Thailand Ministry of Public Health –
U.S. Centers for Disease Control and Prevention

International Emerging Infections Program (IEIP)

POPULATION-BASED SURVEILLANCE FOR MICROBIAL AGENTS OF PNEUMONIA AND SEPSIS WITH DETECTION OF *STREPTOCOCCUS PNEUMONIAE*

STANDARD OPERATING PROCEDURES

FOR CLINICAL AND LABORATORY STAFF

Document History

First version prepared February 2005 (Version 1.0)
Revised version prepared 02 September 2005 (Version 1.1)

International Emerging Infections Program
Thailand MOPH – U.S. CDC Collaboration
Department of Diseases Control, 3rd Floor, Building 7
Ministry of Public Health. Muang, Nonthaburi 11000 Thailand
Tel: 02-5914039, 02-5912182 Fax: 02-5915753

Document prepared by:
Julie E. Fischer
Sonja J. Olsen
Wanna Wongjindanon
Scott F. Dowell
Leonard Peruski

TABLE OF CONTENTS

INTRODUCTION **4**

OVERVIEW OF HEMOCULTURE PROCESS **5**

QUALITY ASSURANCE AND QUALITY CONTROL **6**

A. CLINICAL METHODS **7**

1. Guidelines for obtaining blood cultures **7**
1.1 Clinical indications: identifying patients for blood culture 7

2. Collection of Patient Specimens **9**
2.1 Precautions for safe collection of blood 9
2.2 Blood culture supplies 10
2.3 Good blood collection techniques 11
2.4 Collecting blood for cultures 12

3. Record-keeping and Reporting Results **15**
3.1 Record Keeping – Patient Information 15
3.2 Record Keeping – Laboratory Information 16
3.3 Record Keeping – Reporting Results 20

4. Transport of clinical materials **24**
4.1 Post-collection process at district (community) hospitals 24
4.2 Post-collection process at provincial (general) hospitals 26
4.3 Transporting isolates and blood culture materials to IEIP laboratory 27

5. Processing patient blood cultures and sera **28**
5.1 Preparing MB bottles for incubation 28
5.2 Loading and removing bottles from BacT/ALERT 3D instrument 29
5.3 Preparing patient serum disks for pre-hospital antibiotic use testing 31

B. MICROBIOLOGICAL METHODS **33**
6.1 Preparing solid media and slides for initial identification of positive isolates 33
6.2 Gram stain procedure for positive blood cultures (Hucker modification) 36
6.3 Acid-fast bacilli staining for positive MB bottles 38
6.4 Alarm-positive blood cultures with no apparent growth 40
6.6 Confirmatory identification of *S. pneumoniae* 45
6.7 Antimicrobial susceptibility testing of *S. pneumoniae* 48
6.8 Confirmatory identification of *H. influenzae* 52
6.9 Shipping clinically significant isolates for further testing 55

APPENDIX I: QUALITY CONTROL AND QUALITY ASSESSMENT **57**

APPENDIX 2: TESTING PATIENT SERUM FOR PRE-HOSPITAL ANTIBIOTIC USE **66**

APPENDIX 3: PREPARING MEDIA AND REAGENTS **68**

References **81**

Introduction

Twelve hospitals in the Thai province of Nakhon Phanom, on the Lao border, and 8 hospitals in the eastern Thai province of Sa Kaeo on the Cambodian border currently participate in a pneumonia disease surveillance network directed through a Thai Ministry of Public Health (MOPH) - U.S. Centers for Disease Control and Prevention (CDC) collaboration. This Thai-U.S. collaboration uses active surveillance to determine the population-based incidence of radiographically confirmed pneumonia.

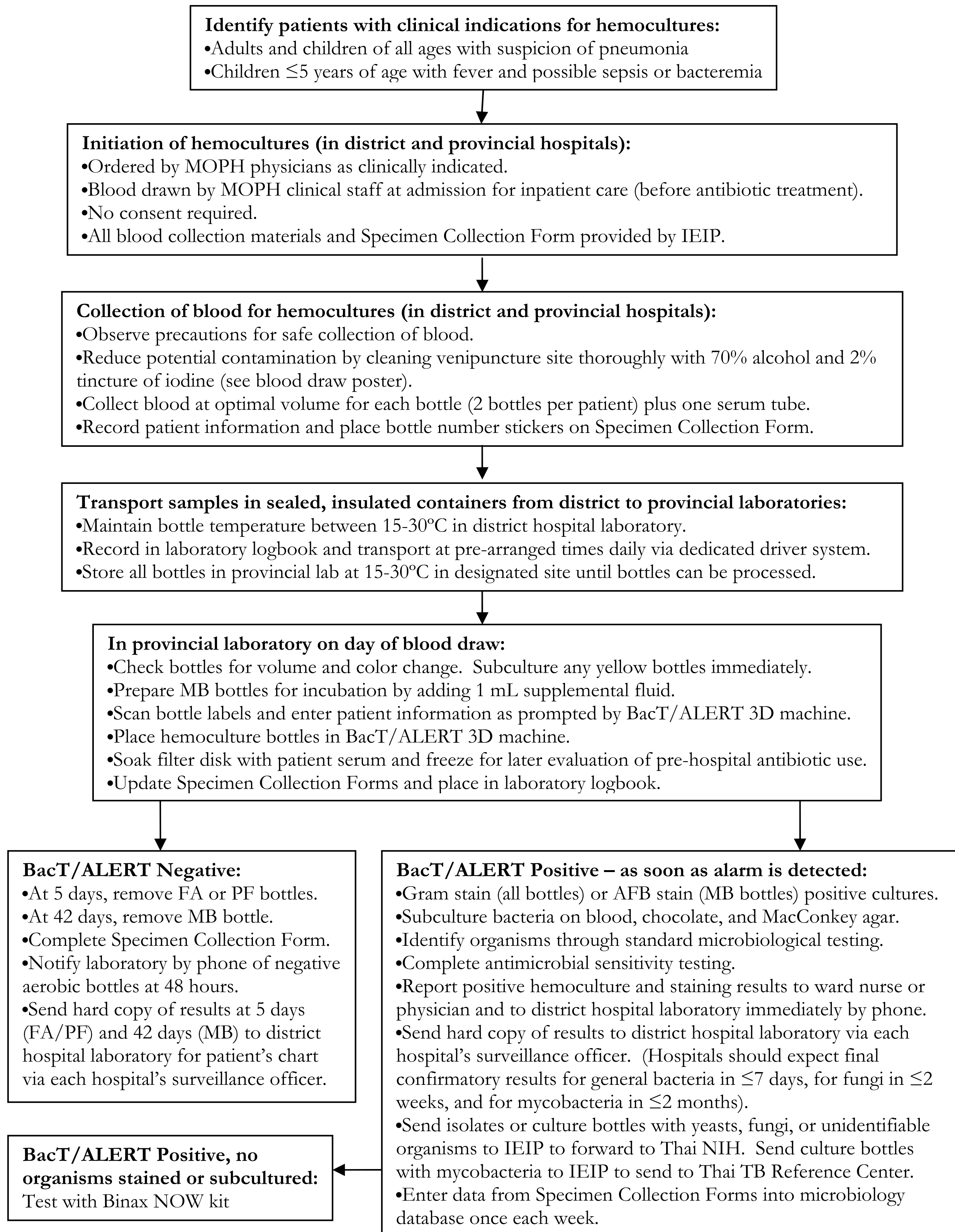
With the support of the PneumoADIP program at Johns Hopkins University and the Global Alliance for Vaccines and Immunization (GAVI), the Thai-U.S. CDC collaboration now aims to strengthen microbiology laboratory capacity in Nakhon Phanom and Sa Kaeo. This support will allow MOPH clinicians to request sensitive blood cultures as clinically indicated, with special emphasis on patients with suspected pneumonia and children with fever and possible bacteremia. This increased microbiology capacity will:

- strengthen the Thai MOPH system for population-based pneumonia surveillance;
- allow clinicians to meet the recommended standards of clinical care for pneumonia and sepsis;
- help define the population-based incidence of invasive bacterial disease, including *Streptococcus pneumoniae*; in these two provinces; and
- provide a profile of antimicrobial sensitivity for *S. pneumoniae* and other bacteria.

Using pre-treatment blood cultures to identify disease etiology for patients who require hospitalization for community-acquired pneumonia, as emphasized by internationally accepted practice guidelines, can improve care of individual patients by allowing selection of the most effective therapies. Microbiological diagnosis can also advance knowledge and improve care of other patients by detecting pathogens of potential epidemiological importance and patterns of antibiotic resistance, and can help physicians select antibiotics to limit development of microbial resistance in the community.[1],[2]

Streptococcus pneumoniae (or pneumococcus) affects children and adults worldwide, causing invasive diseases such as pneumonia, meningitis, and sepsis, and accounting for about two-thirds of all cases of community-acquired bacterermic pneumonia.[3] An accurate assessment of the disease burden from pneumococcus in these two provinces will allow for better characterization of potential benefits from routine use of existing or new pneumococcal vaccines in Thailand. Therefore, the collection, transport, culture, and storage protocols described in this manual have been designed to collect potential pneumococcus isolates as efficiently as possible. The techniques adopted to preserve notoriously fastidious pneumococcus cultures for comprehensive analysis should allow for high-throughput, high-sensitivity detection of a wide spectrum of invasive bacterial and mycotic diseases.

Overview of Hemoculture Process



Quality Assurance and Quality Control

Throughout these standard operating procedures (SOPs), boxed sections refer to Quality Control and Quality Assurance steps. These boxes serve as reminders of specific actions that must be taken to assure clinicians and patients that the laboratory results can be trusted.

Quality Assurance (QA) can be defined as the overall system for assuring a reliable standard of work. QA includes internal quality control (defined below), comprehensive record-keeping to demonstrate the validity and performance of tests, training of personnel, monitoring of results to improve laboratory efficiency and reliability, and external assessments to test laboratory proficiency. QA exists not only to detect errors when they occur, but to improve the laboratory system continuously. A comprehensive QA program spans the entire laboratory process, from collection of specimens to feedback from clinical staffs.

Quality Control (QC) refers to the steps that laboratory technicians take with every procedure to demonstrate that their tests provide reliable results. QC includes selecting the right assays, media, and reference control strains for every situation; assessing whether media, reagents, equipment, and assays perform correctly; including appropriate controls to confirm that microbiological techniques yield results that make sense and fall within normal parameters; maintaining essential environmental conditions in media quality, temperature, humidity, CO_2 levels, and sterility; and keeping accurate records that allow technicians to share and validate their results quickly.

Many comprehensive references to QA/QC systems exist. The American Society for Microbiology and the Clinical Laboratory Standards Institute (formerly NCCLS) offer numerous publications on general and specific QA/QC procedures. The *Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World* (World Health Organization, Geneva, 2003) describes QC practices for many common bacterial causes of pneumonia and sepsis. The World Health Organization's Southeast Asian Regional Office has also posted *Blood Safety and Clinical Technology: Quality Assurance in Bacteriology and Immunology* online at: http://w3.whosea.org/EN/Section10/Section17/Section53/Section375.htm.

A. Clinical Methods

1. Guidelines for obtaining blood cultures

With the PneumoADIP/GAVI funding, IEIP will support the routine performance of blood cultures as clinically indicated in all 20 participating hospitals in Nakhon Phanom and Sa Kaeo. Clinicians are urged particularly to obtain blood cultures for all patients admitted for inpatient care with suspected pneumonia, and for all children five years of age and younger admitted with fever and possible sepsis or bacteremia.

All blood cultures will be initiated at the request of hospital clinicians with materials, equipment, technical training, and staff support provided by IEIP. Neither the patient nor the hospital will incur any cost. Because Thai and international standards of care support blood cultures for diagnosis of community-acquired pneumonia and sepsis, and because the Thai MOPH performs pneumonia surveillance as a core function, written informed consent is NOT required to perform these blood cultures. A patient who receives a blood culture at the order of a hospital clinician with the support of IEIP resources may enroll subsequently in a research study requiring consent.

1.1 Clinical indications: identifying patients for blood culture

1) Blood cultures are indicated for **patients of all ages who are admitted for inpatient care with suspected pneumonia**. Suspicion of pneumonia, as per the case definition used by the IEIP pneumonia surveillance network, can be based upon one or more signs of acute infection and one or more signs and symptoms of respiratory illness, as described below.

a) Signs of acute infection:
- Fever (>38.2°C within 24 hours, including by patient history)
- Hypothermia (<35.5°C within 24 hours, including by patient history)
- Abnormal WBC
- Abnormal differential

b) Signs or symptoms of respiratory illness:
- Cough
- Sputum production
- Hemoptysis
- Chest pain
- Dyspnea
- Tachypnea
- Abnormal breath sounds

Because blood for cultures should be drawn prior to antibiotic treatment, clinicians should **not** wait for evidence of a new infiltrate on a chest radiograph to begin blood cultures for patients whose clinical signs and symptoms suggest pneumonia.

2) Cultures should also be obtained for **all children ≤ 5 years of age admitted for inpatient care with fever of >38.2°C or hypothermia (<35.5°C) and suspicion of sepsis or bacteremia**, as determined by the clinician. Clinical signs or symptoms of possible sepsis or bacteremia are often non-specific, especially in small infants, but may include one or more of the following:[4]

- Altered mental state, including lethargy or irritability
- Feeding difficulties
- Vomiting
- Convulsions
- Signs of respiratory distress, including apnea or tachypnea
- Pallor, cyanosis, or jaundice

2. Collection of Patient Specimens

2.1 Precautions for safe collection of blood[5]

Infections may be transmitted between patients and staff during the blood-drawing procedure. Viral agents are the greatest hazard, and can be lethal. Of particular importance are the viruses causing hepatitis and acquired immunodeficiency syndrome (AIDS). The greatest risk to health-care personnel may come from samples from asymptomatic, and therefore unsuspected, carriers of the hepatitis B and human immunodeficiency viruses. To decrease the risk of transmission of these viral agents, the following recommendations should be followed as closely as possible:

1. Wear latex or vinyl gloves impermeable to liquids, and change gloves for each patient.

2. Use a new syringe and needle for each patient.

3. Wipe the gloves with 70% isopropyl alcohol before drawing blood.

4. Disinfect the rubber stopper of the blood culture bottles with 70% alcohol before **and** after puncturing the top with the needle.

5. Inoculate the drawn blood into the culture bottle within one minute to prevent the blood from clotting in the syringe. Do **not** change needles before inoculating the blood culture bottles.

6. Dispose of syringes and needles in a puncture-resistant, autoclavable container. No attempt should be made to re-cap any needle.

7. Transport inoculated blood culture bottles in securely sealed, individual plastic bags. Specimen containers should be individually and conspicuously labeled.

8. Remove gloves and discard in an autoclavable container.

9. Wash hands with soap and water immediately after removing gloves.

10. In the event of a needle stick, or other skin puncture or wound, wash the wound liberally with soap and water. Encourage bleeding.

11. Immediately report any contamination of the hands or body with blood, or any puncture wound or cut to the supervisor and follow hospital guidelines for assessment and treatment.

2.2 Blood culture supplies

All blood culturing will be carried out using the automated BacT/ALERT 3D blood culture system and pre-packaged non-vented blood culture media manufactured by BioMérieux. For each patient, two separate hemoculture bottles will be inoculated and incubated:

- one aerobic medium (FAN for adults and children >5 or Pediatric FAN for children ≤5 years of age), to capture the majority of bacteria likely to cause invasive disease; and

- one medium (BacT/ALERT MB) designed to capture slow-growing mycobacteria, as well as other bacteria, yeasts, and fungi.

Most materials necessary to collect blood for cultures will be pre-packaged by IEIP staff in a sealable plastic bag to make the process as convenient as possible. Each bag will contain a form and tubes pre-labeled with the same running number. IEIP will supply gloves and cotton balls with sterilizing jars in bulk to each hospital. The IEIP microbiology laboratory technologists in the laboratories at Nakhon Phanom Hospital in Nakhon Phanom province and Crown Prince General Hospital in Sa Kaeo province will ensure that each district hospital receives an adequate supply of blood draw kits. These kits, which contain BacT/ALERT 3D blood culture media, should be stored at 15-30ºC, away from direct sunlight. If an air-conditioned room is not available, the kits should be stored in an insulated cold chain box in an interior room (away from open hallways and windows).

The main IEIP laboratory in Nonthaburi will supply two types of pre-packaged blood draw kits, one for children ≤5 years and one for adults and children >5 years.

Blood culture supply kit
Children ≤5 years

Specimen Collection Form
1 sterile 10 cc syringe
1 sterile 23-gauge wing (butterfly) set
1 sterile 24-gauge wing (butterfly) set
3 70% isopropyl alcohol packaged swabs
1 2% tincture of iodine swab stick
Adhesive bandage
1 numbered red-top serum tube
1 numbered cryotube
1 MB blood culture bottle (black lid)
1 PF blood culture bottle (yellow lid)

Blood culture supply kit
Adults and children >5 years

Specimen Collection Form
1 sterile 20 cc syringe
1 sterile 22 gauge needle
1 sterile 23 gauge needle
3 70% isopropyl alcohol packaged swabs
1 2% tincture of iodine swab stick
Adhesive bandage
1 numbered red-top serum tube
1 numbered cryotube
1 MB blood culture bottle (black lid)
1 FA blood culture bottle (pale green lid)

Blood collection should be carried out in the location and by personnel assigned by normal hospital procedures. The person drawing blood should gather everything needed for the process (blood culture supply kit, tourniquet, autoclavable puncture-proof container, gloves, and sterile cotton balls) before beginning.

2.3 Good blood collection techniques

The quality of blood collection techniques affects both the sensitivity and the reliability of any blood culture system.[6,7] Success in detecting clinically significant pathogens relies upon:.

The timing of the culture **–** blood should be drawn **prior** to administration of antibiotics.

The volume of blood collected **–** inadequate blood volumes decrease the likelihood of detecting clinically significant infections in any blood culture system.[8,9] Despite common assumptions that bacterial concentrations are higher in the blood of small children than adults, and reluctance to draw blood from younger patients, low-level bacteremias that might be missed by relying upon small blood volumes can be quite common in infants.[10] Including two or more bottles per patient (rather than a single bottle) appears to increase yield regardless of whether the blood is collected from one or more sites, especially in children.[11]

Thorough skin disinfection at an appropriate blood draw site – contamination of blood cultures with bacteria that commonly reside on the skin can confuse patient care and consume resources. Thorough disinfection of the venipuncture site with a combination of alcohol and iodine solutions can significantly reduce the number of contaminated blood cultures, but only if used correctly.[12,13] Skin should be disinfected first by scrubbing vigorously enough to create friction with a 70% isopropyl alcohol solution (higher concentrations are not effective and should not be used). An iodine solution should then be used and left on the skin for an appropriate length of time before and during the blood draw. A tincture of 2% iodine must be allowed to act for 30 full seconds prior to drawing blood, and iodophors such as 10% povidone-iodine require a full two minutes. Blood should always be drawn directly from a vein rather than through a central venous access catheter.[14]

The ratio of blood to culture medium in the blood culture bottle – the BacT/ALERT hemoculture system depends upon an optimal broth-to-blood ratio. The bottles contain not only concentrated nutrient broth, additional growth factors, and antibiotic-binding activated charcoal, but the anti-coagulant sodium polyanetholesulfonate (SPS). Inoculating the bottles with too little blood might not only exclude the possibility of detecting low-level bacteremias, but might inhibit bacterial growth due to insufficiently diluted media. Filling bottles beyond their optimal amount can lead to detection of CO_2 emitted by blood cells rather than growing bacteria.[15] Thus, under-filling culture bottles can cause significant numbers of false negatives, while over-filling can lead to false positives.

2.4 Collecting blood for cultures

Blood cannot be transported before being placed in the BioMérieux culture bottles because the collection procedure does not use an anticoagulant. Once drawn, blood **must** be inoculated into the bottles within one minute to prevent clotting in the syringe.

a) **For adults and children > 5 years: collect a total of 15.5 mL:**

- 10 mL to inoculate one BacT/ALERT FAN Aerobic (FA) bottle;
- 5 mL to inoculate one BacT/ALERT Mycobacteria Blood (MB) bottle; and
- 0.5 mL to fill one serum (red-top) tube for testing pre-hospital antibiotic use.

b) **For children ≤ 5 years: collect a total of 7.5 mL:**

- 4 mL to inoculate one BacT/ALERT Pediatric FAN (PF) bottle;
- 3 mL to inoculate one BacT/ALERT Mycobacteria Blood (MB) bottle; and
- 0.5 mL to fill one serum (red-top) tube for testing pre-hospital antibiotic use.

