Standard Operating Procedure (SOP) for External Quality Assessment Scheme (EQAS)

Version 1.2 dated 08-08-2018

DEPARTMENT OF CLINICAL MICROBIOLOGY

CHRISTIAN MEDICAL COLLEGE, VELLORE

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1. Background

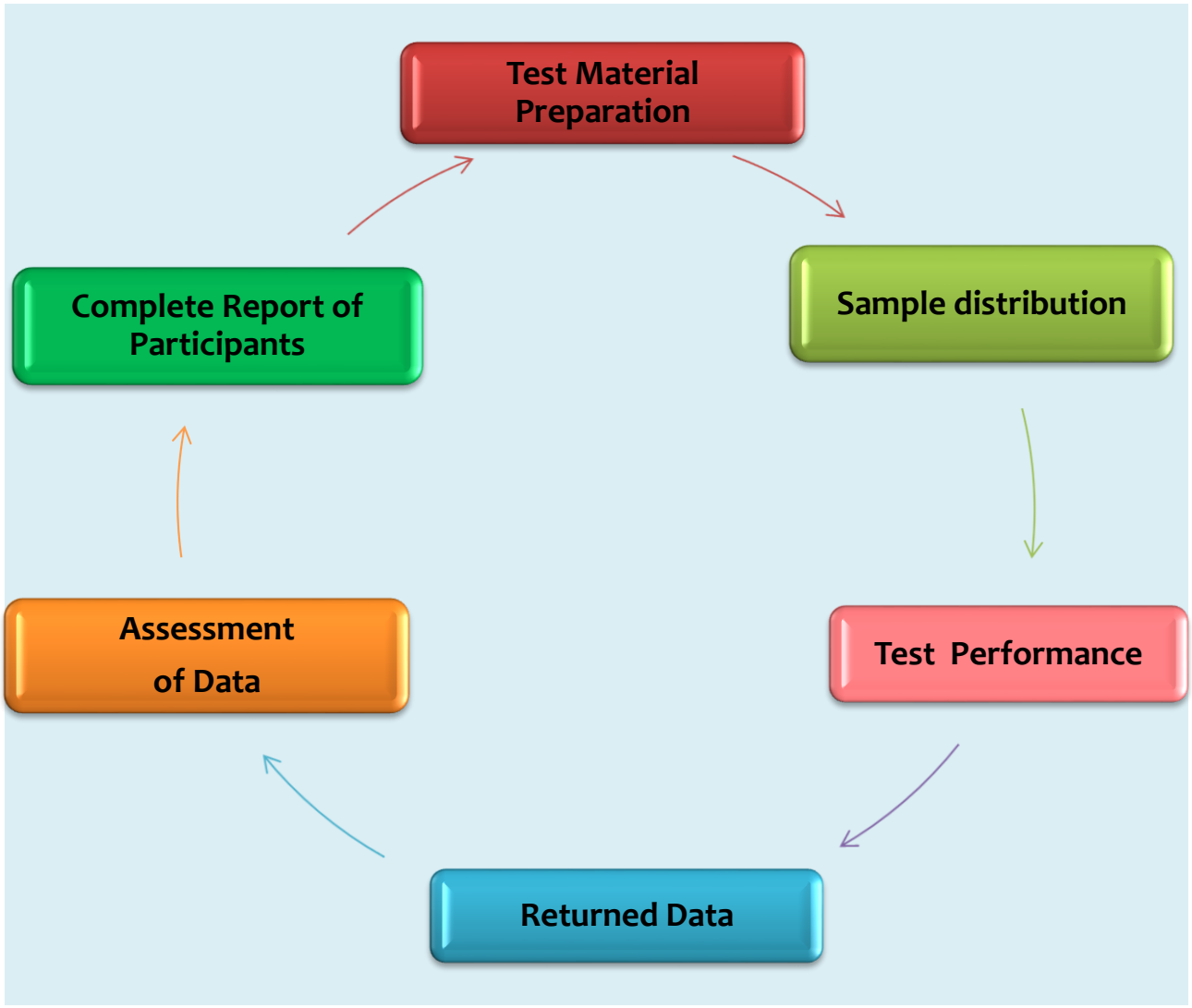
Christian Medical College is pleased to introduce bacteriology, molecular biology laboratory supports through conducting external quality assessment program and invites your laboratory to participate in the EQA survey.

External quality assessment scheme is an important component in any of the health care surveillance schemes to achieve maximum quality and reliable data. When used in conjunction with daily QC, these external programs will assist laboratories in improving analytical quality, inter-laboratory agreement, identify potential equipment or reagent failures, and identify any training deficiencies

2. EQA survey objectives

- To assess the capacity of bacteriology/molecular biology laboratories supporting surveillance network
- To assess the quality of microbiological laboratory work to ensure the data collected by participating laboratories meets set targets and standards

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



4. EQA distribution testing guidelines

The distribution includes smears for gram stain, lyophilized clinical specimens for culture identification, antimicrobial susceptibility testing and genotypic characterization. Non culture specimens for molecular testing and typing will be included in this survey.

The pathogens included in smear testing will have clinical specimens such as:

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

The pathogens included in culture testing will have clinical specimens such as: (Table 1)

Table 1

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	Group A haemolytic *Streptococci*	*E. coli* (EPEC)	**E. coli*	*Staphylococcus aureus (MRSA, VISA)*
Enterococcus spp (VRE)	*Haemophilus influenzae*		*Corynebacterium diphtheriae*	*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*			

Antimicrobial susceptibility testing (AST):

AST to be performed for each of the pathogen for the given list of antimicrobials listed in the protocol

Genotypic testing for antimicrobial resistance (AMR) will include:

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

- ESBL (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{CTX\text{-}M\ 1, 2, 8, 9, 25}$)
- *AmpC* (*bla*$_{DHA}$, *bla*$_{MOX}$, *bla*$_{FOX}$, *bla*$_{CMY}$, *bla*$_{ACC}$, *bla*$_{ACT}$)
- Class A carbapenemase (*bla*$_{KPC}$, *bla*$_{GES}$)
- Class B carbapenemase (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$)
- Class D carbapenemases (*bla*$_{OXA\text{-}48\ like}$, , *bla*$_{OXA\text{-}51\ like}$, *bla*$_{OXA\text{-}23\ like}$, *bla*$_{OXA\text{-}24\ like}$, *bla*$_{OXA\text{-}58\ like}$)

For Gram positive organisms:

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

5. SOP for test material preparation:

5.1 Simulated smear preparation

Scope

All competent technical staff may perform this test. Students may perform this test under supervision of a suitably qualified technical staff member.

Responsibility

It is the responsibility of all the technical staff who are involved in the PT sample preparation to follow the instructions described in this document.

Definitions

- PTS – Proficiency Testing Scheme
- ATCC – American Type Culture Collection
- MIC - Minimum inhibition concentrations
- CMC – Christian Medical College

- ID – Identification
- AST – Antimicrobial susceptibility testing
- N/A – Not applicable
- SOP – Standard Operating Procedure
- BHI – Brain Heart Infusion media

Laboratory equipment:

- Vortex agitator
- Spectrophotometer
- Balance
- O_2 Incubator
- Autoclave
- -70˚C freezer
- -20˚C freezer
- P200 Pipette
- Microtitre plate

Potential sources of variation, limitations and interferences

Contamination of media: all media are quality controlled recorded on the working card.

Homogeneity of samples: the product is places on a magnetic stirrer and is constantly stirred to ensure even distribution of cells and bacteria.

Environmental and safety precautions

Universal safety precautions are followed when preparing samples for the programme. Any broken, damaged or unsuitable slides or samples are disposed of in the appropriate way. Staffs are to wear the necessary personal protective clothing.

Equipment

- Vortex agitator
- Spectrophotometer
- O_2 incubator

- Centrifuge
- Magnetic stirrer
- Microscope

Specimen collection

Selection of pathogen:

Isolates are selected from stored clinical isolates or control organisms – specific isolates that demonstrate aspects of identification, serotyping/grouping, or susceptibility testing are chosen:

Panel decision

The isolate/s are submitted from either a reference laboratory with their laboratory working card confirming identification, serotyping/grouping, susceptibility testing as appropriate (β-lactamase testing, disc diffusion, minimum inhibition concentrations - mics) or obtained from the American Type Culture Collection - ATCC (with relevant documentation) which is managed by CMC (with confirmation of results).

A separate working card is completed where the intended survey number, and any additional information is recorded.

5.1.1. Simulated Cerebrospinal Fluid (CSF) Smear

- Purpose
- Principle of the procedure
- Procedural steps
- Quality control procedures

Purpose

To prepare simulated cerebrospinal fluid (CSF) smears using polymorphonuclear leukocytes (PMN) mixed with an organism.

Principle of the procedure

The examination of CSF smears using the gram stain is helpful in establishing presumptive diagnosis based on the stain reaction and morphology of the organism. Polymorph prep allows for the separation of whole blood into 4 different components: plasma, lymphocytes, polymorphonuclear leukocytes (PMN) and erythrocytes. The PMN can be removed as used to simulate the appearance of CSF smear specimen containing a pathogen.

