Standard Operating Procedures for the LAKANA trial

**SOP Lab-06 *S. pneumoniae* culture**

Version 1.0 (2022-06-22)

# Purpose and overview:

This Standard Operating Procedure (SOP) explains how to grow and identify *Streptococcus pneumoniae* (*S.pneumoniae*) from NPS specimen stored in STGG. This SOP was adapted from the CDC protocol (<https://www.cdc.gov/meningitis/lab-manual/chpt06-culture-id.html>, [https://www.cdc.gov/meningitis/lab-manual/chpt08-id-characterization-streppneumo.html](https://eur01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.cdc.gov%2Fmeningitis%2Flab-manual%2Fchpt08-id-characterization-streppneumo.html&data=02%7C01%7C%7C8021d53789f44bc20bfa08d81db46120%7C1faf88fea9984c5b93c9210a11d9a5c2%7C0%7C0%7C637292007842033954&sdata=Bxi%2Fuh67hLh%2BivDusfYkTv%2Fe6vzCVVwNPVwq04GczsM%3D&reserved=0))

# Applicability to and responsibilities of various staff members

|  |  |
| --- | --- |
| **Staff member** | **Responsibility** |
| Laboratory technician | * Maintaining sufficient supply of culturing materials in the laboratory * Culture of *S.pneumoniae* from stored or fresh NPS specimens using aseptic technique * Recording results |

# Required materials

| **Item** | **Number** | **Specification** |
| --- | --- | --- |
| Disinfectant | 1 | e.g. 10% freshly prepared bleach |
| Biohazard bags and bins | Well supplied | None |
| Bunsen burner | 1 | None |
| CO2 incubator, CO2 sachets | 1 | ~5% CO2; 35-37°C |
| Light microscope | 1 | None |
| Vortex | 1 | None |
| Micropipette | 1 | 10µL sterile tips |
| Forceps | 1 | none |
| Sheep blood agar (BAP) Culture plates | Well supplied | Trypticase soy agar (TSA) plate (e.g. Sigma) containing 5% sheep blood (appendix 1) |
| Todd Hewitt (CM0189 OXOID ) with 0.5% Yeast extract  Rabbit Serum (e.g. by GIBCO by Life Technologies USA Ref # 16120-107 500ml) |  | 5 ml Todd Hewitt THY Broth tubes prepared. Aseptically add 1 ml rabbit serum to each THY tube |
| Optochin Discs | Well supplied | (thylhydrocuprein hydrochloride; 6 mm, 5μg disk (e.g. Oxoid DD0001) |
| Bile solubility reagent | Well supplied | 2% sodium desoxycholate, pH7.0 (e.g. Sigma D6750) |
| Saline solution | Well supplied | 0.85% saline |
| Ethanol-cleaned microscope slides | Well supplied | For Gram stain |
| Crystal violet ammonium oxalate solution | Well supplied | For Gram stain |
| Gram iodine solution | Well supplied | For Gram stain |
| Carbol-fuchsin | Well supplied | For Gram stain |
| Methanol | Well supplied | For Gram stain , 95% methanol solution |
| Acetone/Alcohol | Well supplied | For Gram stain |
| Immersion oil for microscope | Well supplied | For Gram stain |
| Inoculation loops | Well supplied | None |
| Permanent marker | 1 | None |
| Ethanol | Well supplied | None |
| Cryolabels and paper labels | Well supplied | None |
| Bacterial storage bead or Sterile freezing solution (STGG media) 1ml aliquoted in 2ml screw cap tubes or cryovials | Well supplied | To archive *S.pneumoniae* isolates at -800C Freezer |

# Definitions and general instructions

## Definitions

### Laboratory technician/scientist: a staff member in the laboratory responsible for LAKANA study.

## General instructions

### Below is a flowchart for identification of *S. pneumoniae* in NPS specimen. Alpha-hemolytic colonies will be Gram stained and tested for Optochin sensitivity. If the optochin test is sensitive and the Gram shows Gram-positive cocci, report as *S.pneumoniae*. If optochin resistant is found (zone of inhibition 9-13mm), perform a bile solubility test

### Summary of workflow

#### Screen all 1° plates after 24 hours to see which plates i.) require bile solubility test, ii.) have *S.pneumoniae* confirmed, iii.) have no *S.pneumoniae* (and need to be re-incubated).

