2017

External Quality Assessment Scheme: Standard operating procedures for participating sites

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Nodal Center for EQAS
As part of AIIMS-ICMR-CDC

Multi-centric Project entitled "Capacity Building and Strengthening of Hospital Infection Control to detect and prevent antimicrobial resistance in India"


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CHRISTIAN MEDICAL COLLEGE
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शिक्षा EDUCATION
अनुसंधान RESEARCH
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स्वास्थ्य सेवा PATIENT CARE


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CENTERS FOR DISEASE
CONTROL AND PREVENTION


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1. Background

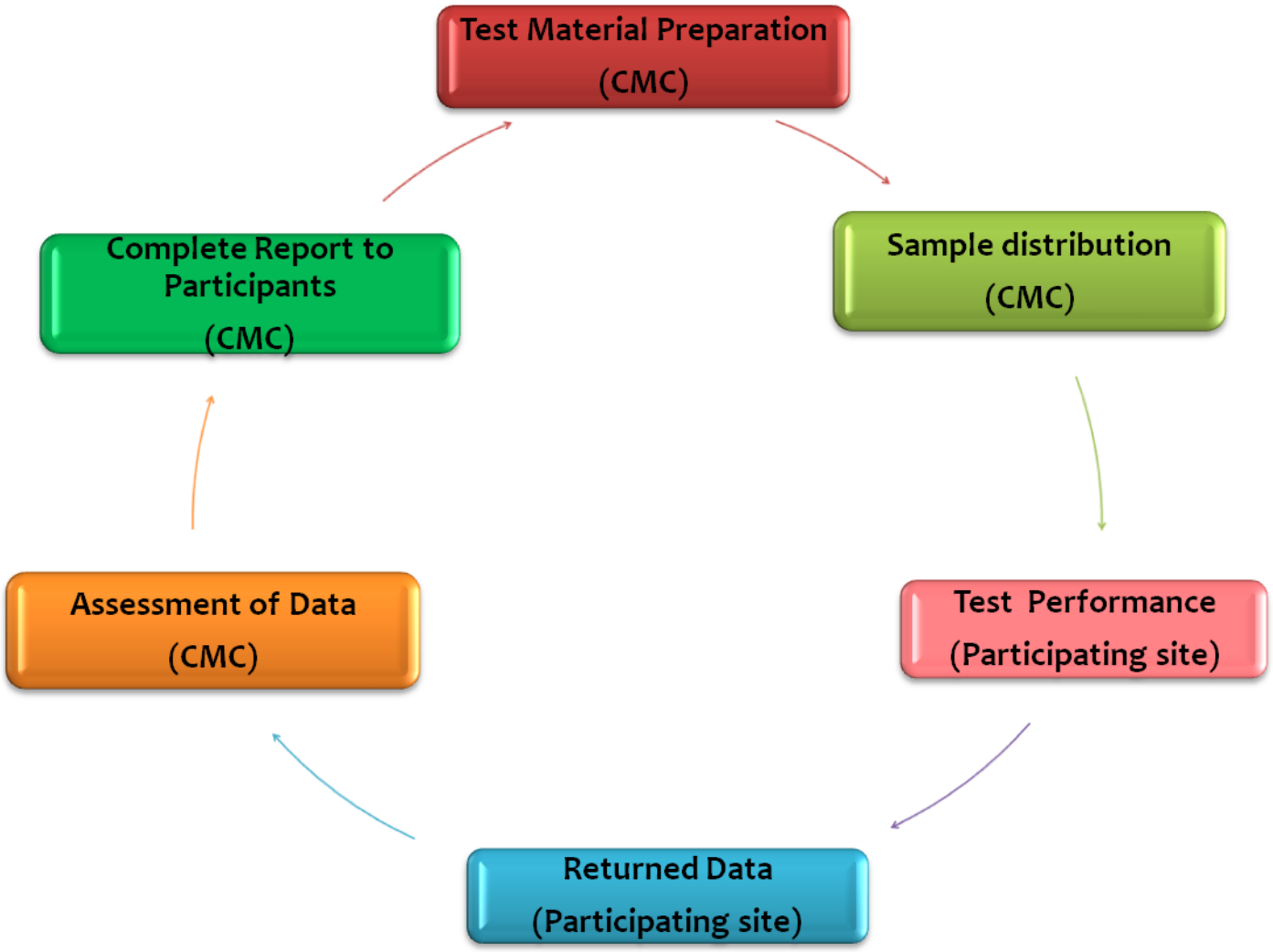
Christian Medical College is delighted to introduce bacteriology and molecular biology laboratory support through conducting an external quality assessment program.

An external quality assessment scheme (EQAS) is an important component in any healthcare or laboratory system to achieve maximum quality and reliable data. When used in conjunction with daily quality control (QC), these external programs assist laboratories in improving analytical quality and inter-laboratory agreement, and in identifying potential equipment or reagent failures and any training deficiencies.

2. EQA survey objective

- To assess the capacity of laboratory procedures on bacteriology for
    - clinical specimen processing
    - bacterial identification
    - antimicrobial resistance testing

3. **Strategy for Proficiency Testing Scheme (PTS**



EQA distribution testing guidelines

- EQAS panels will be conducted in 3 cycles per year, at four month intervals.
- EQAS panels will be sent to each participating microbiology laboratory from the reference laboratory at CMC.
- Each EQAS panels will consist of lyophilized cultures or simulated clinical specimens contain bacterial pathogens that contain challenge strains (bacterial pathogen) that participating sites will need to process, identify, and evaluate.
- The sample for the EQAS panel will be sent through courier mode. Packing and shipment of EQA materials will be as per international guidelines as prescribed by international air transport association (IATA) guidelines.
- CMC will provide an information sheet with each EQAS panel that details the survey number (cycle, batch, year), the date of sample dispatch, the deadline by which a participating site must submit results to CMC, and the date by which CMC will send preliminary feedback reports to a site. An example is listed below:

Survey Number	**Date of sample dispatching**	**Results Dead line**	**Responses of Evaluation**
001 **(Batch 1- 2017)**	**Dispatch from CMC**: 01.March.2017 (5 days for transport time for the participant site to receive the EQAS panel) (06.March.2017 to 20.March.2017 – 2 weeks testing time will be given to the participating sites) 21.March.2017 to 25.March.2017 – transport time of reports from sites to CMC	Result deadline – reports should reach CMC by 26.March.2017	CMC will send the preliminary report to sites in two weeks' time of receipt of report 09. April.2017