Generally, it should be possible to draw this amount of blood, even from small children. **IF it is impossible to draw enough blood to inoculate both bottles, use the volume drawn to fill the FA bottle to 10 mL (adults) or PF bottle to 4 mL (children ≤ 5 years). Do not divide blood evenly among the two bottles.** For example:

Blood draw from 3 year-old = 5 mLs → 4 mLs in PF bottle + 0.5 mLs in serum tube
NOT 2.5 mLs in PF bottle, 2.5 mLs in MB bottle

Blood specimens from a single draw can be divided for multiple uses if necessary, in which case the blood culture bottles should be inoculated before filling any other blood collection tubes. (NCCLS recommends filling tubes from a single blood draw in the following order: blood culture bottles, then citrate, serum, heparin, and finally EDTA tubes.[16])

> **Quality Control (QC), Box 2.1:** Before beginning the blood draw, check that the broth in the hemoculture bottles is clear, and that the color indicator in the bottom is still intact and blue-green in color. If a bottle is cloudy, damaged, or the indicator has turned yellow, do not use. Return to the laboratory and obtain a new bottle.

The following procedure should be followed in collecting blood for hemocultures:[17]

1. Explain the procedure to the patient or guardian.

2. Complete the top (boxed) part of the Specimen Collection Form.

3. Check that the hemoculture bottles are clear and the color indicator intact and blue-green. Label the bottles with the patient's name, hospital number, and date. Do not write over the barcode or lot number. Remove and place the peel-off number from the FA or PF hemoculture bottle under "Bottle Number" on the front of the Specimen Collection Form, and the label from the MB bottle under "Bottle Number" on the back of the form.

4. Check that the running number on the red-top serum tube (for example, N000001) matches the running number on the Specimen Collection Form.

5. If any dirt is visible on the patient's arm, clean with soap and water.

6. Select an arm and apply a tourniquet to restrict the flow of venous blood. The most prominent vein of the forearm is usually chosen.

7. Disinfect the skin at the proposed venipuncture (needle insertion) site as follows:

    a. Scrub the venipuncture site vigorously with a 70% alcohol wipe for 30 seconds.

    b. Apply iodine solution from the packaged swab to an area approximately 5 cm in diameter around the venipuncture site for 30 seconds.

    c. **Allow to dry completely.**

    d. **If the vein is palpated again, repeat the skin disinfection**.

    e. For patients with iodine sensitivity, scrub with 70% alcohol alone for 60 seconds.

8. Remove the protective flip-top cover from the hemoculture bottles.

9. Carefully disinfect the top (rubber septum) of each hemoculture bottle with a 70% alcohol wipe and allow to dry for 1 minute before inoculation.

10. Wipe the gloves with 70% alcohol to disinfect.

11. Insert the needle bevel-upwards into the vein. Once the vein is entered, withdraw the required amount of blood by pulling back the plunger of the syringe at a slow, steady pace. Air must not be pumped into a vein. After the desired amount of blood is obtained, release the tourniquet and place a sterile cotton ball over the insertion site while holding the needle in place. Withdraw the needle and ask the patient or guardian to hold the cotton ball firmly in place until the wound has stopped bleeding.

12. Immediately inject blood into the hemoculture bottles. DO NOT change needles after drawing blood. Swirl the bottle gently several times to mix.

13. Inject 0.5 mLs of blood into the red-top serum tube.

14. Dispose of the needle and syringe in a puncture-resistant, autoclavable container. Do not re-cap the needle.

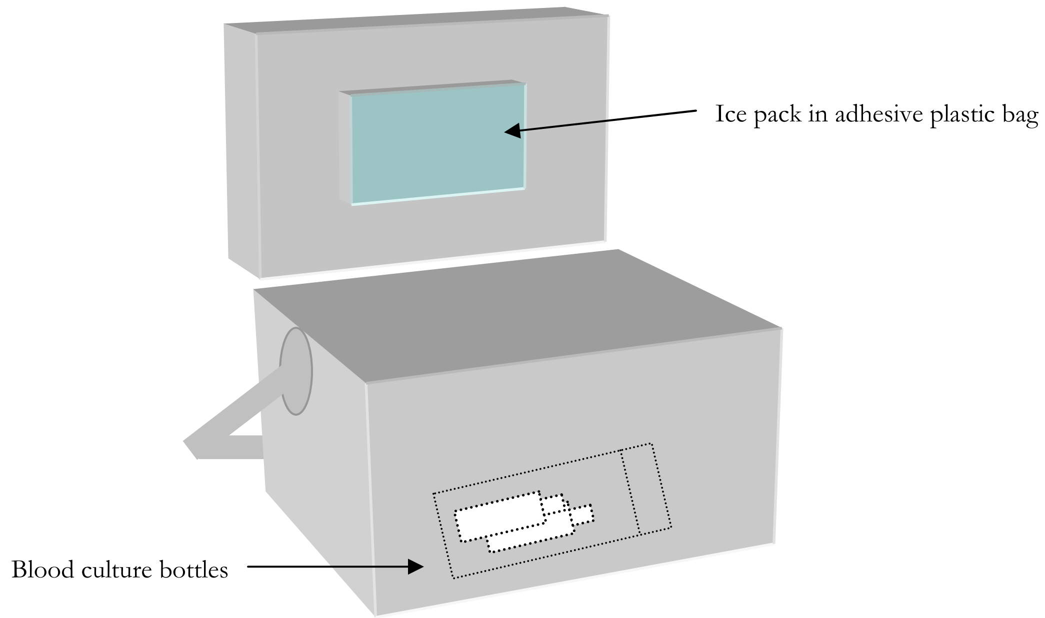
15. Clean the top of each hemoculture bottle and the serum tube with a 70% alcohol wipe.

16. Clean the patient's venipuncture site with 70% isopropyl alcohol, removing iodine to prevent skin irritation. Bandage and ask the patient or guardian to continue to place pressure on the upright arm for a few minutes.

17. Empty the plastic bag of all unused supplies EXCEPT the labeled cryotube.

18. Place the inoculated culture bottles, the red-top tube, and the Specimen Collection Form back into the plastic bag and re-seal.

19. Transport the sealed bag to the clearly labeled designated temperature-controlled storage site (either an air-conditioned room between 15-30ºC or an insulated storage box supplied by IEIP) in the hospital's microbiology laboratory. Bottles must **never** be refrigerated. If room temperature exceeds 30ºC, place a cold pack **inside an adhesive bag (shipping label bag) stuck to the inside of the cold chain box lid,** and then place the samples in the insulated box as far from the cold pack as possible.


Ice pack in adhesive plastic bag
Blood culture bottles

3. Record-keeping and Reporting Results

3.1 Record Keeping – Patient Information

IEIP will provide Specimen Collection Forms for Hemocultures with two sections, one to be filled in by the person drawing blood, and the rest to be completed in the laboratory. All underlined fields on the forms will be used for patient care purposes only.

1) The person performing the blood draw will complete the following information in the top (boxed) section:

Information on Specimen Collection Form	Purpose
Patient's name	Clinical management
Patient's hospital number	Clinical management
Patient's admission number	Clinical management
Name of the person drawing blood	Clinical management
Patient's ward or hospital room location	Clinical management
Name of the physician requesting the hemoculture	Clinical management
Physician's phone number	Clinical management (notification of results)
Name of the hospital where the patient is admitted	Clinical management and disease surveillance
Date and time of specimen collection	Clinical management and disease surveillance
Patient age (in months if less than 1 year)	Disease surveillance
Diagnosis (possible pneumonia, possible sepsis, or other)	Disease surveillance
History of patient's antibiotic use, if known	Disease surveillance

2) The person performing the blood draw will remove the peel-off part of the barcode from each hemoculture bottle, and place it into the appropriate section (FAN/Pediatric FAN or MB) on the Specimen Collection Form. One adhesive label with the running number will already have been attached to the Specimen Collection Form.

Quality assurance (QA) Box 3.1: Placing the peel-off bottle number stickers correctly on the Specimen Collection Form links each bottle to the patient and to the laboratory data, decreasing the chances that results could be separated from critical patient information.

3.2 Record Keeping – Laboratory Information

1) Blood for hemocultures will be collected in both district (community) and provincial (general) hospitals. Any inoculated bottles will be transported to the laboratory of the hospital in which they were collected. In the district hospitals, the bottles will then be picked up once each day by a driver, who will deliver them to the provincial hospital microbiology laboratory. The microbiology laboratory technician from the provincial hospital will phone the district hospital laboratory with the preliminary results, and then send a hard copy of the results to put into the patient's chart.

   Good record-keeping in each laboratory is essential to track the cultures and the results throughout the cycle from district hospital to provincial hospital and back. Each hospital laboratory will keep a bound logbook (supplied by IEIP) in which the laboratory technician records information about blood culture bottles inoculated **in that hospital**. The laboratory technician who receives the specimens (and, later, the results) should record the following information in the blood culture logbook:

<table>
<tr><th colspan="2">Information for Blood Culture Logbook</th></tr>
<tr><td colspan="2">Running number</td></tr>
<tr><td colspan="2">Date and time of specimen arrival in laboratory</td></tr>
<tr><td colspan="2">Patient's name</td></tr>
<tr><td colspan="2">Patient's hospital number</td></tr>
<tr><td colspan="2">Patient's admission number</td></tr>
<tr><td colspan="2">Patient's ward or hospital room location</td></tr>
<tr><td colspan="2">Name of the physician requesting the hemoculture</td></tr>
<tr><td rowspan="2">record date received and:
For positive culture results –</td><td>Gram stain/AFB stain results</td></tr>
<tr><td>Identification of isolate</td></tr>
<tr><td rowspan="2">For negative culture results – make a checkmark in the appropriate column to indicate:</td><td>No growth at 48 hours</td></tr>
<tr><td>No growth at 5 days (FA or PF bottle) or 42 days (MB bottle)</td></tr>
</table>

2) Inoculated blood culture bottles will be transported to the provincial hospital microbiology laboratory (either directly after the blood draw or after transport from the district hospital). The laboratory technician who receives the inoculated cultures bottles will add the following information to the Specimen Collection Form:

   a) Check the box indicating whether the aerobic bottle is FAN or Pediatric FAN
   b) The time that the bottles were received in the provincial microbiology laboratory
   c) The time that the bottles were placed into the BacT/ALERT 3D incubator
   d) The approximate inoculation volume of blood added to each bottle, as compared visually to pre-measured standard bottles.

3) After recording this information, the laboratory technician will place the original form in chronological order in a binder supplied by IEIP.

4) The laboratory technician who removes the bottles from the BacT/Alert 3D incubator and who processes the samples will complete the following information on the original form:

e) The date and time that each bottle became positive, or an indication of "no alarm"
f) Description of any organisms by Gram stain (positive FAN and MB bottles)
g) Description of any organisms by AFB stain (positive MB bottles only)
h) Description of subculture growth results
i) Optochin sensitivity (for suspected *S. pneumoniae* isolates only)
j) Identification – the suspected genus and species of positive cultures
k) Antimicrobial sensitivity testing results (recorded by attaching the laboratory report)
l) Date and time that the technician notified the hospital/physician of results
m) Date that the technician shipped isolates to IEIP for further testing and storage
n) The results of the patient serum bioassay for pre-hospital antibiotic use (to be determined later).

Please collect patient's blood
before antibiotic administration

Running number

Specimen Collection Form for Hemocultures

To be filled out by phlebotomist:

Patient name: ____________________________ HN: ______________________ AN: _________________________

Blood drawn by: __________________________ Physician name: ____________________ Phone: _____________

Hospital name: ____________________________________ Patient Ward/Room: _______________________________

Patient age: _____ years _____ months (if <1 year) Date/Time of blood draw: ___/____/____ ___:___

Diagnosis: ☐ Possible pneumonia ☐ Possible sepsis (≤ 5 years only) ☐ Other_______________

Has patient received any antibiotics within 72 hours of blood draw? ☐ Yes ☐ No ☐ Don't know

Name of antibiotic(s): 1) _______________________ 2) _______________________ 3) ___________________________

Blood drawn using: ☐ needle/wing set from blood culture kit ☐ IV catheter ☐other (please specify)______________________

To be filled out by lab technician:

Received in NP Microbiology Lab: Date ____/____/_____Time ___:___ by ________________

Placed in BacT/ALERT machine: Date ____/____/_____Time ___:___ by ________________

☐ Pediatric FAN (4 mL) or ☐ FAN Aerobic (10 mL) -- one bottle per patient

Bottle Number	Blood volume	Positive alarm (date and time)	At 5 days
	mLs	_____ /_____ _____ : _____	☐ No alarm

Removed from BacT/ALERT machine: Date ____/____/_____Time ___:___ by ___________________________

Culture and Gram stain results

Gram stain 1: Gram reaction	Gram stain 1: Shape	Gram stain 1: Arrangement	Gram stain 2: Gram reaction	Gram stain 2: Shape	Gram stain 2: Arrangement
☐ Positive	☐ Bacilli	☐ Pairs	☐ Positive	☐ Bacilli	☐ Pairs
☐ Negative	☐ Cocci	☐ Chains	☐ Negative	☐ Cocci	☐ Chains
☐ Not found	☐ Other______	☐ Clusters	☐ Not found	☐ Other______	☐ Clusters
		☐ None			☐ None
		☐ Other_______			☐ Other_______
Culture results	**Hemolysis**	**Optochin-sensitive**	**Culture results**	**Hemolysis**	**Optochin-sensitive**
☐ No growth	☐ None	☐ Yes	☐ No growth	☐ None	☐ Yes
☐ Growth on BAP	☐ Alpha	☐ No	☐ Growth on BAP	☐ Alpha	☐ No
☐ Growth on CAP	☐ Beta	☐ Not tested	☐ Growth on CAP	☐ Beta	☐ Not tested
☐ Growth on MAC			☐ Growth on MAC		

Gram stain 3:____

Plated: Date ____/____/_____Time ___:___ Plates examined: Date ____/____/_____Time ___:___

Identification (Genus/Species): 1)_________________________2)___________________________3)_____________________

Reported by: __________________________________ Approved by: ______________________________________

Hospital/physician notified: Date ____/____/_____Time ___:___

Isolate sent to IEIP: Date ____/____/_____

PLEASE ATTACH ALL MICROBIOLOGY REPORTS WITH ANTIBIOTIC SUSCEPTIBILITY

Running number

Specimen Collection Form for Hemocultures (Page 2)

HN: ____________________ Patient name:________________________ Hospital name: ____________________

Mycobacteria (MB) Bottle -- one bottle per patient

(Children ≤ 5 years = 3 mL, children and adults >5 years = 5 mL)

Bottle Number	Blood volume	Positive alarm (date and time)	At 42 days
	mLs	_____ /_____ _____ : _____	☐ No alarm

Removed from BacT/Alert machine: Date ____/____/_____Time ___:___ by ________________________________

Gram stain results					
Gram stain 1			**Gram stain 2**		
Gram reaction	**Shape**	**Arrangement**	**Gram reaction**	**Shape**	**Arrangement**
☐ Positive	☐ Bacilli	☐ Pairs	☐ Positive	☐ Bacilli	☐ Pairs
☐ Negative	☐ Cocci	☐ Chains	☐ Negative	☐ Cocci	☐ Chains
☐ Not found	☐ Other_____	☐ Clusters	☐ Not found	☐ Other_____	☐ Clusters
		☐ None			☐ None
		☐ Other_______			☐ Other_______
Culture results	**Hemolysis**	**Optochin-sensitive**	**Culture results**	**Hemolysis**	**Optochin-sensitive**
☐ No growth	☐ None	☐ Yes	☐ No growth	☐ None	☐ Yes
☐ Growth on BAP	☐ Alpha	☐ No	☐ Growth on BAP	☐ Alpha	☐ No
☐ Growth on CAP	☐ Beta	☐ Not tested	☐ Growth on CAP	☐ Beta	☐ Not tested
☐ Growth on MAC			☐ Growth on MAC		

Gram stain 3:____

Acid-fast Bacilli (AFB) stain results		
☐ Negative	☐ AF Bacilli positive	☐ Other AFB forms (yeast/fungi)
All AFB + samples should be sent to IEIP for subculture and identification		

Plated: Date ____/____/_____Time ___:___ Plates examined: Date ____/____/_____Time ___:___

Identification (Genus/Species): 1)______________________2)_______________________3)_____________________

PLEASE ATTACH ALL MICROBIOLOGY REPORTS WITH ANTIBIOTIC SUSCEPTIBILITY

Reported by: ______________________________ Approved by: ________________________
Hospital/physician notified: Date ____/____/_____Time ___:___
Isolate sent to IEIP: Date ____/____/_____

Disk test for serum antimicrobials	Zone of inhibition: ☐ None ☐ Positive __________ mm			
Binax test for *S. pneumoniae* Ag	☐ Positive	☐ Weakly positive	☐ Negative	☐ ND

3.3 Record Keeping – Reporting Results

Prompt feedback to clinicians, whether results are positive or negative, is a critical factor in both good clinical practice and in establishing strong trust in the microbiology laboratory's reliability and utility. The following system outlines directions for communication between the provincial microbiology laboratory, the district hospital microbiology laboratories, and the clinicians who requested the hemocultures. The IEIP microbiologist in each province will be responsible for ensuring that clinicians receive preliminary and confirmatory results in a timely way.

Reporting positive results:

1) The results of the Gram staining should be recorded on the Specimen Collection Form.

2) Once isolates from a positive blood culture have been identified by Gram stain, the provincial laboratory microbiologist should immediately report the preliminary results (positive blood culture, and Gram reaction and morphology of the isolate) **by phone** to the ward nurse or the physician who requested the blood culture, by clinician preference.

3) At this time, the microbiologist should explain to the ward nurse or physician that more information confirming the identity of the organism will be available in 24 hours and the antimicrobial susceptibility testing results will be available in 48 hours. The microbiologist should explain that all results will be sent to the hospital's microbiology laboratory by phone call and then in a hard copy via the driver system (to place in the patient's chart) within another day. The hard copy of the results of confirmatory testing for general bacteria will arrive within one week (or 2 weeks for fungi or yeast, or 2 months for mycobacteria).

4) After notifying the clinician, the provincial laboratory microbiologist should report preliminary results by phone to the district microbiology laboratory. The laboratory technician in the district microbiology laboratory should note the preliminary results in the bound blood culture logbook. For patients in the provincial hospital, the results should be recorded in the provincial hospital's bound blood culture logbook.

5) Once the results of overnight growth on solid media and standard microbiological testing have confirmed the identity of the isolate, the microbiologist should repeat steps 1-4 above. (Report the results by phone to the ward nurse or physician, then to the district microbiology laboratory by phone to be recorded in the bound logbook.)

6) The original Specimen Collection Form should be completed, removed from the binder, copied 2 times, and returned to the same chronological order in the binder.

   a) For patients in the provincial hospitals, one copy should be sent directly to the ward (according to hospital procedure) to be placed in the patient's chart.

   b) For patients in the district hospitals, one copy should be sent via the driver system to the hospital's surveillance officer, who will bring the copy to the hospital laboratory to be placed in the patient's chart according to hospital procedure.

   c) The second copy should be placed in a folder to send to IEIP for later data entry.

7) The results of antimicrobial sensitivity testing should be recorded on the standard form used regularly by the provincial hospital's microbiology laboratory. (In Nakhon Phanom and Sa Kaeo, this may be hand-written or a print-out from existing clinical microbiology software.)

8) The antimicrobial testing results should also be copied twice. The original report should be attached to the original Specimen Collection Form in the provincial laboratory's binder. The second copy should be sent to the ward or to the district hospital microbiology laboratory to place in the patient's chart, and the third copy should be attached to the copy of the patient's Specimen Collection Form in the IEIP data folder.

9) If the second bottle becomes positive, the process above should be repeated EXCEPT that it is not necessary to phone the clinician or ward nurse if the organism appears to be the same by Gram stain, growth on solid media, and other standard microbiological testing.