#### For those with no *S.pneumoniae* confirmed, record information in the lab book and dispose of plates in biohazard bin.

#### Separate the remaining types of plates on the bench.

#### Initiate bile salt solubility test on all plates requiring confirmatory testing and place in incubator.

#### Re-incubate 1° plates with inadequate or contaminated growth for a further 24 hours.

#### Create 2° plates from 1° plates with adequate growth.

#### Store isolates

|  |  |  |  |
| --- | --- | --- | --- |
| **Plate** | **Type** | **storage** | **Vial No** |
| 10 | sweep | Yes | Vial 13, 10 BAP, ID, date |

# Step-by-step procedures

## Preparation of work area

1. Note: Before starting, ensure all supplies (incl. BAP plates and Optochin discs, are in good condition and not expired. BAP plates should be labeled with plate type (‘5% BAP’) and date of manufacture/expiration. If plates are not labeled, expired or are contaminated (visible growth), do not use.
2. Confirm the incubator is at correct temperature (acceptable range: 35-37°C) and ~5% CO2.
3. Clean all working surfaces with 10% bleach.

## Pre-incubation step

### Remove selected NPS-STGG specimen for culture (vial 5) from the -80°C freezer or the fridge if stored less than 48 hours.

### Allow NPS-STGG specimen to fully thaw at room temperature (~25°C) Place these in a clean rack on the bench. Limit time samples are at room temperature and store samples in the refrigerator until culture is completed to avoid freeze thaw cycles. Once culture and identification is completed, freeze back sample at -80oC

### Vortex each specimen for 15 seconds

### Place one Todd Hewitt Broth tube per specimen in a rack

### Aseptically add 1ml of thawed and mixed rabbit or sheep serum to each 5ml Todd Hewitt broth (THY)

### Label each THY tubes with the details of the specimen to be inoculated

### Open a single labeled THY tube, maintaining precautions to minimize risk of contamination; using a sterile micropipette transfer 200 µl of the NP‐STGG into each corresponding matched ID THY Tube

### Vortex and incubate the THY tube rack at 370C Incubator for 4-6 hours

### While the incubation of THY is in process, remove BAP plates (1 per specimen) from the refrigerator and lay them on the bench surface and allows plates to reach room temperature (~25°C)

### Using a permanent marker, label the bottom of each plate with the details of the specimen to be inoculated and with ‘1o’ to indicate that this is the primary culture plate. Labels can also be printed if this option is available

## Culturing *S. pneumoniae* Culture: Creating Primary Plates

### Stack the labeled non-inoculated BAP plates on the bench; ensuring the order of the stack corresponds to the order of the specimen samples to be used.

### After 4-6 hours incubation take out the THY rack from the incubator

### Open a single THY tube, maintaining precautions to minimize risk of contamination.

### Using a sterile 10µl plastic inoculation loop, take 2 full loops (20µl) of the THY broth and streak this, in a quadrant fashion, on the BAP plate (Fig 1 and 2).

Fig 1: Quadrant streak method

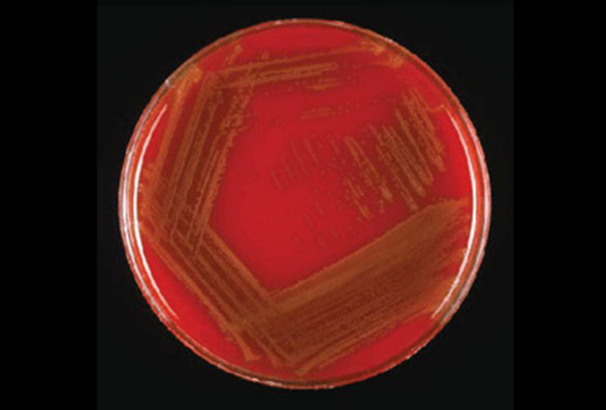


Fig 2: Properly steaked *S.pneumoniae* on BAP

### After streaking the plate, dispose the inoculation loop in 10% bleach.

### Replace the lid on the inoculated plate and place, upside down, on the bench apart from the non-inoculated plates.

### Repeat steps 5.3.3-5.3.6 with the remaining THY tubes until all specimen have been plated, all the time **ensuring Lab ID No. on BAP plate corresponds to Lab ID No. on the THY tube**

### 01b_IMG_1702Using sterilized (with Bunsen burner or bench top bio-incinerator) or sterile disposable forceps a sterilized needle or similar, place an Optochin disc on all inoculated 1° plates where streaks 1 and 2 intersect (Fig 2). Forceps must be sterilized every time an Optochin disc is placed on a new plate.