- Participants are requested to check the package upon receipt and inspect for any breakages, possible deterioration during transportation or missing samples. If samples are not suitable for testing, please inform CMC, Vellore immediately at 0416-2282588 or e-mail to vbalaji@cmcvellore.ac.in / esbl@cmcvellore.ac.in

Each EQAS panel includes:

1. Smears for gram stain
2. Lyophilized clinical cultures/simulated specimens with pathogens

   4.2.1. Culture identification

   4.2.2. Antimicrobial susceptibility testing

   4.2.3. Antimicrobial resistance characterization

   - Phenotypic characterization
   - Genotypic characterization

4.1 Smear testing: Sites are expect to report Gram stain results. The following pathogens and clinical specimen combinations may be included in smear testing:

- CSF (*Streptococcus pneumoniae, Haemophilus influenzae, Neiserria meningitidis, Listeria monocytogenes*)
- Sputum (*Streptococcus pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa, Moraxella cattarhalis, Staphylococcus aureus, Viridians streptococci*)

4.2 Lyophilized clinical cultures/specimens with pathogens: The following pathogens and clinical specimen combinations may be included in culture identification and antimicrobial susceptibility testing and characterization **(*Refer to Table 1*).**

4.2.1 Culture Identification

Identification of the bacteria is to be performed up to the species level ***(Refer to section 6.2).***

4.2.2 Antimicrobial susceptibility testing (AST):

AST to be performed for each of the pathogen for the given list of antimicrobials listed in the protocol ***(Refer to section 6.3).***

Table 1: Pathogen and Clinical Specimens Combinations for Culture Identification and AST

Bloodstream pathogens	**Cerebro spinal fluid pathogens**	**Respiratory pathogens**		**Faecal pathogens**	**Urinary pathogens**	**Skin and Soft tissue pathogens**
Staphylococcus aureus (MRSA, VISA)	*Streptococcus pneumoniae*	Upper Respiratory Infection	Group A haemolytic *Streptococci*	*E. coli* (EPEC)	*E. coli*	*Staphylococcus aureus (MRSA, VISA)*
Enterococcus spp (VRE)	*Haemophilus influenzae*		*Corynebacterium diphtheria*	*Shigella spp.*	*Klebsiella spp.*	Coagulase negative *Staphylococci* (CONS)
Streptococcus pneumoniae	*E. coli*	Upper and Lower Respiratory	*Haemophilus influenzae*	*Salmonella* Typhi	*Proteus spp.*	Group A beta haemolytic *Streptococci (Streptococcus pyogenes)*
E. coli	*Klebsiella spp*		*Streptococcus pneumoniae*	*Vibrio cholerae*	*Staphylococcus aureus (MRSA, VISA)*	Group B beta haemolytic *Streptococci*
Klebsiella spp.	*P. aeruginosa*	Lower Respiratory Infection	*Staphylococcus aureus (MRSA, VISA)*	*Aeromonas spp.*	*Enterococcus spp. (VRE)*	*E. coli*
Salmonella Typhi			*Pseudomonas aeruginosa*		*Enterobacter spp.*	*Klebsiella spp.*
Pseudomonas aeruginosa			*Klebsiella spp.*			*Pseudomonas spp.*
Acinetobacter baumannii			*Moraxella catarrhalis*			*Enterococcus spp. (VRE)*
			Acinetobacter baumannii			

4.3.3. Antimicrobial Resistance

Phenotypic testing for antimicrobial resistance will include:

For Gram negative organisms: as recommended by CLSI guidelines

- ESBL – combination disk testing:
    - Cefotaxime and cefotaxime/clavulanic acid
    - Ceftazidime and ceftazidime/clavulanic acid

- Carbapenemase
    - Modified Hodge Test
    - CarbaNP
    - Modified Carbapenem Inactivation Method (mCIM)

For Gram positive organisms: as recommended by CLSI guidelines

- β lactamase – Nitrocefin disk (For *Staphylococcus aureus*)
- Vancomycin agar screen (For *Staphylococcus aureus* and *Enterococcus* spp)
- Inducible clindamycin resistance (For *Staphylococcus aureus* and *Streptococcus pneumoniae*)
- High level aminoglycoside resistance (HLAR) detection (For *Enterococcus* spp)

Genotypic testing for antimicrobial resistance will include:

For Gram negative organisms: (plasmid mediated antimicrobial resistance determinants)

- ESBL (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{PER}}$, *bla*$_{\text{VEB}}$, *bla*$_{\text{CTX-M 1, 2, 8, 9, 25}}$)
- *AmpC* (*bla*$_{\text{DHA}}$, *bla*$_{\text{MOX}}$, *bla*$_{\text{FOX}}$, *bla*$_{\text{CMY}}$, *bla*$_{\text{ACC}}$, *bla*$_{\text{ACT}}$)
- Class A carbapenemase (*bla*$_{\text{KPC}}$, *bla*$_{\text{GES}}$)
- Class B carbapenemase (*bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{SPM}}$)
- Class D carbapenemases (*bla*$_{\text{OXA-48 like}}$, *bla*$_{\text{OXA-23 like}}$, *bla*$_{\text{OXA-24 like}}$, *bla*$_{\text{OXA-58 like}}$)

For Gram positive organisms:

- MR - *S. aureus* (*mecA*)
- Vancomycin resistant *Enterococcus* (*vanA*, *vanB*)
- *S. pneumoniae* (*pbp2b*, *ermB*, *mefA*)

5. Sample Distribution (EQAS Testing Scheme)

EQAS panels will be sent to the participating sites every four months. This includes:

- 4 smear
- 4 isolates/cultures

EQAS will be conducted in 3 cycles per year, with a total of 12 smears and 12 samples/isolates per year. In the first year of EQAS participation, the source specimens will be lyophilized cultures. In the subsequent cycles, simulated clinical specimens may also be sent to sites.

	Testing scheme	Test performance source
Cycle 1	• Smear, Culture, Identification, Antimicrobial Susceptibility Testing	Lyophilized cultures
Cycle 2	• Smear, Culture, Identification, Antimicrobial Susceptibility Testing	Lyophilized cultures
Cycle 3	• Smear, Culture, Identification, Antimicrobial Susceptibility Testing, Antimicrobial Resistance – Phenotypic tests	Lyophilized cultures

NOTE: Molecular tests for antimicrobial resistance characterization will be assessed for the selected sites in the third cycle.

