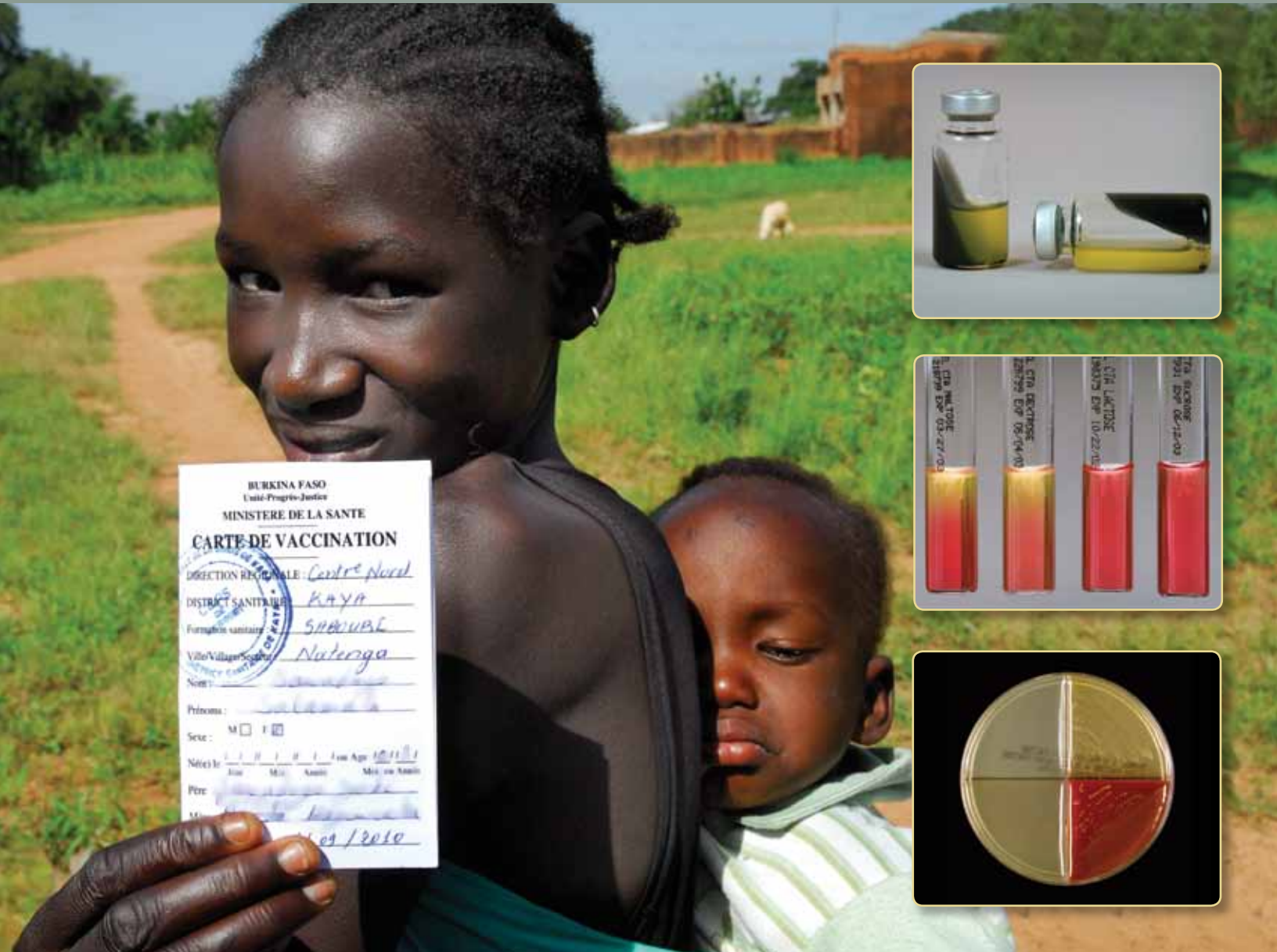


Laboratory Methods for the Diagnosis of Meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*

W H O M A N U A L , 2 N D E D I T I O N



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Laboratory Methods for the
Diagnosis of Meningitis caused by
Neisseria meningitidis, *Streptococcus pneumoniae*,
and *Haemophilus influenzae*

W H O M A N U A L , 2 N D E D I T I O N ¹



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¹ The first edition has the WHO reference WHO/CDS/CSR/EDC/99.7: Laboratory Methods for the Diagnosis of Meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, http://whqlibdoc.who.int/hq/1999/WHO_CDS_CSR_EDC_99.7.pdf

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Acknowledgements

The World Health Organization and the Centers for Disease Control and Prevention express their gratitude to those who have contributed their time and experience to this 2nd edition of the "Laboratory Methods for the Diagnosis of Meningitis caused by *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*".

Special thanks should be given to **Dr. Leonard Mayer**, The Centers for Disease Control and Prevention (CDC), WHO collaborating center for Meningitis, Atlanta, USA, for taking the lead in the process of developing as well as compiling and editing this Manual.

WHO manual – Foreword

Tanja Popovic, MD, PhD, Deputy Associate Director for Science, CDC

Microbiology and microbiologists have made extraordinary contributions to humanity saving hundreds of millions of lives over the past few centuries. These contributions span from developing and improving diagnostic assays and procedures to identify the causative agents, to discovering antimicrobial agents for treatment and manufacturing of vaccines for disease prevention. The first observations of living organisms were made in 1683 by Anthony von Leeuwenhoek. In 1876, almost 200 years later, Robert Koch provided the first proof linking a specific disease (anthrax) to a specific microorganism. From then on, a succession of subsequent discoveries followed, three of which are the focus of this manual: in 1884, pneumococcus by Albert Fraenkel; in 1887, meningococcus by Anton Weichselbaum; in 1892, *Haemophilus influenzae* by Johannes Pfeiffer.

Almost 15 years ago, when we started working on developing the 1st edition of this manual, there was an extraordinary amount of excitement about the work. We realized how important it was to provide microbiologists, worldwide, with guidance for performing simple and reliable procedures to isolate and characterize these microorganisms. Having this resource available, empowers countries to build and strengthen their own laboratory capacities and capabilities. The experience of having worked and providing trainings to many colleagues in Nigeria, Ghana, Egypt, and South Africa have brought to me a sense of accomplishment and satisfaction of making an important contribution. But, much more than that, this work made all of us realize how proud these microbiologists are of their own work and what they can do to help people in their countries when empowered with this skill and knowledge. Ultimately, it brought a renewed sense of humility to all of us and a strong commitment to work continuously on strengthening those relationships. This much enriched edition of the manual is the result of that commitment.

In response to the success of the first edition, we have updated this manual to include current microbiological approaches to isolation and identification of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. In addition, ever-expanding knowledge and consequent progress on laboratory methods, epidemiology and surveillance, and development of vaccines are all reflected in several new chapters. Microbiology laboratories have always played a critical role in prevention and control of bacterial meningitis. Today, that very role in rapid detection and surveillance cannot be overemphasized. Recently, in June 2011, a group of leaders in the global immunization area provided their insights into the importance of microbiological monitoring after implementation of major vaccine efforts (1). They defined surveillance as a critical component of broader efforts that are essential following the introduction of new vaccines to detect any vaccine-induced serogroup or serotype replacement.

And a final word to those who will be using this manual – not only will you find a wealth of useful information and procedures to guide you as you do the work yourself, but also be able to successfully spread that knowledge to your colleagues!

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WHO Manual, 2nd Edition**

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CHAPTER 1

Introduction

Bacterial meningitis remains a serious global health problem. The laboratory plays a crucial role in diagnosing this devastating disease. By identifying the causative organism and determining antimicrobial susceptibility, laboratorians provide clinicians with the information required to deliver appropriate treatment to their patients. Laboratories play a crucial role for communities and populations as laboratory data are the foundation of public health surveillance for bacterial meningitis. These surveillance data guide ministries of health when responding to epidemics, making decisions about the introduction and use of vaccines, and properly allocating resources according to the needs of the population. Thus, a well-trained and equipped diagnostic laboratory is critical for the health of individuals and populations.

In 1999, the World Health Organization published the first edition of “Laboratory Methods for the Diagnosis of Meningitis Caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*.” That manual aimed to provide laboratories with a clear, concise guide to the basic procedures for isolating and identifying *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* from the blood or cerebrospinal fluid of patients with bacterial meningitis. The focus was on including laboratory procedures chosen for their utility, ease of performance, and ability to give reproducible results; while taking into account the diversity of laboratory capabilities, availability of materials and reagents, and their cost. Since its publication, that manual has been widely adopted by laboratories worldwide.

