control result and document; proceed with corrective action, if necessary.
- Perform daily and weekly QC testing.

Procedure

1. Antibiotic powder, solvents and diluents

- Obtain standard powder from the pharmaceutical company or a reputable supplier such as Sigma (Poole, Dorset, UK)
- Obtain the information from the supplier regarding expiry date, potency, solubility, stability as a powder and in the solution, storage conditions and any relevant information.

- Always prepare stock solutions following the manufacturer's recommendations.
- Freeze and thaw stock solution only once and then discard them.

2. Preparation of antibiotic stock solutions and dilution range

- Choose a suitable range of antibiotic concentration for the organisms to be tested.
- Prepare stock solution using the formula 1000/P×V×C = W. Where P = potency given by the manufacturer (µg/ml), V = volume required (ml), C = final concentration of solution (multiples of 1000) (ml), and W = weight of antibiotic in mg to be dissolved in volume V (ml).
- For the preparation of further stock solutions and dilution range, from the solution, prepare as described in chart below.
- Dilution range for each antibiotic is prepared similarly. Solvents, diluents, dilution range and storage condition for antibiotic solution is described in appendix.

3. Preparation of agar dilution pates

- Prepare Mueller-Hinton agar following the manufacturer's instructions.
- Add 1 ml of working antibiotic solution to each container containing 19 ml of cooled agar (ensure that the medium is cooled to be 45 degree Celsius before adding to the antibiotic), including the antibiotic-free control. Mix well before pouring into 90mm Petri dishes.
- Allow agar to set and then dry surface of the plates for 10 min in a fan assisted drying cabinet (without ultraviolet light) or in an incubator (time needed depend on the efficacy of incubator).
- Store plates at 4-8ºC protected from light until inoculated. Ideally, plates should be used on the day of preparation. If plates are to be stored at 4-8ºC before use, the stability of the drug must be determined by individual laboratories as part of the routine quality control programme.

4. Preparation of inoculum

The inoculum should be adjusted so that 10^4 cfu/spot are applied to the plates. The following procedure describes a method for preparing the desired inoculum by comparison with a 0.5 McFarland standard.

4.1. Preparation of the McFarland standard

- Add 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl2.2H_2O$) to 99.5 ml of 0.18 M H_2SO_4 (1% v/v) with constant stirring. Distribute the standard into screw cap tubes of the same size and with the same volume as those used in growing the broth cultures.
- Seal the tubes tightly to prevent loss by evaporation. Store protected from light at room temperature. Vigorously agitate the turbidity standard on a vortex mixer before use. Standards may be stored for up to six months after which time they should be discarded.

4.2. Preparation of inoculum

Touch at least four morphologically similar colonies with a sterile loop. Transfer growth into Tryptone Soya broth and incubate broth with shaking at 35-37ºC until the visible turbidity is equal to or greater than the 0.5 McFarland standard. Alternatively an overnight broth culture can be used.

4.3. Adjustment of the organism suspension to the density of the 0.5 McFarland standards. Adjust the density of the organism suspension prepared to equal that of the 0.5 McFarland standards by adding sterile distilled water. To aid comparison, compare the test and standard against a white background with a contrasting black line. Suspensions should contain between 10^7 and 10^8 cfu/ml depending on genera. For the agar dilution method further dilution of suspension in sterile distilled water (1: 10 for *S. aureus*) is carried out before inoculation.

5. Quality control

E. coli ATCC 25922

6. Inoculation

Use a multipoint inoculator to deliver 10µl of suspension on to the surface of the agar. Allow the inoculums to be absorbed into the agar before incubation.

7. Incubation conditions

Incubate at 35ºC in air for 24-48 hours.

8. Reading and interpretation

After incubation ensure that all of the organisms have grown on the antibiotic-free control plate. The MIC is defined as the lowest concentration of antibiotic at which there is no visible growth of the organism. The growth of one or two colonies or a fine film of growth should be disregarded. The MIC for the control strain should be within plus or minus one two-fold dilution of the expected MIC.