6. Test Performance (site specimen processing)

6.1. Smear processing

Gram stain

1. Flood the slide with crystal violet for 1 minute to stain. Rinse with distilled water. Shake off excess water
2. Flood the slide with Gram's iodine for 1 minute
3. The iodine acts as a mordant as it binds the alkaline crystal violet dye to the cell wall. Rinse with distilled water. Shake off excess water
4. Decolorize with acetone until no more stain washes off (5-10 seconds may be enough). Rinse with distilled water. Shake off excess water
5. It is essential to view decolorization closely: gram-positive bacteria can be made to appear gram-negative by over-decolorization and gram-negative bacteria can be made to appear gram-positive by under decolorization
6. Counterstain with safranin for 30 seconds or with carbol-fuchsin for 10-15 seconds. Rinse with distilled water. Shake off excess water
7. Gently blot the slide using bibulous paper or a clean paper towel. Let air dry

8. When dry, examine the stained smear under a microscope with 100X oil immersion objective.

- **Record the reading in the reporting sheet (under microscopic examination):**

  Gram-positive organisms will appear dark violet or purple

  Gram-negative organisms will appear red or pink (from the counter stain)

6.2. Lyophilized culture processing

Recovery of Lyophilized Cultures

Opening of an Ampoule

1. Make a file mark on the ampoule about the middle of the cotton wool plug and apply a red-hot glass rod at that site to crack the glass.
2. Allow time for air, filtered by the plug to seep into the ampoules. Otherwise when the pointed end is snapped off, the plug will be drawn in. hasty opening may release aerosols.
3. The plug should be removed with forceps. The discarded plug and the pointed end of the ampoule should be put into a lotion jar.

Re-hydration and Recovery

1. Flame the open end of the tube and add about 0.3-0.5ml of nutrient broth / trypticase soy broth / chocolate broth using a sterile Pasteur pipette.
2. Mix the contents carefully so as to avoid frothing.
3. Subculture a loop-full of broth suspension onto appropriate media.
4. Transfer the rest to a tube containing 1ml of appropriate broth.
5. Incubate both broth and plate cultures in aerobic / CO_2 tin/incubator at 35-37°C.
6. Follow up with the growth in agar media as per individual labs routine procedures

Test Media:

- Blood Agar
- MacConckey Agar
- Chocolate Agar
- Nutrient agar

Culture identification

- After overnight incubation, the media are to be checked for visible growth and individual colony smears made for Gram staining
- Colony and smear morphology to be recorded as observed

Biochemical tests to be set up as per the standard methods and/or automated identification system can also be used for the bacterial identification

For Gram negative organisms:

- Oxidase
- Catalase
- Motility
- Indole
- Citrate
- Tripe Sugar Iron test
- Carbohydrate utilization (glucose, lactose, sucrose, mannitol, xylose, arabinose, dulcitol)
- Enzyme production (gelatinase, urease and phenyl pyruvic acid)
- MR-VP
- Amino acid utilization (Nitrate, Lysine, Ornithine, Arginine)

For Gram positive organisms:

- Oxidase
- Catalase
- Optochin
- Bacitracin
- Novobiocin
- DNAse
- Indole

The participating laboratories are expected to identify and report the organism up to the species level.

6.3. Antimicrobial Susceptibility testing:

- **Disk diffusion methods:**

The Kirby-Bauer method is to be used for antimicrobial susceptibility testing as recommended by CLSI guidelines.

Antimicrobial agents to be tested for each of the clinical specimen are given below:

Antimicrobial agents panel for: Cerebro Spinal Fluid (CSF) pathogens

Enterobacteriaceae	Cefpodoxime Chloramphenicol Cefoperazone/sulbactam Piperacillin/tazobactam Imipenem Meropenem Ciprofloxacin
NFGNB & *Pseudomonas*	Ceftazidime Cefepime Aztreonam Cefoperazone/sulbactam Piperacillin/tazobactam Meropenem Levofloxacin Netilmicin Polymyxin B 300
S. pneumoniae	Oxacillin Chloramphenicol Levofloxacin Vancomycin Linezolid Optochin Penicillin & Cefotaxime (MIC to be done)
H. influenzae	Ampicillin Chloramphenicol Ceftriaxone Meropenem

Antimicrobial agents panel for: Urinary tract pathogens

Gram negative bacilli	Cefpodoxime Amoxicillin/sulbactam Cefoperazone/sulbactam Piperacillin/tazobactam Imipenem Meropenem Ertapenem Ciprofloxacin Co-Trimoxazole Nitrofurantoin Amikacin Netilmicin Gentamicin PB 300
NFGNB & *Pseudomonas* spp	Ceftazidime Cefepime Cefoperazone/sulbactam Piperacillin/tazobactam Imipenem Meropenem Aztreonam Levofloxacin Amikacin Netilmicin Tobramycin PB 300
S. aureus / Staphylococci	Cefoxitin Norfloxacin Cotrimoxazole Nitrofurantoin Rifampicin Novobiocin
Enterococci	Ampicillin Gentamicin (HLG) Nitrofurantoin Vamcomycin Teicoplanin Linezolid

Antimicrobial agents panel for: Skin and Soft tissue pathogens

S. aureus / Staphylococci	Cefoxitin Erythromycin Tetracycline Chlorapmphenicol TMP-SXT Netilmicin Clindamycin Limezolid
Enterococci	Ampicillin Chloramphenicol Gentamicin Linezolid Tetracycline Vancomycin Teicoplanin
Gram negative bacilli	Cefpodoxime Amoxicillin/clavulanic acid Piperacillin/tazobactam Imipenem Meropenem Chloramphenicol Co-Trimoxazole Amikacin Gentamicin
Pseudomonas & NFGNB	Ceftazidime Cefepime Cefoperazone/sulbactam Piperacillin/tazobactam Imipenem Meropenem Aztreonam Levofloxacin Amikacin Netilmicin Tobramycin PB 300

Antimicrobial agents panel for: Faecal pathogens

Shigella / EPEC	Ampicillin Cefixime Cefotaxime Cotrimoxazole Imipenem Meropenem Nalidixic acid Ofloxacin Norfloxacin Chloramphenicol Gentamicin Azithromycin Ciprofloxacin Tetracycline
S. Typhi, S. Paratyphi A & Other Salmonella spp	Ampicillin Ceftriaxone Chloramphenicol Cotrimoxazole Nalidixic acid Pefloxacin Ciprofloxacin Azithromycin
Aeromonas & *Vibrio cholerae*	Ampicillin Cefotaxime Imipenem Meropenem Tetracycline Cotrimoxazole Nalidixic acid Ofloxacin Norfloxacin Ciprofloxacin (only for *Aeromonas*) Gentamicin Amikacin

Procedure for Performing the Disk Diffusion Test:

Materials required:

- Sterile broth medium in 1.5 ml quantities. (Nutrient broth / Mueller Hinton broth)
- MHA for Non-fastidious organisms
- MHBA for *S. pneumoniae* and other *Streptococci*

- HTM for *Haemophilus* spp.
- GC agar with 1% growth supplements for *Neisseria* spp.
- Calibrated loop of 2 mm diameter
- Antibiotic solution
- Sterile filter paper disks / Commercial disks
- Pasteur pipettes sterile
- Cotton swabs sterile
- Normal saline and / Nutrient broth
- Mc Farland BaSO4 turbidity standard 0.5 and 1.0
- Sterile forceps / needle / disk dispenser
- 12 x 100 mm sterile test tubes
- Measuring scales / sliding callipers
- Table lamp
- Zone diameter interpretation charts
- Quality control reference strains
- Discard jar with disinfectant

Quality control strains:

- ATCC 25922 *Escherichia coli*
- ATCC 27853 *Pseudomonas aeruginosa*
- ATCC 25923 *Staphylococcus aureus*
- ATCC 19606 *Acinetobacter baumannii*
- ATCC 700603 *Klebsiella pneumoniae (ESBL)*
- ATCC 35218 *Escherichia coli (ESBL)*
- ATCC BAA 1705 *Klebsiella pneumoniae* (carbapenemase positive)
- ATCC BAA 1706 *Klebsiella pneumoniae* (carbapenemase negative)

Inoculum Preparation:

Growth Method:

1. With a sterile needle / loop, touch four or five well isolated colonies of the same morphological type

2. Inoculate into 1.5 ml of a sterile suitable broth. Incubate at 35 – 37ºC for 2 – 6 hours to produce a bacterial suspension of moderate turbidity

3. Adjust the density of the suspension to Mc Farland barium sulphate standard, 0.5 (for gram negative organisms), 1.0 (for gram positive organisms) with sterile saline / broth.

(Adequate light is needed to visually compare the inoculum tube and the 0.5 Mc Farland standard against a card with a white background and contrasting black lines)

Inoculation of Test Plates:

1. Mark the plates into sections according to the number of antibiotics to be used
2. Inoculate the plates within 15 minutes of preparation of bacterial suspension
3. Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level
4. Inoculate the dried surface of the appropriate agar plate by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60º each time to ensure an even distribution of inoculum. As a final step, the rim of the agar has to be swabbed.

Note: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of Disks to Inoculated Agar Plates:

1. Dispense antimicrobial disks with appropriate concentrations onto the surface of the inoculated agar plate
2. Each disk must be pressed down to ensure complete contact with the agar surface.
3. Make sure that they are no closer than 24 mm from center to center. Ordinarily, no more than 6 disks should be placed on a 90-mm plate.
4. As some of the drug diffuses almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.
5. Incubate the plates in an inverted position in an incubator set to 37ºC at appropriate conditions

Note: With the exception of *Haemophilus* spp., *Streptococci* and *N. gonorrhoeae*, the plates should not be incubated in an increased CO2 atmosphere, because the interpretive standards were developed by using ambient air incubation, and CO2 will significantly alter the size of the inhibitory zones of some agents.

Reading Plates and Interpreting Results:

- Examine each plate after 16 – 18 hours of incubation
- If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated
- The point of abrupt diminution of growth, which in most cases corresponds with the point of complete inhibition of growth, is taken as the zone edge
- Measure the diameters of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk.
- Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted petri plate, with reflected light. Zones can be measured easily by holding the petri plate a few inches above a black, non-reflecting background, illuminated with reflected light.
- If blood was added to the agar base (as with *Streptococci*), the zones are measured from the upper surface of the agar illuminated with reflected light, with the cover removed
- The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be sub-cultured, re-identified, and retested. Strains of *Proteus* spp. may swarm into areas of inhibited growth around certain antimicrobial agents. With *Proteus* spp, the thin veil of swarming growth in an otherwise obvious zone of inhibition should be ignored. When using blood-supplemented medium for testing *Streptococci*, the zone of growth inhibition should be measured, not the zone of inhibition of haemolysis.
- Refer CLSI guidelines for interpreting the susceptibility pattern

Minimum Inhibitory Concentration (MIC):

Methods of determining MIC:

- Broth dilution method
- E- test

Broth dilution method

Materials:

- Sterile graduated pipettes – 10 ml, 5 ml, 2 ml and 1 ml.
- Sterile capped 75 x 12 mm tubes / small screw-capped bottles
- Sterile Pasteur pipettes
- Overnight broth culture of test and control organisms (same as for disk diffusion tests)
- Required antibiotic in powder form (either from the manufacturer or standard laboratory accompanied by a statement of its activity in mg/unit or per ml. Clinical preparations should not be used for reference technique)
- Required solvent for the antibiotic
- Sterile Distilled Water - 500 ml
- Suitable nutrient broth medium (Muller Hinton Broth Medium).
- Test tube rack to hold 22 tubes in two rows i.e. 11 tubes in each row.

Method:

1. Prepare stock dilutions of the antibiotic of concentrations as required
2. Arrange two rows of 11 sterile 75 x12 mm capped tubes in the rack.
3. Prepare 8 ml of broth containing the concentration of antibiotic required for the first tube in each row from the appropriate stock solution already made, in a sterile 30 ml (universal) screw capped bottle.
4. Mix the contents of the universal bottle using a pipette and transfer 2 ml to the first tube in each row.
5. Using a fresh pipette, add 4 ml of broth to the remaining 4 ml in the universal bottle mix and transfer 2ml to the second tube in each row.
6. Continue preparing dilutions in this way.

7. Where as many as 10 or more are required the series should be started again half the way down.
8. Place 2 ml of antibiotic free broth to the last tube in each row.
9. Inoculate one row with one drop of an overnight broth culture of the test organism diluted approximately to 1 in 1000 in a suitable broth and the second row with the control organism of known sensitivity similarly diluted.
10. Incubate tubes for 18 hours at 37^{o}C.
11. Inoculate a tube containing 2ml broth with the organism and keep at +4^{o}C in a refrigerator overnight to be used as standard for the determination of complete inhibition.