In the twelve years since the first edition of this manual, important changes have occurred both in the epidemiology of bacterial meningitis and in the available laboratory techniques for isolating, identifying, and characterizing the causative organism. In recent years, great progress has been made in increasing worldwide access to vaccines to prevent meningococcal, pneumococcal, and *H. influenzae* type b (Hib) disease. Most recently, the historic development and implementation of a new meningococcal conjugate vaccine for serogroup A has the potential to eliminate epidemic meningitis in sub-Saharan Africa. Surveillance for diseases caused by infectious agents that are targeted by newer vaccines will likely require a syndromic approach. Patients diagnosed with meningitis syndrome may all exhibit similar symptoms (i.e., fever, headache, stiff neck) but each individual’s disease could be caused by a variety of organisms, including the bacterial meningitis pathogens *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. Hence, clinical syndromic surveillance must be complemented by a strong laboratory component to allow for diagnostic confirmation of the specific disease agent. Laboratory networks supporting surveillance, such as the Invasive Bacterial Vaccine Preventable Diseases (IB-VPD) Surveillance Network and Integrated Disease Surveillance and Response (IDSR), have helped to improve data quality to expedite and sustain evidence-informed decisions at the global, regional, and national levels.

These developments prompted a revision of the manual to produce this second edition. The revision follows the format of the first edition, but has been expanded to include Results Management and Reporting of Data (Chapter 3); Biosafety (Chapter 4); PCR for Detection and Characterization of Bacterial Meningitis Pathogens (Chapter 10); Antimicrobial Susceptibility

Testing (Chapter 11); Characterization by Molecular Typing Methods (Chapter 12); and Quality Control/Quality Assurance (Chapter 13).

CHAPTER 2

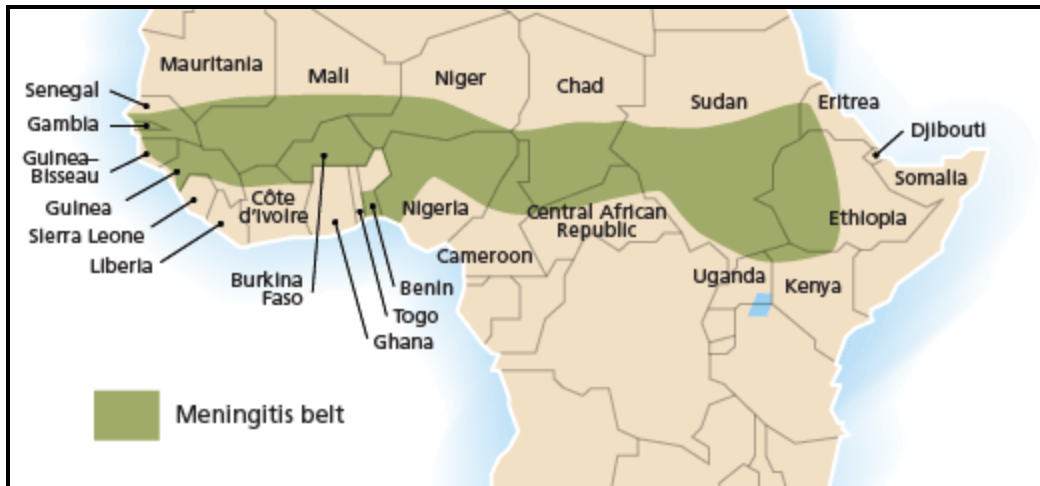
Epidemiology of Meningitis Caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*

The term “meningitis” describes inflammation of the membranes (meninges) and/or cerebrospinal fluid (CSF) that surrounds and protects the brain and spinal cord. Meningitis can result from many causes, both infectious and non-infectious. Bacterial meningitis is a life-threatening condition that requires prompt recognition and treatment. Beyond the newborn period, the most common causes of bacterial meningitis are *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. All three of these organisms are respiratory pathogens. They are spread from person to person by close contact with respiratory secretions. Once acquired, each species can colonize the mucosa of the nasopharynx and oropharynx, which is known as pharyngeal carriage. From there, they may cross the mucosa and enter the blood. Once in the blood, they can reach the meninges, causing meningitis, or other body sites causing other syndromes. Over 1.2 million cases of bacterial meningitis are estimated to occur worldwide each year (24). The incidence and case-fatality rates for bacterial meningitis vary by region, country, pathogen, and age group. Without treatment, the case-fatality rate can be as high as 70 percent, and one in five survivors of bacterial meningitis may be left with permanent sequelae including hearing loss, neurologic disability, or loss of a limb (18).

Neisseria meningitidis

N. meningitidis may either be encapsulated or unencapsulated. However, nearly all invasive *N. meningitidis* organisms are encapsulated, or surrounded by a polysaccharide capsule. This capsular polysaccharide is used to classify *N. meningitidis* into 12 serogroups. Six of these serogroups cause the great majority of infections in people: A, B, C, W135, X, and Y (12). Incidence rates of *N. meningitidis* meningitis are generally highest in children less than five years of age and in adolescents. *N. meningitidis* can also cause a severe bacteremia, called meningococemia. The worldwide distribution of serogroups of *N. meningitidis* is variable. In the Americas, Europe, and Australia, serogroups B and C are the most common, while serogroup A causes the majority of disease in Africa and Asia (7). Sometimes serogroups can emerge, increasing in importance in a specific country or region, like serogroup C in China (20) or serogroup Y in North America (15, 17, 23).

Worldwide, the incidence of meningitis due to *N. meningitidis* is highest in a region of sub-Saharan African known as the “meningitis belt” (Figure 1). This hyper-endemic region extends from Senegal to Ethiopia, and is characterized by seasonal epidemics during the dry season (incidence rate: 10-100 cases per 100,000 population), punctuated by explosive epidemics in 8-12 year cycles (incidence rates can be greater than 1,000 cases per 100,000 population). Across the meningitis belt, at least 350 million people are at risk for meningitis during these annual epidemics. Meningitis epidemics are generally caused by serogroup A, although outbreaks have also been caused by serogroups C, W135, and X (1-3, 7, 13, 21, 28). Outbreaks of different serogroups may overlap, therefore, laboratory confirmation is important both to recognize and monitor the progression of outbreaks (5-7).



Source: Control of epidemic meningococcal disease, WHO practical guidelines, World Health Organization, 1998, 2nd edition, WHO/EMC/BAC/98.3

Figure 1. The African meningitis belt. These sub-Saharan countries are at high epidemic risk for meningococcal meningitis.

Haemophilus influenzae

H. influenzae, like *N. meningitidis*, may be either unencapsulated or encapsulated with a polysaccharide capsule. The makeup of this polysaccharide capsule allows encapsulated *H. influenzae* isolates to be classified into six serotypes (a, b, c, d, e, and f) with the most common cause of invasive disease being *H. influenzae* type b (Hib). Though *H. influenzae* meningitis is rare in adolescents and adults, rates of meningitis due to Hib are highest in children less than five years of age, with an estimated incidence rate of 31 cases per 100,000 (22). In young children, the case-fatality rate for meningitis due to *H. influenzae* is generally higher than that for meningitis due to *N. meningitidis*. In addition to meningitis, *H. influenzae* is also an important cause of pneumonia as well as epiglottitis. While the worldwide burden of disease caused by *H. influenzae* is not completely understood, lab networks supporting surveillance systems such as Paediatric Bacterial Meningitis (PBM) and Invasive Bacterial Diseases (IBD) contribute standardized disease burden data.

Streptococcus pneumoniae

S. pneumoniae, like *N. meningitidis* and *H. influenzae*, is an encapsulated bacterium. The diversity of capsular types is large, with at least 93 serotypes recognized based on the composition of the capsular polysaccharide. Many *S. pneumoniae* serotypes are capable of causing invasive disease, including meningitis, bloodstream infections, and pneumonia; however, most disease worldwide is caused by a small number of common serotypes (8). The relative contribution of each serotype to the local burden of disease varies globally, with serotypes 1 and 5 more prominent in developing countries. *S. pneumoniae* and Hib disease may vary seasonally, and while they do not cause epidemics like *N. meningitidis*, large outbreaks do occur rarely (4, 12). Meningitis due to *S. pneumoniae* occurs most commonly in the very young and the very old, with an estimated incidence rate of 17 cases per 100,000 population in children

less than five years of age (14). The case fatality rate for meningitis due to *S. pneumoniae* in children less than five years of age exceeds 73% in some parts of the world.