Reagents

- Blood agar
- Minimum Essential media
- Polymorph prep
- EDTA blood
- Sterile beaker
- Sterile swabs
- Sterile Pasteur pipettes
- Sterile 15ml centrifuge tubes
- Disposable cuvettes
- Microscope slides
- Phosphate buffered saline 7.2 %
- Mueller Hinton broth
- Pipette and tips
- Magnetic stirrer bar

Procedural steps

Polymorphonucleocytes preparation:

1. Place 6mls of polymorph prep™ into each labeled 15ml sterile screw-capped centrifuge tube
2. Carefully layer 6mls of fresh EDTA blood (approximately 2 tubes per isolate) onto the polymorph prep without mixing the two solutions

3. Spin the tube for 35mins at 1600 rpm
4. After spinning, the layers are observed: there is a large plasma layer, a very dense and fluffy looking lymphocyte layer, a whitish layer with a very faint cloud of denser material in it (PMNs), next the reagent layer with a button of erythrocytes
5. Each layer is carefully removed and placed in a separate clearly labeled 15ml tube
6. Add 4mls of minimum essential medium (mem), which has been brought to room temperature, to the tube containing the PMNs
7. The fluids are gently mixed and then spun at 22-2300 rpm's for 10 minutes.
8. Pour off the supernatant and add another 4mls of MEM to the tube. Gently resuspend the pellet. The tube is again spun as in step 7
9. Pour off the supernatant and dribble 1ml of MEM into the tube. Using a sterile pipette gently resuspend the cells
10. This is the PMN mixture and must be kept on ice or in a fridge if not been used immediately. It is preferred to use freshly prepared cells,
11. Place 10ul of the cell suspension onto a clean glass slide and stain with gram-stain to check the number of cells present - there should be 6-10 PMNs/field under the x100 oil immersion objective
12. Dilute the PMN to give an approximate count of approximately 3 ml.

Organism suspension preparation:

1. The day before the PMN preparation, two tubes of Mueller-Hinton broth/ BHI broth are inoculated with a colony of the organism, and incubated overnight.
2. If the organism does not require growth in a broth, it can be plated onto blood or chocolate agar (preparation on blood agar or in broth will depend on pathogen)
3. The next day the tubes containing the broth and the organism are centrifuged for 10mins at 3300rpm's.the supernatant is discarded and 6mls of PBS is added.
4. The concentration of the suspension is then read on a spectrophotometer at 570 nm with PBS as the blank
5. An OD of between 0.2 and 0.4 is suitable with 0,3 been the most ideal.

6. The organism from the plate can be emulsified directly into the 6mls of PBS and an OD reading taken.
7. One to two smears are made of the suspension and gram stained to assess concentration and microscopic appearance of bacteria. Include positive & negative controls

Preparation of the simulated smear:

1. Make a 1:5 dilution of PMNs and bacterial suspension, (1 part bacteria to 4 parts PMNs). Mix well using a pipette.
2. Pipette 10µl onto a clean glass slide. The slide is heat-fixed and gram stained.
3. The slide is examined to check the number of cells and organisms per 100× field. Varying dilutions can be made to obtain the best picture. Smear needs to resemble a CSF smear as closely as possible.
4. A small stirrer bar must be added to keep the product stirring while making the slides so as to give an even distribution of cells and bacteria on all the slides. This gives a more homogenous result. Slides can be stored at 4°c for up to 3 months.
5. Prepare enough slides so that each participant receives 2 slides each per organism

Quality control procedures

1. A sterility check must be carried out on all media and reagents used in the preparation of the simulated CSF smear
2. Timing of QC of the slides (depending on the number of slides being sent out, 5-10% are kept back for qc purposes. This is based on a military standard)
    - 5 random slides are used for immediate qc: 2 staff members will review all 5 slides for quality of the smear, characteristics of the neutrophils and bacteria
    - Any additional slides are kept at room temperature in case of a discrepant result between referee's
    - Slides are kept in the laboratory until a review of responses and qc results are reconciled

3. Failure of QC:
    - Need 100% compliance: slides need to demonstrate the characteristics required by the case
    - If any slide fails, hold shipment, review type of failure
    - If review exposes a shortcoming in the smear preparation, postpone shipment and start from the beginning

5.1.2 Simulated sputum smear

Purpose

To provide detailed instruction on how to manufacture simulated sputum smears that contains polymorphonuclear leukocytes (PMN), epithelial cells and normal flora of the oral cavity, together with mucous strands. Smears can be used as part of a bacteriology survey

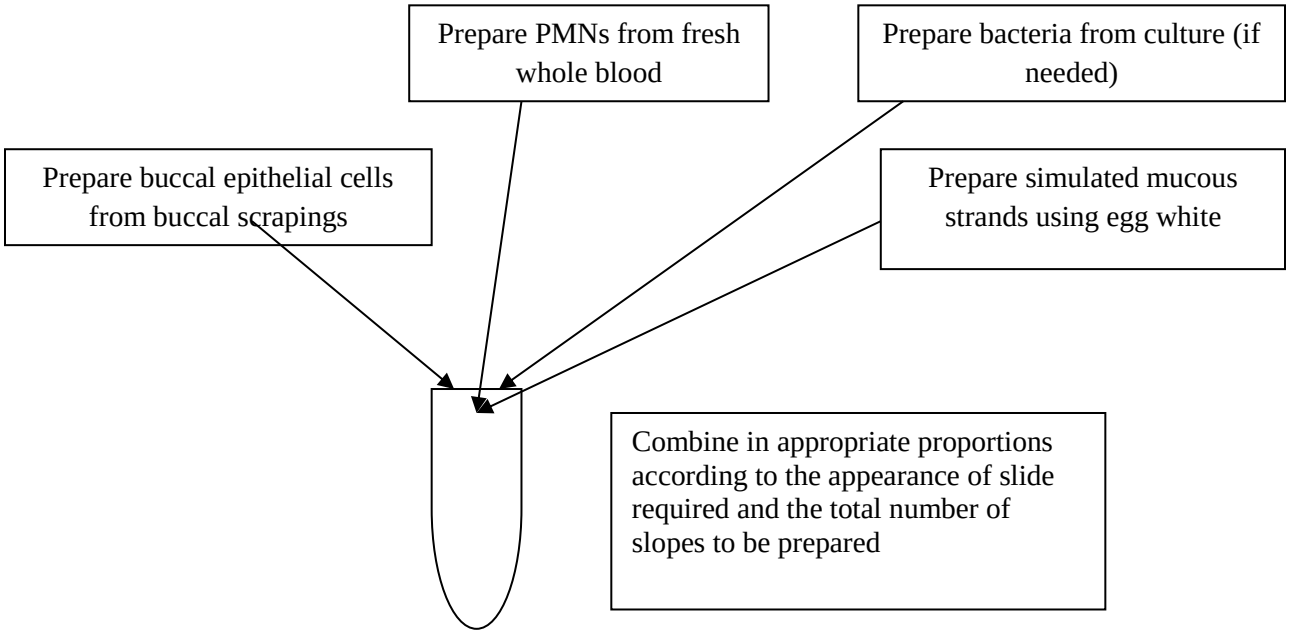
Principle of the procedure

Simulated sputum smears allows for the microscopic identification of possible respiratory infections compared to normal flora that is present in sputum. The addition of albumin of the egg white simulates the mucous present in sputa.

Reagents

- Polymorphprep
- 15ml fresh human blood collected in EDTA tubes
- Minimum Essential Medium (MEM)
- Phosphate buffered saline ph 7.2
- One fresh egg
- Bacteria in Mueller-Hinton broth
- Sterile swabs
- 5 ml syringes
- Hypodermic needles
- Slides

Procedural steps:



Polymorphonucleocytes preparation:

1. Place 6mls of Polymorphprep™ into each of the labeled sterile 15ml screw-capped tube. Prepare 3 tubes to have sufficient cells
2. Carefully layer 6mls of fresh EDTA blood onto the Polymorphprep without mixing the 2 solution
3. Spin the tubes for 35mins at 1600 rpm
4. After spinning, the layers are observed: there is a large serum layer, a very dense and fluffy looking lymphocyte layer, a whitish layer with a very faint cloud of denser material in it (PMNs), next the reagent layer with a button of erythrocytes
5. Each layer is carefully removed and placed in a separate tube and clearly labeled as to what is in the tube
6. Add 4mls of minimum essential medium (MEM), which has been brought to room temperature, to the tube containing the PMNs
7. The fluids are gently mixed and then spun at 22-2300 rpm's for 10 minutes

8. Pour off the supernatant and add another 4mls of MEM to the tube. Gently resuspend the pellet. The tube is again spun as in step 7
9. Pour off the supernatant and dribble 1ml of MEM into the tube. Using a sterile pipette gently resuspend the cells
10. Place 10ul of the cell suspension onto a clean glass slide and stain with Gram-stain to check the number of cells present

Epithelial cell preparation

1. Request 2 volunteers to rinse out their mouths with sterile PBS ph 7.2, which they can spit into a basin. This is just to wash out the mouth prior to obtaining the epithelial cells.
2. Dip a sterile swab into a sterile bottle of PBS pH 7.2
3. Vigorously brush buccal cavity with the swab
4. Again rinse out the mouth with 10ml PBS, and spit the mouth contents into a 50ml labeled conical centrifuge tube.
5. Rotate and press the sterile swab against the sides of the 50ml tube to release all the buccal epithelial cells into the PBS
6. Vortex the tube (containing saliva, PBS and cells) vigorously for 10 seconds
7. Centrifuge the tube at 2200 rpm for 10 minutes
8. Discard the supernatant
9. Resuspend the pellet in PBS to make up 1ml of solution.
10. Gram stain the slide to confirm the presence and number of epithelial cells.