Reading of result:

- MIC is expressed as the lowest dilution which inhibited growth judged by lack of turbidity in the tube. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition. A standard strain of known MIC value that is run with the test is used as the control to check the reagents and conditions.
- Check the control tube for the presence of growth by turbidity. Only then the test is read.
- First note the concentration of antibiotic in the last tube with no turbidity as the end point.
- If there is no turbidity in 0.25 µg/ml tube and if there is turbidity in the 0.125 µg/ml tube, then 0.25 µg/ml is taken as the MIC.
- The quality control strain should show the MIC within the acceptable range.
- Expected value of MIC range for ATCC *P. aeruginosa* (27853) is **0.25 – 1.0 µg/ml**
- Record the results and report

E-test:

Materials required:

- Sterile broth medium in 1.5 ml quantities. (Nutrient broth / Mueller Hinton broth)
- MHA for Non-fastidious organisms.
- MHBA for *S. pneumoniae* and other *Streptococci*
- HTM for *Haemophilus* spp.
- GC agar with 1% growth supplements for *Neisseria* spp.

- E-test strips
- Pasteur pipettes sterile
- Cotton swabs sterile
- Normal saline and / Nutrient broth
- Mc Farland $BaSO_4$ turbidity standard 0.5 and 1.0
- Sterile forceps / needle / disk dispenser
- 12 x 100 mm sterile test tubes
- Measuring scales / sliding callipers
- Table lamp
- Quality control reference strains
- Zone diameter interpretation charts
- Discard jar with disinfectant

Inoculum Preparation:

Growth Method:

1. With a sterile needle / loop, touch four or five well isolated colonies of the same morphological type

2. Inoculate into 1.5 ml of a sterile suitable broth. Incubate at 35 – 37^{o}C for 2 – 6 hours to produce a bacterial suspension of moderate turbidity

3. Adjust the density of the suspension to Mc Farland barium sulphate standard, 0.5 (for gram negative organisms), 1.0 (for gram positive organisms) with sterile saline / broth.

(Adequate light is needed to visually compare the inoculum tube and the 0.5 Mc Farland standard against a card with a white background and contrasting black lines)

Inoculation of Test Plates:

1. Mark the plates into sections according to the number of antibiotics to be used
2. Inoculate the plates within 15 minutes of preparation of bacterial suspension
3. Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level

4. Inoculate the dried surface of the appropriate agar plate by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60º each time to ensure an even distribution of inoculum. As a final step, the rim of the agar has to be swabbed.

Note: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inocula for streaking plates.

Application of E-test strip on to inoculated agar plates:

1. Place the strip onto the surface of the inoculated agar plate
2. Strips must be pressed down to ensure complete contact with the agar surface
3. As some of the drug diffuses almost instantaneously, a strip should not be relocated once it has come into contact with the agar surface
4. Incubate the plates in an inverted position in an incubator set to 37ºC at appropriate conditions

Reading Plates and Interpreting Results:

1. Examine each plate after 16 – 18 hours of incubation
2. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be precise. If individual colonies are apparent, the inoculum was too light and the test must be repeated

Results reading and interpretation

- CLSI guidelines criteria to be followed

6.4. Simulated clinical specimen processing

The simulated clinical specimens are to be processed as per the participating laboratory standard operating procedures. This includes:

- Smear processing
    - Prepare a smear using an isolate by adding a small drop of physiological saline to the slide and create a slightly turbid, uniform suspension of cells from an overnight culture.

- Let the suspension air dry. The suspension MUST be completely dry before proceeding.
- Heat -fix the smears by quickly passing the slide through a flame three times.
- Do not over-heat the slide as over-heating will cause significant distortion or destruction of the cells.
- Follow steps from 6.1 protocol for smear

- Culture of clinical specimens

  The specimen has to be processed using the media given below:

Specimen	**Media to be used**
Blood	Blood agar, McConkey agar, Mannitol salt agar
Respiratory	Blood agar, Chocolate agar, McConkey agar, Nutrient agar
CSF	Blood agar, Chocolate agar, McConkey agar, Nutrient agar
Faeces	Blood agar, McConkey agar, Xylose lysine deoxycholate agar (XLD), Deoxycholate citrate agar (DCA), Thiosulfate citrate bile salt sucrose agar (TCBS)
Urine	Blood agar, McConkey agar
Pus	Blood agar, McConkey agar, Mannitol salt agar

- Sample processing is to be done as per the participating laboratory standard operating procedures.
- Incubate the plates at 37^{o}C for 16 - 18 hours
- Examine the colony morphology and record the results in the reporting sheet

Proceed with protocols for processing the smear, culture identification and susceptibility testing.

6.4 Phenotypic testing for antimicrobial resistance (from bacterial cultures)

Phenotypic testing for antimicrobial resistance will include:

For Gram negative organisms: as recommended by CLSI guidelines

- ESBL – combination disk testing:
    - Cefotaxime and cefotaxime/clavulanic acid
    - Ceftazidime and ceftazidime/clavulanic acid
- Carbapenemase
    - Modified Hodge Test
    - CarbaNP
    - Modified Carbapenem Inactivation Method (mCIM)

For Gram positive organisms: as recommended by CLSI guidelines

- β lactamase – Nitrocefin disk (For Staphylococcus aureus)
- Vancomycin agar screen (For Staphylococcus aureus and Enterococcus spp)
- Inducible clindamycin resistance (For Staphylococcus aureus and Streptococcus pneumoniae)

Participating centers has to perform these tests as recommended by CLSI guidelines

Phenotypic testing for antimicrobial resistance will include:

For Gram negative organisms: as recommended by CLSI guidelines

I) ESBL – combination disk testing: (Cefotaxime and cefotaxime/clavulanic acid, Ceftazidime and ceftazidime/clavulanic acid)

The combined disk method depends on comparing the inhibition zones around disks containing an indicator cephalosporin with and without clavulanic acid.

Test procedure:

- With a sterile loop touch eight or ten well isolated colonies of the same morphological type
- Inoculate into 1.5 ml of a sterile suitable broth
- Incubate at 35 – 37^{o}C for 2 – 6 hours to produce a bacterial suspension of moderate turbidity
- Adjust the density of the suspension to Mc Farland standard - 0.5 with sterile broth

- Dip a sterile cotton swab into the suspension and remove the excess fluid by rotating the swab against the side of the tube above the fluid level
- Inoculate the dried surface of a Mueller-Hinton agar plate by streaking the swab over the entire sterile agar surface
- Place 30 µg of cefotaxime and 30 µg of ceftazidime with and without 10 µg clavulanic acid
- Incubate the plates at 37oC for 16-20 hours at ambient temperature

Results and interpretation

If ESBL is produced, the zone diameters given by the disks with clavulanate are >5 mm larger than those without the inhibitor.