Fig 2: Position of Optochin disc between the first and second streak intersect

### Place all inoculated 1° plates, upside down, into the incubator for 18-24 hours (35-37°C, ~5% CO2). Do not place more than 8 plates in a single stack.

### Record all necessary information in a lab book, including

#### Lab ID No. (using second printed label)

#### Date 1° plate streaked

#### Time placed into incubator

#### Any relevant observation/incident

## Day 2 Checking colony morphology

### Clean all working surfaces with 10% bleachsolution or 70% ethanol.

### Remove all 1° plates from the incubator and place them on the bench.

### mage result for pneumococcal coloniesPresumptive identification of S. pneumoniae can be made on the basis of growth and colony morphology on a BAP and a Gram stain of the organisms. Examine a single 1° plate for growth of pneumococcal colonies; **small, greyish, moist, watery colonies surrounded by a greenish zone of alpha-hemolysis**, **draughtsman like in appearance either showing rough or smooth morphology** (Fig 3 and 4).

Fig 3: *S.pneumoniae* colonies on BAP

### **Reading plate**: **This MUST be done in good light**, moving the plate in such a way to catch reflection of light across the entire plate.

Fig 4: S. pneumoniae colonies have a flattened and depressed center after 24-48 hours of growth on a BAP, whereas the viridans streptococci retain a raised center

## Gram-stain

### With a bacterial loop add a few drops of water or saline to a microscope slide

### With a fresh loop aseptically transfer a small amount of a colony presumed to be *S. pneumoniae* to the wetted part of the microscope slide to create a slightly turbid, uniform suspension of cells

### Let the suspension air dry. The suspension MUST be completely dry before proceeding.

### Fix the smear by the flooding the slide with 95% methanol for a minimum of 2 minutes. Rinse with water. Shake off excess water.

### If methanol is not available, heat-fix the smears by quickly passing the slide through a flame three times. Do not over-heat the slide as over-heating will cause significant distortion or destruction of the cells.

### Flood the slide with crystal violet ammonium oxalate for 1 minute to stain. Rinse with water. Shake off excess water.

### Avoid touching the slide with the tip of the reagent bottle or applying liquid directly onto the smear.

### Flood the slide with Gram’s iodine for 1 minute. 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.

### Briefly flood the slide evenly with Acetone/Alcohol for 5 – 10 seconds being aware that over decolourisation will lead to false readings. Rinse with water. Shake off excess water.

### 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.

### Counterstain with carbol-fuchsin for up to 1 minute. Rinse with distilled water. Shake off excess water.

### Gently blot the slide using bibulous paper or a clean paper towel. Let air dry

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

### Reading Gram-stain under microscopic examination (Fig 5)

#### Gram-positive organisms will appear dark violet or purple.

#### Gram-negative organisms will appear red or pink (from the counterstain).

#### 

Fig.5: *S.pneumoniae* appears as lancet-shaped cocci (elongated cocci with a slightly pointed outer curvature). Usually, they are seen as pairs of cocci (diplococci), but they may also occur singly and in short chains

## Checking for Optochin sensitivity

### Check for Optochin sensitivity. If there is a Zone of inhibition (ZOI) around the Optochin disk, measure with a ruler or callipers from the back of the plate. If the ZOI is not uniformly circular, measure the shortest distance.

### If colonies are Optochin susceptible (ZOI ≥14mm), allows presumptive identification of *S. pneumoniae****.***

### If colonies are Optochin resistant (no ZOI) or indeterminate (ZOI <14mm), perform the bile solubility test (BST)

## Performing the bile solubility test

### With a loop, add bacterial growth from the overnight BAP to 1.0 ml of 0.85% saline to achieve turbidity in the range of a 0.5-1.0 McFarland standard.