Organism suspension preparation (only required if simulated material is intended to represent sputum from a patient with a lower respiratory infection):

1. The day before the PMN preparation; two tubes of Mueller-Hinton broth are inoculated with a colony of the organism, and incubated overnight.
2. If the organism does not require growth in a broth, it can be plated onto blood agar (preparation on blood agar or in broth will depend on pathogen)

3. The next day the tubes containing the organism in broth are centrifuged for 10mins at 3300 RPM's.The supernatant is discarded and 6mls of PBS is added.
4. The concentration of the suspension is then read on a spectrophotometer at 570 nm with PBS as the blank
5. An OD of between 0.2 and 0.4 is suitable with 0,3 been the most ideal.
6. The organism from the plate can be emulsified directly into the 6mls of PBS and an OD reading taken.
7. One to two smears are made of the suspension and Gram stained to assess concentration and microscopic appearance of bacteria.

Preparation of simulated mucous

1. Label a 50ml sterile plastic centrifuge tube with 1:2 Egg white
2. Separate the white of an uncooked egg into the labeled tube
3. Combine 10ml of PBS pH 7.2 with 10ml of fresh egg white Vortex the egg white dilution vigorously using a high speed vortex for about 2 minutes (until foam appears on the surface)
4. Remove the white foam with sterile swabs and discard
5. The remaining suspension is then used as part of the background solution.

Preparation of the simulated smear:

1. Combine the PMN mixture with the epithelial cells, egg white background material and AFB Smear fixative in appropriate proportions.
2. Pipette 10µl of the mixture onto a clean glass slide. The slide is heat-fixed and stained with Gram method
3. The slides are examined to check the number of cells and organisms per 100× field. Varying dilutions can be made to obtain the final acceptable dilution. The final dilution is recorded on the working card
4. If bacteria are required, make a 1:5 dilution of PMNs and bacterial suspension, (1 part bacteria to 4 parts PMNs). Mix well using a pipette.

5. Make a test slide to confirm that appearance is appropriate
6. Use a magnetic stirrer to ensure even distribution of cells and bacteria on all the slides. This is for homogeneity

Quality control:

1. A sterility check must be carried out on all media and reagents used in the preparation of the simulated sputum smears
2. The amount of slides that are sent for QC depends on the size of the batch that is to be made. On average 5-10% of the batch is quality controlled.
3. 5 random slides are used for immediate QC: 2 staff members will review all 5 slides for quality of the smear, characteristics of the Neutrophil and bacteria (if necessary) this is only done for routine sputum slides.
4. Slides are kept in the laboratory until a review of responses and QC results are reconciled
   - *Failure of QC:*
   - Need 100% compliance:
   - If any slide fails, hold shipment, review type of failure
   - If review exposes a shortcoming in the smear preparation, postpone shipment and start from the beginning

5.2 Lyophilized culture (For Identification and Antimicrobial Susceptibility testing)

The organisms/pathogens likely to grown in the clinical specimens such as Blood, CSF, Respiratory, Urine, Faeces and Skin and soft tissue infections will be lyophilized as per the standard procedures and sent to the participating centers for testing.

Lyophilization

Lyophilization procedures to be performed under aseptic conditions (Skim milk to be used for lyophilization)

Principle of the procedure

Freeze-drying is a three step process:

1. The product is frozen solid so that the water present in the material is converted to ice.
2. The freeze drying process – the ice formed in the freezing is removed from the product by direct conversion from solid to vapour (sublimation)
3. The water which was strongly bound to the solids of the sample is converted to vapour and removed from the product.

The main goal for freeze drying is to preserve materials in their natural states and to keep them stable. Organisms that are unable to be sent in a simulated form are more fastidious and require lyophilisation.

Procedural steps

Preparation of vials:

The glass injection vials are packed into the stainless trays, placed in an aseptor bag and autoclaved at 121°C for 15 minutes. The rubber stoppers are also autoclaved

Preparation of isolate:

All media and tubes must be clearly marked. Refer to SOP "Labeling of media and consumables used during PT sample preparation" QASM0025

Day 1:

Plate the organism onto suitable media and incubate overnight at an atmosphere and temperature optimum to the growth of the organism. This is also a QC of media to see that it does support the growth of the organism

Day 2:

If there is no sign of contamination on the original plate from the day before, set up 5-10 plates of a pure culture of the organism on suitable media to obtain a

heavy growth (make sure that an individual colony is picked off rather that a sweep at this stage). Avoid the edges of the agar plate. Incubate overnight (or longer if necessary) at an atmosphere optimum to the growth of the organism.

Day 3:

1. Observe for any growth on sterility plates of skim milk subcultures from Day 1 (after 48 hours incubation)
2. Observe plates from Day 2 (isolate to be lyophilised) for pure growth and no contamination
3. Quality control for sterility of skim milk (subculture an aliquot of skim milk onto non-selective media and incubate at room temperature at 37°C for 24-48 hours)
4. Working in a class 2 biosafety cabinet : Place all autoclaved injection vials, beakers, rubber stoppers, skim milk, un-inoculated media plates, sterile loops, pipette and tips in the hood and switch on the UV light for a minimum of I hour.
5. When the staff are ready to start the process the UV light is switched off and the plates with the pure growth of organism are placed in the hood
6. Aliquot the required amount of skim milk with 5% inositol in to a sterile beaker (150-200ml).
7. Wash off the organism into the skim milk using a sterile loop for each plate
8. Subculture a aliquot of the bacterial suspension on non-selective media, prior to and after dispensing into the injection vials and incubate under conditions optimal for the growth of the isolate at 37°C
9. Dispense 1.0ml of the inoculated skim milk into sterile glass injection vials and cap with sterile rubber stoppers
10. The vials are ready for freeze-drying.
11. Place vials in -70°C freezer for two-three hours, while the freeze-drier is set up
12. Vials are left in the freeze-drier overnight: ensure that the temperature and vacuum gauges are at correct settings

Day 4:

1. Seal all vials under vacuum
2. Remove vials from freeze drier.
3. Cap with aluminium seal
4. Re-suspend the appropriate number of vials for QC with 1.0ml of appropriate broth
5. Incubate at room temperature for +/- 15-30 minutes.
6. Subculture the sample to confirm growth and contamination: number of vials to be sampled depends on number to be sent

Quality control

1. A sterility check must be carried out on all media and reagents used in the preparation of the organism
2. The skim milk must be checked for sterility prior to the addition of the organism.
3. The skim milk mixture with the organism is plated onto non-selective media at the start and at the end of the dispensing procedure. This is to confirm growth as well as to check for contamination of the samples.
4. All lyophilized samples are kept at room temperature until shipping.
5. Timing of QC (Depending on the number of samples being prepared, 5-10% random samples are kept back for QC purposes)
    - 2 vials are sub-cultured immediately after the batch has been prepared , check for predominant growth on selective and non-selective media
    - Samples that are inhomogeneous, show instability, damaged or broken are disposed of in the appropriate way
    - All bottles will be numbered and dated, identifying at which stage they were sub-cultured. This is to be recorded on the attached working card.
    - At closure of a shipment all remaining bottles are sub-cultured where possible

- At the closure of survey and QC the organism is again confirmed for identification, serotyping/grouping, susceptibility testing as appropriate (β-lactamase testing, disc diffusion, Minimum inhibition concentrations - MICs)
- According to these results the sample will be deemed acceptable or unacceptable for evaluation
- Bottles are kept in the laboratory at room temperature until a review of responses and QC results are reconciled

6. **Failure of QC:**
    - Need 100% compliance
    - Any non-viability or contamination of QC samples 1 and 2 constitutes failure (mixed cultures – need to evaluate predominance of pathogen if appropriate to the specimen)
    - Hold shipment, repeat test with a larger number of vials (dependent on shipment size)
    - If repeat test consistent with the first non-compliance, stop shipment, evaluate necessary corrective action and start from the beginning
    - Subsequent vials are tested for viability over time: non-viability will determine the inability to evaluate laboratories with no growth
    - The organism is stored at -70ºC, indefinitely and can then be used in the event of a discrepant result from one of the referee laboratories.
    - Sometimes an independent quality control is performed by designated laboratories when there are discrepant results

5.3 Simulated clinical specimens (For culture, Identification and Antimicrobial susceptibility testing

5.3.1. Simulation of CSF specimen

Purpose

To simulate a CSF specimen with a specific pathogen

Principle of the procedure

Cerebrospinal fluid (CSF) is a clear colourless liquid bodily fluid found in the brain and spine. CSF can be tested for the diagnosis of a variety of neurological diseases, usually obtained by a procedure called lumbar puncture. CSF is extracted through the needle, and tested. Cells in the fluid are counted, as are the levels of protein and glucose. These parameters alone may be extremely beneficial in the diagnosis of subarachnoid hemorrhage and central nervous system infections (such as meningitis). Moreover, a CSF culture examination may yield the microorganism that has caused the infection. The process detailed in this document described the production of simulated CSF samples in order for pts participants culture the pathogen in their laboratories.