Prevention and control

The risk of secondary cases of meningococcal disease among close contacts of someone with meningococcal disease (i.e., household members, day-care center contacts, or anyone directly exposed to the patient's oral secretions) is high. In non-epidemic settings, antimicrobial chemoprophylaxis is effective in preventing secondary cases among close contacts by eliminating nasopharyngeal carriage if administered rapidly after the index case is identified. Such intervention may not be feasible in many countries. Mass chemoprophylaxis to prevent/control epidemics is not recommended. Secondary cases are also seen for Hib meningitis, particularly in unvaccinated children less than 4 years of age who are exposed to someone with Hib disease. Oral rifampin is recommended to eliminate nasopharyngeal carriage and prevent disease in these children. Secondary meningitis cases are very rare among those exposed to a patient with pneumococcal disease.

Laboratory surveillance data are critical to tracking the spread of less susceptible strains and to providing guidance in the empirical selection of antimicrobial agents. For all three bacterial meningitis pathogens, antimicrobial resistance has been identified, affecting the treatment of patients and chemoprophylaxis of close contacts. *N. meningitidis* isolates resistant to sulfonamides are common in many countries. Isolates resistant to rifampicin, penicillin, chloramphenicol, cotrimoxazole, ceftriaxone, and ciprofloxacin have also been identified (27). One report from the United States described 2 isolates which were rifampin resistant (16). Resistance to beta-lactam antimicrobials is common in *H. influenzae* isolates; the majority of which produce beta-lactamase. *S. pneumoniae* isolates have been reported with resistance to beta-lactams, macrolides, tetracycline, and trimethoprim/sulfamethoxazole. The increasing proportion of pneumococci resistant to penicillin and the development of resistance to ceftriaxone has huge implications for treatment and makes prevention through vaccination that much more important. The introduction of vaccine in the United States has resulted in a decreasing proportion of invasive isolates that are antibiotic-resistant, thus vaccine may have a role in controlling the spread of antibiotic resistance (10).

Vaccines are the cornerstone of prevention and control of bacterial meningitis. Vaccines for *N. meningitidis* made up of capsular polysaccharide have been available and used since the 1970s. These include a bivalent vaccine (serogroups A and C), a trivalent vaccine (A, C, Y), and a quadrivalent vaccine (A, C, W135, and Y). Timely mass-vaccination campaigns using polysaccharide vaccines can effectively interrupt the course of meningitis epidemics, but they are less effective in young children, do not provide long duration of protection, do not have sustained impact on nasopharyngeal carriage, and therefore do not interrupt person to person transmission. For this reason, they do not result in "herd immunity", which is the extension of protection to unvaccinated people in the community.

In 2010, a new serogroup A meningococcal conjugate vaccine was licensed, pre-qualified by WHO, and introduced in Burkina Faso, Mali, and Niger (11). Conjugate vaccines generally result in higher levels of protection, longer duration of protection, protection of children less than

2 years of age, and may interrupt nasopharyngeal carriage and transmission, resulting in herd immunity. When implemented in national preventive vaccination programs across the meningitis belt, it is hoped that the vaccine will prevent the occurrence of serogroup A epidemics. Traditional public health and bacteriologic surveillance, as well as molecular epidemiology, will play a crucial role in evaluating both the short- and long-term impact of these vaccination programs. For example, the need for vaccines to other serogroups, the potential re-emergence of serogroup A due to waning vaccine-induced immunity, or the emergence of new serogroups will only become evident through ongoing, high-quality surveillance.

Polysaccharide-protein conjugate vaccines for Hib are available for young children. In most industrialized countries, these vaccines have dramatically decreased the burden of Hib meningitis and virtually eliminated it as a public health problem through direct effects and induction of herd immunity without significant strain replacement. More recently, many developing countries have introduced, or plan to introduce, Hib vaccines through various global initiatives, such as the Hib Initiative and the GAVI Alliance, whose goals are to accelerate introduction of Hib vaccines in low and middle income countries.

A 23-valent polysaccharide vaccine is available for *S. pneumoniae*. Like other polysaccharide vaccines, it is not effective in children younger than two years of age; the group with the highest risk of *S. pneumoniae* meningitis. Newer polysaccharide-protein conjugate vaccines have been introduced in many industrialized countries, leading to dramatic declines in pneumococcal meningitis in infants and young children and in adults through induction of herd immunity (9). Currently, 7-valent, 10-valent, and 13-valent pneumococcal conjugate vaccines have been developed and have received WHO prequalification. In some settings, serotypes not covered by the 7-valent conjugate vaccine have increased somewhat following 7-valent conjugate vaccine introduction (25). As with Hib vaccine, global initiatives such as PneumoADIP and the GAVI Alliance have helped to accelerate introduction of these vaccines in low and middle income countries. As of the end of 2010, 42 countries were using a pneumococcal conjugate vaccine for routine infant immunization, including 3 low-income countries, and as many as 15 more low-income countries are slated to introduce vaccine in 2011 (26).

Role of the laboratory

Microbiologists play a critical role in gathering data both for clinical and public health decision making. Efficient and accurate microbiologic diagnosis of bacterial meningitis guides the choice of antibiotics and other treatment options for the patient. Collectively, serogroup or serotype results from isolates of bacterial meningitis in an effected population guide response efforts and determine the appropriate vaccine to be used. Similarly, microbiologic surveillance is critical to guide appropriate antibiotic therapy through the identification of local resistance profiles. Thus, the role of the microbiology laboratory is essential to preventing morbidity and mortality from bacterial meningitis.

Infection with *N. meningitidis* may be acquired through working with bacterial isolates in the microbiology laboratory if appropriate protective procedures are not followed (19). Microbiologists who routinely work with these isolates are at increased risk for infection. This risk highlights the importance of consistent adherence to biosafety procedures. In addition,

vaccination against meningococcal disease is recommended for microbiologists who routinely work with *N. meningitidis*, and antimicrobial chemoprophylaxis should be used if lapses in biosafety procedures result in exposure to the organism.

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CHAPTER 3

Results Management and Reporting of Data

I. Data management and reporting

Proper laboratory procedures are essential for correctly identifying and characterizing pathogens from patients with bacterial meningitis. However, even the best laboratory efforts are not useful if the results are not accurately reported to those who make policy and epidemic response decisions. The development of an accurate data reporting system requires quality data management: collection, recording, validation, and results feedback of important information about patients, specimens, and laboratory results.

II. Data management systems

A data management system can be as simple as a laboratory logbook or as complex as a computerized information system. Laboratories with small workloads may find that paper records are sufficient for their data management requirements. However, computerized information systems are recommended for laboratories that handle larger numbers of clinical specimens and isolates. Regardless of whether the data management system is paper or computerized, it should allow clinical and laboratory data to be accurately recorded, easily accessed and reported, and reliably stored. It is also important to keep laboratory data linked with epidemiological data to ensure the quality of results reported.

When considering computerized information systems, laboratory directors should consider the user preferences, hardware and maintenance requirements, software costs, local expertise needed to develop, install, refine, and maintain the system, and the costs of routine data backups. Computerized information systems should allow easy recording of data, formatting and editing of reports, and simple analyses such as frequencies and workload calculations. These basic analyses can help laboratory managers estimate operating costs and supply needs, and can also provide surveillance systems with useful summary information.