Reporting negative results:

1) The provincial laboratory microbiologist should report negative (no-growth) culture results by phone to the district microbiology laboratory after 48 hours of culture for PF and FA bottles. The laboratory technician in the district microbiology laboratory should note the results in the bound specimen log-book.

2) The district microbiology laboratories (and the provincial microbiology laboratories for patients in the provincial hospitals) should notify clinicians of negative results through normal hospital procedures at 48 hours and 5 days.

3) The original Specimen Collection Form indicating "No growth" should be completed, removed from the binder, copied twice, and returned to the same chronological order in the binder.

   a) For patients in the provincial hospitals, one copy should be sent directly to the ward (according to hospital procedure) to be placed in the patient's chart.

   b) For patients in the district hospitals, one copy should be sent via the driver system to the hospital's surveillance officer, who will bring the copy to the hospital laboratory to be placed in the patient's chart according to hospital procedure..

   c) The extra copy should be placed in a folder to send to IEIP for later data entry.

Capturing results for the Pneumonia Surveillance Network

1) The IEIP Microbiology Database will contain no personally identifiable information.

2) Every Friday, the laboratory microbiologist in each provincial laboratory will download the records captured by the BioMerieux BacT/ALERT 3D system's Observa software, and use the IEIP Microbiology Database Access forms to enter the remaining information from Specimen Collection Forms completed that week. IEIP staff in Nonthaburi will provide

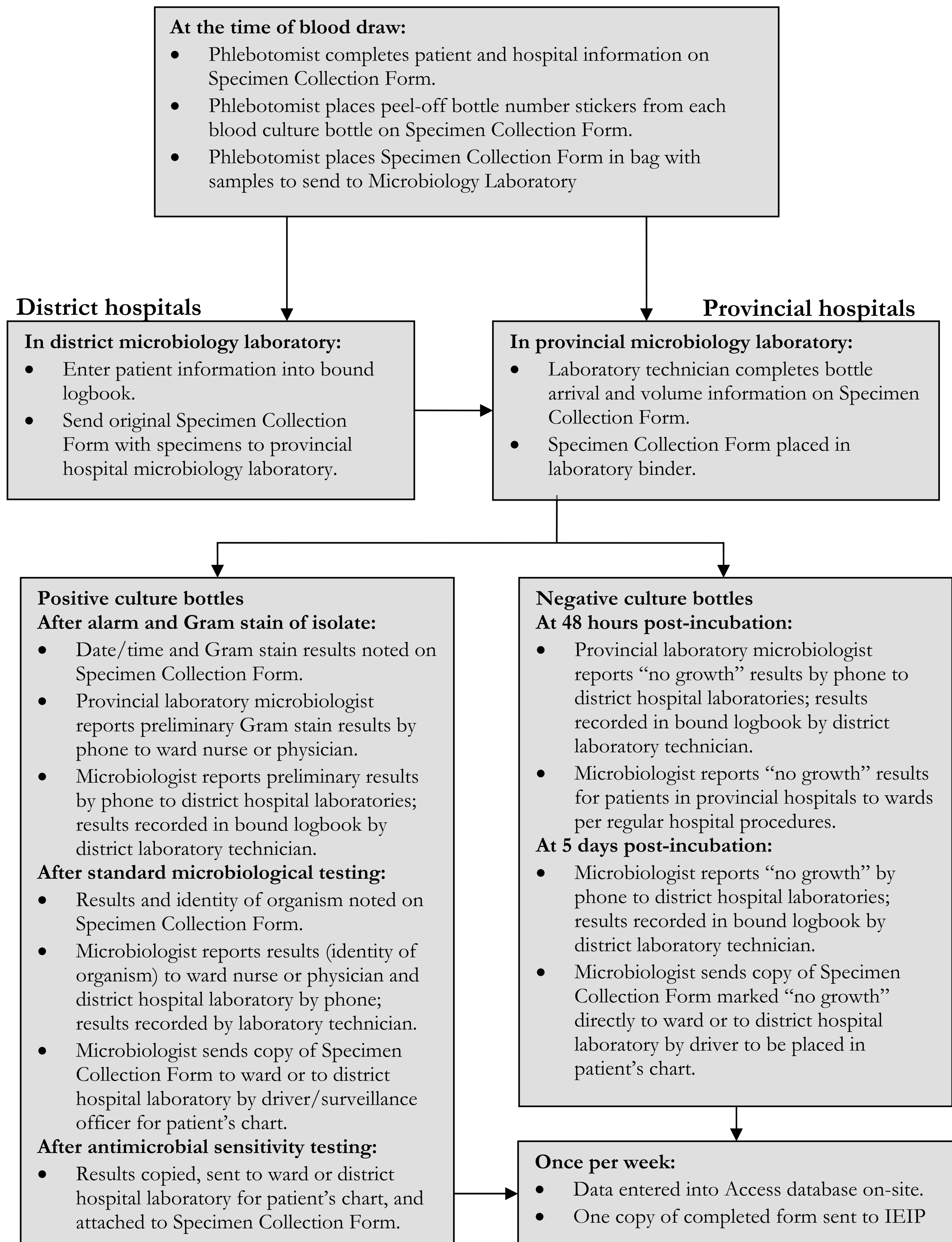
support for converting Observa files into entries that can be completed using Microsoft Access.

3) Every Tuesday, the laboratory microbiologist should send the folder with copies of all completed Specimen Collection Forms and antimicrobial sensitivity reports to the IEIP office in Nonthaburi. The data will be entered a second time at IEIP.

4) Each hospital's IEIP disease surveillance officer already identifies cases of suspected pneumonia by routinely screening inpatient charts. The disease surveillance officer will be able to determine whether a patient who meets the case definition for pneumonia had a blood culture by looking for a completed Specimen Collection Form in the patient's chart. The Pneumonia Surveillance Network database contains fields for entering blood culture results as part of regular data entry. (The Specimen Collection Form may not have been placed in a patient's chart by the time that the disease surveillance officer reviews it for the first time. Regular reviews of the bound blood culture logbooks in each hospital's laboratory combined with review of post-discharge patient charts should capture these results.)

5) The accuracy and completeness of data captured for the Pneumonia Surveillance Network (which should include hemoculture results for every patient with clinical signs and symptoms of pneumonia) and the GAVI Microbiology Database (which should include the results of all hemocultures conducted for patients with suspected pneumonia or children ≤5 years of age with fever and possible sepsis, without any personally identifiable information) will be validated by regular audits using the original Specimen Collection Forms and patient logbooks as references.

Overview of Record-Keeping and Reporting



4. Transport of clinical materials

S. pneumoniae and some other common disease agents of pneumonia and sepsis, such as *Haemophilus influenzae*, are fastidious and fragile bacteria. The ability to culture and identify *S. pneumoniae* successfully can be complicated by the tendency of densely grown cultures to undergo autolysis or self-destruction. Delaying the entry of blood culture bottles into the BacT/ALERT 3D instrument for up to 12 hours post-inoculation falls within the manufacturer's normal operating parameters and requires no special precautions, as long as the inoculated bottles can be maintained at 15-30ºC.[18] Therefore, the following process has been developed to protect potential *S. pneumoniae* isolates against extremes of temperature or incubation past the log phase of growth that might cause false negatives and lost cultures.

4.1 Post-collection process at district (community) hospitals

All blood culture bottles inoculated at the district (community) hospitals will be transported to the provincial hospital microbiology laboratory by car at pre-assigned times for incubation and identification of any isolates through standard microbiological techniques.

The IEIP provincial Surveillance and Research Coordinator will oversee the transportation system. Drivers will pick up bottles daily at a scheduled time and place coordinated by the director of the district hospital microbiology laboratory, the hospital's IEIP surveillance officer, the Surveillance and Research Coordinator, and the driver. Together, this team will decide:

- What time the driver will arrive each day, based on his route and the goal of collecting the most freshly drawn cultures possible;
- Who in the district hospital will meet the driver each day to transfer the insulated box containing the sealed bags of inoculated hemoculture bottles and blood tubes to the car, and to carry fresh media, the empty insulated box, and hard copies of completed Specimen Collection Forms back to the microbiology laboratory; and
- Who in the district hospital will be responsible for calling the driver and the provincial hospital laboratory if there are NO inoculated bottles for transport.

The microbiology laboratory technician in each provincial hospital should contact the district hospital laboratories if expected shipments do not arrive. The IEIP microbiology laboratory technician should also arrange for the driver to pick up the empty cold chain transport boxes, new hemoculture media bottles, and copies of completed Specimen Collection Forms to transport to the district hospitals.

Inoculated hemoculture bottles should be protected from temperature extremes (<15ºC or >30ºC) prior to and during transport in an insulated cold chain box, supplied by IEIP. The inoculated hemoculture bottles must **never** come into contact with a cold pack, or be placed in a refrigerator. IEIP will supply thermometers to measure ambient room temperatures. The district laboratory technician should note and record the room temperature daily at the warmest time of the day. If the insulated box will sit where temperatures exceed 30ºC, place a cold pack **in the adhesive plastic bag inside the lid of the cold chain box.** All inoculated blood culture bottles should be received by the provincial microbiology laboratory between 14:00 and 15:00 each day.

QC 4.1: Each district hospital laboratory technician should check and record the peak room temperatures in his/her laboratory daily, using the room thermometer supplied by IEIP. In addition, each driver should place a TempTale device, set to detect temperature at 10-minute intervals, into the insulated transport box with the first bottles collected each day. The temperature record should be checked by the laboratory technician who receives samples at the provincial hospital, and any temperature changes <15ºC or >30ºC noted on the Specimen Collection Form. If a bottle has been subjected to serious temperature fluctuations during transport or storage (held at <15ºC or >30ºC for more than 30 minutes in total, or exposed to any temperatures >35ºC), it should be rejected. Another hemoculture should be requested, if possible, and the clinician notified using the Specimen Rejection Form. The microbiology laboratory technician should advise the driver and the IEIP Surveillance and Research Coordinator when out-of-range temperature measurements occur.

Checklist for drivers

_____ Each driver has an assigned weekday route clearly mapped by the IEIP Surveillance and Research Coordinator, with expected distance in kilometers indicated.

_____ Each driver has a plan to:

- Begin at the provincial microbiology laboratory every Monday – Friday between 11:00 and 12:00 (depending on the length of the route) to pick up supplies and completed Specimen Collection Forms for each district hospital laboratory;
- Travel to the most distant hospital on the route **first** to drop off supplies and pick up patient specimens, and then stop by each subsequent hospital on the way back to the provincial hospital;
- Drop off supplies and pick up patient specimens at each district hospital laboratory;
- Keep supplies and patient specimens between 15-30ºC in the passenger compartment of the vehicle with air-conditioning on, stopping for the minimum necessary amount of time at each district hospital;
- Deliver the patient specimens, in their insulated cold boxes, to the provincial hospital microbiology laboratory by 14:00 (Sa Kaeo) or 15:00 (Nakhon Phanom) each day.

_____ Each driver has a mobile phone where he/she can be contacted by the district hospital laboratories. The district hospital laboratory technician must notify the driver if there are no patient specimens on a weekday (Monday – Friday). .

_____ A monthly schedule assigns one driver in each province to be "on call" for picking up patient specimens by request each Saturday and Sunday. Each laboratory has a copy of the current drivers' schedule and contact information.

_____ The driver in each province scheduled to be "on call" on weekends has a mobile phone where he/she can be contacted by the district hospital laboratories. On Saturday and Sunday, the district hospital technician must request a patient specimen pick-up by calling the driver before the normal pick-up time.

4.2 Post-collection process at provincial (general) hospitals

Drivers will deliver all inoculated blood culture bottles from the district hospitals to the provincial hospital laboratories each day. Clinicians at the two general hospitals will also be encouraged to request blood cultures for patients seen on-site, which may represent as many as one-third of all blood culture specimens collected in each province.

The chief of each provincial hospital's microbiology laboratory should designate a specimen receiving site for inoculated cultures in an air-conditioned room (15-30ºC). IEIP will supply thermometers, to be calibrated yearly, in order to measure ambient room temperatures. If room temperatures exceed 30ºC, the specimens should be stored in an insulated cold chain box. Inoculated culture bottles should **never** come into direct contact with a cold pack or be placed in a refrigerator.

The Specimen Collection Form should be kept with the inoculated bottles until the laboratory technician records the date and time received, date and time placed in the BacT/ALERT 3D, the approximate blood volume, and the type of aerobic bottle (FAN or Pediatric FAN), and then the form should be placed in the three-ring binder.

All inoculated bottles should be placed in the BacT/ALERT 3D instrument just before the close of normal laboratory working hours. For every patient specimen, a disk should be prepared using the serum in the red-top tube and the disk should be frozen for later testing (see section B.6.1 below for details).

4.3 Transporting isolates and blood culture materials to IEIP laboratory

Once the blood culture process and standard microbiological testing have been completed in the provincial hospital microbiology laboratory, three types of samples will need to be shipped to the IEIP laboratory in Nonthaburi for preservation or further testing:

- **Positive blood cultures (by alarm) that cannot be identified conclusively through standard microbiological testing in the laboratory.** The provincial laboratories should ship the original blood culture bottle to IEIP, along with Gram stains and the isolate inoculated into two nutrient agar stabs and onto two Dorset Egg media slants if it has been successfully cultured on solid media (see Microbiology section 6.8 for details). Specimens should be accompanied by a copy of the original Specimen Collection Form and the Specimen Referral Form for the Thai National Institutes of Health (NIH). IEIP will forward these to the Thai NIH for identification and antibiotic susceptibility testing.

  If no organisms can be identified by Gram stain or isolated on solid media from a blood culture bottle despite a positive alarm, the bottle should be tested using the modified Binax NOW *Streptococcus pneumoniae* Test® protocol (see Microbiology Section 6.5).

- **Any clinically significant isolates from positive blood cultures that have been identified** (see Section 6.8 for an explanation of clinical significant pathogens for shipping). The provincial laboratories should ship any Gram stains, as well as the isolate inoculated into two nutrient agar stabs and onto two Dorset Egg media slants. These should be labeled with the running number, patient name, and hospital ID number, listed on the "Specimen Referral Form/Bacterial isolate list for confirmatory identification," and sent in batches to IEIP every Tuesday along with a shipment cover sheet.

  IEIP will forward these to the Thai NIH for preservation at -70ºC in TSB media containing 15% glycerol and further testing at the discretion of the Thai MOPH (all *S. pneumoniae* isolates will be serotyped at CDC-Atlanta and tested for penicillin susceptibility using the E-test®, while a selection of other specimens will be tested for confirmatory identification as a QC method).

- **Positive blood cultures that contain acid-fast bacilli, yeasts, or fungi, as determined by initial staining and microscopy.** The provincial laboratories should ship the original blood culture bottle and any Gram- or AFB-stained organisms fixed on slides, but should **not** attempt to culture such organisms on solid media. Specimens should be shipped with a copy of the Specimen Referral Form for the Thai National TB Reference Laboratory Center or Thai NIH (as appropriate) attached. IEIP will forward suspected mycobacteria to the Thai National TB Reference Laboratory Center and yeasts or fungi to the Thai NIH for confirmatory identification and antibiotic sensitivity testing.

Specimens should be packed according to international safety guidelines. All specimens should be clearly labeled. Bottles and tubes should be placed in a sealed plastic secondary container, packed into a specimen box to protect the specimens during transport; and finally placed in an external cardboard box shipping box. IEIP will supply all shipping containers, labels, and forms.

5. Processing patient blood cultures and sera

Once patient specimens have been received in the provincial microbiology laboratories, from patients at either the same site or from the district hospitals, the following steps must be taken:

1. Recording of specimen and patient information in the bound blood culture logbook and/or on Specimen Collection Forms (see Sections 3 and 4 above).
2. Preparing MB bottles for incubation by supplementing with MB/BacT Enrichment Fluid.
3. Loading blood culture bottles into the BacT/ALERT 3D automated blood culture machine.
4. Preparing a patient serum disk to use in evaluating the impact of pre-hospital antibiotic use on the sensitivity of the blood culture system.

Several of these steps can be completed at the same time with careful attention to keeping the Specimen Collection Forms with the blood culture bottles until all information has been recorded. For example, the red-top serum tubes for the last step could be placed in the centrifuge to spin while the laboratory technician completes the Specimen Collection Forms and the laboratory logbook. The MB bottles can be prepared for incubation in the biosafety cabinet at the same time "assembly line-style," with the technician changing needles carefully between bottles.

5.1 Preparing MB bottles for incubation

To ensure optimal growth of mycobacteria, all inoculated MB bottles must be supplemented with 1 mL of MB/BacT Enrichment Fluid, supplied by BioMérieux in vials with a total fluid volume of 5.5 mLs each, prior to incubation. Inoculated MB bottles can sit at room temperature (15-30ºC) for up to 24 hours prior to the addition of MB Enrichment Fluid, although delayed supplementation can lead to concomitant delay in time to detection of positive organisms.[19]

To supplement inoculated MB bottles:

1. Place inoculated MB bottles in a biosafety cabinet to avoid accidental aerosolization of potentially infectious materials. Use standard precautions for safe handling of blood.

2. Wipe gloves with 70% isopropyl alcohol, then carefully disinfect the top (rubber septum) of each MB bottle and each MB/BacT Enrichment Fluid vial with 70% isopropyl alcohol.

3. Using a sterile needle and 1 cc syringe, aseptically transfer 1 mL of MB/BacT Enrichment Fluid into one MB bottle.

4. Discard the needle and syringe to prevent cross-contamination of hemoculture bottles.

5. Repeat this process until all inoculated MB bottles have been supplemented and then load the inoculated, supplemented MB bottles into the BacT/ALERT 3D instrument.

5.2 Loading and removing bottles from BacT/ALERT 3D instrument

The operator's manual to the BacT/ALERT 3D automated blood culturing machine, provided by BioMérieux, contains full instructions for operating the instrument's hardware and software. The following information highlights only an overview of operating procedures and QC issues.

The BacT/ALERT 3D system detects bacterial growth through continuous monitoring of a chemical reaction in which the rising levels of CO_2 emitted by multiplying microorganisms causes a fall in pH, and the subsequent change of the colorimetric indicator from blue-green to light green or yellow. The instrument uses the same algorithm to detect growth in both aerobic and mycobacterial media for the first four days (allowing detection of rapidly growing bacteria in the MB bottles), then switches to a more sensitive mechanism for detecting the growth of slow-growing organisms. Once the instrument has been calibrated to 35ºC and set to the correct parameters, daily use should involve the following steps (within the operating guidelines provided by BioMérieux).

QC 5.1: Before loading each bottle, the laboratory technician should check to see if the bottle has been damaged in transit, under- or over-filled, or has already turned yellow. Damaged bottles should not be incubated. Yellow cultures may have become positive due to rapid bacterial growth, and should be subcultured and stained immediately. The approximate volume of blood added to each bottle should be noted on the Specimen Collection Form. Under- or over-filled bottles (compared to a standard bottle filled to the correct volume) should be incubated, but the problem recorded in the Microbiology Database and reported to the clinician with the results.

Loading bottles:

1. Press the "Load Bottles" button on the main screen.
2. Use the barcode reader to scan the first bottle. The instrument will determine the type of bottle, and whether it falls into the 5-day (FA or PF) or 42-day (MB) protocol.
3. Enter the hospital ID number, accession number, running number, hospital name, and patient's first and last name in the fields on the screen.
4. Open a drawer and insert the bottle, bottom-first, into any empty cell (indicated by a light).
5. Go to the next bottle. Repeat until all bottles are loaded.
6. Check that all incubator drawers are closed and press the "Check" button.

Removing bottles:

1. The instrument will indicate, by audible alarm and a change in screen color, when a bottle has become positive. Generate an "Unload Report" from the main screen and locate the Specimen Collection Forms that go with each bottle to be unloaded.
2. From the main screen, press the "Unload Positives" button. Green lights will indicate the drawers that contain positives, and lights will also indicate the positive cells within each

drawer. Remove each positive bottle and scan with the barcode reader again. Note the time and date of positive alarm on the Specimen Collection Form, and proceed with subculture and staining. **Positive bottles should be removed as promptly after the indication of a positive alarm as possible, and processed immediately.**

3. Finish unloading the positive bottles and press the “Check” button.

4. From the main screen, select the “Unload Negatives” button. Lights will indicate the appropriate drawers and cells, and those bottles can be removed and scanned with the barcode reader. For negative bottles, check “No alarm at 5 days” for FA or PF media, or “No alarm at 42 days” for MB media, on the correct Specimen Collection Form. Negative bottles should still be treated as potentially infectious waste, and autoclaved prior to disposal.