Reagents:

- Blood agar
- Brain Heart Infusion Broth
- Sterile swabs
- Sterile pasteur pipettes
- Sterile 50ml centrifuge tubes
- Disposable cuvettes
- Pipette and tips
- Cryovials

Procedural steps: Isolate preparation

1. Subculture the isolate onto 2-3 blood agar plates (non-selective medium) and incubate overnight at 37^{o}c aerobically (optimal incubation dependent on pathogen)
2. Observe for pure growth after incubation
3. Inoculate a sweep of the organism (using a sterile cotton wool swab) into a 50ml sterile plastic centrifuge tube containing a sufficient amount of BHI broth needed for the sample preparation.
4. Vortex
5. Confirm/adjust to a turbidity of 0.2 absorbance spectrophotometrically at 570 wavelength (mcfarland 1,0)

6. This is the working solution

Method of sample preparation

1. Working in a biohazard cabinet, 1ml of the prepared bacterial suspension is dispensed into cryo-tubes or suitable vials.
2. The prepared specimens are then left at room temperature until shipping.

Quality control procedures

1. A sterility check must be carried out on all media and reagents used in the preparation of the organism and sample
2. The BHI and organism mixture is plated onto non-selective media at the start and at the end of the dispensing process. This is to confirm growth as well as to check for contamination of the samples.
3. All inoculated bottles are stored at room temperature until shipping.
4. Timing of qc (depending on the number of samples prepared, between 10-20% are kept back for qc purposes)

- 2 vials are sub-cultured immediately after the batch has been prepared.
- Samples that are inhomogeneous, show instability, damaged or broken are disposed of in the appropriate way
- 3 samples are sub-cultured at weekly intervals. This can vary depending on the number of samples that were prepared
- All bottles will be numbered, identifying at which stage they were sub-cultured
- At closure of a shipment (when the eqa panel decides that no more responses are outstanding or expected): all bottles are sub-cultured
- Bottles are kept in the eqa laboratory until a review of responses and qc results are reconciled

Failure of QC:

- Need 100% compliance
- Any contamination or non-viability of pathogen in any of the samples constitutes failure
- Hold shipment, repeat test

- If repeat test is consistent with the first non-compliance, stop shipment, evaluate necessary corrective action and start from the beginning.
- Subsequent samples are tested for viability over time: non-viability will determine the inability to evaluate laboratories with no growth.
- In the event of a discrepant result from one of the referee laboratories, the organism is also stored at -70ºc.

5.3.2. Simulation of Urine specimen

Purpose

To provide instructions on how to produce simulated urine specimen with a specific pathogen

Principle of the procedure

Urinary tract infections can be classified into uncomplicated and complicated categories. Uncomplicated infection is acute cystitis or pyelonephritis in women without underlying urinary tract or systemic infection. Cystitis or pyelonephritis in males, children, chronically catheterized patients and women with recurrent infection urologic abnormalities or underlying disease is considered as complication infection. The process detailed in this document described the production of simulated urine in order for PTS participants culture the pathogen in their laboratories.

Reagents:

- Blood agar / MacConkey agar
- Mueller-Hinton broth
- Sodium formate
- Boric acid
- Sterile loops
- 2ml cryovials or other appropriate vials
- Sterile 15ml centrifuge tubes
- Disposable cuvettes

- Appropriate media for quality control samples

Procedural steps: Media: Stabilizer

A fresh stabilizer is prepared, autoclaved and checked for sterility before use.

- 9g Sodium Formate made up to 1liter.
- 18g Boric Acid made up to 1liter.
- Mueller Hinton Broth (obtained in 100ml quantities.)
- Equal volumes of the above are added together and then autoclaved.

2ml of the sterile stabilizer is dispensed into cryo-tubes or suitable vials and can be stored in the fridge until required. It is best to prepare fresh stabiliser the day before the samples are prepared.

Isolate Preparation:

All media and tubes must be clearly marked.

1. Subculture the isolate onto 2-3 blood agar plates (non-selective medium) and incubate overnight at 37ºC aerobically (optimal incubation dependent on pathogen)
2. Observe for pure growth after incubation
3. Sterility QC of Mueller Hinton stock broth is performed.
4. Inoculate a sweep of the organism (using a sterile loop) into a sterile plastic centrifuge tube containing 6mls of Mueller Hinton broth
5. Vortex
6. Confirm/adjust to a turbidity of 0.1 absorbance spectrophotometrically at 570 wavelength (McFarland 0.5) equivalent to 1×10^8cfu/ml.
7. Make further dilutions (1:10) of the stock solution until the desired CFU concentration is obtained.
8. If the final concentration in the simulated specimen is to be $1x10^5$, then the stock solution should have a concentration of $1x10^6$
9. Confirm the cfu/ml of this suspension by inoculating a blood agar and MacConkey plate (90mm) with a urine loop (1:1000); perform a colony count after incubation: if individual colonies visible - count colonies; if numerous – count a quarter plate and

multiply by 4, multiply by 5 for cfu/ml; if confluent growth – document as too numerous to count (TNTC)

Method for sample preparation:

1. Dispense 200µl of the final stock dilution ($1x10^6$) as calculated above into 2ml of the stabiliser which has already been dispensed in to the cryo vials.
2. The sample will then have a concentration of $1x10^5$ cfu/ml

Quality control

- A sterility check must be carried out on all media and reagents used in the preparation of the organism
- All vials are kept at room temperature until shipping
- Timing of QC (Depending on the number of samples being prepared, 5-10% random samples are kept back for QC purposes)
- Isolates are initially confirmed for identification, serotyping/grouping, susceptibility testing as appropriate (β-lactamase testing, disc diffusion, Minimum Inhibition Concentrations - MICs). Isolates are confirmed using API, disc susceptibility, Microscan and Vitek 2 vials are sub-cultured immediately after the batch has been prepared, check for number of CFU per ml on selective and non-selective media
- 2 vials are sub-cultured immediately after sample preparation.
- Samples that are inhomogeneous, show instability, damaged or broken are disposed of following the safety manual.
- Vials are sub-cultured weekly to determine viability.
- All bottles will be numbered and dated, identifying at which stage they were sub-cultured. This is to be recorded on the attached working card.
- At closure of a survey all samples are sub-cultured
- At the closure of the survey, QC is again performed on the isolate to confirm identification, serotyping/grouping, susceptibility testing as appropriate (β-lactamase testing, disc diffusion, Minimum Inhibition Concentrations - MICs)
- According to these results the sample will be deemed acceptable or unacceptable for evaluation

- All documents are attached to the working card
- Samples are kept in the laboratory at room temperature until a review of responses and QC results are reconciled.

Failure of QC:

- Need 100% compliance
- Any contamination or non-viability of pathogen in any of the samples constitutes failure
- Hold shipment, repeat test
- If repeat test is consistent with the first non-compliance, stop shipment, evaluate necessary corrective action and start from the beginning.
- Subsequent samples are tested for viability over time: non-viability will determine the inability to evaluate laboratories with no growth.
- Sometimes it is necessary to include a semi-solid or lyophilised pure culture of the pathogen if there is any doubt of the pathogen's survival over prolonged time.
- In the event of a discrepant result from one of the referee laboratories, the organism which is also stored at -70ºC will be retested.

5.3.3. Simulation of Pus swab:

Purpose

To simulate a pus, urethral or vaginal swab by inoculating a cotton-tipped swab with a suspension of the required organism and then placing the swab in suitable transport media (with or without charcoal)

Principle of the procedure

Swabs are commonly used for obtaining many cultures; however are generally inferior to other methods for collecting specimens. Swabs placed in a transport medium prevent drying and death of bacteria. Good recovery of most bacterial species from these tubes has been demonstrated for up to 48 hours or longer. The use of Stuart or Amies' transport medium serves as an adequate means for holding swab cultures during

transport. The process detailed in this document described the production of simulated swabs in order for PTS participants culture the pathogen in their laboratories.

Reagents:

- Blood agar and any other suitable agar
- Mueller- Hinton, brain heart infusion or serum broth
- Sterile beaker or bottle
- Sterile loop
- Tran swab
- Disposable cuvettes
- Appropriate media for quality control samples (See attached working card)

Procedural steps

Organism suspension preparation:

All media and tubes must be clearly marked.

1. **Day 1**: Set up 2-3 plates of a pure culture of the organism on blood agar or chocolate agar to obtain a heavy growth. Incubate overnight at temperature and atmosphere depending on organism.
2. **Day 2:** A sweep of organism is taken from the blood plate or chocolate agar with a sterile loop. Emulsify the organism in the required amount of suitable broth in a sterile beaker or bottle. The amount of broth required is dependent on the number of sample to be prepared.
3. Vortex.
4. Take the OD reading at 570 nm using the spectrophotometer and suitable broth as a blank control. There is no need for an specific OD reading in this sample, but an OD of between 0.6 and 0.8 is normally sufficient for most organisms
5. This is the working bacterial suspension.