- Positive – zone of inhibition of combination is >5 mm than the single agent
- Negative – zone of inhibition of combination is <5 mm than the single agent
- With each test, positive and negative controls use is mandatory
    - *Klebsiella pneumoniae* ATCC 700603 - positive control
    - *E. coli* ATCC 25922 – negative control

Test for carbapenemase detection:

II) Modified Hodge Test (MHT)

- Carbapenemase production is detected by the MHT when the test isolate produces the carbapenemase enzyme and allows growth of a carbapenem susceptible strain (*E. coli* ATCC 25922) towards a carbapenem disk. The result is a characteristic cloverleaf-like indentation. A positive MHT indicates that this isolate is producing a carbapenemase. A negative MHT indicates that this isolate is not producing a carbapenemase. The class of carbapenemase cannot be determined by the results of the MHT

Test procedure:

- Prepare 0.5 McFarland dilution of the *E. coli* ATCC 25922 in broth or saline
- Dilute the adjusted suspension in the ratio of 1:10 (culture + saline)
- Make a lawn culture on a Mueller Hinton agar plate and allowed to dry 3–5 minutes

- Place a meropenem (10 μg) or ertapenem (10 μg) disk in the centre plate
- Streak the test organism from the edge of the disk to the edge of the plate in a straight line
- Up to four organisms can be tested on the same plate with one drug
- Incubated the plates overnight at 35ºC ± 2ºC in ambient air for 16–24 hours
- Examine the plates after incubation for a clover leaf-type indentation at the intersection of the test organism and the *E. coli* 25922, within the zone of inhibition of the carbapenem disk

Test result interpretation:

- The test organism having a clover leaf-like indentation of the *E. coli* 25922 growing along the test organism growth streak within the disk diffusion zone will be considered as MHT Positive.
- Isolates showing a slight indentation will not be considered to produce carbapenemase.
- With each test, positive and negative controls use is mandatory
    - *Klebsiella pneumoniae* ATCC BAA-1705 - positive control
    - *Klebsiella pneumoniae* ATCC BAA-1706 – negative control

III) Carba NP test

- Take 100 μl of B-PER II (lysis buffer) in two eppendorf tubes and mark it as control and test
- Inoculate 10 μl of the strains to be tested directly from blood agar or MHA plate
- Vortex the suspension for one minute
- Add 100 μl of phenol red solution with mg/ml imipenem to test solution tube
- Add 100μl of phenol red without imipenem to control solution tube
- Incubate at 37ºC for a maximum of 2 hours

Preparation of Phenol red solution

1. Prepare a concentrated solution of red phenol 0.5% w/v
2. Mix 2 ml of concentrated red phenol solution in 16.6 ml of distilled water

3. Adjust the pH at 7.8 by adding drops of a NaOH solution (1 N)
4. Add 180µl of 10 mM $ZnSO_4$ (to obtain a final concentration of 0.1 mM)

Results and interpretation

	Non carbapenemase producer	**Carbapenemase producer**
Control	Red	Red
Test	Red	Yellow

- With each test, positive and negative controls use is mandatory
    - *Klebsiella pneumoniae* ATCC BAA-1705 - positive control
    - *Klebsiella pneumoniae* ATCC BAA-1706 – negative control

IV) Modified Carbapenem Inactivation Method (mCIM)

- Emulsify 1 µl loop full of the test isolate colonies grown on blood agar overnight, in to 2 ml of trypticase soy broth
- Vortex it for one minute
- Add 10 µg meropenem disk to the tube using sterile forceps (Ensure the entire disk is immersed in the broth)
- Incubate at 37oC for four hours
- Prepare 0.5 McFarland suspension of *E. coli* ATCC 25922 in nutrient broth and make a lawn culture on the Mueller Hinton agar plate
- Remove the meropenem disk from the broth and place it on the lawn culture plate of *E. coli* ATCC 25922
- Invert and incubate the plate at 37^{o}C for 18-24 hours
- Following incubation, measure the zone of inhibition

Test results Interpretation:

- Positive: zone 6 – 15 mm or presence of colonies within 16 – 18 mm zone
- Negative: if zone of inhibition is ≥ 19 mm

With each test, positive and negative controls use is mandatory.

- *Klebsiella pneumoniae* ATCC BAA-1705 - positive control
- *Klebsiella pneumoniae* ATCC BAA-1706 – negative control

For Gram positive organisms: as recommended by CLSI guidelines

I) β lactamase – Nitrocefin disk (For *Staphylococcus aureus*)

- Nitrocefin disk test is to be performed as per manufacturer's instructions

Test results interpretation:

- Positive - Color conversion from yellow to red/pink
- Negative – Absence of color change

With each test, positive and negative controls use is mandatory

- *Staphylococcus aureus* ATCC 29213 - positive control
- *Staphylococcus aureus* ATCC 25923 - negative control

II) Vancomycin agar screen (For *Staphylococcus aureus* and *Enterococcus spp*)

- This is a agar dilution based test
- Prepare Brain hear infusion agar supplemented with 6 µg/ml vancomycin
- Spot 1 – 10 µl of a 0.5 McFarland suspension onto agar surface
- Invert and incubate the plate at 37^{o}C for 24 hours

Test results interpretation

- Positive - presence of >1 colony or light film of growth (> 1 colony – presumptive reduced susceptibility to vancomycin) – *S. aureus*
- Positive - > 1 colony – presumptive vancomycin resistance – *Enterococcus* spp

With each test, positive and negative controls use is mandatory

- *E. faecalis* ATCC 51299 - positive control
- *E. faecalis* ATCC 29212 - negative control

III) Inducible clindamycin resistance (For *Staphylococcus aureus* and *Streptococcus pneumoniae*) Disk diffusion – D Zone test

- Prepare Mueller hinton blood agar plates with the lawn culture of the test organism as per standard disc diffusion testing protocol
- Place 15 µg erythromycin and 2 µg clindamycin disks spaced 15-26 mm apart for *S. aureus* and 12 mm apart for *S. pneumoniae*
- Invert and incubate the plate at 37ºC for 16 - 18 hours for *S. aureus* and 20 – 24 hours for *S. pneumoniae*

Test results interpretation

- Positive – flattening of zone of inhibition adjacent to the erythromycin disk (referred to as D-Zone) = inducible clindamycin resistance (for *S. aureus*)
- Hazy growth within the zone of inhibition around clindamycin disk – taken as positive for clindamycin resistance, even if no D zone is apparent

With each test, positive and negative controls use is mandatory

- *S. aureus* ATCC BAA 977 – D zone test positive
- *S. aureus* ATCC BAA 976 – D zone test negative

IV) High level aminoglycoside resistance (HLAR) detection in *Enterococcus* spp – Disk diffusion

- Prepare Mueller hinton agar plates with the lawn culture of the test organism as per standard disc diffusion testing protocol
- Place 120 µg gentamicin disks on to the lawn culture
- Invert and incubate the plate at 37ºC for 16 - 18 hours

Test results interpretation

- Zone of inhibition is 6 mm – Resistant
- Zone of inhibition is 7 to 9 mm – Inconclusive
- Zone of inhibition is ≥10mm – Susceptible

With each test, use of quality control strain is mandatory

- *E. faecalis* ATCC 29212 (16-23 mm)

6.5. Molecular testing for antimicrobial resistance (From bacterial cultures/DNA spiked clinical specimens) - only for selected sites

DNA isolation: Extract whole genomic DNA from overnight grown colonies on blood agar using the QIAamp DNA Mini Kit (or) protocols followed as per kit insert.