5. *IF the BacT/ALERT 3D instrument becomes completely full,* remove the **oldest** MB bottles from the instrument and continue incubating in a standard 35ºC incubator for the remainder of the 42 days. Any bottles removed in this fashion will need to be Gram- and AFB-stained and subcultured, whether or not color change occurs.

QC 5.2: The BacT/ALERT 3D’s calibrated temperature of 35ºC should be checked daily and noted on a log sheet kept with the instrument. Follow the Operator’s Manual to check the internal reference thermometer, and to adjust the temperature from the “Calibrate Module Temperature” screen if the actual temperature is more than 0.5ºC above or below 35ºC.

5.3 Preparing patient serum disks for pre-hospital antibiotic use testing

The patient's use of antibiotics (either self-prompted or at the direction of private clinicians) prior to hospital admission can have a significant impact on the sensitivity of a blood culture system.[20] The prevalence of pre-hospital antibiotic use in the Nakhon Phanom and Sa Kaeo in patients presenting with possible pneumonia or sepsis is unknown. As a QC measure to evaluate whether pre-hospital antibiotic use will regularly affect the yield and sensitivity of blood cultures in these two provinces, serum collected at the time of blood culture inoculation from all patients will be used to test for the presence of antibiotics.

IEIP will supply the 6-mm paper filter disks in autoclave-ready sleeves of 20 disks each, marked with autoclave indicator tape. The sleeves should be autoclaved by the provincial microbiology laboratories as needed, and carefully re-sealed after aseptic removal of disks with sterilized forceps.

1. Remove the red-top serum tube and cryovial from the bag containing the inoculated blood culture bottles. Confirm that running number on the red-top serum tube matches the running number on the cryovial and the Specimen Collection Form.

2. If the clot has contracted sufficiently to remove 20 µL of serum easily with a pipet tip, continue. If not, centrifuge the red-top serum tube(s), with appropriate balance tubes, for 10 minutes with no braking in a swinging bucket centrifuge at 1300 x g or less.

3. Line up all red-top serum tubes and corresponding cryovials in the biosafety cabinet to avoid accidental aerosolization of potentially infectious materials. Use standard precautions for safe handling of blood.

4. Remove one 6-mm paper filter disk per patient aseptically from the autoclaved sleeve of disks using sterilized forceps, and place on a sterile surface in front of each tube and vial set.

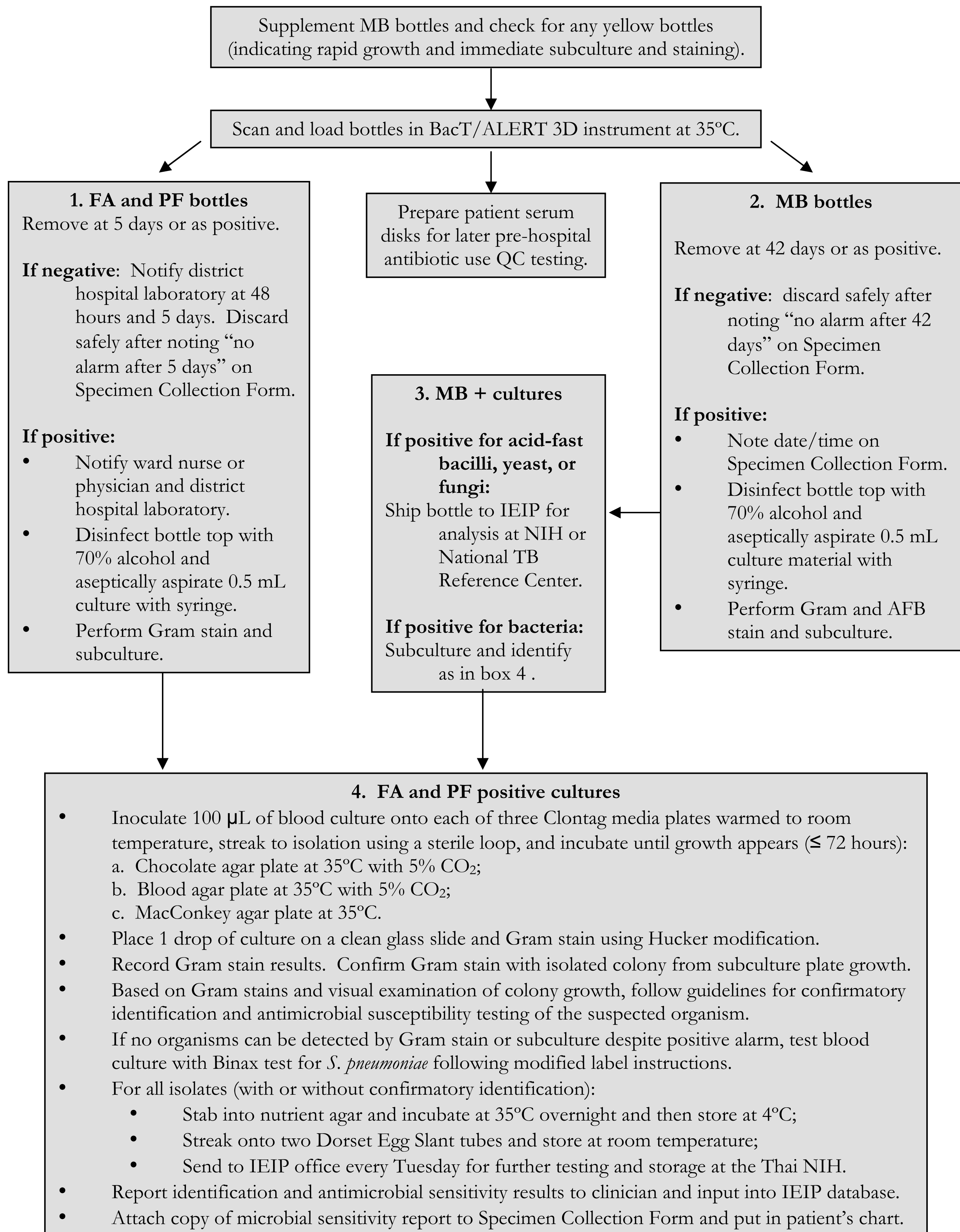
5. Remove the stopper from the first patient's red-top serum tube. Aspirate 20 µL of serum from the separated blood and dispense onto the paper filter disk. Repeat for each patient.

6. Air-dry all disks for 5 minutes.

7. Using sterile blunt-edged forceps, place each dried serum-soaked filter disk into the correct cryovial and secure the top.

8. Store all patient serum disks at −70ºC for later testing of pre-hospital antibiotic activity as a QC measure (see Appendix 2).

Overview of culture process in provincial laboratories



B. Microbiological Methods

In order to determine the burden of invasive bacterial and mycotic disease through routine blood cultures, laboratory staff in the provincial hospital laboratories must be able to isolate bacteria from positive culture bottles on solid media, and subculture isolates for confirmatory identification and antimicrobial sensitivity testing.[21]

The procedures that follow begin by outlining techniques common to all potential isolates, but focus primarily on identifying the importat causes of invasive bacterial disease *S. pneumoniae*, *H. influenzae* and *N. meningitidis*. This section does not comprehensively address techniques to identify other bacteria that may be of clinical importance. Microbiologists should refer to clinical microbiology manuals such as the American Society for Microbiology's *Manual of Clinical Microbiology* (Washington, DC, USA; 1992) or the World Health Organization's *Basic Laboratory Procedures in Clinical Bacteriology* (Geneva; 2001) for other identification procedures.

The appearance and growth characteristics of isolated bacterial colonies, combined with the microscopic experience of a Gram-stained specimen, often allow preliminary identification of an isolate. Gram stains of colonies and broths may be especially helpful if atypical or aberrant reactions are observed on the tests to identify *H. influenzae* and *S. pneumoniae*. *S. pneumoniae* are lancelate gram-positive diplococci, sometimes occurring in short chains, and *H. influenzae* are small gram-negative rods with random arrangement. A more comprehensive microbiology manual should be consulted for Gram stain reactions of other bacteria.

6.1 Preparing solid media and slides for initial identification of positive isolates

After a positive bottle has been signaled and unloaded from the BacT/ALERT 3D machine, and the Specimen Collection Form completed appropriately, a small amount of media from the blood culture bottle will be aspirated to inoculate solid media, perform a Gram stain (for FAN, Pediatric FAN, and MB bottles) and perform an acid-fast bacillus (AFB) stain for positive MB bottles only.

All media plates for isolating samples on solid media will be purchased from Clinag (Clinical Diagnostics, Ltd., Bangkok, Thailand), and supplied with appropriate documentation of QC testing for each batch. Plates should be stored at 4ºC until ready for use. All plates should be examined for signs of visible contamination prior to use; IEIP technicians should be notified of any media plates that demonstrate contamination prior to opening, or fail internal QC testing.

1. For every positive blood culture bottle, prepare the following by labeling each with the patient's name, hospital number, and the date:

    a. One clean (unused) glass slide for Gram stain;
    b. A second clean (unused) glass slide for AFB stain (MB bottles only);
    c. One sheep blood agar plate (pre-warmed to room temperature);
    d. One chocolate blood agar plate (pre-warmed to room temperature); and
    e. One MacConkey agar plate (pre-warmed to room temperature).

2. Place positive blood culture bottles in a biosafety cabinet to avoid accidental aerosolization of potentially infectious materials. Use standard precautions for safe handling of blood.

3. Wipe gloves with 70% isopropyl alcohol, then carefully disinfect the top (rubber septum) of each bottle with 70% isopropyl alcohol.

4. Using a sterile needle and syringe, aseptically remove 0.5 mLs of blood culture media from one positive bottle.

5. Inoculate 100 µL of blood culture onto each of the three solid media plates and streak to isolation using a flamed sterile inoculating loop.

6. Place one drop of blood culture media onto each clean glass slide as indicated and allow to air-dry in the biosafety cabinet.

7. Invert and incubate media plates for up to 48 hours (or until the growth of colonies is observed) at 35ºC, placing the blood and chocolate agar plates in a 5% CO_2 incubator.

8. Store the original blood culture bottle at room temperature until bacterial growth has been confirmed by Gram stain and subculture. Bottles should be kept for at least one week. If no organisms can be subcultured from the blood culture bottle despite a positive alarm, follow the instructions in Appendix 3 for using the Binax NOW *S. pneumoniae* antigens kit.

QC 6.1: The quality of nutrient media, storage conditions, incubation temperatures, and CO_2 levels can all affect laboratory consistency in successfully culturing and subculturing microbial isolates.

Blood agar and Mueller-Hinton blood agar plates containing sheep blood should be used to culture or subculture clinical isolates. Human blood should never be used in preparing solid media for isolating microbial agents from blood cultures, as both natural and pharmaceutical antibiotic factors in human blood can inhibit the growth of pathogens.

The temperature of refrigerators, freezers, and incubators should be checked daily and any deviations of more than 1°C from their respective correct temperatures (such as -80, -20, 4, or 35°C) noted on a log sheet kept with the instrument. 5% CO_2 incubators should be checked daily to be sure that tanks still have sufficient pressure and are functioning normally. Steps should be taken to calibrate or repair the equipment as necessary, and specimens moved to an appropriate back-up instrument if possible until the conditions can be restored to normal. Each laboratory should have plans for what to do with patient specimens (preserving them if possible, but discarding them and notifying the clinician if necessary) in case of an equipment failure.

Although IEIP will supply commercially prepared media, standard bacterial reference strains should be streaked to isolation on solid media – just like patient specimens – at least once per week and every time a new batch of solid media is received to confirm that the environmental conditions and media remain of high enough quality to sustain the growth of fastidious bacteria (see Appendix 1 for the list of appropriate reference strains). In addition, sterility testing should be performed. If more than 1% of plates from a batch grow colonies during sterility testing, the batch should be rejected.

If media or equipment fail to support the growth of control strains on blood, chocolate, or MacConkey agar, all conditions should be checked carefully and corrected where necessary. If required, new media should be obtained through IEIP and tested again until predictable and reliable growth of reference strains on selective media can be demonstrated. Any patient isolates that show no growth on solid media, despite a positive alarm in the BacT/ALERT 3D machine, should be regarded as false negatives if the standard reference strains cultured in parallel do not show adequate growth and all steps to isolate strains on solid media should be repeated.

See Appendix 1 for a protocol for performing QC testing for growth on solid media with standard reference strains, and for maintaining such strains. The results of the weekly QC testing and assessment of each new batch of media should be recorded on the provided QC forms, sent to IEIP every two weeks, and stored in laboratory logbook which will be audited quarterly as part of external QA reviews

6.2 Gram stain procedure for positive blood cultures (Hucker modification)

Positive cultures from either the aerobic (FAN or Pediatric FAN) or the mycobacterial (MB) bottle for each patient should be Gram stained as part of preliminary identification of isolates. Gram staining divides bacteria into two groups based on the composition of their cell walls: those that retain the primary stain crystal violet and appear purple on examination (Gram positive) and those that are de-colorized by an ethanol rinse and instead take up the counterstain, appearing pink on examination (Gram negative). Gram staining also increases the visibility of bacterial morphology, making it easier to identify the shape of an isolate.

Gram staining should always be performed using a clean new slide. Debris from previous tests may remain on washed and re-used slides, making identification of organisms difficult, and can falsely suggest multiple isolates in a single bottle. Overheating during the flaming process, excessive washing with acetone, or attempting to stain old cultures can also confuse results, as any of these may result in Gram positive bacteria staining poorly and appearing to be Gram negative.

All Gram stain reagents will be provided by IEIP. If commercially prepared reagent kits include specific instructions, follow these. Otherwise, take the following steps:

1) Pass the slide with the **air-dried** drop of blood culture medium quickly through a flame three times to fix the smear. Flame only briefly. Slide should not be hot enough to cause discomfort when touched to back of hand. Alternatively, bacteria can be fixed using methanol (95%-100%).

2) Flood the smear with ammonium oxalate-crystal violet and let stand for 1 minute.

3) Rinse gently with tap water by running a gentle stream across the edge or back of the slide. Drain off excess water by tapping the slide on a paper tower.

4) Flood the smear with Gram's iodine solution and let stand for 1 minute.

5) Rinse gently with tap water and drain.

6) Decolorize with 95% ethyl alcohol for 5-10 seconds.

7) Counterstain with safranin for 20-30 seconds, or with carbol-fuchsin for 10-15 seconds.

8) Rinse the slide with tap water and blot dry.

9) Examine the stained smear microscopically, using a bright-field condenser and an oil-immersion lens. NOTE: The BioMérieux blood culture media contain activated charcoal particles, intended to bind antibiotic factors in blood that might impair bacterial growth. These may be aspirated from the culture bottle with the isolates and be deposited on the slide. These particles will appear dark, and may be mistaken for heavily stained Gram positive organisms initially. A negative control slide should be prepared by Gram stain techniques using un-inoculated BioMérieux blood culture media and compared to slides from positive blood cultures to help identify the charcoal particles more easily.

10) Describe the observed organisms by morphology (shape) and color (indicating Gram positive or negative). The majority of organisms will fall into a few categories:

Color	Shape	Arrangement
Purple (Gram positive)	**Cocci (spheres)** **Bacilli (rods)** **Coccobacilli (intermediate forms)**	Chains Pairs Clusters None
Pink (Gram negative)		

11) Record observations on Specimen Collection Form and use to guide confirmatory identification steps. If observed organisms do not fall into the categories above, select "Other" and describe the appearance.

12) Slides should be kept until colony growth and the microscopic appearance of organisms from an isolated colony grown on solid media can be described and confirmed (at least one week).

QC 6.2: Gram stain reagents and techniques should be tested at least once weekly, or when new staining reagents are opened, by staining isolated colonies from one Gram positive (*S. aureus* ATCC 25923) and one Gram negative (*E. coli* ATCC 25922) reference strain. Results should be recorded on the supplied forms and kept in a laboratory logbook. If the reference strains do not produce clearly observable predicted results, the reagents and protocol should be checked again by an experienced microbiologist and new materials obtained if necessary. If dark particulate matter appears on the control slides, the reagents should be filtered and the Gram stain QC procedure repeated.

6.3 Acid-fast bacilli staining for positive MB bottles

For all MB bottles that signal a positive alarm in the BacT/ALERT 3D machine, the laboratory technician should prepare two slides: one for Gram staining, and the other for AFB staining to detect mycobacteria. MB bottles that become positive within 72 hours of inoculation most likely contain typical bacterial pathogens, but bottles that become positive after a much longer period (up to 42 days post-inoculation) should be suspected of containing slow-growing mycobacteria (*M. tuberculosis, M. avium,* or other mycobacterial pathogens), yeast, or fungi. These isolates should be treated as potentially highly infectious.

IEIP will supply reagents for AFB staining through the Ziehl Neelsen technique. If commercially prepared reagent kits include specific instructions, follow these. Otherwise, take the following steps:[22]

1) Pass the slide with the **air-dried** drop of blood culture medium through a flame three times to fix the smear. Flame for 3-4 seconds each time.

2) Place the slide, smear side up, on a staining rack over a heat source.

3) Flood the smear with filtered 1% carbol fuchsin.

4) Heat the slide from below until vapors start to rise. Do not let carbon fuchsin boil, or slide dry completely. Continue to heat for up to five minutes.

5) Allow the slide to cool completely (5-7 minutes).

6) Rinse the slide gently with tap water to remove excess carbol fuchsin stain and drain (the blood culture smear will still appear red).

7) Decolorize by flooding the slide with 25% sulphuric acid. Let the sulphuric acid sit on the slide for 2-4 minutes.

8) Rinse gently with tap water and drain.

9) If the slide still appears red, repeat the decolorization process, leaving sulphuric acid on the slide for 1-3 minutes and rinsing gently with tap water.

10) Counterstain with 0.1% methylene blue solution for one minute.

11) Rinse gently with tap water and drain.

12) Examine the stained smear microscopically, using a bright-field condenser and an oil-immersion lens. NOTE: The BioMérieux blood culture media contain activated charcoal particles, intended to bind antibiotic factors in blood that might impair bacterial growth. These may be aspirated from the culture bottle with the isolates and be deposited on the slide. A negative control slide should be prepared by AFB stain techniques using un-inoculated BioMérieux blood culture media and compared to slides from positive blood cultures to help identify the charcoal particles more easily.

13) Describe the observed organisms by morphology (shape) and color (indicating AFB positive or negative). The majority of organisms will fall into a few categories:

Color	Shape	Arrangement
Pink or red (acid-fast)	**Bacilli (rods)** **Cocci (spheres)** **Coccobacilli (intermediate forms)** **Branched or hyphae (suggesting yeast or fungi)**	Chains Pairs Clusters None
Blue (negative)		

14) Record observations on Specimen Collection Form, determining whether organisms can be classified as acid-fast bacilli, other acid-fast forms, or negative.

15) All AFB negative samples that appear to be bacteria by morphology and Gram stain should be processed as per standard bacterial protocols. All acid-fast bacilli and suspected yeasts or fungi should be shipped to IEIP in the original culture bottles. These will be forwarded to the Thai National TB Reference Center (mycobacteria) or the Thai NIH (fungi or yeasts), for further testing. These should not be sub-cultured in the provincial laboratories. Clinicians should be notified that positive specimens appear to be acid-fast bacilli (mycobacteria), yeast, or fungi, and that confirmatory identification and testing will be performed by one of the two reference laboratories.

QC 6.3: AFB stain reagents and techniques should be tested at least once weekly, or when new staining reagents are opened, by staining isolated colonies from one AFB positive (*Mycobacterium spp.* ATCC 25177) and one AFB negative (*S. aureus* ATCC 25923) reference strain. Results should be recorded on the provided forms and kept in a laboratory logbook. If the reference strains do not produce clearly observable predicted results, the reagents and protocol should be checked again by an experienced microbiologist and new materials obtained if necessary.