III. Request form and record keeping

Quality data management begins with the clinical request for laboratory testing. All clinical specimens and isolates should be accompanied by a standardized request form that includes the following information:

- Patient's name, date of birth, sex, and residence address
- Unique Identification Number. Examples include, Patient Hospital Number, Case Identification Number (for patients included in epidemiologic investigations or research studies), or Surveillance Identification Number (such as an EPID number).
- Patient's hospital, hospital address, and room number
- Physician's name and contact information
- Clinical diagnosis and relevant patient history

- Specimen type (clinical specimen or isolate)
- Anatomical site of specimen collected (CSF, blood, other)
- Date and time of specimen collection
- Test(s) requested
- Antimicrobial therapy the patient is receiving or has received, if any
- Immunization status for meningitis pathogens (*N. meningitidis*, *H. influenzae* serotype b, *S. pneumoniae*)
- Name and address for report recipient

Each specimen should have a label firmly attached to the specimen container bearing the following information:

- Patient's name
- Unique Identification Number
 - Be sure this number matches the number on the request and report forms.
- Date and time of specimen collection

Upon receiving a clinical specimen or isolate, the laboratory staff should enter the above information into the laboratory data management system. Additional information to be recorded includes:

- Date and time the clinical specimen or isolate arrives to the laboratory
- Number of items received in the laboratory
- Gram stain result
- Whether or not the treating clinician was notified of the Gram stain result within one (1) hour of the test result
- The date the specimen or isolate was stored by the laboratory
- The date the specimen or isolate was sent the national laboratory or regional reference laboratory

Other important information to record includes transport conditions (e.g., conservation of ideal temperature for transporting specimens and maintaining appropriate shipping conditions according to guidelines), condition of the specimen upon arrival (e.g., volume, possible contamination, compromised container, etc.), and any preparatory actions taken (e.g., aliquoting or centrifugation). The specimen should be given a unique laboratory identification number to be used in all subsequent procedures. It is important that the identification number be recorded on the request form, the specimen container, and in the data management system so that results are linked to the patient information.

Results for all tests performed on the clinical specimens and isolates should be entered into the data management system as soon as they are obtained. Any information regarding quality control and quality assurance (see Chapter 13: Quality Control and Quality Assurance) related to the tests should be recorded in the appropriate logbook or database.

IV. Data reporting

Another important function of the laboratory is to provide users with timely and accurate laboratory results. Users include physicians, surveillance units, disease control programs, local, state, district, regional, and national health departments, and outbreak investigation teams. Each of these users will need different information from the laboratory. For example, a surveillance unit in the African meningitis belt may need summaries of all patients with confirmed *N. meningitidis* by serogroup, while a physician treating a patient with bacterial meningitis may need rapid reporting of the pathogen and antimicrobial susceptibility testing results. Laboratory directors should work with users to develop a standardized report form, data formats, and regular reporting frequencies. The expected flow of information and communication method should be clearly agreed upon by both the laboratory and the data recipients. It is essential that efficient reporting mechanisms are established as the best laboratory efforts are futile if the information is not reported back to the patient's physician, surveillance team, etc. Furthermore, surveillance and study information must be reported to public health officials in order to affect public policy decisions.

Recommended reading

- ISO 15189:2007. Medical laboratories: requirements for quality and competence. Geneva, Switzerland: International Organization for Standardization; 2007.
- Polio laboratory manual, fourth edition. WHO/IVB/04.10. World Health Organization, Geneva, 2004, p 157.

CHAPTER 4

Biosafety

Laboratorians working with infectious agents are at risk of laboratory-acquired infections as a result of accidents or unrecognized incidents. The degree of hazard depends upon the virulence and dose of the biological agent, route of exposure, host resistance, proper biosafety training, and experience with biohazards. Laboratory-acquired infections occur when microorganisms are inadvertently ingested, inhaled, or introduced into tissues. Multiple instances of laboratory-acquired meningococcal infection have been reported with a case fatality rate of 50% (1, 2). While laboratory-acquired *H. influenzae* and *S. pneumoniae* infections are not as extensively reported, deadly infections with any of these organisms are possible if appropriate biosafety procedures are not strictly followed in a properly equipped laboratory. Biosafety Level 2 (BSL-2) practices are required for work involving these agents as they present a potential hazard to personnel and the environment. The following requirements have been established for laboratorians working in BSL-2 facilities:

- Laboratory personnel must receive specific training in handling pathogenic agents and be directed by fully trained and experienced scientists.
- Access to the laboratory must be limited to personnel who have a need to be in the laboratory and have undergone proper training when work is being conducted.
- Extreme precautions must be taken with contaminated sharp items and sharps must be disposed of in labeled appropriate hardened plastic containers.
- Personal protective equipment (PPE) must be worn at all times, and particular care must be taken when performing procedures that have the potential to create aerosols.

I. Protective clothing and equipment

A. Laboratory coats

Protective coats, gowns, smocks, or uniforms designated for laboratory use must be worn while working in the laboratory. Laboratory coats should fit properly and should cover arms to the wrist. This protective clothing must be removed and left in the laboratory before leaving for non-laboratory areas, such as offices or eating areas. All protective clothing is either disposed of in the laboratory or laundered by the institution; it should never be taken home by personnel.

B. Gloves

Regardless of the type of infectious material, gloves should be worn when performing potentially hazardous procedures involving infectious materials in which there is a risk of splashing or skin contamination or when the laboratory worker has cuts or broken skin on his or her hands. Gloves should always be worn when handling clinical specimens, body fluids, and tissues from humans and animals. These specimens should be handled as if they are positive for hepatitis B virus, human immunodeficiency virus (HIV), or any bloodborne pathogens. Gloves must be removed when contaminated by splashing or spills or when work with infectious materials is

completed. When removing gloves, avoid touching any areas of the gloves that may have come in contact with infectious material.

Gloves should not be worn outside the laboratory. Personnel should not use the telephone, computer, or open doors with gloves that have been used in laboratory procedures. All used gloves should be disposed of by discarding them with other disposable materials and autoclaving. Hands should be washed immediately after removing gloves.

C. Barrier precautions

Clinical specimens, body fluids, and tissues from humans and animals should be assumed to be positive for human pathogens. These materials should be handled in a biosafety cabinet (BSC) or using other barrier precautions (e.g., goggles, mask, face shield, or other splatter guards) whenever a procedure is performed that can potentially create an aerosol. However, manipulating suspensions of *N. meningitidis* outside of a biosafety cabinet is associated with a high risk for contracting meningococcal disease (2) and using only a splatter guard on the bench top does not provide adequate protection (1).

D. Foot Protection

Closed-toe comfortable shoes that have low heels should be worn in the laboratory or other areas where chemicals are present. This will reduce injuries that may occur from spills, splashes, falling objects, slipping, and broken glass.

II. Standard microbiological safety practices

The following safety guidelines listed below apply to all microbiology laboratories, regardless of biosafety level. All procedures requiring handling of infectious materials, potentially infectious materials, or clinical specimens should be performed while wearing appropriate PPE.

A. Limiting access to laboratory

Sometimes non-laboratorians attempt to enter the laboratory to obtain test results. Although this occurs more frequently in clinical laboratories, access to the laboratory should be limited to trained personnel with a need to work in the laboratory, regardless of the setting.

Biohazard signs or stickers should be posted near or on all laboratory doors and on all equipment used for laboratory work (e.g., incubators, hoods, microwaves, ice machines, refrigerators, and freezers). Children who have not reached the age of adulthood and pets are not allowed in laboratory areas. All laboratories should be locked when not in use. In addition, all freezers and refrigerators located in corridors should be locked, especially those that contain infectious organisms or other hazardous materials.

B. Disinfectants

Organisms may have different susceptibilities to various disinfectants. As a surface disinfectant, 70% isopropyl alcohol is generally effective. However, 70% alcohol is not the disinfectant of choice for decontaminating spills. It should be noted that 100% alcohol is not as effective a disinfectant as 70% alcohol. Phenolic disinfectants, although expensive, are effective against many organisms. Always read disinfectant labels for manufacturers' recommendations for dilution and for exposure times for efficacy. An effective general disinfectant is a 1:100 (1%) dilution of household bleach (sodium hypochlorite) in water; at this dilution, bleach can be used for wiping surfaces of benches, hoods, and other equipment. A 1:10 (10%) dilution of bleach should be used to clean up spills of cultured or concentrated infectious material where heavy contamination has occurred; however, it is more corrosive, will pit stainless steel, and should not be used routinely. If bleach is used, wipe down the area with 70% alcohol to inactivate the bleach. If bleach is used as a disinfectant, the diluted solutions should be made weekly from a concentrated stock solution.

C. Decontamination of spills

The following procedure is recommended for decontaminating spills:

- Isolate the area to prevent anyone from entering.
- Wear gloves and protective clothing such as a gown or lab coat, shoes, and a mask (if the spill may contain a respiratory agent or if the agent is unknown).
- Absorb or cover the spill with disposable towels, but do not wipe up the spill or remove the towels.
- Saturate the towels and the affected area with an appropriately diluted intermediate or high-level disinfectant (e.g., a phenolic formulation or household bleach) and leave them in place for at least 15 minutes.
- Wipe area using clean disinfectant-soaked towels and allow area to air dry.
- Place all disposable materials used to decontaminate the spill into a biohazard container. If broken glassware is involved, use mechanical means to dispose of it.
- Handle the material in the same manner as other infectious waste.