Precautions

- The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use.
- All the materials used within their expiration dates and stored at the proper temperature.
- The incubator is at proper temperature and atmosphere.
- The control strain has not changed and is not contaminated.
- Inoculum suspensions were prepared and adjusted correctly.
- Inoculum for the test was prepared from a plate incubated for the correct length of time and in no case more than 24 hours old.

Table 13: Preparation of dilution of agents for using in agar dilution susceptibility tests

Table 2 Preparation of dilutions of agents for use in agar dilution susceptibility tests

Antimicrobial concentration (mg/L) in stock solution	Volume stock solution (mL)	Volume distilled water (mL)	Antimicrobial concentration obtained (mg/L)	Final concentration in medium after addition of 19 mL of agar
10 240	1	0	10 240	512
10 240	1	1	5120	256
10 240	1	3	2560	128
2560	1	1	1280	64
2560	1	3	640	32
2560	1	7	320	16
320	1	1	160	8
320	1	3	80	4
320	1	7	40	2
40	1	1	20	1
40	1	3	10	0.5
40	1	7	5	0.25
5	1	1	2.5	0.125
5	1	3	1.25	0.06
5	1	7	0.625	0.03
0.625	1	1	0.3125	0.015
0.625	1	3	0.1562	0.008
0.625	1	7	0.0781	0.004

APPENDIX- VIII

Table **14**: **Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae**

Species	Test/ substrate[a]											
	lac	mot	gas	ind	VP	cit	PDA	ure	lys	H_2S	inos	ONPG
Escherichia coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. Sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
Salmonella typhi	-	+	-	-	-	-	-	-	+	+	-	-
Salmonella paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
Citrobacter freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
Klebsiella pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
Enterobacter aerogens	+	+	++	-	+	+	-	-	+	-	+	+
Ent. Cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens[b]	-	+	±	-	+	+	-	-	+	-	±	+
Proteus mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
Morganella morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
Prov. Stuartii	-	+	-	+	-	+	+	±	-	-	+	-
Prov. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica[c]	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

[a] lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl-β-D-galactopyranoside.

[b] Some strains of *Serratia marcescens* may produce a red pigment

[c] *Yersinia* are motile at 22°C.

{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; 16-84% of strains are positive after 24-48 hour at 36°C}

(Source: Collee *et al.,* 1996)

APPENDIX-VIII

Table 15: Distribution of patients requesting for urine culture, growth pattern and MDR percentage in different age group and sex

Age group	ng Patients requesti for urine cuture		Significant Growth				strains MDR Total (%)
	Male	Female	Male		Female		
			.No (%)	MDR strain (%)	.No (%)	MDR strain (%)	
≤14	8	12	2	1(50)	2	2(100)	3(75)
15-24	18	22	2	2(100)	5	3(60)	5(71.42)
25-34	52	33	4	4(100)	2	2(100)	6(100)
35-44	42	43	8	7(87.5)	9	7(77.77)	14(82.35)
45-54	41	40	6	4(66.66)	9	9(100)	13(86.66)
55-64	44	40	6	6(100)	13	13(100)	19(100)
≥65	62	39	15	15(100)	17	13(76.47)	28(87.5)
Total	267	229	43(16.10)	39(86.66)	56(24.45)	49(87.5)	88 (88.88)

Table 16: Age-wise distribution of different pathogens and MDR strains from different sample

	Age group							Total
	less than 14	15-24	25-34	35-44	45-54	55-64	More than 65	
Escherichia coli	1 (1)	5(3)	3(3)	13(12)	11(9)	17(17)	21(18)	71(63)
Klebsiella pneumoniae	2(2)	2(2)	3(3)	3(2)	3(3)	2(2)	6(5)	21(19)
Pseudomonas aeruginosa	0	0	0	1(0)	0	0	2(2)	3(2)
Proteus vulgaris	0	0	0	0	1(1)	0	3(3)	4(4)