### Divide the cell suspension equally into 2 tubes (0.5 ml per tube).

### Add 0.5 ml of 2% sodium deoxycholate (bile salts) to one tube. Add 0.5 ml of 0.85% saline to the other tube. Mix each tube well.

### Incubate the tubes at 35-37°C in CO2 initially for 10 minutes

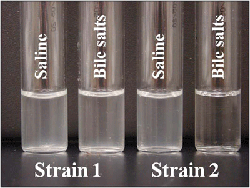
### Vortex the tubes.

### Observe the tubes for any clearing of turbidity after 10 minutes. Continue to incubate the tubes for up to 2 hours at 35-37°C in CO2 if negative after 10 minutes. Observe again for clearing.

### Reading the bile solubility test results

### A clearing of the turbidity in the bile tube but not in the saline control tube indicates a positive test (Fig5).

Fig 5: Results of the bile solubility test are shown for two different strains of bacteria. For strain 1, a slight decrease in turbidity is observed in the tube containing the bile salts (2nd from left), but the contents are almost as turbid as the control tube (far left); therefore, strain 1 is not S. pneumoniae. For strain 2, all turbidity in the tube containing the bile salts (far right) has cleared, indicating that the cells have lysed, in contrast to the control tube (2nd from right), which remains turbid; therefore, strain 2 is S. pneumoniae.



### **Partial clearing (partial solubility) is not considered positive** for pneumococcal identification. Partially soluble strains that have optochin zones of inhibition of less than 14 mm are not considered pneumococci.

### Bile soluble (positive test) confirms *S. pneumoniae*.

### Bile insoluble (negative test) confirms negative for *S. pneumoniae*

## Re-incubating 1° Plate:

### If the growth on the 1° plate is inadequate (for development of a 2° plate), create a new 1° plate. Sweep as much *S. pneumoniae* as possible (avoiding contaminants) and streak a new 1° BAP plate. Label this as above and the plate ‘1°R’ (Re-plated) and place in incubator.

### When collecting growth (e.g. for a sweep, re-plating, etc.), do not cross the Zone of Inhibition (ZOI), as this will risk contaminating the sample with the anti-microbial agent of the Optochin Disk.

## Record results of the 1° plate in the logbook, labelled, including:

### Time 1° plate was taken out of incubator

### Date 1° plate was read

### Growth type (incl. No Growth, White colonies, Alpha-haemolytic, and/or βeta-haemolytic). Record all growth types on 1° plate, even if re-plating 1° plate.

### Gram-stain result

### Optochin result (diameter of ZOI, mm)

#### 1° plate outcome (Optochin sensitive, *S.pneumoniae*, Optochin resistant, not *S.pneumoniae*, Indeterminate)

### Bile solubility test result

### Any relevant observation/ incident (incl. re-plating if performed)

### Note: Record all growth types, even if re-plating.

### If *S.pneumoniae* is confirmed, proceed making of a AMR plate and storage

### If plate is negative for *S. pneumoniae*, re-incubate 18-24 hours (35-37°C, ~5% CO2).

## Obtaining *S. pneumoniae* growth for AMR testing and storage.

### Re-incubated plates: If re-incubated plates are still negative for growth*,* dispose of the plates in a biosafety bin. Record in this in the logbook

### From the 10 plate, pick *S. pneumoniae* colonies with normal morphology to prepare suspension as of AMR SOP. Set up separate AMR plates for each morphology type (up to 3 different morphologies, see table below)

### The following samples should be stored or tested further for their antimicrobial resistance profile

|  |  |  |  |
| --- | --- | --- | --- |
| **Plate** | **Type** | **storage** | **Vial No** |
| 10 | sweep | Yes | Vial 13, 10 BAP, ID, date |
| 10 | Optochin sensitive, colonies | No, carry on with ID and AMR | N/A |
| 10 | *S.pneumoniaeb* colonies (Optochin sensitive), alternate morphology, confirmed up to 3 colonies | No, carry on with ID and AMR | N/A |
| 10 | Optochin resistant,  Colonies, normal morphology | No, carry on with ID and AMR | N/A |
| 10 | Optochin resistant,  Colonies, alternate morphology, | No, carry on with ID and AMR | N/A |

a only if enough colonies are available, if not use for AMR

bif present

### Storage of bacteria

#### With a permanent marker label the storage vials accordingly (see table).

#### With a 10μl bacterial loop take a sweep from the 1o plate and under aseptic conditions open the screwcap of the storage vial and inoculate the cryopreservative fluid by twisted the loop in the fluid multiple times. Make sure not to spill the fluid. Discard the loop in 10% bleach.