D. Hand washing

All laboratories should contain a sink with running water and soap for hand washing. Frequent hand washing is one of the most effective procedures for avoiding laboratory-acquired infections. Hands should be washed for at least one minute with an appropriate germicidal soap after infectious materials are handled and before exiting the laboratory. If germicidal soap is unavailable, then use 70% isopropyl or ethyl alcohol to cleanse hands.

E. Eating

Eating, drinking, and smoking are not permitted in laboratory work areas. Food must be stored and eaten outside of the laboratory in designated areas used for that purpose only. Personal articles (e.g., handbags, eyeglasses, or wallets) should not be placed on laboratory workstations.

F. Mouth pipetting

Mouth pipetting is strictly prohibited. Rubber bulbs or mechanical devices must be used.

G. Sharps

A high degree of precaution must always be taken with any contaminated sharp items, including needles and syringes, slides, glass pipettes, capillary tubes, broken glassware, and scalpels. Sharps should be disposed of in designated puncture-proof, leak-proof, and sealable sharps containers. To minimize finger sticks, used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal. Non-disposable sharps should be placed in a labeled discard pan for decontamination before cleaning. Broken glassware should not be handled directly by hand but should be removed by mechanical means (e.g., brush and dustpan, tongs, or forceps).

H. Aerosols

All procedures must be carefully performed to minimize splashes or aerosolization. When procedures with a high potential for creating infectious aerosols are conducted or when a procedure that can result in splashing or spraying of the face with infectious or other hazardous materials is used, laboratory work should be conducted in a biosafety cabinet or by laboratorians wearing the appropriate face protection equipment (e.g., goggles, mask, face shield, or other splatter guards). Face protection should also be used when working with high concentrations or large volumes of infectious agents. Procedures that pose such a risk may include:

- Centrifugation, vortexing, and vigorous mixing: these procedures should be performed in closed containers. If safety capped tubes are not available, sealed tubes should be used. All body fluids and infectious materials should only be centrifuged in carriers with safety caps.
- Handling tissue specimens or bodily fluids: gauze should be used to remove the tops on blood specimens and should be placed around the top of blood culture bottles to minimize aerosol production during removal of the needle. Grinding of tissue specimens should be performed in a biosafety cabinet.
- Sonic disruption: infectious materials that undergo sonic disruption should be placed in a sealed container within the sonicator.
- Opening containers of infectious materials whose internal pressures or temperatures may be different from ambient pressures or temperatures.
- Loops containing infectious material should be dried in the hot air above a burner before flaming.
- Inoculating wires and loops should be cooled after flame sterilization by holding them still in the air for 5-10 seconds before they touch colonies or clinical material. Disposable loops are preferred if resources are available.

I. Decontaminating bench tops and other surfaces

Bench tops and other potentially contaminated surfaces should be wiped with a phenolic disinfectant (10% bleach) routinely after working with infectious agents or clinical specimens or after spills, splashes, or other contamination by infectious materials. Following disinfection with

10% bleach, the surface must then be wiped down with 70% isopropyl or ethyl alcohol to inactivate the bleach and prevent corrosion of the work surface. Solutions of disinfectants should be maintained at each work station (see Disinfectants, Section II.B.).

J. Disposal of contaminated materials

All discarded plates, tubes, clinical samples, pipettes, gloves, and other contaminated materials should be placed in disposal containers at each bench. Special disposal containers typically constructed of puncture-proof plastic must be used for sharps to minimize the risk of injury. Avoid overfilling disposal containers. The lids should rest flush with the top of the container. Containers of contaminated material should be carefully transported to the autoclave room and autoclaved before disposal. Water should be added to each container to be autoclaved for optimal sterilization. Waste disposal containers in the laboratory should be clearly labeled for disposal of infectious items or non-infectious items. Waste disposal containers for infectious or potentially infectious items should be lined with a plastic biohazard or otherwise specially marked bag.

K. Autoclaving

An autoclave must be available for the BSL-2 laboratory and must be operated only by personnel who have been properly trained in its use. To verify that each autoclave is working properly, spore strips (such as *Bacillus stearothermophilus*) or other biological indicators designed to test for efficiency of sterilization should be included in autoclave loads on a regular basis (i.e., monthly). Each autoclave load should be monitored with temperature-sensitive tape, thermograph, or by other means (i.e., biological indicators). A logbook should be maintained for each autoclave to record the date, times, and indicator of sterilization of each autoclave run.

L. General laboratory cleanliness

All areas of the laboratory must be kept clean and orderly. Dirt, dust, crowding, or clutter is a safety hazard, may lead to contamination of specimens, isolates, and/or biological assays, and is not consistent with acceptable biological research. Floors should be kept clean and free of unnecessary clutter and should be washed with a germicidal solution on a regular basis and after any spill of infectious material.

M. Refrigerators and freezers

The temperature of laboratory refrigerators and freezers should be monitored daily to ensure that they are functioning properly. They should also be regularly inspected for the presence of broken vials or tubes containing infectious agents. When removing and discarding broken material, laboratorians should wear gloves and PPE. If the broken material is suspected of being infectious, disinfectant should be applied to the affected area and kept in place for at least 15 minutes before removal of the broken material. Refrigerators and freezers should be regularly cleaned with a disinfectant and defrosted to prevent possible contamination or temperature failure.

N. Fire prevention

Burners should be used away from light fixtures and flammable materials. Bulk flammable material must be stored in a safety cabinet. Small amounts of these flammable materials (e.g., ethyl acetate, ethyl alcohol, and methanol) can be stored in safety containers such as a safety bench can or dispenser can. Burners must be turned off when not in use. All laboratorians must know the location of fire extinguishers, fire blankets, alarms, and showers, and fire safety instructions and evacuation routes should be posted.

III. Special Practices

A. Accidents

All injuries or unusual incidents should be reported immediately to the supervisor. When cuts or puncture wounds from potentially infected needles or glassware occur, the affected area should be promptly washed with disinfectant soap and water for 15 minutes. Report a needle-stick injury, any other skin puncture, to the supervisor and appropriate health officials immediately as prophylactic treatment of the personnel performing the procedure may be indicated. In the event of a centrifuge accident in which safety carriers have not been used, other personnel in the area should be warned immediately and the area should be isolated to prevent anyone from entering.

B. Laboratory design and equipment

The laboratory should be designed to avoid conditions that pose biosafety problems. Ample space should be provided to allow for safe circulation of staff when working and cleaning. There should be clear separation of areas for infectious and non-infectious work. Illumination should be adequate. Walls, ceiling, floors, benches, and chairs must be easy to clean, impermeable to liquids, and resistant to chemicals and disinfectants. Hand-washing basins with running water and soap and disinfectant must be provided in each room. An autoclave or other means of decontamination must be available close to the laboratory. Adequate storage space for specimens, reagents, supplies, or personal items should be provided inside and outside the working area, as appropriate. Safety systems for fire, chemicals, electrical, or radiation emergencies, and an emergency shower and eyewash facilities should be in place. Security measures should also prevent theft, misuse, or deliberate release of the infectious materials.

C. Medical surveillance of laboratory workers

The employing authority is responsible for providing adequate surveillance and management of occupationally acquired infections. Pre-employment and periodic health checks should be organized and performed. Prophylaxis or other specific protective measures may be applied after a risk assessment of possible exposure and a health check of the individual or individuals. Special attention should be paid to women of childbearing age and pregnant women as some microorganisms present a higher risk for the fetus (i.e., rubella virus).

Immunization of the laboratory workers can also be proposed taking into account the following criteria:

- Conclusion of the risk assessment.
- Verification by serology of the immunization status of the worker (some workers may be already immunized from prior vaccination or infection).
- The local availability, licensing state, and utility of vaccines (i.e., does the vaccine provide protection against the prevalent serogroups or serotypes circulating in the region?).
- The availability of therapeutic drugs (i.e., antibiotics) in case of accident.
- The existence of national regulations or recommendations.

A first-aid box containing basic medical supplies should be available along with a written emergency procedure to access a doctor for definitive treatment of the injury. First aid kits should be periodically checked to ensure contents are within the expiration date.

D. Biosafety management and implementation

The laboratory director is responsible for implementation of biosafety measures. He or she can delegate tasks to a qualified individual or a group of individuals who perform them on a part-time basis, or even assign a biosafety officer with the appropriate background and knowledge.