6.4 Alarm-positive blood cultures with no apparent growth

Occasionally, the BacT/ALERT 3D machine will signal that an inoculated blood culture bottle has become positive, but no organism can be identified by Gram stain or isolated on solid media. If positive standard reference strains included for QC purposes show that all reagents and media are performing as expected, and that conditions are optimal for bacterial growth (see QC references in sections 6.1, 6.2, and Appendix 1), three main possibilities should be considered:

1. A "false positive" has occurred due to over-filling of the bottle, temperature fluctuations affecting the media or color indicator, or machine error;

2. The culture has yielded an extremely fastidious and hard-to-identify organism that might be viable in small numbers (more likely if the alarm happens late in the incubation period);

3. An isolate of *S. pneumoniae* has been cultured past the log phase of growth, resulting in a real alarm but inability to locate or culture organisms due to autolysis (self-destruction) of the previously viable isolate.

To test whether an apparent false positive culture represents an autolysed *S. pneumoniae* isolate, IEIP will supply the NOW *Streptococcus pneumoniae* Test® manufactured by Binax (Portland, Maine, USA). This rapid immunochromatographic assay is designed to detect *S. pneumoniae* antigens in the urine of patients with pneumonia and cerebrospinal fluid from patients with meningitis, but the test can be modified to detect pneumococcal antigens in blood culture medium even in no organisms can be Gram stained or cultured.[23] Binax NOW kits should be stored between 15-30ºC.

This test should be used for **every apparent false positive blood culture bottle**. If the results are positive, the laboratory microbiologist should report a preliminary identification of *S. pneumoniae* to the clinician who ordered the blood culture and the district hospital laboratory, and explain that no further testing will be possible. This result should also be recorded on the Specimen Collection Form, and sent to the patient's chart through the normal mechanisms.

<u>Modified Binax NOW *S. pneumoniae* Test protocol for apparent false positive blood cultures:</u>

1) Place blood culture bottles in a biosafety cabinet to avoid accidental aerosolization of potentially infectious materials. Use standard precautions for safe handling of blood.

2) Allow blood cultures to equilibrate to room temperature (15-30ºC) and swirl gently to resuspend antigens before beginning tests.

3) Wipe gloves with 70% isopropyl alcohol, then carefully disinfect the top (rubber septum) of each bottle with 70% isopropyl alcohol.

4) Unwrap one testing device for each specimen to be tested and lay flat in biosafety cabinet without touching the reaction area of the testing device.

5) Remove one Binax swab per sample from the kit, and use the foil package from the testing device as a tray for the swab. **Do not use other swabs for this test.**

6) Using a sterile needle and syringe, aseptically remove 0.5 mLs of blood culture media from each positive bottle.

7) Drop culture media from the needle onto a Binax swab until the swab head is completely soaked, but not lying in a puddle of excess media. If the swab head drips when picked up, remove the excess liquid by pressing against inside edge of the foil package.

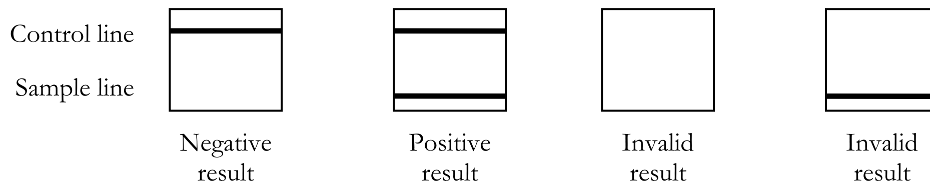
8) Insert the swab into the **bottom hole** (swab well) on the inner right panel of the testing device. Firmly push upwards so that the swab tip is fully visible in the top hole. Do not remove the swab.

9) Hold the Reagent A vial vertically (**straight up and down**) 1-2 cm above the device. Slowly let 3 drops of Reagent A fall into the bottom hole.

10) Immediately remove the adhesive liner from the right edge of the test device, and close and seal the device. Repeat all steps for each apparent false positive bottle.

11) Read the result in the window 15 minutes after closing the device. Results read after 15 minutes may not be accurate; strongly positive samples may produce a visible sample line in less than 15 minutes.

12) One or two lines should appear in the window on the testing device. A single pink-to-purple colored Control Line in the top half of the window means that the test was performed correctly, but no pneumococcus antigens were detected. The appearance of two pink-to-purple colored lines, the Control Line and a Sample Line, indicated a positive result even if the sample line is very faint. If no lines appear, or only the bottom Sample Line appears, the test results are not reliable. If this happens, the test should be repeated using three samples: the pre-packaged positive and negative control swabs, and the blood culture again.



<u>To use the positive and negative control swabs with the Binax NOW Tests:</u>

13) Unwrap one testing device for each specimen to be tested and lay flat in biosafety cabinet without touching the reaction area of the testing device. Unwrap the prepackaged negative control swab and insert it into the **bottom hole** of a testing device. Firmly push upwards until the swab tip is fully visible in the top hole. Do not remove the swab. Repeat with a positive control swab and another testing device.

14) Hold the Reagent A vial vertically (**straight up and down**) 1-2 cm above the device. Slowly let 6 drops of Reagent A fall into the bottom hole.

15) Immediately close and seal the device.

16) Read the result in the window 15 minutes after closing the device. Results read after 15 minutes may not be accurate; strongly positive samples may produce a visible sample line in less than 15 minutes.

QC 6.4: Two kinds of QC testing can be applied to the Binax NOW Test. The first relies upon the internal procedural control – that is, the pink-to-purple Control Line that should appear with every test to demonstrate that the device functions as expected, and that enough reagent was added to ensure sufficient capillary flow through the device. The background of the window should be light pink to white within 15 minutes – if the background is dark enough to interfere with the reading of the test result, the test should be repeated.

The second type of QC testing involves the external positive and negative controls supplied as pre-packaged swabs with the Binax NOW Test kit. The positive and negative controls should be used **every time a new Binax NOW Test kit is opened, if the kit has not been used in more than a week, or if an invalid result occurs with a patient sample**. Although, ideally, the positive and negative control should be run with every patient sample, each kit includes only five control swab sets. If possible, apparent false positive bottles should be tested in batches with one external positive and negative control set for the entire batch. If the provincial laboratory consumes all of the pre-packaged swabs before the expiration date of the kits, the microbiologist should consult with the IEIP microbiology chief about additional controls.

If the Binax NOW Tests do not give expected results with the external or internal controls, do not report the patient results to the clinician, and notify IEIP to request a new testing kit.

6.5 Presumptive identification of *S. pneumoniae, H. influenzae,* and *N. meningtidis* isolates[24]

The primary purpose of this section is to aid in preliminary identification of isolates of *S. pneumoniae, H. influenzae,* and *N. meningitidis* from positive blood cultures. The methods described here will not apply to other suspected bacterial agents of pneumonia and sepsis; clinical microbiology textbooks and manuals referenced in the introduction to this section provide detailed protocols for isolating and characterizing the range of other possible pathogens.

Presumptive identification of these three organisms can be made on the basis of differential growth on blood and chocolate agar, macroscopic examination of colonies, and microscopic examination of Gram-stained organisms. After growth is observed on solid media inoculated from the positive blood culture bottles, a second Gram stain should be prepared from each pure culture through the following steps:

1) Place one drop of physiological saline or distilled water on a clean, unused slide labeled with the patient's name, hospital ID number, and the date.

2) With a flamed sterile inoculating loop, touch the center of an isolated bacterial colony from one of the three solid media plates.

3) Add the bacteria from the inoculating loop to the saline or distilled water with a gentle tap, using the loop to mix the organisms into suspension.

4) Spread the suspension and allow to air dry for approximately ten minutes.

5) Follow the steps for Gram staining described in section 6.3 (above).

<u>Presumptive identification of *S. pneumoniae*</u>

S. pneumoniae appear as Gram positive pairs of cocci (diplococci) or chains of cocci on microscopic examination. When examined macroscopically on solid media, S. *pneumoniae* appears as small, greyish, moist (sometimes mucoid), watery colonies with a greenish zone of α-hemolysis surrounding them on blood and chocolate agar plates. Some serotypes appear more mucoid than others, an effect exaggerated by fresh culture medium. Young pneumococcal colonies appear raised, and can be difficult to differentiate from α-hemolytic viridans streptococci, which also produce a greenish zone of hemolysis on a blood or chocolate agar plate. As the culture ages 24-48 hours, the colony becomes flattened and the central part of each colony becomes depressed, which does not occur with the viridans streptococci. A hand lens or microscope (30X-50X) can prove useful in distinguishing pneumococci from viridans streptococci.

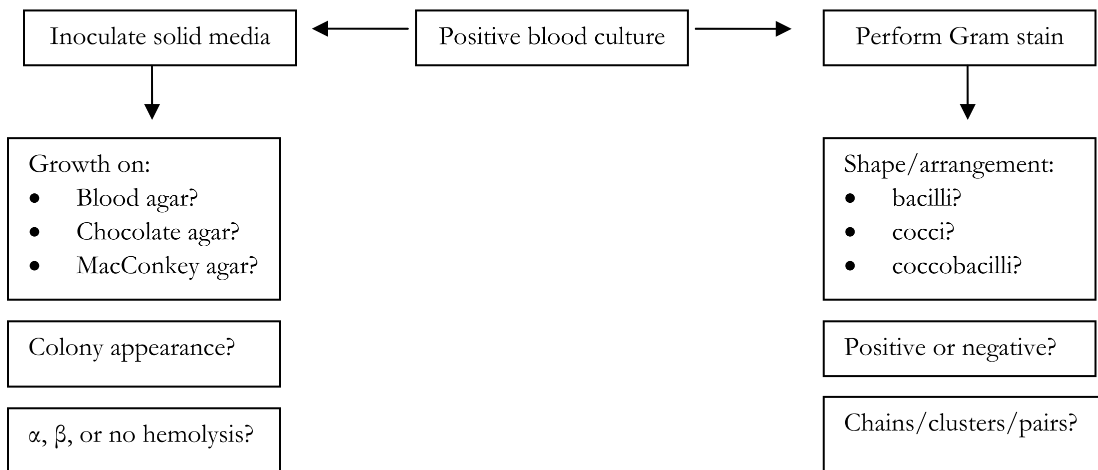
<u>Presumptive identification of *H. influenzae*</u>

H. influenzae appear as small, Gram-negative bacilli or coccobacilli on microscopic examination. When examined macroscopically on chocolate agar plates, *H. influenzae* appears as large, flat, colorless-to-grey opaque colonies on chocolate agar. No hemolysis or discoloration of the medium is apparent. Encapsulated strains appear more mucoidal than non-encapsulated strains, which appear as compact grayish colonies.

Presumptive identification of *N. meningitidis*

N. meningitidis appear as Gram-negative, coffee bean-shaped diplococci on microscopic examination. When examined macroscopically on blood agar plates, young colonies of *N. meningitidis* are round, smooth, moist, glistening and convex, with a clearly defined edge. Some colonies appear to coalesce with other nearby colonies. Growth of *N. meningitidis* on blood agar appears greyish and unpigmented; older cultures become more opaquely grey and sometimes cause the underlying agar to turn dark. Well-separated colonies can grow from about 1 mm in diameter in 18 hours to as large as 4 mm, with a somewhat undulating edge, after several days.

N. meningitidis grows on blood agar, whereas *H. influenzae* will not grow without supplements found in chocolate agar. When grown on chocolate agar, *H. influenzae* and *N. meningitidis* look similar. However, *H. influenzae* gives off a pungent smell of indol, while *N. meningitidis* does not.



Growth on		Gram stain morphology	Presumptive identification	Confirmatory tests
Chocolate agar	**Sheep blood agar**			
+	+	Gram – coffee bean-shaped diplococci	*N. meningitides*	Kovac's oxidase test, carbohydrate utilization test
+ (α-hemolysis)	+ (α-hemolysis)	Gram + diplocci or cocci	*S. pneumoniae*	Optochin sensitivity, bile solubility
+	-	Gram – small pleomorphic bacilli/coccobacilli	*H. influenzae*	X and V factor dependency, serum agglutination

The Thai NIH will confirm identification of all potential isolates of *N. meningitides*, *S. pneumoniae*, and *H. influenzae* (see Section 6.9).

6.6 Confirmatory identification of *S. pneumoniae*

Confirmatory identification of pneumococcus follows the presumptive identification, described above, based on the microscopic identification of Gram-positive cocci in pairs or chains, and the macroscopic appearance of the small, grayish, and mucoid colonies demonstrating α-hemolysis on blood and chocolate agar plates incubated in a 5% CO_2 atmosphere.

Laboratory differentiation between *S. pneumoniae* and viridans streptococci can be accomplished by optochin and bile solubility testing. Pneumococci are susceptible to optochin and are bile soluble, while viridans streptococci are not. The bile solubility test can be particularly helpful when the results of the optochin susceptibility test appear ambiguous.

<u>Performance of the optochin susceptibility test</u>

1) Touch the suspect α -hemolytic colony with a sterile bacteriological loop and streak onto a blood agar plate in a straight line. Because the inoculum is streaked in a straight line, several 3-4 colonies can be tested on the same plate, streaked in parallel lines and properly labeled.

2) Aseptically place an optochin or "P" disk with a diameter of 6 mm (and containing 5 μg ethylhydrocupreine) on the end of the streak where the wire loop was first placed.

3) Incubate the plates in a CO_2 incubator or candle-jar at 35°C for 18-24 hours.

4) Read, interpret, and record the results on the Specimen Collection Form:

   a) α-hemolytic strains with a zone of inhibition of growth greater than 14 mm in diameter (or >16 mm in diameter if using a 10-mm optochin disk) are considered susceptible to optochin, and are therefore pneumococci;

   b) α-hemolytic strains with no zones of inhibition are considered resistant to optochin, and are viridans streptococci;

   c) α-hemolytic strains with zones of inhibition ranging from 9 to 13 mm show intermediate resistance to optochin, and should therefore be tested for bile solubility.

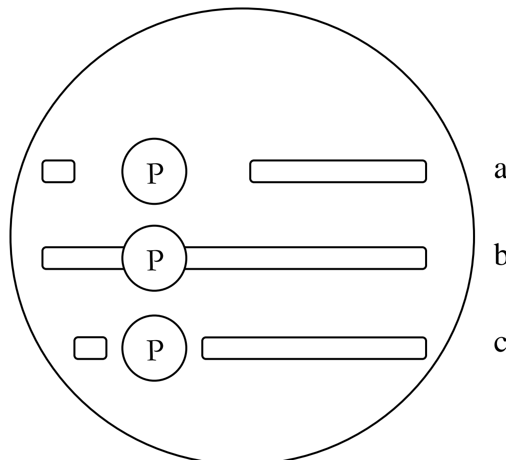


Diagram of optochin susceptibility test showing three streaks (three different strains) with varying susceptibilities, as described above..

Performance of the bile solubility test

The bile solubility test is performed on isolates with small zones of inhibition in the optochin sensitivity test. It can be performed using either the "tube method" or the "plate method."

1) Using the tube method, two tubes are required for bile solubility testing of each suspect strain of *S. pneumoniae.*

2) Take a loop of the suspect strain from fresh growth on a blood agar plate and prepare a bacterial cell suspension in 0.5 ml of sterile saline. The suspension of cells should be equal to that of a 0.5 McFarland density standard (see Appendix 3).

   a) If growth on the optochin test plate is sufficient, the suspension can be made with the bacterial cells collected from the specific streak of suspect *S. pneumoniae.*

   b) When there is insufficient growth to make a suspension of the proper density in 0.5 mL of sterile saline, inoculate a blood agar plate with the suspect growth and incubate overnight at 35ºC in a 5% CO_2 incubator to prepare a fresh culture.

3) Divide the suspension into two equal amounts (0.25 ml per tube). Add 0.25 ml of saline to one tube and 0.25 ml of 2% deoxycholate (bile salts) to the other.

4) Shake the tubes gently and incubate them at 35 - 37°C for up to 2 hours
.
5) Examine the tubes periodically for lysis of cells in the tube containing the bile salts. A clearing of the tube, or a loss in turbidity, is a positive result.

6) For the plate method, place a drop of 10% sodium deoxycholate solution directly on a colony of a freshly prepared culture of the suspect isolate. (To prepare the 10% solution of bile salts, add 1 g of sodium desoxycholate bile salts to 10 mL of sterile saline.)

7) Keep the plate at room temperature (18-25ºC) or place it agar-side up on a level surface in an ambient air (non-CO_2) incubator at 35ºC for approximately 15 minutes, or until the 10% bile salt reagent dries.

8) When the reagent is dry, read, interpret, and record the results.

Pneumococcal colonies are bile soluble and will disappear or appear as flattened colonies, while bile-resistant streptococcal colonies will be unaffected.

QC 6.5: The quality of the conditions and the reagents for both the optochin susceptibility test and the bile solubility test should be evaluated every time either test is performed. The standard reference strain *S. pneumoniae* ATCC 49619 can be used as a positive control strain for both tests, and should be run in parallel with every suspect clinical isolate. The results of the reference standard should be recorded on the provided forms and kept in a bound laboratory logbook with the date and the results from any suspect clinical isolates for each test. If the positive control reference strain does *not* show optochin sensitivity and bile solubility, the tests should be repeated using new reagents, if necessary, to achieve the expected result with the reference strain.

Optochin susceptibility (diameter of zone of inhibition with 6-mm, 5 µg optochin disk)	Bile solubility	Most likely identification
Susceptible (≥14 mm)	**Not necessary to test**	*S. pneunomiae*
Intermediate/definite (9-13 mm)	**Yes**	*S. pneunomiae*
Intermediate/small (≤8 mm)	**No**	Viridans streptococci
Resistant (no zone of inhibition)	Not necessary to test	Viridans streptococci

Serotyping *S. pneumoniae* isolates

The Quellung reaction is traditionally used to identify the serotypes of pneumococcal isolates due to its ease of use, speed, and accuracy. A Quellung reaction results when a type-specific antibody bound to the pneumococcal capsular polysaccharide causes a change in the refractive index of the capsule so that it appears "swollen" and more visible. The pneumococcal cell stains dark blue and is surrounded by a sharply demarcated halo, which represents the outer edge of the capsule. The light transmitted through the capsule appears brighter than either the pneumococcal cell or the background. Single cells, pairs, chains, and even clumps of cells may have Quellung reactions. However, most laboratories do not type pneumococcal isolates because of the large number of diagnostic antisera required.

All confirmed isolates of *S. pneumoniae* identified through the routine use of blood cultures for patients with suspected pneumoniae, or in children ages 5 and under with fever and possible sepsis, in Nakhon Phanom and Sa Kaeo will be identified through the Quellung typing technique. These tests will be performed by technicians from the Thai NIH using facilities and reagents at the CDC (Atlanta, Georgia, U.S.) as per existing arrangement. *S. pneumoniae* isolates will be shipped to the IEIP laboratory each Tuesday (described in Sections 4.3 and 6.8), and transported to the Thai NIH for cryopreservation. Twice each year, these samples will be shipped to CDC and will be serotyped there using the Quellung reaction by Thai MOPH researchers who have already received training and conduct these tests routinely with IEIP support.

6.7 Antimicrobial susceptibility testing of *S. pneumoniae*

The results of antimicrobial susceptibility testing can be used to help make recommendations for clinical treatment for individual patients, and to help predict which antibiotics might be effective for patient populations in general. The disk diffusion method presented here represents a modification of the Kirby-Bauer technique standardized by NCCLS, as well as the Etest (AB BIODISK, Solna, Sweden) antimicrobial gradient agar diffusion technique for testing sensitivity to penicillin. If performed precisely according to the following protocol, this method will yield data that can reliably predict the *in vivo* effectiveness of specific antimicrobial agents against *S. pneumoniae*.

The WHO recognizes three classes of antimicrobial susceptibility: susceptible, resistant, and intermediate. These can be used to describe the results of the disk diffusion testing. All results from antimicrobial susceptibility testing should be attached to the original Specimen Collection Form for each patient, and included when entering results each week into the GAVI database using Microsoft Access. The results should also be reported to the ward nurse or physician and the district hospital laboratory immediately by phone as soon as they are interpreted.

IEIP will provide reagents necessary to perform the disk diffusion method – valid for certain antibiotics – and the Etest to gather data about the minimal inhibitory concentration (MIC) of penicillin for bacterial isolates.