Method for the inoculation of the sterile swabs

1. Working in a safety cabinet and using a pipette, dispense 200µl of the bacterial suspension into a sterile, round bottomed, microtitre plate.
2. Using sterile trans swabs, remove the swabs form the sterile package.
3. Place sterile cotton-tipped swabs into the inoculated wells.
4. Leave the swabs in the wells for approximately 5 minutes to absorb the bacterial suspension.
5. Place the inoculated swabs into supplied transport media tube. The specimens are then stored at room temperature until shipping.

Quality control procedures

All QC results must be recorded on the working card

4. A sterility check must be carried out on all media and reagents used in the preparation of the organism.
5. All swabs remain at room temperature until shipping.
6. Timing of QC (Depending on the number of samples prepared, between 5-10% are kept back for QC purposes)
    - 2 swabs are sub-cultured immediately after sample preparation.
    - Swabs are sub-cultured at weekly intervals. Samples that are inhomogeneous, show instability, damaged or broken are disposed of in the appropriate way
    - Swabs are sub-cultured weekly onto appropriate media.
    - All swabs will be numbered, identifying at which stage they were sub-cultured
    - At closure of a shipment a swab is sub cultured.
    - At the closure of survey and QC the organism is again confirmed for identification, serotyping/grouping, susceptibility testing as appropriate (β-lactamase testing, disc diffusion, Minimum inhibition concentrations - MICs)
    - According to these results the sample will be deemed acceptable or unacceptable for evaluation
    - Swabs are kept in the laboratory at room temperature until a review of responses and QC results are reconciled

Failure of QC:

- Need 100% compliance
- Any non-viability of pathogen constitutes failure (mixed cultures – need to evaluate predominance of pathogen)
- Hold shipment, repeat test
- If repeat test consistent with the first non-compliance, stop shipment, evaluate necessary corrective action and start from the beginning
- Subsequent swabs are tested for viability over time: non-viability will determine the inability to evaluate laboratories with no growth.
- Sometimes it may be necessary to include a semisolid or lyophilised pure culture of the pathogen if there is any doubt of the pathogen's survival over prolonged time.
- The organism is stored at -70ºC, indefinitely and can then be used in the event of a discrepant result from one of the referee laboratories.
- Sometimes an independent quality control is performed by designated laboratories when there are discrepant results

5.3.4. Simulated Faeces specimen

Purpose

To provide detailed instructions on how to prepare a simulated stool specimen by mixing lentils with the pathogen (e.g. *Shigella dysenteriae* type 1) in Cary-Blair transport medium.

Principle of the procedure

Diarrhoeal stool samples allows for the detection of stool pathogens. Pathogens in stool samples are not viable for long periods of time. Boiled lentils are mixed into a pulp visually appear like stool. The enteric pathogen is added to the lentils to simulate a stool sample. The lentils and pathogen are added to Cary-Blair medium which allows for the transportation and viability of the pathogen.

Reagents:

- Blood agar
- Mueller-Hinton broth
- Lentils
- Cary-Blair transport medium in bijou bottles
- Sterile beaker
- Wooden spatula
- Sterile swabs
- Sterile 15ml centrifuge tubes
- Disposable cuvettes
- Appropriate stool media for quality control samples (See attached working card)

Procedural steps

Lentil preparation:

1. **Day 1:** Boil lentils, and then blend into a pulp with a liquidizer.
2. **Day 2:** Using a wooden spatula weigh out the required amount of lentils, on a balance into a beaker which has been autoclaved in an Aseptor bag prior to use. Approximately 42g of lentils will yield 35 samples when inoculated into the Cary-Blair medium. If the lentil mixture is too dry, add some broth (Mueller-Hinton) before autoclaving. Cover with the same Aseptor bag and autoclave. Once cool, the lentils can be stored, covered in the fridge at 4˚C until required. Make sure that the beakers are clearly marked with the date of preparation.
3. Have some sterile broth on hand as after autoclaving the mixture can sometimes again be too dry and the addition of broth will make the lentil mixture creamier.
4. Any lentils remaining are then stored in a plastic container in the -20˚C freezer until required for another survey.

Organism preparation:

All media and tubes must be clearly marked. Refer to labeling of media and consumables used during PT sample preparation SOP

Day 1:

1. Plate the organism onto a blood agar plate and incubate overnight at an atmosphere and temperature optimum to the growth of the organism. This is also a QC of media to see that it does support the growth of the organism

Day 2:

2. If there is no sign of contamination set up 1-2 plates of a pure culture of the organism on blood agar to obtain a heavy growth (make sure that an individual colony is picked off rather that a sweep at this stage). Incubate overnight (or longer if necessary) at an atmosphere optimum to the growth of the organism.

Day 3

3. The following day a sweep of the organism is taken from the blood plate with a sterile swab. Emulsify the organism in 6mls of Mueller-Hinton broth, in a sterile plastic centrifuge tube. Conduct a sterility test on the broth by placing a small drop on blood agar and the plating this out. Incubate for 24-48hrs at 37˚C under aerobic conditions
4. Vortex on vortex mixer for a few seconds.)
5. Confirm the optical density (OD) of the suspension by reading it on the Secomam Prism spectrophotometer at an absorbance wavelength of 570 with Mueller Hinton-broth as the blank. The OD reading can vary depending on the organism to be used (between 0.3 and 0.8). This can be determined by doing trials.
6. Adjust to obtain the required OD reading by adding Mueller-Hinton broth or more organisms to the suspension. Record the final OD reading on the working card.
7. This is the working bacterial suspension.
   If preparing a Vibrio species using Alkaline Peptone Water instead of Mueller Hinton Broth.

Method of inoculating the Cary-Blair medium:

1. Do a sterility test on the autoclaved lentils by plating out a pea-sided portion on blood agar. Incubate for 24 – 48 hrs at 37˚C under aerobic conditions
2. With a sterile disposable pipette dispense 2ml of the working bacterial suspension per 45g of lentils into the beaker containing the autoclaved lentils. These amounts can vary depending on the number of samples to be prepared
3. Mix well and carefully using a wooded spatula.
4. Inoculate generous pea-sized portions of the lentil and organism mixture into each Cary-Blair vial using a sterile disposable pipette where the tip has been cut off just before the bulb. Stir the mixture regularly for homogeneity.
5. Seal by closing the screw cap top.
6. Store at room temperature (18-24˚C) until shipping.

Quality control procedures

All QC results must be recorded on the working card

1. A sterility check must be carried out on all media and reagents used in the preparation of the organism and lentils.
2. The lentils must be checked for sterility after autoclaving and prior to the addition of the organism.
3. The lentil and organism mixture is plated onto non-selective media at the start and at the end of the inoculation of the Cary Blair media. This is to confirm growth as well as to check for contamination of the samples.
4. All bottles are kept at room temperature until shipping.
5. Timing of QC (Depending on the number of samples prepared, between 5-10% randomly selected samples are kept back for QC purposes)
   a. 2 bottles are sub-cultured immediately after sample preparation.
   b. Samples that are inhomogeneous, show instability, damaged or broken are disposed of in the appropriate way
   - 2 bottles are sub-cultured at weekly intervals. This can vary depending on the number of samples that were prepared

- Ideally there should be a (++) to (+++) growth of the organism/s on the non-selective media after 24-48hrs incubation. This growth is recorded on the working card
- For sample traceability all bottles will be numbered and dated, identifying at which stage they were sub-cultured. This is to be recorded on the attached working card.
- At closure of a shipment all bottles are sub-cultured where possible
- At the closure of survey and QC the organism is again confirmed for identification, serotyping/grouping, susceptibility testing as appropriate (β-lactamase testing, disc diffusion, Minimum inhibition concentrations - MICs)
- According to these results the sample will be deemed acceptable or unacceptable for evaluation
- Bottles are kept in the laboratory at room temperature until a review of responses and QC results are reconciled

Failure of QC:

- Need 100% compliance
- Any non-viability of pathogen with initial sub-culture prior to inoculation in Cary-Blair constitutes failure (mixed cultures – need to evaluate predominance of pathogen)
- Hold shipment, repeat test
- If repeat test is consistent with the first non-compliance, stop shipment, evaluate necessary corrective action and start from the beginning
- Subsequent bottles are tested for viability over time: non-viability will determine the inability to evaluate laboratories with no growth.
- Sometimes it may be necessary to include a semisolid or lyophilised pure culture of the pathogen if there is any doubt of the pathogen's survival over prolonged time.
- The organism is stored at -70ºC, indefinitely and can then be used in the event of a discrepant result from one of the referee laboratories.
- Sometimes an independent quality control may need to be performed by designated reference/referee laboratories when there are discrepant results

Validation of simulation clinical specimen preparations:

5.3.1 Preparation of samples and Quality Control of Simulated CSF isolate in BHI broth

Survey no: _____
Organism: _____
Date of preparation: _____ Initial: _____

Quality control of un-inoculated media:

Media/Reagents	Date of subculture	Date read	initial	BA / Choc	BA / Choc
BHI					

Quality control of inoculated media:

Media/Reagents	Date of subculture	Date read	initial	BA / Choc	BA / Choc
Inoculated CSF (at start)					
Inoculated CSF (at end)					

Stability and Viability QC (performed during shipping and closure of survey
All media incubated for duration and atmosphere optimal for growth of the organism