E. Other sources of biosafety information

For more information, please review:

Centers for Disease Control and Prevention. 1999. Biosafety in microbiological and biomedical laboratories, 5th ed. Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

Clinical and Laboratory Standards Institute. Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guidelines—Third Edition. CLSI document M29-A3. [ISBN 1-56238-567-4]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898. USA, 2005.

Laboratory Biosafety Manual, third edition. WHO/CDS/CSR/LYO/2004.11. World Health Organization, Geneva, 2004, p 178.

Laboratory Biosafety Guidelines, third edition. Public Health Agency of Canada, 2004, p 113.

Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world. Centers for Disease Control and Prevention and World Health Organization, 2003, p 359.

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2. **Boutet, R., Stuart, J. M., Kaczmarek, E. B., Gray, S. J., Jones, D. M., and Andrews, N.** 2001. Risk of Laboratory-Acquired Meningococcal Disease. *Journal of Hospital Infection*, **49**:282-284.

CHAPTER 5

Collection and Transport of Clinical Specimens

The proper collection and transport of clinical specimens is critical for the isolation, identification, and characterization of agents that cause bacterial meningitis. Optimally, clinical specimens should be obtained before antimicrobial therapy commences in order to avoid loss of viability of the etiological agents. Treatment of the patient, however, should not be delayed while awaiting collection of specimens or results from the laboratory and a specimen should be obtained in all suspect cases as bacterial pathogens can still be detected even after antimicrobial therapy has begun. *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* are fastidious and fragile bacteria. They are more reliably isolated if the clinical specimens are examined as soon as possible after collection. Cerebrospinal fluid (CSF) should be processed in a microbiology laboratory within 1 hour after collection or inoculated into Trans-Isolate (T-I) medium for transport to the laboratory if processing within 1 hour is not feasible. Blood specimens should be immediately inoculated into a blood culture bottle and transported to a microbiology laboratory as soon as possible for overnight incubation and growth of bacteria.

I. Biosafety

It is important to adhere to proper biosafety guidelines while handling potentially infectious clinical specimens in order to maintain a safe working environment for patients, health care workers, and laboratorians. Infection may be transmitted from patient to staff and from staff to patient during the procedures described below. In addition to the agents that cause bacterial meningitis, the patient could have other bacterial or viral agents in either the CSF or blood and both are a great hazard and potentially lethal. Of particular importance are the viruses causing hepatitis and acquired immunodeficiency syndrome. To decrease the risk of transmission of these agents, the recommendations below should be followed:

- Wear latex or nitrile gloves that are impermeable to liquids and change gloves between every patient.
- Dispose of syringes and needles in a puncture-resistant, autoclavable discard container. Do not attempt to re-cap, shear, or manipulate any needle. A new sterile syringe and needle must be used for each patient.
- For transport to a microbiology laboratory, place the specimen in a container that can be securely sealed. Wipe any bottles with CSF or blood on the outside thoroughly with a disinfectant, such as a 70% alcohol swab.
 - Do not use povidone-iodine on the rubber septum of a T-I or blood culture bottle.
- Remove gloves and discard in an autoclavable container.
- Wash hands with antibacterial soap and water immediately after removing gloves.

- In the event of a needle-stick injury or other skin puncture or wound, wash the wound liberally with soap and water. Encourage bleeding.
- Report a needle-stick injury, any other skin puncture, or any contamination of the hands or body with CSF to the supervisor and appropriate health officials immediately as prophylactic treatment of the personnel performing the procedure may be indicated.

II. Collection and transport of CSF

The collection of CSF is an invasive procedure and should only be performed by experienced personnel under aseptic conditions. If bacterial meningitis is suspected, CSF is the best clinical specimen to use for isolation, identification, and characterization of the etiological agents. Suspected agents should include *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* and other pathogens in some cases.

A. Preparing for lumbar puncture

If possible, three tubes (1 ml each) of CSF should be collected for microbiology, chemistry, and cytology. If only one tube of CSF is available, it should be given to the microbiology laboratory. Because the presence of blood can affect cultures of CSF, if more than one tube of CSF is collected from a patient, the first tube collected (which could contain contaminating blood from the lumbar puncture) should not be the tube sent to the microbiology laboratory.

The kit for collection of CSF should contain (Figure 1):

- Skin disinfectant: 70% alcohol swab and povidone-iodine
 - Alcohol with concentrations greater than 70% should not be used because the increased concentrations result in decreased bactericidal activity. Do not use alcohol with glycerol added to it.
- Sterile gloves
 - Be sure to check the expiration date.
- Sterile gauze
- Surgical mask
- Adhesive bandage
- Lumbar puncture needle
 - 22 gauge/89 mm for adults
 - 23 gauge/64 mm for children
- Sterile screw-cap tubes
- Syringe and needle
- Transport container
- T-I medium (if CSF cannot be analyzed in a microbiological laboratory immediately)
 - T-I should be refrigerated at 4°C and added to the kit immediately before use in the field.
- Venting needle (only if T-I is being used)
- Instructions for lumbar puncture and use of T-I medium



Figure 1. Kit for collection of cerebrospinal fluid (CSF)

B. Lumbar puncture procedure

Follow all appropriate biosafety precautions (see Section I).

1. Gather all materials from the CSF collection kit and a puncture-resistant autoclavable container for used needles.
2. Wear surgical mask and sterile latex or nitrile gloves that are impermeable to liquids and change gloves between every patient.
3. Label the collection tubes with appropriate information: patient's name, date and time of specimen collection, and Unique Identification Number. Be sure this number matches the number on both the request and report forms.
4. Ensure that the patient is kept motionless during the lumbar puncture procedure, either sitting up or lying on the side, with his or her back arched forward so that the head almost touches the knees in order to separate the lumbar vertebrae during the procedure (Figure 2).
5. Disinfect the skin along a line drawn between the crests of the two ilia with 70% alcohol and povidone-iodine to clean the surface and remove debris and oils. Allow to dry completely.
6. Position the spinal needle between the 2 vertebral spines at the L4-L5 level and introduce into the skin with the bevel of the needle facing up.
 - Accurate placement of the needle is rewarded by a flow of fluid, which normally is clear and colorless.

7. Remove CSF (1 ml minimum, 3-4 ml if possible) and collect into sterile screw-cap tubes. If 3-4 ml CSF is available, use 3 separate tubes and place approximately 1ml into each tube.
8. Withdraw the needle and cover the insertion site with an adhesive bandage. Discard the needle in a puncture-resistant, autoclavable discard container.
9. Remove mask and gloves and discard in an autoclavable container.
10. Wash hands with antibacterial soap and water immediately after removing gloves.
11. Transport the CSF to a microbiology laboratory within 1 hour for culture and analysis.
 - If that is not possible, inoculate CSF into T-I medium (see Section I.C. below).
 - If T-I is not available, incubate CSF at 35-37°C with ~5% CO₂ (see Section I.D. below) and store in an approved location if the laboratory is closed.
12. In the event of a needle-stick injury or other skin puncture or wound, wash the wound liberally with soap and water. Encourage bleeding.
13. Report a needle-stick injury, any other skin puncture, or any contamination of the hands or body with CSF to the supervisor and appropriate health officials immediately as prophylactic treatment of the personnel performing the procedure may be indicated.