QC 6.6: To verify the accuracy of antimicrobial susceptibility testing results, a standard reference strain should be tested regularly in parallel with patient isolates. The positive control strain *S. pneumoniae* ATCC 49619 should be used when performing antimicrobial sensitivity tests with *S. pnemoniae* isolates. Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits (described in Appendix 1). If zones produced by the control strain lie outside of the expected ranges, the technician should evaluate the following factors, which can affect antimicrobial susceptibility tests:

- Variations in media, inoculum size, incubation time, temperature, or other environmental factors, such as CO_2 levels;
- Whether the depth of the agar in the plate varies from a uniform 3-4 mm, required to allow consistent diffusion of the antimicrobial agents;
- If the pH of the test medium lies outside of the optimal range (7.2-7.4);
- If the inoculum is not a pure culture, or does not contain a concentration of bacteria approximately equivalent to the 0.5 McFarland turbidity standard.

The reference strain should be included with every group of tests until the laboratory has demonstrated 30 consecutive days of in-control results. QC testing with the positive control strain can then be performed weekly, or each time the laboratory uses a new batch of testing medium or lot of disks. If testing is done less frequently than every day, the reference strain should be included with every group of tests.

Antimicrobial sensitivity testing by disk diffusion

Mueller-Hinton agar medium supplemented with 5% sheep blood, poured at a uniform depth of 3-4 mm, is the recommended medium for determining the antimicrobial sensitivity of *S. pneumoniae* isolates by disk diffusion.

Susceptibility testing with penicillin disks does not provide reproducible results. Results of screening with the 1 μg oxacillin disk can be generalized across the β-lactam drugs for *S. pneumoniae*, allowing the technician to conclude whether a strain is susceptible (but not resistant) to penicillin. If the oxacillin disk produces a zone of inhibition of less than 20 mm, additional MIC testing by Etest® must be performed to determine whether the isolate is resistant or susceptible to penicillin.

1) Suspend viable colonies from a fresh pure culture of *S. pneumoniae* (grown overnight on blood or chocolate agar) in Mueller-Hinton broth or sterile physiological saline.

2) Adjust the cell suspension to the density of a 0.5 McFarland turbidity standard (described in Appendix 2), carefully avoiding froth or bubbles while mixing. Compare the density of the suspension to the 0.5 McFarland turbidity standard by holding the suspension and the McFarland standard in front of a light against a white background with black lines. Add more saline or broth if the density appears too heavy, and more bacteria if the density appears too light.

3) Dip a sterile cotton swab into the bacteria suspension, removing excess fluid by rotating against the sides of the dilution tube.

4) Use the same swab (without re-dipping in the bacterial cell suspension) to inoculate the entire surface of the 5% sheep blood-Mueller Hinton agar three times, rotating the plate 60 degrees between each inoculation to achieve even coverage.

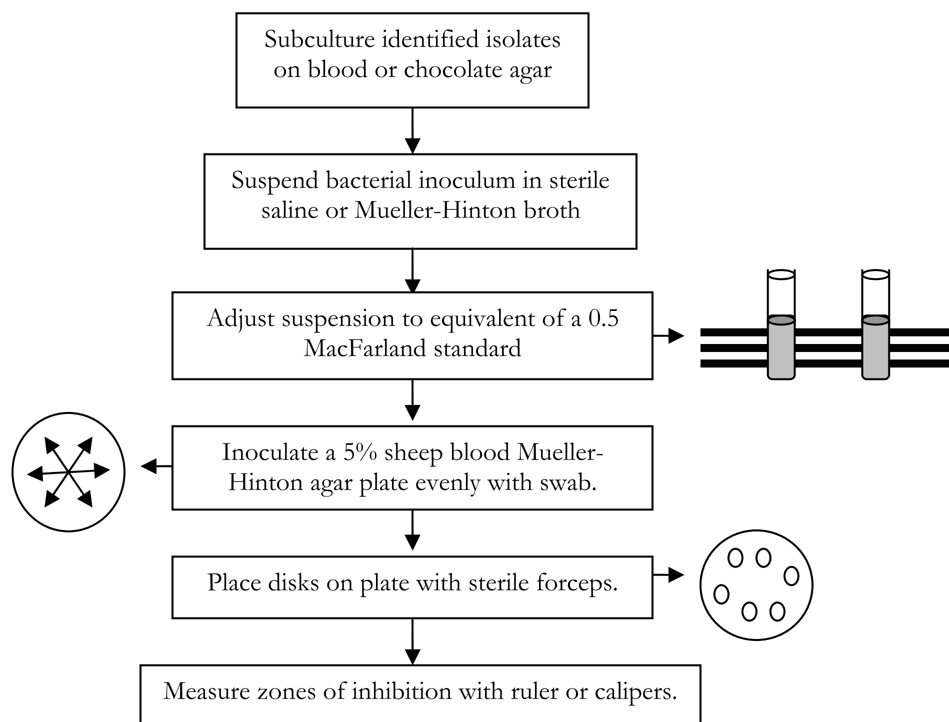
5) Allow the inoculum to dry before placing disks on the plates (5-15 minutes).

6) Place the antimicrobial disks on the plates with a dispenser or sterile forceps, tapping them gently to ensure that they adhere to the agar. Once a disk contacts the agar surface, diffusion of the drug begins immediately, and the disk should not be moved. For suspected *S. pneumoniae* isolates, four disks can be placed on each plate.

7) Incubate the plates in an inverted position (upside down) at 35°C in a 5% CO_2 incubator for 20-24 hours.

8) Measure the diameter of the zone of inhibition with a rule or calipers, measuring the distance from the last visible colony on each side of the disk from the top surface of the plate with the lid removed. Care should be taken not to touch the disk or surface of the agar with the ruler or calipers.

9) Interpret the antimicrobial susceptibility of the isolate according the NCCLS performance standards, checking to be sure that the results for the positive control reference strain *S. pneumoniae* ATCC 49619 lie within the acceptable control range.



Etest® for MIC testing of *S. pneumoniae*

Disk diffusion testing with oxacillin and *S. pneumoniae* isolates allows the laboratory to screen for susceptibility to penicillin, but does not allow technicians to distinguish whether strains that appear **not** to be susceptible exhibit complete or intermediate resistance. Therefore, all *S. pneumoniae* should also be tested for penicillin susceptibility using the commercially available Etest® to determine MIC. All reagents will be supplied by IEIP, and the tests will be performed at the Thai NIH reference laboratory.

Etests® to determine penicillin resistance in *S. pneumoniae* isolates should be performed using Mueller-Hinton agar supplemented with 5% sheep blood.

1) Suspend viable colonies from a freshly grown overnight blood agar plate in a Mueller-Hinton broth tube, adjusting the suspension to the equivalent of a 0.5 MacFarland turbidity standard.

2) Dip a sterile cotton swab into the bacterial suspension, removing excess fluid against the side of the tube, and inoculate the entire surface of the 5% sheep blood Mueller-Hinton agar plate evenly with the same swab, rotating the plate 60 degrees after each inoculation to ensure confluent growth.

3) Allow the plate to dry completely (5-15 minutes). While the plate is drying, remove the Etest strips from the -20°C freezer and allow the strips that will be used to warm to room temperature. Unused strips must always be stored at -20°C.

4) Place the Etest® strip onto the inoculated agar plate with sterile forceps. Make sure that the printed MIC values face upward. Once applied, do not move the antimicrobial strip.

5) Incubate the plates in an inverted position (upside down) at 35°C in a 5% CO_2 incubator for 20-24 hours, following the manufacturer's instructions.

6) MICs are read by examining the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip, read using oblique light, with a magnifying glass if necessary. The intersection should be determined from the point of inhibition of **all** growth, including hazes and isolated colonies. Follow the manufacturer's instructions in the package insert for interpreting and reporting antimicrobial susceptibility results. If the intersection of the printed MIC value and the zone of inhibition lies between two markings, read the next highest standard value.

7) Interpret the antimicrobial susceptibility of the isolate, checking to be sure that the results for the positive control reference strain *S. pneumoniae* ATCC 49619 lie within the acceptable control range.

QC 6.7: In addition to checking that the Etest® results for the standard reference strain *S. pneumoniae* ATCC 49619 fall within the acceptable control range (as described above), performing and reading the Etest® correctly also requires careful attention. After placing the strip on the inoculated agar, check to be sure that the numbers appear to be facing the correct direction for easy reading. If the strip is facing backwards (i.e., the numbers appear reversed), the antibiotic is not placed against the agar, and results will not be valid; if this occurs, start again with a fresh plate and Etest® strip. If the intersection is different on either side of the strip, read the higher value; if the difference is > 1 dilution, repeat the test. A very thin line of growth along the edge of the strip is probably caused by organisms growing in a tunnel of water, and should be ignored.

6.8 Confirmatory identification of *H. influenzae*

Confirmatory testing for *H. influenzae* follows the identification of small, pleomophic Gram-negative bacilli or coccobacilli which grow on chocolate but not blood agar plates and have a pungent indol smell. Laboratory testing for *H. influenzae* includes testing for X and V factor requirements and serotyping using an agglutination reaction. In the absence of vaccination, most invasive *H. influenzae* disease is caused by organisms with the type b polysaccharide capsule (*H. influenzae* b, or Hib).

Identification of X and V growth factor requirements

H. influenzae is a fastidious organism that can be distinguished from other strains of *Haemophilus* on the basis of its growth requirements for media containing haemin (X factor) and nicotinamide adenine dinucleotide (NAD, V factor). *H. hemolyticus* is the only other species requiring X and V factors, and can be distinguished from *H. influenzae* by looking for hemolysis on a blood agar plate. The standard medium for growth is chocolate agar, because the processing of heating the blood during preparation makes both X and V factors available to the organism. IEIP will supply supplemented, commercially prepared chocolate agar plates.

1) Prepare a heavy suspension of cells (No. 1 McFarland) from a primary isolation plate in a suitable broth (tryptone-based soy broth or TSB, or heart infusion broth). If the primary isolation plate contains insufficient growth or is contaminated, make a subculture on a chocolate agar plate. When preparing the media, avoid transfer of agar medium to the broth; even the smallest sample of agar will affect the test and may lead to misidentification of the bacteria because the agar contains X and V factors.

2) Inoculate a heart infusion or tryptone-based soy agar plate by streaking a sterile swab of the suspension over one-half of the plate in at least two directions to ensure confluent growth. Allow the plate to dry.

3) Using sterile forceps, place paper strips or disks containing X, V, and XV factors on the inoculated plate spaced equally far apart from each other and press gently against the agar.

4) Incubate the plates in an inverted position (upside down) at 35°C in a 5% CO_2 incubator for 18-24 hours.

5) *H. influenzae* will grow only around the XV disk (i.e., the disk containing both X and V factors).

Species	X and V Factor Requirements X	 V	β-hemolysis on blood agar
H. influenzae	+	+	-
H. parainfluenzae	-	+	-
H. hemolyticus	+	+	+
H. parahemolyticus	-	+	+
H. aphrophilus	+	-	-
H. paraphrophilus	-	+	-

Identification of the *H. influenzae* serotype

H. influenzae is currently recognized to have 6 serotypes (a, b, c, d, e, and f). *H. influenzae* type b (Hib) is a major cause of invasive bacterial disease, and the major cause of meningitis, in unvaccinated children in many parts of the world. All *Haemophilus* isolates should be tested with Hib antiserum, an antiserum to one of the other groups, and saline. A strongly positive (3+ or 4+) agglutination reaction with type b antiserum and no agglutination with an antiserum to the other serotypes and saline is evidence of Hib. All identification testing for *H. influenzae* serotypes will be conducted at the Thai NIH.

Antisera should be stored in the refrigerator at 4ºC when not in immediate use. Screening an isolate first with polyvalent antiserum (which contains antisera to all six recognized serotypes) and a saline control will be performed to save resources. If the isolate is positive with a polyvalent antiserum and negative in the saline control, technicians will proceed by testing the isolate with type b antiserum. If the serotype b reaction is negative, technicians will then test with the remaining type-specific antisera (a, c, d, e, and f). If an isolate is non-agglutinating in the polyvalent antiserum, the assumption will be made that it is either non-typeable, or that it is not *H. influenzae* and steps will be taken to re-confirm the species identity.

Slide agglutination test for serotyping suspected *H. influenzae* isolates

1) Divide a new, pre-cleaned glass slide into equal sections with a wax pencil or other marker.

2) Collect a small portion of growth from the surface of an overnight culture on chocolate agar (without bacitracin), a *Haemophilus* ID plate, or a *Haemophilus* test medium plate with a sterile inoculating loop. Make a moderately milky suspension of the test culture (roughly equivalent to a 6 MacFarland turbidity standard) with 250 µl of 0.5% formalinized physiological saline and vortex the suspension.

3) For the agglutination reaction, transfer a loopful (5-10 µl) of the cell suspension to the lower portion of two sections of the prepared slide. Use enough suspension so that it does not dry before testing.

4) Add 5-10 µl of polyvalent antiserum above the drop of suspension in one test section on the slide. In an adjacent section of the slide, use the same method to add a 5-10 µl drop of saline above the final drop of suspension. The loop used in the antiserum must not touch the cell suspension or the other antiserum being tested; if it does, it must not be placed back into the source bottle of antiserum to prevent contamination of the source bottle.

5) Using a sterile toothpick or sterile loop for each section, mix the antiserum or saline with the corresponding drop of cell suspension. Avoid contamination across sections of the slide.

6) Gently rock the slide back and forth for up to 1 minute, being careful to avoid motions that cause the mixtures to run together. After one minute of rocking, observe the mixed drops and read the slide agglutination reactions under a bright light against a black background.

7) In a strong reaction, all the bacterial cells will clump and the suspension fluid will appear clear. Only strong agglutination reactions (3+ or 4+) are read as positive. If strong agglutination occurs in the polyvalent antiserum, use the same methods to test the isolate with type b and other type-specific antisera. If agglutination does not occur, continue testing the isolate to confirm whether it belongs to another *Haemophilus* species.

Antimicrobial susceptibility testing of *H. influenzae*

Antimicrobial susceptibility testing for isolates of *H. influenzae* will be performed as described above for *S. pneumoniae* in Section 6.6, with the following exception:

- The recommended medium for antimicrobial susceptibility testing for *H. influenzae* is *Haemophilus* test medium (HTM), which will be prepared by the Thai NIH and supplied to the provincial laboratories by IEIP. The zone diameter sizes can be interpreted correctly **only when HTM is used**, as per NCCLS standards.

- The 10 µg ampicillin disk predicts both intrinsic (penicillin-binding protein-mediated) and β-lactamase-mediated penicillin and ampicillin resistance and should be used when testing *H. influenzae*.

QC 6.8: To verify the accuracy of antimicrobial susceptibility testing results, a standard reference strain should be tested regularly in parallel with patient isolates. The positive control strain *H. influenzae* ATCC 49247 should be used when performing antimicrobial sensitivity tests for most antimicrobial agents (such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole). Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits (described in Appendix 1). If zones produced by the control strain lie outside of the expected ranges, the technician should evaluate the following factors, which can affect antimicrobial susceptibility tests:

- Variations in media, inoculum size, incubation time, temperature, or other environmental factors, such as CO_2 levels;
- Whether the depth of the agar in the plate varies from a uniform 3-4 mm, required to allow consistent diffusion of the antimicrobial agents;
- If the pH of the test medium lies outside of the optimal range (7.2-7.4);
- If the inoculum is not a pure culture, or does not contain a concentration of bacteria approximately equivalent to the 0.5 McFarland turbidity standard.

The reference strain should be included with every group of test until the laboratory has demonstrated 30 consecutive days of in-control results. QC testing with the positive control strain can then be performed weekly, or each time the laboratory uses a new batch of testing medium or lot of disks. If testing is done less frequently than every day, the reference strain should be included with every group of tests.

6.9 Shipping clinically significant isolates for further testing

Any clinically significant isolates obtained through patient hemocultures should be shipped to the IEIP laboratory in Nonthaburi (as described in Section 4.3), which will forward them to the Thai NIH for long-term storage at -70ºC and/or further testing (see flow chart below).

Careful disinfection of the patient's skin, the nurse or laboratory technician's gloves, and the surface of the blood culture bottles prior to inoculation and incubation of the blood culture bottles can reduce the numbers of contaminated blood cultures. Despite these precautions, some proportion of inoculated blood cultures will yield positive growth of bacteria commonly found on the skin or in the environment and rarely (if ever) associated with serious diseases.

No absolutely conclusive method exists to differentiate a contaminant from a true bacteremia, although comparing multiple blood cultures from a single patient (a technique employed in the U.S., but not recommended by this protocol) can help. Even organisms known as common skin contaminants can cause disease in severely immunocompromised patients, particularly in those with indwelling central venous catheters. **The final conclusion on whether an isolate represents a clinically significant pathogen or a culture contaminant must be made by the clinician who ordered the blood culture, on the basis of the laboratory and clinical information available.**

However, the laboratory microbiologist can use some existing tools to determine the likelihood that a microorganism represents a true bacteremia rather than a blood culture contaminant:[25],[26],[27]

1. The isolation of virulent human pathogens such as *S. pneumoniae*, *Staphylococcus aureus,* Klebsiella species, Pseudomonas species, *E. coli* and other Enterobacteriacae species, *H. influenzae*, or *N. meningitides* from blood culture generally indicates true bacteremia.

2. In contrast, the following suggest the possibility of contaminated cultures:
    a. Prolonged incubation before the culture becomes alarm-positive;
    b. Multiple organisms from a single culture (unless the patient is severely immunocompromised);
    c. The organism isolated appears inconsistent with the patient's clinical condition;
    d. Growth yields bacteria that frequently reside on the skin (such as *Corynebacterium* species, *Bacillus* species, or *Propionibacterium acnes*) or in the local environment (such as Brevibacterium species in an area with many dairy farms) and rarely cause human disease. Although coagulase-negative staphylococci usually represent contaminants, up to 15% of isolates may be true bacteremias; this is more likely in patients with indwelling catheters or other implanted devices.

The laboratory microbiologist will have to decide whether isolates are as likely as not to represent clinically significant isolates when shipping these to IEIP for preservation and possible further testing at the Thai NIH. All isolates of *S. pneumoniae* should be assumed to be clinically significant..

<u>Preparing isolates for shipping</u>

Because of the particular focus on *S. pneumoniae* and, to a lesser extent, *H. influenzae* isolates, Dorset Egg media slants have been selected as a standard storage and transport medium to increase the chances of preserving these two fastidious organisms. Dorset Egg medium slants will be prepared by the Thai NIH and supplied by IEIP to the provincial laboratories monthly.

1) Using a sterile inoculating wire loop, touch a colony from a freshly prepared overnight culture of the isolate (as a pure culture grown on blood agar at 35ºC with 5% CO_2).