Sample no	Date of subculture	Date read	Initial	Blood agar	Choc agar	Other	MALDI/ Vitek2 result
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							

Closure of Survey:
Final ID: _____ Date: _____QC of survey: Acceptable / Unacceptable
Were survey results evaluable: Yes / No

5.3.2. PREPARATION OF SAMPLES AND QUALITY CONTROL OF SIMULATED URINE

Survey no: _____

Organism used: _____

OD reading: _____

Final dilution used: _____

Date of preparation: _____

CFU/ml from stock solution: _____ Initial:_____

Quality control of un-inoculated media:

Media/Reagents	Date of subculture	Date read	24hrs incubation	48hrs incubation	Initial
Mueller Hinton + stabiliser					

Quality control of inoculated media:

Media/Reagents	Date of subculture	Date read	24hrs incubation cfu/ml	48hrs incubation cfu/ml	Initial
Inoculated urine (before dispensing)					
Inoculated urine (after dispensing)					

Stability and Viability QC (performed during shipping and closure of survey
All media incubated for duration and atmosphere optimal for growth of the organism

Sample no	Date of subculture	Date read	Blood cfu/ml	MacConkey cfu/ml	MALDI/Vitek 2 Result	Initial
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						

Closure of Survey:

Final ID:_____ Date: _____ Initial:_____

QC of Survey : Acceptable / Unacceptable

Were the survey results evaluable: Yes / No

5.3.3. PREPARATION OF SAMPLES AND QUALITY CONTROL FOR SIMULATED SWABS

Survey No: _____
Organism used: _____
BA/Choc (QC) - batch no_____ expiry:_____
OD reading: _____
Preparation Date: _____ Initial:_____
Swabs batch number and expiry date:_____

1. **<u>Sterility check</u>**
(0n blood agar)

	24 hrs	48 hrs	Date	initial
Serum, BHI, Mueller-Hinton broth prior to addition of organism				

2. <u>Contamination QC</u>: After addition of organism and during the sample inoculation procedure

	24 hrs	48 hrs	Date	Initial
Serum, BHI, Mueller-Hinton broth prior to addition of organism (Start)				
Serum, BHI, Mueller-Hinton broth prior to addition of organism (End)				

3. <u>Stability and Viability QC (performed during shipping and closure of survey</u>
All media incubated for duration and atmosphere optimal for growth of the organism

Sample No	Date of subculture	Date Read	Initial	Blood agar	Mac	XLD	DCA	TCBS	Camp media	MALDI/ Vitek 2 result
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										

<u>Closure of Survey:</u>

Final ID: **_____ Date: _____ Initial_____**
QC of survey: Acceptable / Unacceptable
Were survey results evaluable: Yes / No

5.3.4. PREPARATION OF SAMPLES AND QUALITY CONTROL FOR SIMULATED STOOL SPECIMENS

Survey No: _____
Organism used: _____
BA/Choc (QC) - batch no_____ expiry:_____
OD reading: _____ Lentils weighed out:_____g
Preparation Date: _____ Organism added:_____ml
Cary-Blair media (batch no and expiry date): _____

1. **Sterility check of reagents** (0n blood agar)

	24 hrs	48 hrs	Date	initial
a. Mueller Hinton Broth				
b. Lentils after autoclaving				

2. Contamination QC: After addition of organism and during the sample inoculation procedure

	24 hrs	48 hrs	Date	initial
c. Lentils with organism prior to inoculation of Cary-Blair media				
d. Lentils with organism after inoculation of all Cary-Blair media				

3. Stability and viability QC (performed during shipping and closure of survey
All media incubated for duration and atmosphere optimal for growth of the organism

Sample No	Date of subculture	Date Read	initial	Blood agar	Mac	XLD	DCA	TCBS	Camp media	MALDI/ Vitek Results (if applicable)
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										

Closure of Survey:

Final ID: _____ **Date:** _____ **Initial**_____
QC of survey: Acceptable / Unacceptable
Were survey results evaluable: Yes / No

5.4. Molecular testing (from bacterial cultures)

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 **7.6**

6. Sample distribution

- The sample pack will be sent through courier mode, while packing and shipment of EQA materials will be as per international guidelines as prescribed by international air transport association (IATA) guidelines
- An EQAS pack containing challenge strains will be sent to each participating microbiology laboratory in from the reference laboratory at CMC, every 4 months with three cycles per year

Survey Number	Date of sample dispatching	Results Dead line	Responses of Evaluation

7. Test Performance (site specimen processing)

7.1 Smear processing

Gram stain

1. 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.

2. Let the suspension air dry. The suspension MUST be completely dry before proceeding.
3. Heat -fix the smears by quickly passing the slide through a flame three times.
4. Do not over-heat the slide as over-heating will cause significant distortion or destruction of the cells.
5. Flood the slide with crystal violet for 1 minute to stain. Rinse with distilled water. Shake off excess water.
6. Flood the slide with Gram's iodine for 1 minute.
7. 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.
8. Decolorize with acetone until no more stain washes off (5-10 seconds may be enough). Rinse with distilled water. Shake off excess water.
9. 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.
10. Counterstain with safranin for 30 seconds or with carbol-fuchsin for 10-15 seconds. Rinse with distilled water. Shake off excess water.
11. Gently blot the slide using bibulous paper or a clean paper towel. Let air dry.
12. When dry, examine the stained smear under a microscope with 100X oil immersion objective.

- **Reading the Gram stain results (under microscopic examination):**

  Gram-positive organisms will appear dark violet or purple.

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

7.2. Lyophilized culture identification

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

For Gram negative organisms:

- Oxidase

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

For Gram positive organisms:

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

Antimicrobial Susceptibility testing methods:

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 calipers
- Table lamp
- Zone diameter interpretation charts
- Quality control reference strains
- 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 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, nonreflecting 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 subcultured, 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 +4ºC 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. Standard strain of known MIC value 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 ciprofloxacin 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**

Tube No.	1	2	3	4	5	6	7	8	9	10	Control
MH broth	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ciprofloxacin in serial dilutions	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
Discard										0.5	
Culture	Add 0.05 ml or 1 Drop of approximately 0.05 ml to each tube Mix gently, incubate 16 – 18 hours at 37°C, read										
Final concentration of Ciprofloxacin µg / ml	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.015	0.0078	0.0039	

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 BaSO4 turbidity standard 0.5 and 1.0
- Sterile forceps / needle / disk dispenser
- 12 x 100 mm sterile test tubes
- Measuring scales / sliding calipers
- 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

- Refer CLSI guidelines for interpreting the susceptibility pattern

7.3. Simulated clinical specimen processing

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

- Smear processing
- Culture of clinical specimens
- Identification
- Antimicrobial Susceptibility testing

Refer 7.1 and 7.2 protocols for processing the smear, culture identification and susceptibility testing.

7.4 Commercial Antimicrobial Susceptibility (cAST):

VITEK 2:

Antimicrobial susceptibility test based on broth micro dilution with specific cards

Materials Required:

1. For QC organisms use ATCC lyophilized ampoules or other quality source of ready-to-use microorganism (e.g., REMEL disposable Culti-Loops)

2. Sterile Inoculating Loops
3. Supplemental Media: nutrient agar (NA), tryptic soy broth (TSB), trypticase soy agar with 5% Sheep Blood (BAP), and trypticase soy agar (TSA).
4. VITEK 2 Identification cards (for example, GP, ANC, BCL, and GN): store at 2-8°C in unopened original liner.
5. 75 mm × 12 mm clear polystyrene tubes (single use only)
6. DensiCHEK Plus Meter with McFarland Standards for calibration (0.0, 0.5, 3.0, and 5.0 McF) (see section 15).
7. Sterile saline solution (aqueous 0.45% to 0.50% NaCl, pH 4.5-7.0)
8. Bar-coded 10 well cassette card holders
9. Internal Carousel for card processing

Preparation of inoculum

Select the appropriate card based on the Gram stain reaction and the organism's microscopic appearance.

- First, bring card to room temperature before use
- Aseptically transfer at least 3 mL of sterile saline (0.45 % Nacl) into a clear polystyrene 12×75 mm test tube
- Prepare a homogenous organism suspension by transferring several isolated colonies from the plates to the saline tube
- Adjust the suspension to the McFarland standard required by the ID reagent using a calibrated DensiCHEK Plus Meter according to the standard given below in the table

Card	McF Range
GN	0.5-0.63
GP	0.5-0.63
ANC	2.7-3.3
BCL	1.8-2.2

NOTE: the age of the suspension before loading the instrument for AST testing must be less than 30 minutes.