Molecular testing includes characterization of antimicrobial resistance in gram negative and gram positive organisms. Multiplex PCRs to be done to screen for the presence of resistant genes responsible for antibiotic resistance. PCR protocol and the primers are summarized in Table 2 to 6.

Table 2: PCR targets for determination of AmpC enzymes: CMY, CIT, DHA, ACC, ACT and FOX

Target gene	Primer Name	Primer sequence	PCR product size
MOX-1, MOX-2, CMY-1, CMY 8-11	*MOXMF*	GCTGCTCAAGGAGCACAGGAT	520 bp
	MOXMR	CACATTGACATAGGTGTGGTGC	
LAT-1to LAT-4, CMY 2 to CMY-7, BIL-1	*CITMF*	TGGCCAGAACTGACAGGCAAA	462 bp
	CITMR	TTTCTCCTGAACGTGGCTGGC	
DHA-1, DHA-2	*DHAMF*	AACTTTCACAGGTGPGCTGGGT	405 bp
	DHAMR	CCGTACGCATACTGGCTTTGC	
ACC	*ACCMF*	AACAGCCTCAGCAGCCGGTTA	346 bp
	ACCMR	TTCGCCGCAATCATCCCTAGC	
MIR-1T, ACT-	*EBCMF*	TCGGTAAAGCCGATGTTGCGG	302 bp
	EBCMR	CTTCCACTGCGGCTGCCAGTT	

FOX-1 to FOX-5b	*FOXMF*	AACATGGGGTATCAGGGAGATG	190 bp
	FOXMR	CAAAGCGCGTAACCGGATTGG	

Table 3: PCR targets for extended spectrum beta-lactamases (ESBL) and carbapenemases [Reaction 1]: SPM, VIM, PER, OXA-48 and KPC

Primer name	**Primer sequence**	**PCR product size**
SPMf	AAAATCTGGGTACGCAAACG	271 bp
SPMr	ACATTATCCGCTGGAACAGG	
VIM-F	GATGGTGTTTGGTCGCATA	390 bp
VIM-R	CGAATGCGCAGCACCAG	
PERf	GCTCCGATAATGAAAGCGT	520 bp
PERr	TTCGGCTTGACTCGGCTGA	
OXA-48A	TATATTGCATTAAGCAAGGG	800 bp
OXA-48B	CACACAAATACGCGCTAACC	
KPCyF	TGTCACTGATCGCCGTC	1011 bp
KPCyR	CTCAGTGCTCTACAGAAAACC	

Table 4: PCR targets for extended spectrum beta-lactamases (ESBL) and carbapenemases [Reaction 2]: IMP, GES and NDM

Primer name	**Primer sequence**	**PCR product size**
IMP2-F	GGAATAGAGTGGCTTAAYTCTC	232 bp
IMP2R-2	CCAAACYACTASGTTATCT	
GESf	AGTCGGCTAGACCGGAAAG	399 bp

GESr	TTTGTCCGTGCTCAGGAT	
NDMf	CACCTCATGTTTGAATTCGCC	984 bp
NDMr	CTCTGTCACATCGAAATCGC	

Table 5: PCR targets for CTX-M enzymes: CTX-1, CTX-2, CTX-8, CTX-9 and CTX-25

Target gene group	Primer name	Primer sequence	PCR product size
CTX-M-1	CTX-M1f2	AAAAATCACTGCGCCAGTTC	415 bp
	CTX-M1r2	AGCTTATTCATCGCCACGTT	
CTX-M-2	CTX-M2f2	CGACGCTACCCCTGCTATT	552 bp
	CTX-M2r2	CCAGCGTCAGATTTTTCAGG	
CTX-M-9	CTX-M9f2	CAAAGAGAGTGCAACGGATG	205 bp
	CTX-M9r2	ATTGGAAAGCGTTCATCACC	
CTX-M-8	CTX-M8A	TCGCGTTAAGCGGATGATGC	666 bp
	CTX-M8-25B	AACCCACGAGTTGGGTAGC	
CTX-M-25	CTX-M25A	GCACGATGACATTCGGG	337 bp
	CTX-M8-25B	AACCCACGATGTGGGTAGC	

Table 6: PCR targets for resistance genes in gram positive organisms

Target gene group	**Primer sequence**	**PCR product size**
Pbp2b	GATCCTCTAAATGATTCTCAGTGG	415 bp
	CAATTAGCTTAGCAATAGGTGTGG	

vanA	CATGACGTATCGGTAAAATC	885 bp
	ACCGGGCAGRGTATTGAC	
vanB	CATGATGTGTCGGTAAAATC	885 bp
	ACCGGGCAGRGTATTGAC	
ermB	CTGTATGGAGCTACCTGTCTGG	224 bp
	CCCAGCTTAGGTATACGTAC	
mefA	CGTACCTTGGATATTCACCG	294 bp
	GTAAACAGTTGACGATATTC	
mecA	AGTTGTAGTTGTCGGGTTTGG	454 bp
	GGCCAATTCCACATTGTTTC	

PCR cycling conditions for gram negatives:

Initial Denaturation	95 º C	1 cycle
Denaturation	95º C	35 cycles
Annealing	65º C (for *ampC*) 60º C (for Reaction 1) 60 º C (for Reaction 2) 52º C (for *CTX-M*)	
Extension	72º C	
Final Extension	72º C	1 cycle

Post PCR analysis (Agarose Gel electrophoresis): Amplicons to be visualized in a 2% agarose gel containing ethidium bromide. Respective positive controls and appropriate DNA ladder to be used for analyzing the presence of any particular gene.

7. Returned Data (Result Submission)

- Participants are requested to submit the report in two weeks' time of receipt of the test materials.
- The reports are to be submitted to CMC, through hard copies in the recommended format given below.
- Online submission database will subsequently be developed and informed to the participating centers.