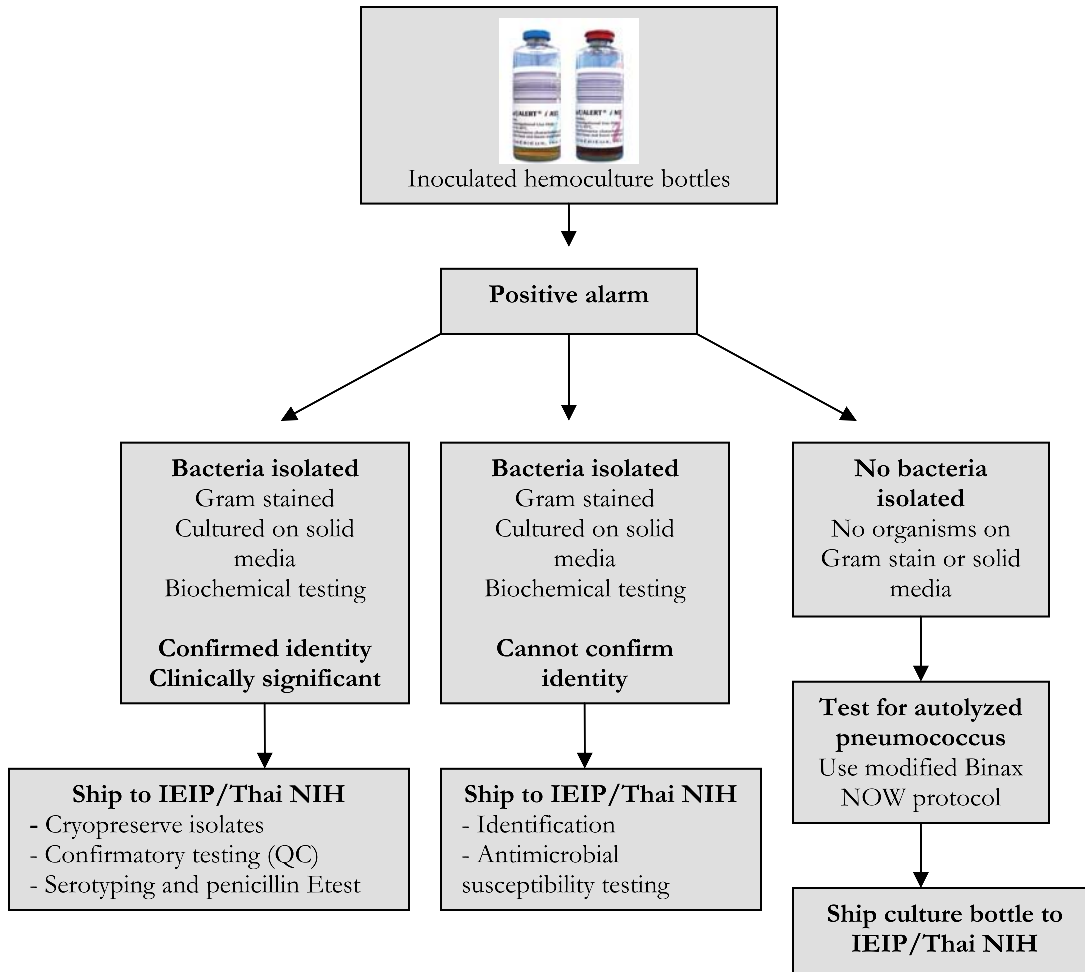
2) Inoculate a 4 mL Dorset Egg media slant by streaking the isolate onto the surface and store at room temperature (18-25ºC).

3) Repeat with a second colony from the same plate and a second slant.

4) With a sterile inoculating wire, remove another colony from the freshly prepared overnight culture and plunge the wire into the center of the prepared nutrient agar tube.

5) Repeat with another colony and a second nutrient agar tube.

6) Ship all isolates to IEIP every Tuesday at room temperature with the provided shipping list.



Appendix 1:
Quality Control and Quality Assessment

A stringent QC/QA program relies upon consistent inclusion of appropriate reference strains in standard microbiology testing; systematic monitoring of environmental conditions and equipment; careful record-keeping; and regular training and refinement of laboratory protocols. Throughout these Standard Operating Procedures, specific actions to establish the quality of media, reagents, conditions, and testing procedures have been described. The list below provides a brief overview of critical components of general QC testing.

In order to demonstrate that blood culture process implemented in the provinces of Nakhon Phanom and Sa Kaeo can be trusted by clinicians and patients, IEIP will support the provincial hospital microbiology laboratories in establishing a rigorous QC/QA system.

- The IEIP laboratory microbiologist will oversee the routine performance of daily and weekly internal QC procedures;
- IEIP staff will visit each provincial microbiology laboratory monthly to review the records documenting internal QC procedures, to discuss any modifications necessary to improve protocols, and to arrange regular training sessions as indicated;
- IEIP staff will also conduct a quarterly external QA audit of each provincial microbiology laboratory, including a review of record-keeping and data entry, and introduction of blinded test samples into the blood culture system in the form of spiked control bottles. Several national and international external quality assessment schemes will be considered as an additional layer of monitoring; the laboratories at Crown Prince Hospital in Sa Kaeo have already contracted with a Thai MOPH-sponsored QA auditing program, and plan to use this program as an external check for at least the first year of the project.

The development of criteria for success will be an ongoing collaboration between the laboratory personnel on-site in the district and provincial hospitals, and those at IEIP's Bangkok facilities. Nonetheless, it is expected that laboratories will show a consistent trend toward improvement in conducting internal QC testing and meeting external QA challenges.

Expected accomplishments in QC/QA testing

Action/Milestone	Year 1	Year 2	Year 3
Documentation of daily and weekly QC monitoring, including: • environmental conditions • equipment function	90% complete	95% complete	99% complete
Documentation of all steps in specimen processing, including: • transport temperatures • specimen volume • specimen rejection forms	90% complete	95% complete	100% complete
Documentation of inclusion of standard reference strains as appropriate (weekly, daily, or with each test) for all: • inoculation of solid media • antimicrobial susceptibility • biochemical tests • biological tests	85% complete	90% complete	95% complete
Accuracy and completeness of record-keeping and information-sharing, including: • Completion of Specimen Collection Forms in real time, with related forms attached • Prompt placement of all forms in patient charts • Prompt notification of clinicians (as recorded and through clinician feedback) • Completion of bound logbooks in real time	90% complete	95% complete	99% complete

Maintenance of the GAVI Microbiology Database (as determined by quarterly audit): • Accuracy (correspondence of Specimen Collection Forms, laboratory logbooks, blood culture system Observa software, and data entered through Access) • Completeness • Timeliness (completed records entered the first time within one week of recording results in lab, and entered the second time within two weeks after receipt at IEIP offices)	90%	95%	99%
Successful identification and characterization of antimicrobial susceptibility of a panel of 5-10 unknown organisms, introduced as blinded specimens as part of an quarterly external QA review	80%	85%	95%

I.i Recording information on routine internal QC testing

All QC testing should be documented by recording the results, data, and time in the appropriate format. For daily evaluation of equipment and environmental conditions, record sheets (based on the templates provided by Boonchuay Eampokalap, Chief of Microbiology, Bamrasnaradura Institute of Bamrasnaradura Hospital, Thai Ministry of Public Health) should be kept with the instrument or in a nearby, easily accessible location until complete, and then collected in a binder. Personnel should be assigned to complete records on equipment and environmental conditions on a regular basis. Malfunctioning equipment should be reported first to the IEIP microbiologist in each laboratory, with arrangements for repairs referred through the provincial Surveillance and Research Coordinator's office. The IEIP microbiologists will also be responsible for establishing a plan for regular maintenance (most frequently annual) for all equipment, and making logistical arrangements through the Surveillance and Research Coordinator's office.

For results obtained using standard reference strains in evaluating media and growth conditions, antimicrobial susceptibility testing, and biochemical testing, results should be recorded in a bound laboratory logbook, including the date and time. Instructions on performing appropriate QC testing should be kept in the front of each logbook for easy reference.

Equipment/test/condition	Action	Performed
Equipment		
BacT/ALERT 3D automated blood culture system All incubators Refrigerators and freezers	Evaluate whether temperatures are below, within, or above range (using internal thermometer) – calibrate or request technical assistance if necessary. Move samples to back-up instrument if possible until problem is solved.	Daily (2-3 times)
CO_2 incubators	Check CO_2 levels in incubator and pressure remaining within tank – adjust or request technical assistance if necessary. Have candle-jars on hand as back-up if only one CO_2 incubator is available.	Daily (2-3 times)
Power supply	Note all fluctuations and which samples/equipment might have been affected.	As necessary
Autoclaves	Sterilization testing (tape indicator).	With each use
Autoclaves	Biological testing (spore test)	Weekly
Biosafety cabinet	Check air-flow and gauges for air pressure	Daily (2-3 times)
Conditions		
Room temperature	Check to see if temperature is below 15ºC or exceeds 30ºC; this could affect the function of the blood culture machine and incubators. Take action to decrease temperatures (closing blinds, increasing air-conditioning) if necessary. Do not turn off air-conditioning at night.	Daily (2-3 times)
Temperature during shipping of inoculated blood cultures	Check TempTale device in each insulated box upon receipt of specimens, rejecting any specimens that have been too long out of temperature range (18-30ºC – see Section 4).	With each driver's shipment

Test		
Culturing isolates on solid media	Streak appropriate standard reference strain for each media plate.	Weekly, and with each new batch of media
Antimicrobial susceptibility testing	Test a susceptible standard reference strain for the isolate using the same media and antibiotic disks (for example, an *S. pneumoniae* isolate should be compared to an *S. pneumoniae* standard reference strain).	Every day up to 30 consecutive days of in-range results, then weekly and with each new batch of media or disks
Biochemical tests	Include an appropriate standard reference strain for the test (such as a bile soluble *S. pneumoniae* standard reference strain for the bile solubility test).	With every test
Gram and AFB staining	Include one slide with an appropriate positive and negative standard control strain for each type of staining.	Weekly, and with each new staining kit

I.ii Selecting appropriate standard reference strains

For each batch of media, biological, or biochemical assay, standard reference strains should be tested in parallel with strains isolated from patient blood cultures, and the results compared to published observations. Failure of the standard reference strains to perform as expected should prompt a review of the techniques used, the environmental conditions, equipment function, and media or reagent quality until the problem can be identified and corrected.

Organism	Assay	Characteristic
Streptococcus pneumoniae ATCC 49619	Growth on blood agar Antimicrobial susceptibility testing Optochin test Bile solubility test	Growth positive (α-hemolysis) NCCLS standard reference strain Optochin sensitive Bile soluble
Streptococcus pyogenes ATCC 19615	Growth on blood agar Catalase test	Growth positive (β-hemolysis) Catalase negative
Streptococcus bovis (gallolyticus) ATCC 49147	Optochin test Bile solubility test	Optochin resistant Non-bile soluble
Staphylococcus aureus ATCC 25923	Growth on blood agar Coagulase test Catalase test Gram staining Antimicrobial susceptibility testing	Growth positive (γ-hemolysis) Coagulase positive Catalase positive Gram-positive NCCLS standard reference strain
Staphylococcus epidermidis ATCC 12228	Coagulase test	Coagulase negative
Haemophilus influenzae ATCC 49247	Growth on chocolate agar X and V Factor requirements Antimicrobial susceptibility testing	Growth positive X and V Factor dependent NCCLS standard reference strain
Haemophilus influenzae ATCC 49766	Antimicrobial susceptibility testing	Sensitive strain for some antibiotics
Neisseria meningitidis ATCC 13077	Growth on chocolate agar Oxidase test Carbohydrate utilization test	Growth positive Oxidase positive Glucose +/maltose +

Organism	Assay	Characteristic
E. coli ATCC 25922	Growth on MacConkey agar	Growth positive (pink colonies)
	Growth on blood agar	Growth positive (no hemolysis)
	Triple Sugar Iron Agar (TSI)	Acid butt/acid slant/gas +/H_2S +
	Oxidase test	Oxidase negative
	Gram staining	Gram negative
	Urease agar test	Urease negative
	Indole spot test	Indole positive
	Growth on citrate agar	Growth negative
	Lysine iron agar	Acid butt/alkaline slant/H_2S -
	Antimicrobial susceptibility testing	NCCLS standard reference strain
Pseudomonas aeruginosa ATCC 27853	Oxidase test	Oxidase positive
	Indole spot test	Indole negative
	Antimicrobial susceptibility testing	NCCLS standard reference strain
Salmonella typhimurium ATCC 14028	Growth on MacConkey agar	Growth positive (colorless colonies)
	Growth on citrate agar	Growth positive
	Lysine iron agar	Alkaline butt/alkaline slant/H_2S +
	Triple Sugar Iron Agar	Acid butt/alkaline slant/gas +/H_2S +
Klebsiella pneumoniae ATCC 35657	Urease agar test	Urease positive

I.iii Preserving reference strains for routine laboratory use[28]

While many methods exist for preserving reference strains for long periods of time, the bead stock method has many advantages for laboratories with limited resources and freezer space. Bacteria "stick" to the charged beads, allowing retrieval of and quick inoculation of solid media with the preserved strains. Isolates stored at -70°C or below may be stored indefinitely without losing viability; storage at -20°C is not recommended. IEIP will supply glycerol for the freezing medium, cryovials, and beads, as well as the standard reference strains.

<u>Freezing reference strains as bead stocks</u>

1) Stocks should be prepared in biological safety cabinet.
2) Inoculate a non-selective solid media slant (such as tryptone-based soy agar, TSB, or brain-heart infusion agar, BHIB) and incubate overnight (18-24 hours) at 35°C.
3) Harvest the freshly grown bacteria from the surface of the slant with a sterile bacteriological loop and suspend in freezing medium (TSB or BHIB plus 15% glycerol).
4) Label cryovials with the name of the reference strain and the date, and add 20-30 beads to each cryovial.
5) Dispense the bacterial suspension into the cryovials, making sure that the beads are fully submerged in the suspension.
6) Carefully remove the excess suspension, pouring away as much of the suspension as practical without spilling the beads. Seal the cryovial with the included cap.
7) Place dry ice in a leakproof metal container large enough to hold a metal culture rack, and add enough ethyl alcohol to submerge about half of the cryovial. If dry ice cannot be obtained, place a container of the alcohol in the -70°C freezer overnight.
8) Freeze the suspension rapidly by placing the sealed vials in the dry ice bath. Transfer the vials to a -70°C freezer.

<u>Recovery of cultures from frozen storage</u>

1) Place frozen cultures on dry ice or into an alcohol and dry ice bath and transfer to a biological safety cabinet.
2) Using a sterile loop or forceps, remove a bead from the cryovial, being careful not to contaminate the top or inside of the vial. Roll gently on an appropriate solid media plate to inoculate, then streak to isolation.
3) Reseal and return vial to freezer before the contents completely thaw. By maintaining the temperature of the bead stock and using aseptic technique, transfers can be made from the same vial multiple times.

<u>Working reference strain stocks</u>

1) Select a single isolated colony from the newly prepared solid media plate (above).

2) Prepare a working culture by inoculating a non-selective solid media slant (such as TSB) and incubate overnight (18-24 hours) at 35°C. (Use a chocolate agar slant for *H. influenzae* and *Neisseria* strains.)

3) Store the working culture slant at 4°C. Subculture QC testing cultures from the working culture slants prior to schedule testing (i.e., daily or weekly).

4) Discard the working culture slants after one month and replace from reference strain stocks stored at -70°C.

I.iv Antimicrobial susceptibility test breakpoints and quality control ranges

For the expected results and testing ranges for antimicrobial susceptibility testing, please consult the most recent version of the Clinical and Laboratory Standards Institute (formerly NCCLS) "Performance Standards for Antimicrobial Susceptibility Testing" and the *Manual for Identification and Antimicrobial Susceptibility Testing*. Both will be supplied to the provincial hospital microbiology laboratories by IEIP.

Appendix 2:
Testing Patient Serum for Pre-hospital Antibiotic Use

Patient use of antibiotics can affect the sensitivity of any blood culture system. The impact of pre-hospital antibiotic use by patients in rural Thai hospitals (obtained through private clinics or by over-the-counter purchases) is difficult to predict. Therefore, disks soaked in serum will be prepared for each patient receiving a blood culture as ordered by a Thai clinician, and cryopreserved until a convenient time for later testing. A strain of *Staphylococcus* highly susceptible to many commonly used antibiotics will be used to determine qualitatively whether the serum contained antibiotics at the time of the blood draw. The following method will be used to test patient sera for pre-hospital antibiotic use:

1) Suspend viable colonies from a fresh pure culture of *Staphylococcus aureus* ATCC 9144 (NCTC 6571) grown overnight on nutrient agar in TSB or sterile physiological saline.

2) Adjust the cell suspension to the density of a 0.5 McFarland turbidity standard (described in Appendix 3), carefully avoiding froth or bubbles while mixing. Compare the density of the suspension to the 0.5 McFarland turbidity standard by holding the suspension and the McFarland standard in front of a light against a white background with black lines. Add more saline or broth if the density appears too heavy, and more bacteria if the density appears too light.

3) Dip a sterile cotton swab into the bacteria suspension, removing excess fluid by rotating against the sides of the dilution tube.

4) Use the same swab (without re-dipping in the bacterial cell suspension) to inoculate the entire surface of a nutrient agar plate three times, rotating the plate 60 degrees between each inoculation to achieve even coverage.

5) Allow the inoculum to dry before placing disks on the plates (5-15 minutes).

6) Remove the patient serum disks from the -20ºC or -70ºC freezer and allow to warm to room temperature, thawing only the disks to be used that day. Label each plate appropriately. Disks from 3-4 patients can be placed on each plate as long as sufficient space is allowed and they are labeled clearly.

7) Place each patient serum disk on a plate using sterile forceps, tapping it gently to ensure that it adheres to the agar. Once a disk contacts the agar surface, diffusion of any drugs may begin immediately, and the disk should not be moved.

8) Prepare one plate using commercially available antibiotic susceptibility testing disks. Include one untreated paper filter disk soaked only in sterile saline as a negative control.

9) Incubate the plates in an inverted position (upsidedown) in a 35°C incubator overnight (18-24 hours).

10) Examine each patient serum disk for a zone of inhibition indicating antimicrobial activity. Measure the diameter of the zone of inhibition with a rule or calipers, measuring the distance from the last visible colony on each side of the disk from the top surface of the plate with the lid removed. Care should be taken not to touch the disk or surface of the agar with the ruler or calipers.

11) Confirm that results for the positive (commercial antibiotic) and negative disks lie within acceptable standards.

12) Record the diameter of the zone of inhibition around each patient serum disk on the original Specimen Collection Forms. The running number from the cryotube should be used to record results so that these can be tied to the blood culture results. Because the antibiotic used may not be known (precluding comparison to a control), the presence of a zone of inhibition will be classified as suggestive of, not suggestive of, or inconclusive for pre-hospital antibiotic used.

Appendix 3: Preparing Media and Reagents

IEIP will provide all solid media for confirmatory identification and testing, prepared by either a commercial manufacturer (CLINAG, Bangkok, Thailand) or by the Thai National Institutes of Health (Nonthaburi, Thailand). However, laboratory technicians must still include appropriate standard reference strains to test media quality every time a new batch of media is used. Standard protocols for preparing commonly used microbiological media are also included below for reference and for use by the provincial laboratories in preparing supplemental media, or media for uses other than the IEIP Microbiology Project.

III.i Considerations for quality control of media

Each batch of medium should be tested for the ability to support growth of the target organisms as appropriate, in the laboratory conditions within the provincial microbiology laboratories. An appropriate standard reference strain (listed in Appendix 1, and described below specifically for each type of common media) should be inoculated onto each type of media plate one per week, and every time a new batch of medium is used.

For selective media, use at least one organism that will grow on the medium and one that will not grow to test the medium's ability to differentiate between the two. If the medium is also differential, include two organisms that will grow on the medium and produce different reactions.

Methods for quality control of media

1) Inoculate the control strain to non-selective broth (such as tryptone-based soy broth, or TSB) and incubate overnight at 35ºC.

2) Prepare a standardized inoculum for testing the medium:

   a. If testing selective or inhibitory media, dilute the overnight broth culture 1:10 with sterile broth;
   b. If testing nonselective media, dilute the overnight broth culture 1:100 with sterile broth.

3) Inoculate one plate (or tube) of each medium to be tested with 100 µL of the diluted control strain, and streak to isolation.

4) Incubate at 35ºC overnight (18-24 hours) under appropriate CO_2 conditions for the media and organism.

III.ii Preparation and QC testing of commonly used microbiological media

Routine Agar and Broth Media

All agar media can be dispensed into 15x100-mm Petri dishes (15 to 20 ml per dish). After pouring, leave the plates at room temperature for several hours to prevent excess condensation from forming on the covers of the dishes. Store the plates in a secured plastic bag that does not allow movement of plates in an inverted position at 4°C – a re-sealable bag is not an appropriate container, as plates can slide and become uncovered in storage.

Heart Infusion Agar (HIA) and Trypticase Soy Agar (TSA)

These media are general purpose media used with or without blood for isolating and cultivating a number of microorganisms. The media should appear straw colored (a yellowish to gold colouring). HIA and TSA are also used for determining the X- and V-factor requirements of *H. influenzae*. Each freshly prepared or purchased batch of HIA or TSA should be quality control-tested by determining the X and V requirements of *H. influenzae*. A fresh plate is inoculated with a control strain; X, V, and XV disks should be placed on the inoculated plate. *H. influenzae* should grow only around the XV disk.

1) Prepare the volume of HIA or TSA according to the instructions on the label of the dehydrated medium in flasks. Media should be fully dissolved with no powder on the walls of the vessel before autoclaving.

2) Autoclave at 121°C for 20 minutes.