- A test tube containing the microorganism suspension is placed into a special rack (cassette)
- Identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube
- The filled cassette is placed automatically into a vacuum chamber station
- After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels that fill all the test wells
- Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator
- All card types are incubated on-line at 35.5 $\pm$ 1.0ºC
- Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time
- Data are collected at 15-minute intervals during the entire incubation period
- When the cards are loaded, remove the cassette and dispose of the tubes and straws in a biohazard container
- When the cards are processed and results obtained, cards will be automatically ejected into the waste collection bin

Results

- The VITEK 2 using the AES software will release results of an organism ID and or antibiotic susceptibility to the laboratory information system (LIS) automatically unless review is needed
- For reporting the results, the acceptable probability level is 99%
- Results are concurrently printed and the data sent to the Results View folder on the left side of the screen also called the Navigation Tree where the information is archived
- A red cassette in the Navigation Tree is indicative of an error. If an error occurs during processing, refer to the Software User Manual

- Review results printout and file appropriately
- The different levels of identification and its associated information are shown in the table below

Limitations:

1. Very mucoid organism my not provide acceptable result. Alternative methods should be used for these organisms.

2. Colonies grown on EMB plates cannot be used due to carry over of the dye present in the medium.

BD Phoenix system

Inoculum Preparation:

- The isolates to be tested were plated at least once on the sheep blood Columbia agar and incubated at 35-37°C.
- The bacterial suspension for the Phoenix AST test was prepared by adding one drop of AST indicator to 8 ml of Phoenix AST broth.
- The prepared AST suspension was poured into the AST sector of the Phoenix panel.
- Excess suspension was collected by the absorbent pad at the bottom of the panel.
- After sealing with a plastic cover and scanning the panel barcode, the panel was loaded manually into the Phoenix system.

Reading and interpreting results

- Continuous measurements of changes to the indicator as well as bacterial turbidity are used in the bacterial growth determination.
- The instrument takes readings every 20 minutes.
- Organisms growing in the presence of a given antimicrobial agent reduce the indicator, changing it to a pink colour.

- This signals organism growth and resistance to that antimicrobial agent.
- Organisms killed or inhibited by the antimicrobial agent do not cause reduction of the indicator and therefore do not produce a colour change.
- The Phoenix instrument reads and records the results of the antimicrobial tests contained in the panel and interprets the reactions to give a minimal inhibitory concentration (MIC) value and category interpretations (susceptible, intermediate, resistant).
- AST results are available within 4 to 16 hours.
- This is an autoread result; no manual readings are possible with this system.

MicroScan WalkAway system

Inoculum preparation

- The MicroScan® MIC/Panel contains micro-dilutions of each antimicrobial agent in various concentrations with Mueller Hinton Broth and various nutrients which are dehydrated and dried in panels.
- Each panel contains two control wells: a no-growth control well (contains water only/no nutrients or broth), and a growth control well (contains test medium without antibiotic).
- The panel is rehydrated and inoculated at the same time with 0.1 ml of suspension prepared by the turbidity method (inoculum prepared in water,then 0.l ml transferred to 25 ml of inoculum water containing pluronic-D/F-a wetting solution).
- The panels are incubated at 35ºC in a non-CO2 for 16- 20 hours.

Reading and interpreting results

- The panel is read by visual observation for growth.
- Panels may also be read automatically with the WalkAway® and autoSCAN®-4 Systems, which uses an optics system with growth algorithms to directly measure organism growth.
- The results are reported as minimum inhibitory concentration (MIC) and as categorical interpretation (susceptible, intermediate and resistant).

- The MIC for the test organism are read by determining the lowest antimicrobial concentration showing inhibition of growth.

Reporting cAST

Below mentioned criteria should not be reported with cAST results due to 1) Difference in the card and the performance, 2) Major error is common among antimicrobials with narrow breakpoints either in mm or µg/ml

- 3rd gen cephalosporin (SPICE organisms)
- Cefepime – GNB
- Pip/tazo – GNB
- Colistin – GNB
- SXT – *S. maltophilia, S. aureus*
- Clindamycin susceptibility for *S. aureus* should not be reported (Erythromycin resistant and clindamycin susceptible isolates may exhibit inducible clindamycin resistance)
- Meropenem *(A. baumannii* or other than *E. coli, K.pneumoniae, N. meningitidis, P. aeruginosa, and P. mirabilis*

CAUTION: cAST approved by FDA before 2007, thereafter AMR has increased but revision of break points were not done to re-evaluate the performance

Quality control

1. The instrument should be serviced annually under a preventative maintenance agreement
2. Ensure proper functioning of optical, movements and temperature control with monthly maintenance
3. Quality controls run should be done once in a month with the new batch of reagents and with known susceptible and resistant organisms
4. Test minimum of 30 each of susceptible and resistance phenotype to validate the antibiotics used routinely

Calibration

1. Verify the DensiCHEK Plus using the calibration standards on a monthly basis when in use. DensiCHEK Plus instrument verification results should be within the established range of standards used for the verification
2. To use the DensiCHEK Plus meter with the calibration standards:
   a. Ensure the instrument is ON and set to the GLASS tube setting (the default setting is plastic).
   i. To change the tube type, press the Menu key. SEL and a flashing triangle will display under the current tube type setting. Press the Read/Enter key to move the triangle. When the triangle is pointing to the correct setting, press the Menu key to exit configuration.
   b. Clean the outside of the 0.0 McF standard (blank) with lens tissue and gently invert (do not shake) the blank 5-6 times.
   c. Insert the blank and press the "0" key.
   d. Slowly rotate the blank one full rotation. The instrument will display a series of dashes followed by a numerical value (0.00 will be displayed for the blank).
   e. Repeat steps b-d for the remaining standards (0.5 McF, 2.0 McF, and 3.0 McF).
   f. Check that the displayed value is within the acceptable range and record on the DensiCHEK Plus Calibration Log.
   g. If the reading is outside the acceptable range, repeat steps a-e. If still out of range, contact Technical Support.

7.5 Colistin Susceptibility Testing:

Broth-micro dilution (BMD) is currently the only method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for polymyxin antimicrobial susceptibility testing. According to CLSI recommendations, BMD is performed with cation-adjusted Mueller-Hinton broth (CA-MHB), a range of 2-fold dilutions of polymyxins (ranging from 0.12 to 512 g/ml), and a final bacterial inoculum of 5X 10^5 CFU/ml in each well. BMD is

considered to be the optimal method and is currently recommended for susceptibility testing in the recent document proposed by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group

(http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf).

The joint CLSI-EUCAST Polymyxin Breakpoints Working Group doesn't recommend disc diffusion, agar dilution and E-test for colistin/polymyxin B susceptibility testing.

Antibiotic preparation in distilled water:

$$\text{Volume in ml} = \frac{\text{Potency X weight of antibiotic}}{\text{Needed concentration (Higher concentration)}}$$

From the prepared stock antibiotic solution (needed concentration of antibiotic is prepared in distilled water), antibiotic is serially diluted in 1 ml distilled water from higher concentration to the lower concentration.

Antibiotic pure substance:

Colistin sulfate (Sigma Aldrich) – Potency 15,000 IU/mg

Stock solution:

1 µg = 15 IU (X 1024 µg)

1024 µg = 15,360 IU

- Preparation of 1024 µg/ml stock solution,

$$\frac{15{,}000 \text{ X } 5 \text{ mg of colistin}}{15{,}360 \text{ IU}} = 4.88 \text{ ml sterile distilled water (diluent)}$$

Weigh 5 mg of colistin (15,000 IU) and dissolve in 4.88 ml sterile distilled water to make 1024 µg/ml

Quality control (QC) strains:

- *E. coli* ATCC 25922 (QC range, 0.25 - 2 µg/ml)
- *P. aeruginosa* ATCC 27853 QC range, 0.5 - 4 µg/ml)
- MCR-1 positive *E. coli* (MIC, 4-8 µg/ml)

Inoculum preparation:

1. Inoculate 2-3 morphologically similar colonies of 24 hours pure growth from nutrient agar plate into 1.5ml nutrient broth. Incubate at 37^0c for 2 hours and then adjust to 0.5 McFarland standards.
2. 100 µl of 0.5 McFarland adjusted inoculum, is transferred to 2 ml of normal saline and used as final inoculum for performance of MIC testing

Procedure:

- Label the tubes from higher to lower concentration, add 1 ml of cation adjusted Mueller-Hinton broth (CAMHB) to each of these labeled tubes
- Add 1ml of stock solution containing 1024 µg of colistin to the tube labeled as 512 µg/ml and perform serial dilution from higher to lower concentration (labeled as, 512 µg/ml to 0.12 µg/ml)
- Add 100 µl of CAMHB containing different concentration of colistin from the tubes to respective wells of the labelled 96-wells microtiter plate

- Add 10 µl of final inoculum to the wells of the microtiter plate (as mentioned previously)
- Add 100µl of CAMHB into Sterility control well
- Add 100µl of CAMHB (without antibiotic) and 10 µl of final inoculum into growth control well
- Take 10µl of final inoculum and plate on to nutrient agar (tertiary streaking for purity check)
- Transfer 10µl of inoculum from GC well and add to 10ml of saline for count verification
- From the count verification tube, plate 100µl onto nutrient agar and perform criss cross streaking (30 – 50 CFU/ml – satisfactory, less/more CFU/ml than mentioned indicates insufficient or more inoculum)
- Incubate the microtiter plates and nutrient agar plates at 37°C for 16-18 hrs

Reading and Interpretation:

- Check purity of inoculum
- Check sterility control well. It should be clear/non-turbid
- Check growth control for adequate growth of at least 2mm button formation
- The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism in the wells as detected by the unaided eye
- When single skipped well is seen, read the highest MIC
- Do not report results if more than one skipped well is present
- Take readings for the quality control organisms tested
  - *E. coli* ATCC 25922 (QC range, 0.25 - 2 µg/ml)

- *P. aeruginosa* ATCC 27853 (QC range, 0.5 - 4 µg/ml)
- MCR-1 positive *E. coli* (MIC, 4-8 µg/ml)

Note: *Consider taking reading for the test isolates only if the quality control is satisfactory and found within the ranges mentioned*

7.6 Molecular testing for antimicrobial resistance (From bacterial cultures/DNA spike clinical specimens)

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.