MICROSCOPY (GRAM-STAIN) **SMEAR ID: _____**		
Testing not performed on this specimen/isolate □ **Comments / Reasons:** **Smear ID: _____**		
STAIN REACTION	**MORPHOLOGY**	
Gram-negative □	Cocci □	Diplococci □
Gram-positive □	Bacilli □	Yeast □
Gram-Variable □	Cocco-bacilli □	Others □

Culture characteristics **Culture ID/Specimen ID: _____**	
Testing not performed on this specimen/isolate □ **Comments / Reasons:**	
Media	**MORPHOLOGY**
Blood Agar	
MacConkey Agar	
Chocolate Agar	
Nutrient Agar	
Others (if any)	

IDENTIFICATION: Gram Negative organisms **Culture ID/Specimen ID: _____** **(use separate sheet for each of the specimen type)**					
Testing not performed on this specimen/isolate □					
BASIC BIOCHEMICAL IDENTIFICATION					
		Positive		Negative	
Motility		□		□	
Oxidase		□		□	
Catalase		□		□	
Indole		□		□	
Citrate		□		□	
Hemolysis		α □	β □	None □	
TSI		Slope:	Butt:	Gas:	H_2S:
CHO Reaction	Glucose	□		□	
	Lactose	□		□	
	Sucrose	□		□	
	Mannitol	□		□	
	Xylose	□		□	
	Arabinose	□		□	
	Dulcitol	□		□	
Protein Reactions	Gelatinase	□		□	
	Urease	□		□	
	PPA	□		□	
OF Glucose	Oxidative	□		□	
	Fermentative	□		□	
Methyl Red		□		□	
Voges Proskaeur		□		□	
Nitrate		□		□	
Lysine		□		□	
Ornithine		□		□	
Arginine		□		□	
LIA		□		□	

IDENTIFICATION: Gram Positive organisms

Culture ID/Specimen ID: _____

Testing not performed on this specimen/isolate □

BASIC BIOCHEMICAL IDENTIFICATION

	Positive/Sensitive	**Negative/Resistant**
Catalase	□	□
Oxidase	□	□
Optochin	□	□
Bacitracin	□	□
Novobiocin	□	□
DNAse	□	□
Indole	□	□

Haemolysis	α □	β □	None □
X Factor	V Factor	XV Factor	

SPECIES SPECIFIC IDENTIFICATION

Culture ID/Specimen ID: _____

	1st IDENTIFICATION	**2nd IDENTIFICATION**	**3rd IDENTIFICATION**
Conventional Biochemical Methods	□	□	□
API	□	□	□
MICROSCAN	□	□	□
VITEK 2	□	□	□
Other Commercial Kit	□	□	□
PCR	□	□	□
MALDI TOF	□	□	□
16S rRNA	□	□	□

Antimicrobial susceptibility testing
Culture ID/Specimen ID: _____

No	Antibiotic Code (DISC)	Disc Content (µg)	Disc zone diameter (mm)	S	I	R	Comments
1				□	□	□	
2				□	□	□	
3				□	□	□	
4				□	□	□	
5				□	□	□	
6				□	□	□	
7				□	□	□	
8				□	□	□	
9				□	□	□	
10				□	□	□	

Antimicrobial susceptibility testing
Culture ID/Specimen ID: _____

No	Antibiotic Code (MIC)	Tested range (µg/ml)	MIC value	S	I	R	Comments
1				□	□	□	
2				□	□	□	
3				□	□	□	
4				□	□	□	
5				□	□	□	
6				□	□	□	
7				□	□	□	
8				□	□	□	
9				□	□	□	
10				□	□	□	

Antimicrobial Resistance (Phenotypic tests) **Culture ID/Specimen Id: _____**		
Testing not performed on this specimen/isolate □ **Comments / Reasons:** **Culture/Specimen ID: _____**		
	Observation	**Result**
ESBL **Cefotaxime zone (mm)** □		
Cefotaxime/ **clavulanic zone (mm)** □		
Ceftazidime zone (mm) □		
Ceftazidime/ **clavulanic zone (mm)** □		
Carbapenemasese **CarbaNP (color change)**		
mCIM test		
β lactamase – Nitrocefin disk		
Vancomycin agar screen		
Inducible clindamycin resistance		

Antimicrobial Resistance (Molecular tests) **Culture ID/Specimen Id: _____**		
Testing not performed on this specimen/isolate □ **Comments / Reasons:** **Culture/Specimen ID: _____**		
Genes tested for	**PCR positive for**	**Result**
AmpC: CMY, CIT, DHA, ACC, ACT and FOX		
SPM, VIM, PER, OXA-48 and KPC		
IMP, GES and NDM		
CTX-M enzymes: CTX-1, CTX-2, CTX-8, CTX-9 and CTX-25		
Pbp2b, vanA, vanB, ermB, mefA, mecA		

8. Assessment of data (Scoring system)

SCORING

Gram's stain reaction	**Points awarded**
Correct result*	1
Incorrect result	0

* Where discrimination was highly subjective e.g. Gram positive cocci or Gram positive cocci/diplococcic both results are scored as correct

Identification results (lyophilized cultures/simulated clinical specimens)

Level of identification reported	Points awarded
Correct species	2
Correct genus, incorrect species	1
Correct genus, lack of discrimination to species level	1
Incorrect genus	-1
Negative result or no growth	0

Participants are expected to identify these important pathogens to species level. Discrimination of the isolates to genus level only or reporting an incorrect species within the species is unhelpful in the clinical management of the patients and is therefore awarded only one point. Reporting an incorrect genus could lead to inappropriate clinical management and therefore carries a higher penalty score than reporting an incorrect species, hence a score of minus one.

Antimicrobial susceptibility testing (AST) results

Although an identical panel of antibiotic agents is listed for all the EQA specimens, participants are expected to select these agents for antimicrobial susceptibility testing

Assigned scores

Reference results	Participant's result		
	S	I	R
S	2	1	0
I	1	2	1
R	-1	1	2

Summary of scoring criteria for tests for antimicrobial resistance:

Reference results	Participant's result	
	Present	Absent
Present	2	0
Absent	-1	2

9. Complete Report of Participants (Result announcement and feedback)

- Participant will be given two week's time from the date of receipt of EQAS panel to perform the testing. Participants can send the reports either by the posting the reporting sheet to CMC or e-mail the reports
- CMC will evaluate the reports and give preliminary feedback to the participating centers within two week's time of receipt of report
- Subsequently, CMC will submit the cumulative final report to ICMR in the next two week's time
- All performance data is treated as confidential

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