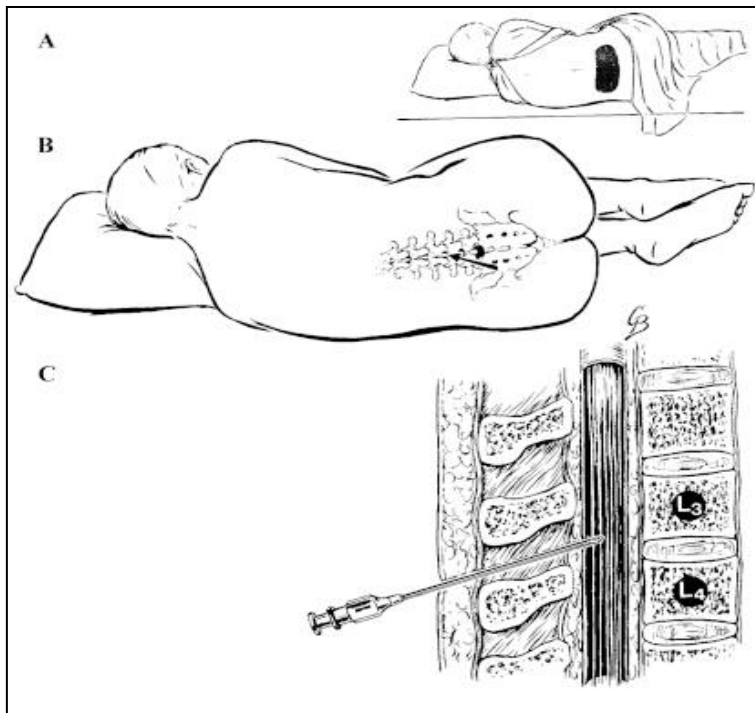


Figure 2. Collection of cerebrospinal fluid (CSF) by lumbar puncture

C. Inoculating and transporting T-I medium

T-I is a biphasic medium that is useful for the primary culture of meningococci and other etiological agents of bacterial meningitis (*S. pneumoniae* and *H. influenzae*) from CSF (Figure 3) (1). It can be used as a growth medium as well as a holding and transport medium. The preparation of T-I media is described in the Annex. T-I media should be stored at 4°C and warmed to room temperature (25°C) before use.

1. Label the T-I bottle with appropriate information: patient name, date and time of CSF inoculation, and Unique Identification Number. Be sure this number matches the number on both the request and report forms.
2. Use sterile forceps to pull the aluminum cover of a T-I bottle away from the rubber stopper and disinfect the stopper with 70% alcohol. Allow to dry.
 - Do not use povidone-iodine as it may be carried into the medium by the passing needle and would inhibit growth of bacteria.
 - Do not completely remove the aluminum cover.
3. Use a sterile syringe and needle to inoculate 0.5-1.0 ml of CSF into the T-I medium. The remaining CSF should be kept in the collection tube. It should not be refrigerated, but should be maintained at room temperature (20-25°C) before Gram staining and other tests. Discard the needle in a puncture-resistant, autoclavable discard container.
4. After inoculation, invert the T-I bottle several times to mix.
5. If transport to a reference laboratory is delayed (next day or longer), insert a venting needle (sterile cotton-plugged hypodermic needle) through the rubber stopper of the T-I bottle, which will encourage growth and survival of the bacteria.
 - Be sure that the venting needle does not touch the broth.
6. Incubate inoculated T-I medium at 35-37°C with ~5% CO₂ (or in a candle-jar) overnight or until transport is possible. If transportation is delayed more than 4 days, remove the vented T-I bottle from the incubator or candle jar and place at room temperature until shipment.
7. Remove the venting needle and wipe the rubber stopper with 70% alcohol before shipping. It is essential to avoid contamination when sampling the bottles to obtain specimens aseptically.
8. If the T-I bottle can be transported to a reference laboratory the same day, do not vent the bottle until it arrives in the receiving laboratory. Upon arrival, vent the T-I bottle, incubate at 35-37°C with ~5% CO₂ (or in a candle-jar), and observe daily for turbidity in the liquid phase for up to 7 days.

- If turbidity is observed, culture onto a blood agar plate (BAP) and a chocolate agar plate (CAP) immediately (see Chapter 6: Primary Culture and Presumptive ID).
- If no turbidity is observed, culture onto a BAP and a CAP on day 4 and day 7.
- If T-I medium appears to be contaminated, selective media such as Modified Thayer-Martin and chocolate agar with bacitracin may be used.



Figure 3. Trans-Isolate (T-I) medium

D. Transporting CSF specimens without T-I media

CSF specimens should be transported to a microbiology laboratory as soon as possible. Specimens for culture should not be refrigerated or exposed to extreme cold, excessive heat, or sunlight. They should be transported at temperatures between 20°C and 35°C. For proper culture results, CSF specimens must be plated within 1 hour (Figure 4). See Chapter 6: Primary Culture and Presumptive ID for instructions on processing CSF once it has arrived in the laboratory.

If a delay of several hours in processing CSF specimens is anticipated and T-I medium is not available, incubating the specimens (with screw-cap loosened) at 35-37°C with ~5% CO₂ (or in a candle-jar) may improve bacterial survival.

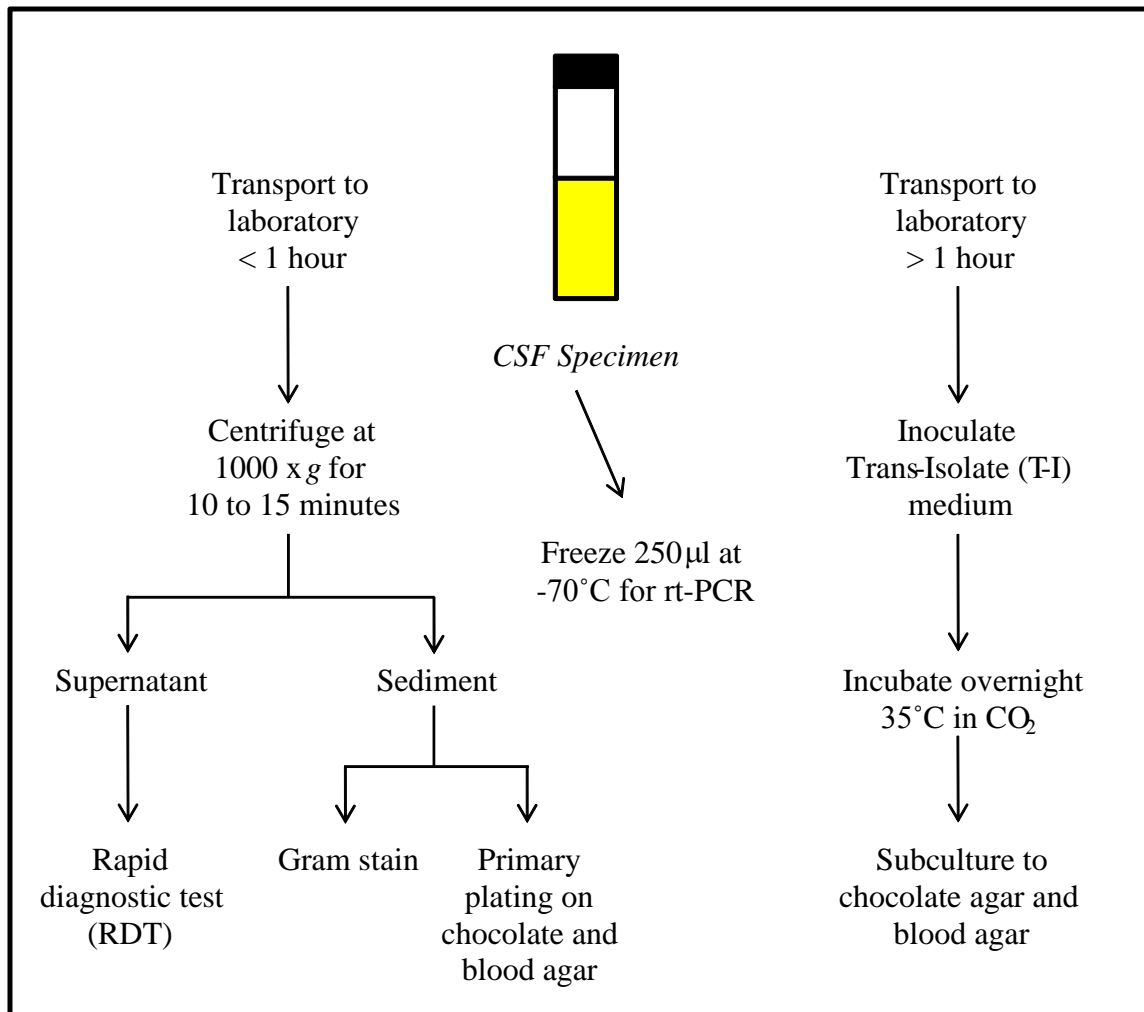


Figure 4. Processing of CSF without T-I media

III. Collection and transport of blood specimens

Blood should be collected when a spinal tap is contraindicated, cannot be performed for technical reasons, or when bacteremia is suspected. Bacteremia can occur with or without meningitis.

A. Sensitivity of blood cultures

Several variables affect the sensitivity of blood cultures: the number of collections, the volume of each collection, the steps taken to inhibit or neutralize bactericidal properties of blood, and the age of the patient. It may be difficult to collect more than 3 ml of blood from a child, but 1-3 ml is considered adequate. Collected blood should be diluted in blood culture broth in order to obtain blood cultures. Typically, 1-2 ml of blood from a child is added to 20 ml of blood culture broth and 5-10 ml of blood from an adult is added to 50 ml of blood culture broth. It is important to use appropriate ratios of blood to culture broth for optimal bacterial growth. The recommendations of the culture broth manufacturer should be closely followed.