#### Close the lid of the storage vial tightly and invert 4-5 times to mix and bind the bacteria to the beads. Do not vortex.

#### Make sure that you have recorded which vials will be stored in the logbook and then immediately freeze the vials at -80oC. Record where vials were frozen (position in cryobox, cyrobox number, position in freezer in the log book)

# Occupational Safety Issues

## The Principal Investigator must ensure that all study team members undertaking this SOP are trained in good clinical laboratory practice

## Handle all specimen and cultures with care and treat them as potentially infectious material.

# Quality Assurance / Quality Control

## Ensure BAP plates are labelled with plate type (incl.: BAP, 5% SB + 5ug gentamicin if available) and expiration date.

## For all new kits/supplies, record batch numbers in the lab book.

## Each new lot of optochin discs should be tested with positive and negative controls.

## Each new lot of sodium deoxycholate should be tested with positive and negative control

# Appendices and other related documents

| **Document number** | **Document content** |
| --- | --- |
| Appendix 1 | Preparation of blood agar plates |
| Appendix 2 | Preparation of freezing media (STGG) |

# Version history, authors and approvals

| **Version (date)** | **Edits to the SOP text (author)** |
| --- | --- |
| 1.0 (2022-06-22) | Authored by Dagmar Alber, Elaine Cloutman-Green and Yuemei Fan, approved by LAKANA PSG. |

**Appendix 1: Preparation of blood agar plates(BAP): trypticase soy agar (TSA) + 5% sheep blood**

A BAP is used as a general blood agar medium. It is used for growth and testing of*S. pneumoniae.* The plate should appear a bright red color. If the plates appear dark red, they are either old or the blood was likely added when the agar was too hot. If so, the media should be discarded and a new batch should be prepared.

Media preparation

1. Prepare the volume of TSA needed in a flask according to the instructions given on the label of the dehydrated powder.
   * It is convenient to prepare 500 ml of molten agar in a l-2 liter flask. If TSA broth powder is used, add 20 g agar into 500 ml of distilled water.
   * The media should be heated and fully dissolved with no powder on the walls of the vessel before autoclaving.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 60°C in a water bath.
4. Add 5% sterile, defibrinated sheep blood (5 ml sheep blood can be added to 100 ml of agar).
   * If a different volume of basal medium is prepared, the amount of blood added must be adjusted accordingly to 5% (e.g., 50 ml of blood per liter of medium). Do NOT use human blood.
5. Dispense 20 ml into 15×100 mm Petri dishes. Allow the media to solidify and condensation to dry.
6. Place the plates in sterile plastic bags and store at 4°C until use.

**Quality control**

1. Grow a *S. pneumoniae* QC strain for 18-24 hours on a BAP at 35-37°C with ~5% CO2.
2. Observe the BAP for specific colony morphology and haemolysis.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO2 (or in a candle-jar).

Passing result:

* *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-haemolysis).
* After 48 hours, the sterility test plate should remain clear.

**Appendix 2: Preparation of freezing media (STGG)**

For 100 ml STGG media

Ingredients:

1. skim milk - 2 gm

2. trypticase soya broth – 3 gm

3. glucose – 0.5 gm

4. glycerol – 10 ml

5. distilled water – 90 ml

Procedure

All ingredients will be mixed together in an autoclavable container and autoclaved at temperature of 116°C, under 15 lbs pressure for 15 minutes. STGG is allowed to cool to room temperature. Date the medium and give it a batch number. Record the expiry date (six months from day of preparation). The medium is now ready for aseptically aliquoting into 2 ml screw cap vials (1ml STGG per vial). Media or tubes prepared in advance should be stored in a refrigerator at 2°-8°C or frozen at -20°C. Prior to aliquoting, vortex the media for 20 seconds.

Test the media 1 week before the batch is used for sterility by plating the entire volume of one vial from each batch number onto Trypticase soy agar with 5% sheep blood and incubating the plate at 37°C for 48 h in a CO2 incubator. If the growth of any organism is observed, discard the batch.