3) Cool to 50°C and pour into 15x100-mm Petri dishes. Allow to solidify and lids to dry before storing in plastic bags at 4°C until used.

Blood Agar Plate (BAP): TSA + 5% Sheep Blood

BAP is used as a general blood agar medium (sheep blood agar plate) for most testing purposes. It is also used for the optochin test and subcultures of *S. pneumoniae.* (Human blood agar should never be used for the isolation or testing of cultured clinical specimens such as *S. pneumoniae* as part of the blood culture process, as natural and pharmaceutical antibiotic factors in human blood can affect the growth of isolates. Only high-quality animal blood should be used; IEIP can provide further guidance on availability as necessary.) The plates should appear a bright red color. A dark red appearance indicates that the blood was added when the agar was too hot. If this happens, discard the medium and prepare another batch. Test each new, freshly prepared or purchased batch of BAP for growth and haemolytic reaction with a strain of *S. pneumoniae*. The colonies are small and should appear grey to grey-green surrounded by a distinct greenish halo in the agar.

1) Prepare TSA according to the instructions given on the label of the dehydrated powder, adding 20 g of agar to 500 mL water in a l-litre flask. Heat to dissolve.

2) Autoclave at 121°C for 20 minutes. Cool to 50°C.

3) Add 5% sterile, defibrinated sheep blood (25 ml sheep blood per 500 ml agar – adjust accordingly for the total volume).

4) Dispense 20 ml into 15x100-mm Petri dishes. Allow to solidify and dry, then store in a plastic bag at 4°C.

Heart Infusion Rabbit Blood Agar Plate (HIA - Rabbit Blood)

HIA-rabbit blood is used for determining the hemolytic reaction of *Haemophilus* species. This medium should appear bright red and look very similar to BAP. If the medium is dark red, discard and prepare a new batch. Horse blood may be substituted for rabbit blood in this medium. A strain of *H. haemolyticus* should be used to quality control the proper growth and haemolytic reactions of the medium. *H. haemolyticus* should grow well and be surrounded by a distinct zone of complete haemolysis which appears as a clear halo surrounding the colonies.

1) Prepare the HIA according to the instructions on the label of the dehydrated medium.

2) Autoclave at 121°C for 20 minutes. Cool to 50°C in a water bath.

3) Add 5% sterile, defibrinated rabbit blood (5 ml/100 ml of medium) and dispense into 15x100-mm Petri dishes. Allow to solidify and dry for a few hours before storing in a plastic bag at 4°C.

Trypticase Soy Broth (TSB)

TSB is a non-selective broth used for making suspensions of *H. influenzae* prior to testing for X and V- factor requirements. Heart infusion broth, sterile saline or phosphate-buffered saline may be substituted for TSB. *H. influenzae* does not grow in TSB but the medium should not be toxic to other bacteria. Therefore, *S. pneumoniae* should be used to quality control for toxicity. Inoculate a tube of medium with a loop of freshly growing strain of *S. pneumoniae*; incubate overnight at 35°C. The broth should be turbid the next day. Subculture the broth to test for proper growth characteristics of *S. pneumoniae* (use a BAP).

1) Prepare the TSB according to the instructions on the label of the dehydrated medium.

2) Dispense 5 ml into 15x125-mm tubes, autoclave at 121°C for 20 minutes, cool, and store at 4 °C.

Chocolate Agar Plate (CAP) with TSA and Growth Supplement

CAP with growth supplements is a medium that supports the special growth requirements needed for the isolation of fastidious organisms, such as *H. influenzae* and *N. meningitidis*, when incubated in a 5% CO_2 atmosphere. CAP has a reduced concentration of agar, which increases the moisture content of the medium. Supplemented chocolate agar should support the growth of *N. meningitidis, H. influenzae,* and *S. pneumoniae.* Chocolate agar slants for transport and short-term storage can be

prepared in the same manner as that described for agar plates, except that the medium is dispensed in 16x125 mm screw-cap tubes and slanted before solidifying. All freshly prepared or purchased chocolate agar media should be tested with the three species of bacteria to determine the medium's capacity to support their growth.

Chocolate agar slants should look brown to brownish-red in colour. *N. meningitidis* and *H. influenzae* should appear as a greyish, almost translucent film on the slant's surface with no discoloration of the medium after 24 hours of incubation. *S. pneumoniae* should appear as small grey to grey-green colonies with a very distinct greenish discoloring of the medium. If the medium does not support the growth of one or all of the bacteria, discard and prepare or purchase a fresh batch. If *H. influenzae* does not grow, the IsoVitaleX or its equivalent may have been inadvertently omitted.

1) Using TSA as the basal medium. Prepare double strength (20 g in 250 ml distilled water).

2) Autoclave, and cool to 50°C. Use the thermometer to verify the cooling temperature.

3) Hemoglobin solution: Prepare a solution of 2% hemoglobin (5 g in 250 ml distilled water). Mix the hemoglobin in 5-6 ml distilled water to form a smooth paste. Continue mixing as the rest of the water is added. Autoclave, and cool to 50°C.

4) Add the hemoglobin solution to the double-strength TSA and continue to hold at 50°C.

5) If a hemoglobin solution is unavailable, an alternative is to add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood per 100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water). DO NOT use human blood. After the base medium has been autoclaved and cooled to 50°C, add the blood and place in a hot water bath at no more than 80°C for 15 minutes or until a chocolate color is achieved. Then cool to 50°C.

6) After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50°C, add growth supplement (such as IsoVitaleX or Vitox) to a final concentration of 1%. Mix the ingredients by gently swirling the flask; avoid forming bubbles.

7) Dispense 15 to 20 ml in each 15x100 mm Petri dish.

<u>Cystine Trypticase Agar (CTA) with 1% Carbohydrate</u>

1) Suspend 28 g of CTA medium or 29.5 g in 900 ml of distilled water. Mix thoroughly, heat with frequent agitation, and bring to a boil for 1 minute to completely dissolve the powder.

2) Autoclave the flask at 121°C for 15 minutes. Cool to 50°C

3) Prepare 10% glucose solution using 10 g glucose in 100 ml of distilled water. Filter sterilize using a 0.22 micron filter.

4) Aseptically add this entire solution (100 ml of 10% glucose solution) to the 900 ml of the CTA medium to obtain a final 1% concentration of the glucose.

5) Dispense 7 ml in 16 x 125 mm glass tubes with screw caps and store at 4°C.

6) Repeat this procedure for the remaining 3 carbohydrates: maltose, lactose and sucrose.

III.iii Preparation of commonly used microbiological reagents

Reagents for Gram Stain (Hucker Modification)

Ammonium oxalate-crystal violet:

1) Solution a: Dissolve 2.0 g crystal violet (certified) in 20 mL 95% ethyl alcohol.

2) Solution b: Dissolve 0.8 g ammonium oxalate in 80 mL distilled water 80.0 ml.

3) Mix solutions a. and b. Let stand overnight. Filter through coarse filter paper before use.

Gram's iodine (protect solution from light)

Dissolve 1 g iodine (crystalline) and 2 g potassium in 300 mL distilled water. Grinding the dry chemicals in a mortar with small additions of distilled water may be helpful in preparing the solution.

Decolorizer

95% ethyl alcohol

Counterstain (safranin)

1) Stock solution: dissolve 2.5 g safranin-O (certified) in 100 mL 95% ethyl alcohol.

2) Working solution: dilute 10 mL safranin stock solution in 90 mL distilled water.

McFarland Turbidity Standards

Commercially prepared 0.5 MacFarland turbidity standards are available from various manufacturers. Alternatively, the 0.5 MacFarland turbidity standard can be prepared by:

1) Add 0.5 mL of a 1.175% (weight/volume, or wt/vol) barium chloride dihydate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 mL of 1% (vol/vol) sulfuric acide (H_2S0_4).

2) Aliquot the turbidity standard into test tubes identical to those that will be used to prepare bacterial suspensions.

3) Mark the level of the liquid, and then seal the McFarland turbidity standard tubes with wax, Parafilm, or other seal that can prevent evaporation.

4) Check the accuracy of the density with a spectrophotometer with a 1-cm light path if possible. For the 0.5 McFarland turbidity standard, the absorbance at a wavelength of 625 nm should be 0.08-0.1. Alternatively, the accuracy of the McFarland turbidity standard can be verified by adjusting a suspension of a control strain (such as *E. coli* ATCC 25922) to the same turbidity, preparing serial 10-fold dilutions, and then performing plate counts of colonies. The adjusted suspension should give a count of 108 colony-forming units/mL.

5) Sealed McFarland turbidity standards may be stored for up to 6 months in the dark at room temperature (22-25°C). Discard after 6 months, or sooner if the level of liquid in the tube drops below the marked level.

6) Before each use, shake the tube containing the turbidity standard well to mix the fine white precipitate thoroughly.

QC Record: BLOOD AGAR PLATES **Test: with EACH NEW BATCH**

Range: Growth/No Growth and Hemolysis

	Plates				**Reference strain growth/hemolysis**							**Results**	**Performed by**	**Comments**
					S. pneumoniae ATCC 49619 (1 plate)		*S. pyogenes* ATCC 19615 (1 plate)		*E. coli* ATCC 25922 (1 plate)		None (sterility)*			
Date	Lot number	Date opened	Expiration date	Supplier	*Growth*	α-*hemolysis*	*Growth*	β-*hemolysis*	*Growth*	γ-*hemolysis*	*Contaminated/ Total plates*	In expected range (Y/N)?	Name	
21/8/05	*A-101*	*15/8*	*31/10*	*MediaCo*	v	v	v	v	v	v	*0/5*	*Y*	*Example*	
28/8/05	*A-101*	*15/8*	*31/10*	*MediaCo*	-	-	v	v	v	v	*3/5*	*N*	*Example*	

Reviewed by: **Date:**

*Incubate plates at 35-37?C for 48 hours. If colonies appear, plate is contaminated and sterility testing must be repeated. If more than 1% of plates in batch grow colonies, reject media.

Number of plates tested (number of plates in batch): 3(=25); 5(26-90); 8(91-280); 13(281-500); 20(501-1200).

WEEKLY QC tests should be repeated for each new batch. Please report out-of-range results to supervisor immediately.

QC Record: CHOCOLATE AGAR PLATES **Test: with EACH NEW BATCH**

Range: Growth/No Growth

	Plates				**Reference strain growth**			**Results**	**Done by**	**Comments**
Date	Lot number	Date opened	Expiration date	Supplier	*H. influenzae* ATCC 49247 Growth	*N. meningitidis* ATCC 13077 Growth	*None (sterility) Contaminated/ total plates*	In expected range (Y/N)?	Name	
21/8/05	*A-101*	*15/8*	*31/10*	*MediaCo*	v	v	*0/5*	*Yes*	*Example*	
Reviewed by:							**Date:**			

*Incubate plates at 35-37?C for 48 hours. If colonies appear, plate is contaminated and sterility testing must be repeated. If more than 1% of plates in batch grow colonies, reject media. Number of plates tested (number of plates in batch): 3(=25); 5(26-90); 8(91-280); 13(281-500); 20(501-1200).

WEEKLY QC tests should be repeated for each new batch. Please report out-of-range results to supervisor immediately.

QC Record: **MACCONKEY AGAR PLATES** **Test: with EACH NEW BATCH**

Range: Growth/No Growth plus Colony Color

	Plates				**Reference strain growth**					**Results**	**Done by**	**Comments**
					E. coli ATCC 25922		*S. typhimurium* ATCC 14028		*None (sterility)*			
Date	Lot number	Date opened	Expiration date	Supplier	Growth	Pink Color	Growth	No color	*Contaminated/ total plates*	In expected range (Y/N)?	Name	
21/8/05	*A-101*	*15/8*	*31/10*	*MediaCo*	v	v	v	*v*	*0/5*	*Yes*	*Example*	

Reviewed by: **Date:**

*Incubate plates at 35-37?C for 48 hours. If colonies appear, plate is contaminated and sterility testing must be repeated. If more than 1% of plates in batch grow colonies, reject media. Number of plates tested (number of plates in batch): 3(=25); 5(26-90); 8(91-280); 13(281-500); 20(501-1200).

WEEKLY QC tests should be repeated for each new batch. Please report out-of-range results to supervisor immediately.

QC Record: **GRAM STAIN**

Range: Positive/Negative plus morphology (cocci, bacilli, other)

Test: WEEKLY and with new stains

Positive = Purple/Negative = Pink

	Reagents				**Reference strain reaction**		**Results**	**Done by**	**Comments**
					Positive	**Negative**			
Date	Gram stain kit (lot number):	Date opened	Expiration date:	Stains last filtered (date):	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	In expected range (Y/N)?	Name	
21/8/05	*A-101*	*15/6/05*	*31/9/06*	*15/8/05*	*Purple cocci*	*Pink bacilli*	*Yes*	*Example*	
Reviewed by:								**Date:**	

WEEKLY QC tests should be repeated for new reagents. Please report out-of-range results to supervisor immediately.

QC Record: **CATALASE TEST**

Range: Positive/Negative

Test: with every set of specimens

Positive = Bubbling/Negative = No bubbling

Read: within 15 seconds

	Reagents			**Reference strain reaction**		**Results**	**Performed by**	**Comments**
				Positive	**Negative**			
Date	Lot number	Prepared on (date)	Prepared by	*S. aureus* ATCC 25923	*S. pyogenes* ATCC 19615	In expected range (Y/N)?	Name	
25/8/05	*A101*		*K. S*	*Bubbles*	*No bubbles*	*Yes*	*Example*	
Reviewed by:						**Date:**		

Please report out-of-range results to supervisor immediately.

QC Record: OXIDASE TEST (Kovac's method)

Range: Positive/Negative

Test: with every set of specimens

Positive = Purple/Negative = No color

Read: within 10 seconds

	Reagents			Reference strain reaction		Results	Performed by	Comments
				Positive	**Negative**			
Date	Lot number	Date thawed	Expiration date	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922	In expected range (Y/N)?	Name	
21/8/05	*ABC101*	*15/8/05*	*31/3/06*	*Blue*	*No change*	*Y*	*Example*	
Reviewed by:						**Date:**		

Please report out-of-range results to supervisor immediately.

QC Record: COAGULASE TEST

Range: Positive/Negative

Test: with every set of specimens

Positive = clotting/Negative = No change

Read: at 4 hours and after 18 hours

	Reagents (rabbit plasma)			**Reference strain reaction**		**Results**	**Performed by**	**Comments**
				Positive	**Negative**			
Date	Lot number	Supplier	Date prepared	*S. aureus* ATCC 25923	*S. pyogenes* ATCC 19615	In expected range (Y/N)?	Name	
21/8/05	*ABC101*	*Innovative Res.*	*15/8/05*	*Clot*	*No clot*	*Y*	*Example*	
Reviewed by:						**Date:**		

Please report out-of-range results to supervisor immediately.

References

[1] Bartlett JG, Dowell SF, Mandell LA, File TA Jr., Musher DM, Fine MJ. Guidelines from the Infectious Diseases Society of America: Practice guidelines for the management of community-acquired pneumonia in adults. *Clin Inf Dis* 2000; 31:347-382.
[2] Mandell LA, Bartlett JG, Dowell SF, File TM Jr, Musher DM, Whitney C. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. *Clin Infect Dis* 2003;37(11):1405-33.
[3] File TM. Community acquired pneumonia. *Lancet*. 2003 Dec 13;362(9400):1991-2001.
[4] Management of the child with a serious infection or severe malnutrition : guidelines for care at the first-referral level in developing countries. World Health Organization, Department of Child and Adolescent Health and Development. WHO/FCH/CAH/00.1 (Geneva: 2000).
[5] Adapted from: Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae.* World Health Organization, Communicable Disease Surveillance and Response. WHO/CDS/CSR/EDC/99.7. (Geneva: 2000).
[6] Buttery JP. Blood cultures in newborns and children: optimizing an everyday test. *Arch Dis Child Fetal Neonatal Ed* 2002: 87:F25-F28.
[7] Weinstein MP. Blood culture contamination: persisting problems and partial progress. *J Clin Microbiol* 2003; 41(6)22-75-78.
[8] Isaacman DJ, Karasic RB, Reynolds EA, Kost SI. Effect of number of blood cultures and volume of blood on detection of bacteremia in children. *J Pediatr* 1996; 128:190-5.
[9] Mermel LA and Maki DG. Detection of bacteremia in adults: consequences of culturing an inadequate volume of blood. Ann Intern Med. 1993;119(4):270-2.
[10] Kellogg JA, Manzella JP, Bankert DA. Frequency of low-level bacteremia in children from birth to 15 years of age. *J Clin Microbiol* 2000;38(6):2181-85.
[11] Isaacman DJ, Karasic RB, Reynolds EA, Kost SI. Effect of number of blood cultures and volume of blood on detection of bacteremia in children. *J Pediatr* 1996; 128:190-5.
[12] Little JR, Murray PR, Traynor PS, Spitznagel E. A randomized trial of povidone-iodine compared with iodine tincture for venipuncture site disinfection: effects on rates of blood culture contamination. Am J Med. 1999 Aug;107(2):119-25.
[13] Calfee DP and Farr BM. Comparison of Four Antiseptic Preparations for Skin in the Prevention of Contamination of Percutaneously Drawn Blood Cultures: a Randomized Trial. *J Clin Microbiol*. 2002 May; 40(5): 1660–1665.
[14] Everts RJ, Vinson EN, Adholla PO, Reller LB. Contamination of catheter-drawn blood cultures. *J Clin Microbiol* 2001. 39:3393-94.
[15] Technical literature, "BacT/ALERT Blood Culture Collection," BioMérieux Inc. (2003).
[16] National Committee for Clinical Laboratory Standards. Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture. Approved Standard, H3-A5, Wayne, PA, 2003.
[17] Adapted from: Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae.* World Health Organization, Communicable Disease Surveillance and Response. WHO/CDS/CSR/EDC/99.7. (Geneva: 2000).
[18] Technical literature, BacT/ALERT 3D automated blood culturing machines, BioMérieux Inc..
[19] Personal communication, technical staff, BioMérieux Inc..
[20] Berkley JA, Loew BS, Mwangi I, Williams T, Bauni E, Mwarumba S, Ngetsa C, Slack MPE, Njenga S, Hart CA, Maitland K, English M, Marsh K, Scott JAG. Bacteremia among children admitted to a rural hospital in Kenya. *N Engl J Med* 2005;352:39-47.
[21] Adapted from *Manual for the Laboratory Identification and Antimicrobial Sensitivity Testing of Bacterial Pathogens of Public Health Importance in the Developing World.* World Health Organization, Centers for Disease Control and Prevention, and U.S. Agency for International Development. (Geneva: 2003) and Laboratory methods for the diagnosis of meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae.* World Health Organization, Communicable Disease Surveillance and Response. WHO/CDS/CSR/EDC/99.7. (Geneva: 2000).
[22] Adapted from *Guidelines for Standard Operating Procedures for Microbiology*. World Health Organization, Southeast Asia Regional Office. Available at http://w3.whosea.org/microbio/ackf.htm (Geneva: 2001).
[23] Petti CA, Woods CW, Reller LB. *Streptococcus penumoniae* antigen test using positive blood culture bottles as an alternative method to diagnose pneumococcal bacteremia. *J Clin Microbiol* 2005;43(5)2510-12.
[24] *Manual for the Laboratory Identification and Antimicrobial Sensitivity Testing of Bacterial Pathogens of Public Health Importance in the Developing World.* World Health Organization, Centers for Disease Control and Prevention, and U.S. Agency for International Development. (Geneva: 2003)
[25] Weinstein MP. Blood culture contamination: persisting problems and partial progress. *J Clin Microbiol* 2003; 41(6)22-75-78.

[26] Richter SS, Beekmann SE, Croco JL, Diekema DJ, Koontz FP, Pfaller MA, Doern GV. Minimizing the workup of blood culture contaminants: implementation and evaluation of a laboratory-based algorithm. *J Clin Microbiol 2002;* 40(7):2437-44.
[27] Shafazand S and Weinacker AB. Blood cultures in the critical care unit: improving utilization and yield. *Chest*2002;122: 1727-46.
[28] Kay BA. Cryopreservation of reference strains (protocol). U.S. Centers for Disease Control and Prevention, 1995; International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), 1986.