Table 17: Antibiotic susceptibility pattern of *E. coli* isolated from different samples

Antibiotics used	Sensitive	Intermediate	Resistant	Total
	No of isolates (%)	No of isolates (%)	No of isolates (%)	
Imipenem	58 (77.3)	9 (12)	8 (10.7)	75
Doxicycline	26 (34.7)	17 (22.7)	32 (42.6)	75
Norfloxacin	21 (28)	7 (9.3)	47 (62.7)	75
Cephalexin	4 (5.3)	0	71(94.7)	75
Nitrofurantoin	32 (42.7)	14 (18.7)	29 (38.6)	75
Ciprofolxacin	20 (26.7)	9 (12)	46 (61.3)	75
Cotrimoxazole	34 (45.3)	2 (2.7)	39 (52)	75
Amikacin	47 (62.7)	14 (18.6)	14 (18.7)	75
Ofloxacin	26 (34.8)	2 (2.7)	47 (62.7)	75
Nalidixic Acid	3 (4)	13 (17.3)	69 (78.7)	75
Ceftazidime	17 (22.7)	7 (9.3)	51 (68)	75
Ceftriaxone	13 (17.3)	3 (4)	59 (78.7)	75

Table 4. MIC value of ciprofloxacin for different isolates (Range 0.004-128 and Break Point 0.015 µg/ml)

SN	Sample	MIC value
1	12	4
2	13	0.03
3	28	8
4	32	4
5	33	4
6	36	16
7	37	128
8	38	8
9	39	32
10	43	8
11	45	32
12	49	32
13	50	2
14	52	32
15	53	0.004
16	54	2
17	55	4
18	56	8
19	57	4
20	59	4
21	41	4
22	61	64
23	63	32
24	64	32
25	66	16
26	67	0.004
27	70	64
28	72	8
29	73	32
30	76	8
31	82	4
32	79	8
33	80	8
34	81	16
35	82	8
36	83	16
37	84	0.004
38	85	0.004
39	86	8
40	88	4
41	89	1
42	90	0.125
43	91	0.004
44	68	16

APPENDIX-IX

1. Association between culture positivity among genders

		Growth		Total
		Positive	Negative	
Gender	Male	44	233	277
	Female	59	181	240
Total		103	414	517

Test statistics is $\aleph^2$

H_O: There is no significant association between culture positivity among genders.

H_1: There is significant association between culture positivity among genders.

From $\aleph^2 = \frac{(O-E)^2}{E}$ we find $\aleph^2 = 6.099$

Thus $\aleph^2_{cal}$ (6.099) $>\aleph^2_{tab}$ at α =0.05 and d.f = 1 i.e.3.841

Hence, H_O is rejected i.e. there is significant association between culture positivity among male and female patients i.e higher proportion of culture positivity seen among female patient is statistically significant.

2. Association between MDR and Non MDR strains among gender

		Bacterial status		Total
		MDR Strain	Non MDR strain	
Gender	Male	39	5	44
	Female	49	10	59
Total		88	15	103

Test statistics is $\aleph^2$

H_O: There is no significant association of multidrug resistance among male and female patients.

H_1: There is significant association of multidrug resistance among male and female patients.

From $\aleph^2 = \frac{(O-E)^2}{E}$ we find $\aleph^2 = 0.631$

Thus $\Re^2_{cal}$ (0.631) $<\Re^2_{tab}$ at α =0.05 and d.f = 1 i.e.3.841

Hence, H_O is accepted i.e. there is no significant association between multidrug resistance among male and female patients i.e higher proportion of MDR seen among female patient is statistically insignificant.

3. Association between culture positivity among sample types

		Growth		Total
		Positive	Negative	
Sample	Urine	99	397	496
	Blood	4	17	21
Total		103	414	517

Test statistics is $\Re^2$

H_O: There is no significant association between culture positivity among different sample types.

H_1: There is significant association between culture positivity among sample types.