AMR gene characterization by PCR:

ESBL multiplex reaction (SHV, TEM, VEB, PER, GES)

The primer mix is prepared in TE and contains 2 µM of each primer (SHV-5/SHV-6; TEM-3/TEM-4; VEB-F/ VEB-R; PER-F/ PER-R; GES-F/GES-R)

Primers for ESBL genes (*SHV, TEM, VEB, PER, GES*)

Primers	Primer sequence	Expected product size
SHV – 5	CCTTTAAAGTAGTGCTCTGC	119 bp
SHV – 6	TTCGCTGACCGGCGAGTAGT	
TEM – 3	CATTTCCGTGTCGCCCTTATTC	800 bp
TEM – 4	CGTTCATCCATAGTTGCCTGAC	
VEB – F	CATTTCCCGATGCAAAGCGT	648 bp
VEB – R	CGAAGTTTCTTTGGACTCTG	
PER – F	GCTCCGATAATGAAAGCGT	520 bp
PER – R	TTCGGCTTGACTCGGCTGA	
GES – F	AGTCGGCTAGACCGGAAAG	399 bp
GES – R	TTTGTCCGTGCTCAGGAT	

Reaction volume

2x multiplex master mix (Qiagen)	10 µl
Primer mix (2 µM of each primer)	2 µl
5x Q solution	2 µl
DNA template (< 0.4 µg/ 20 µl reaction)	Variable
Water	Up to 20 µl

Cycling conditions

95°C	15 mins	1 cycle
94°C	30 sec	30 cycles
59°C	1.5 mins	
72°C	1.5 mins	
72°C	10 mins	1 cycle

ESBL multiplex reaction (CTX-M & its variants)

Primers for ESBL genes (*CTX-M*)

Primers	Primer sequence	Expected product size
CTX-M1f2	AAAAATCACTGCGCCAGTTC	415 bp
CTX-M1r2	AGCTTATTCATCGCCACGTT	
CTX-M2f2	CGACGCTACCCCTGCTATT	552 bp
CTX-M2r2	CCAGCGTCAGATTTT TCA GG	
CTX-M9f2	CAAAGAGAGTGCAACGGATG	205 bp
CTX-M9r2	ATT GGA AAG CGT TCATCACC	
CTX-M8A	TCGCGTTAAGCGGATGATGC	688 bp
CTX-M25A	GCACGATGACATTCGGG	347 bp
CTX-M8-25B	AACCCACGATGTGGGTAGC	

Reaction volume (For *CTX*-M 1, 2 & 9)

2x multiplex master mix (Qiagen)	10 µl
Primer mix (2 µM of each primer)	2 µl
5x Q solution	2 µl
DNA template (< 0.4 µg/ 20 µl reaction)	Variable
Water	Up to 20 µl

Cycling conditions

95°C	15 mins	1 cycle
94°C	30 sec	30 cycles
58°C	1.5 mins	
72°C	1.5 mins	
72°C	10 mins	1 cycle

Reaction volume (For *CTX*-M 8 & 25)

- Qiagen FAST PCR master mix to be used

2x multiplex master mix (Qiagen)	10 µl
Primer mix (5-10 µM of each primer)	2 µl
DNA template (< 0.4 µg/ 20 µl reaction)	Variable
Water	Up to 20 µl

Cycling conditions

95°C	5 min	1 cycle
96°C	5 sec	30 cycles
58°C	5 sec	
68°C	30 sec	
72°C	1 min	1 cycle

AmpC multiplex reaction (CMY/MOX, CIT, DHA, ACC, ACT/MIR, FOX)

Primers used to detect *AmpC* genes

Primers	Primer sequence	Expected product size
CMY/MOX-M – F	GCTGCTCAAGGAGCACAGGAT	520 bp
CMY/MOX-M – R	CACATTGACATAGGTGTGGTGC	
CITM – F	TGGCCAGAACTGACAGGCAAA	462 bp
CITM – R	TTTCTCCTGAACGTGGCTGGC	
DHAM – F	AACTTTCACAGGTGTGCTGGGT	405 bp
DHAM – R	CCGTACGCATACTGGCTTTGC	
ACCM – F	AACAGCCTCAGCAGCCGGTTA	346 bp
ACCM – R	TTCGCCGCAATCATCCCTAGC	
EBCM – F	TCGGTAAAGCCGATGTTGCGG	302 bp
EBCM – R	CTTCCACTGCGGCTGCCAGTT	
FOXM – F	AACATGGGGTATCAGGGAGATG	190 bp
FOXM – R	CAAAGCGCGTAACCGGATTGG	

Reaction volume

2x multiplex master mix (Qiagen)	10 µl
Primer mix (2 µM of each primer)	2 µl
5x Q solution	2 µl
DNA template (< 0.4 µg/ 20 µl reaction)	Variable
Water	Up to 20 µl

Cycling conditions

95°C	15 min	1 cycle
94°C	30 sec	30 cycles
65 °C	1.5 min	
72 °C	1.5 min	
72 °C	10 min	1 cycle

CRO multiplex reaction (IMP, VIM, NDM, OXA-48, KPC)

The primer mix is prepared in TE and contains 2 µM of each primer (IMP2f/IMP2r2; VIMf/VIMr; NDMf/ NDMr; OXA-48f/ OXA-48r; KPCy-F/KPCy-R)

Primers used to detect carbapenemases genes

Primers	Primer sequence	Expected product size
IMP2-F	GGAATAGAGTGGCTTAAYTCTC	232 bp
IMP2-R	GGTTTAAYAAAACAACCACC	
VIM-F	GATGGTGTTTGGTCGCATA	390 bp
VIM-R	CGAATGCGCAGCACCAG	
NDM-F	CACCTCATGTTTGAATTCGCC	984 bp
NDM-R	CTCTGTCACATCGAAATCGC	
OXA-48-F	TATATTGCATTAAGCAAGGG	800 bp
OXA-48-R	CACACAAATACGCGCTAACC	
KPCy-F	TGTCACTGTATCGCCGTC	1011 bp
KPCy-R	CTCAGTGCTCTACAGAAAACC	
SPM-F	AAAATCTGGGTACGCAAACG	271 bp
SPM-R	ACATTATCCGCTGGAACAGG	

Reaction volume

2x multiplex master mix (Qiagen)	10 µl
Primer mix (2 µM of each primer)	2 µl
5x Q solution	2 µl
DNA template (< 0.4 µg/ 20 µl reaction)	Variable
Water	up to 20 µl

Cycling conditions

95°C	15 mins	1 cycle

94°C	30 secs	30 cycles
59°C	1.5 mins	
72°C	1.5 mins	
72°C	10 min	1 cycle

Multiplex PCR for acquired OXA genes in *Acinetobacter baumannii*:

Primers for blaOXA-23/24/58 like PCR:

Primer name	Primer sequence (5'-3')	PCR product size (bp)
OXA-23 like	GATCGGATTGGAGAACCAGA	501
	ATTTCTGACCGCATTTCCAT	
OXA-24/40 like	GGTTAGTTGGCCCCCTTAAA	246
	AGTTGAGCGAAAAGGGGATT	
OXA-58 like	AAGTATTGGGGCTTGTGCTG	599
	CCCCTCTGCGCTCTACATAC	

Reaction volume

2x multiplex master mix (Qiagen)	10 µl
Primer mix (2 µM of each primer)	2 µl
5x Q solution	2 µl
DNA template (< 0.4 µg/ 20 µl reaction)	Variable
Water	up to 20 µl

Cycling conditions

95°C	15 mins	1 cycle

94°C	30 secs	30 cycles
52°C	1.30 mins	
72°C	1.30 mins	
72°C	5 min	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.

8. Returned Data (Result Submission)

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	□		□
	Mannite	□		□
	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				□	□	□	

9. 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 were scored as correct

Identification results (viable and non viable 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

Rationale:

Participants were 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 was 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 was listed for all the EQA specimens, participants were expected to select appropriate agents for antimicrobial susceptibility testing depending on the organism identified. Some participants tested all the agents in the panel regardless of the type of organism, whilst others noted where an agent was not tested because it was inappropriate for the organism identified. In the list of AST results on pages 1, 2 and 3 only agents that were appropriate for the organism are listed. All the agents are listed in the tables on pages 21 to 23 with associated results. Results for appropriate agents are scored unless the participant consensus fell below 80%, in which case the agent is excluded from scoring. Lack of concordance was more commonly associated with agents where only a small number of results were returned and therefore further analysis was considered inappropriate.

Please note for beta lactamase reporting, a negative result is denoted by sensitive and positive result is denoted by resistant.

Summary of scoring criteria for the antimicrobial susceptibilities:

Assigned scores

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

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

From the closure of results deadline an intented report will be sent to the participating centers in two weeks time. Final report will be sent to the participating centers soon after the data compilation and analysis when available