From $\Re^2 = \frac{(O-E)^2}{E}$ we find $\Re^2 = 0.23$

Thus $\Re^2_{cal}$ (0.23) $<\Re^2_{tab}$ at α =0.05 and d.f = 1 i.e.3.841

Hence, H_O is accepted i.e. there is no significant association between culture positivity among the isolates from blood and urine sample. i.e higher proportion of culture positivity seen among urine sample is statistically insignificant.

4. Association among culture positivity and patients with different morbidity

		Growth		Total
		Positive	Negative	
Morbidity	Kidney Patients	81	303	384
	Hemodialysis	21	108	129
	Transplant	1	3	4
Total		103	414	517

Test statistics is χ^2

H_O: There is no significant association between culture positivity among types of patients

H_1: There is significant association between culture positivity among types of patients

From $\chi^2 = \frac{(O-E)^2}{E}$ we find $\chi^2 = 1.28$ Thus χ^2_{cal} (1.28) $< \chi^2_{tab}$ at $\alpha = 0.05$ and d.f = 2 i.e.5.99

Hence, H_O is rejected i.e. there is significant association between culture positivity among different types of patients i.e higher proportion of culture positivity seen among Chronic Kidney patients is statistically insignificant.

5. Association between ESBL production and susceptibility to ciprofloxacin

		ESBL production		Total
		Positive	Negative	
Ciprofloxacin Susceptibility	Sensitive	4	39	68
	Resistant	31	29	35
Total		35	68	103

Test statistics is χ^2

H_O: There is no significant association between ESBL production and reduced susceptibility to ciprofloxacin.

H_1: There is significant association between ESBL production and reduced susceptibility to ciprofloxacin.

From $\aleph^2 = \frac{(O-E)^2}{E}$ we find $\aleph^2 = 20.03$

Thus $\aleph^2_{cal}$ (20.03) $>\aleph^2_{tab}$ at α =0.05 and d.f = 1 i.e.3.841

Hence, H_O is rejecteted i.e. there is a significant association between ESBL production and reduced susceptibility to ciprofloxacin i.e higher rate of resistance to ciprofloxacin seen among ESBL producers is statistically significant.

6. Association between ESBL production and susceptibility to amikacin

		ESBL production		Total
		Positive	Negative	
Amikacin Susceptibility	Sensitive	20	45	65
	Resistant	15	23	38
Total		35	68	10

Test statistics is $\aleph^2$

H_O: There is no significant association between ESBL production and reduced susceptibility to Amikacin.

H_1: There is significant association between ESBL production and reduced susceptibility to Amikacin.

From $\aleph^2 = \frac{(O-E)^2}{E}$ we find $\aleph^2 = 0.81$

Thus $\aleph^2_{cal}$ (0.81) $<\aleph^2_{tab}$ at α =0.05 and d.f = 1 i.e.3.841

Hence, H_O is accepted i.e. there is no significant association between ESBL production and reduced susceptibility to amikacin i.e higher rate of resistance to amikacin seen among ESBL producers is statistically insignificant.

7. Determination of the sensitivity of the screening methods for ESBL detection

i. Ceftazidime

		Confirmatory Test		Total
		Positive	Negative	
Screening test	Positive	33	10	43
	Negative	2	14	16
Total		35	24	59

Sensitivity= a/a+c thus, sensitivity= 94.2%
Specificity=d/b+d thus, specificity= 58.33%
Positive predictive value (PPV) thus, a/a+b= 76.7 %
Negative predictive value (NPV) thus, d/c+d= 87.5%

ii. Cefotaxime

		Confirmatory Test		Total
		Positive	Negative	
Screening test	Positive	32	20	52
	Negative	3	4	7
Total		35	24	59

Sensitivity= a/a+c thus, sensitivity= 91.42%
Specificity=d/b+d thus, specificity= 16.66%
Positive predictive value (PPV) thus, a/a+b= 61.53%
Negative predictive value (NPV) thus, d/c+d= 57.14%