Blood should be cultured in trypticase soy broth (TSB) or brain heart infusion (BHI) broth with a growth supplement (such as IsoVitaleX or Vitox) to support growth of other fastidious organisms such as *H. influenzae*. Neutralization of normal bactericidal properties of blood and potential antimicrobial agents is accomplished by adding chemical inhibitors such as 0.025% sodium polyanetholesulfonate (SPS) to culture media and by diluting the blood. SPS, which has anticoagulant, antiphagocytic, anticomplementary, and antilysozymal activity, may be inhibitory to bacterial growth of *Neisseria* species if used in higher concentrations. The preparation of blood culture media is described in the Annex.

B. Preparing for venipuncture

The materials required for venipuncture are:

- Skin disinfectant: 70% alcohol swab and povidone-iodine
 - Alcohol with concentrations greater than 70% should not be used because the increased concentrations result in decreased bactericidal activity. Do not use alcohol with glycerol added to it.
- Sterile gloves
 - Be sure to check the expiration date
- Sterile gauze
- Adhesive bandage
- Cotton ball
- Tourniquet
- Syringe
- Needle
 - A 21 gauge/20-25 mm needle is generally used for adults
 - A 23 gauge/20-25 mm needle is generally used for children
- Puncture-resistant, autoclavable container
- Thermal insulated transport carrier (such as extruded polystyrene foam)
- Blood culture bottle

C. Venipuncture and inoculating blood culture bottles

If possible, 1-3 ml of blood should be collected from a child, though it may be difficult to collect more than 1 ml, and 5-10 ml of blood should be collected from an adult. Collected blood should be diluted in blood culture broth in order to obtain blood cultures. Blood cannot be transported before being placed in a blood culture bottle because the syringes do not contain any anticoagulant and the blood will coagulate within a few minutes. A diagram of the proper method for collecting blood from the arm is shown in Figure 5.

If using alternative blood collection methods, such as a blood collection tube or a partially evacuated blood collection tube, immediately inoculate specimens into the blood-culture bottle using a needle and syringe after disinfecting the top of the bottle with 70% alcohol.

1. Gather all materials required for venipuncture (listed above).

- Note: blood culture bottles should be stored at 4°C when not in use and pre-warmed to room temperature (25°C) or 37°C before inoculation.
2. Label the blood culture bottle with appropriate information: patient name, date and time of blood culture bottle inoculation, and Unique Identification Number. Be sure this number matches the number on both the request and report forms.
 3. Disinfect the rubber septum of the blood culture bottle with a 70% alcohol swab and allow it to dry.
 - Do not use povidone-iodine on the rubber septum as it may be carried into the medium by the passing needle, thus inhibiting the growth of bacteria.
 4. Select an arm and apply a tourniquet to restrict the flow of venous blood. The large veins of the forearm are illustrated in Figure 5. The most prominent vein is usually chosen.
 5. Wipe the skin with a 70% alcohol swab and then povidone-iodine. Allow to dry. If the vein is palpated again, change gloves and repeat the skin disinfection.
 6. Insert the needle into the vein with the bevel of the needle face up. Once the needle has entered the vein, withdraw the blood by pulling back on the barrel of the syringe in a slow, steady manner. **Air must not be pumped into the vein.** After the desired amount of blood is obtained, release the tourniquet and place a sterile cotton ball or gauze over the insertion site while holding the needle in place.
 7. Withdraw the needle and have the patient hold the cotton ball or gauze firmly in place until the wound has stopped bleeding. Cover the insertion site with an adhesive bandage.
 8. Immediately (within 1 minute) inoculate the blood into the blood culture medium to prevent the blood from clotting in the syringe. Do not attempt to recap the needle and discard it in a puncture-resistant, autoclavable discard container. Wipe the surface of the blood culture bottle with a 70% alcohol swab.
 - In general, follow the manufacturer's instructions for inoculating the specific size of blood culture bottle with the correct volume of blood.
 - For blood from young children, add 1-2 ml of blood into 20 ml of blood culture broth (approximately a 1:10 to 1:20 dilution).
 - For blood from adults, add 5-10 ml of blood into 50 ml of blood culture broth (approximately a 1:5 to 1:10 dilution).
 9. After inoculation, swirl the bottle several times to mix and transport to a microbiology laboratory immediately (See section III.D.)

- If immediate transport to a microbiology laboratory is not feasible, place the inoculated blood culture bottle in an incubator at 35-37°C with ~5% CO₂ (or in a candle-jar) until transport to a microbiology laboratory is possible.
- Inoculated blood culture bottles should not be placed in the refrigerator.

10. Remove gloves and discard in an autoclavable container.

11. Wash hands with antibacterial soap and water immediately after removing gloves.

D. Transporting blood culture bottles

Ideally, inoculated blood culture bottles should be transported to a microbiology laboratory immediately for overnight incubation at 35-37°C with ~5% CO₂ (or in a candle-jar) and subsequent culture onto a BAP and CAP. All inoculated blood culture media should be protected from temperature extremes (not less than 18°C or more than 37°C) with a transport carrier and thermal insulator (such as extruded polystyrene foam).

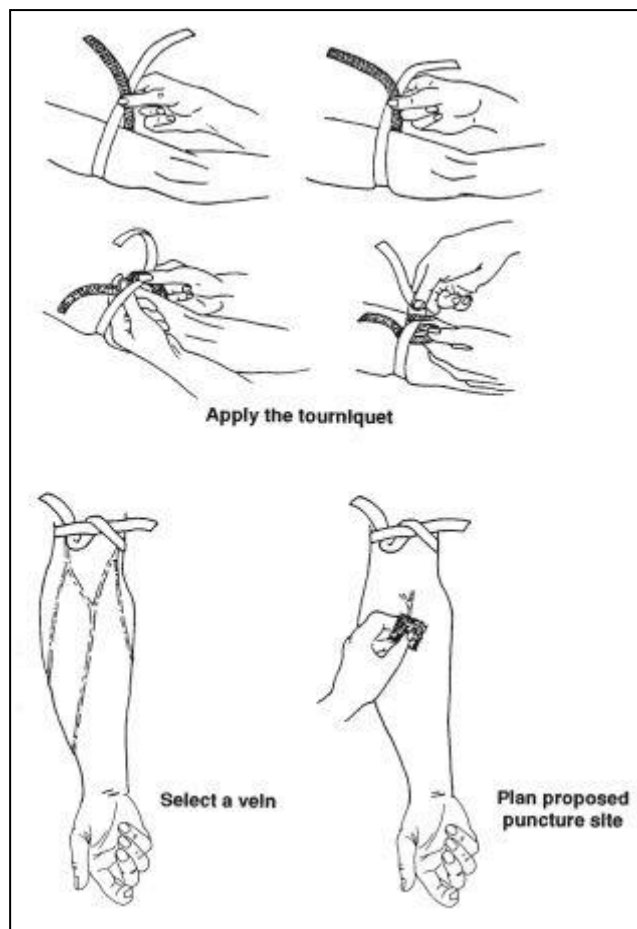


Figure 5. Collection of blood from an arm

References

1. **Ajello, G. W., Feeley, J. C., Hayes, P. S., Reingold, A.L., Bolan, G., Broome, C. V., et al.** 1984. Trans-Isolate medium: A new medium for primary culturing and transport of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. *Journal of Clinical Microbiology* **20**:55-58.

CHAPTER 6

Primary Culture and Presumptive Identification of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*

Microbiology laboratories commonly receive cerebrospinal fluid (CSF) or blood specimens from patients with meningitis, pneumonia, or unexplained febrile illness. Laboratories may also receive joint fluid, pleural fluid, or other sterile site specimens from these patients. Presumptive identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* can be made on the basis of a cytological examination of the CSF, specific colony morphology on blood and/or chocolate agar, staining properties on a Gram stain, or by detection of specific antigens in the CSF by a latex agglutination test or using a rapid diagnostic test (RDT). Methods for confirmatory identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* are presented in the next chapters of this laboratory manual (Chapters 7: Identification and Characterization of *N. meningitidis*, 8: Identification and Characterization of *S. pneumoniae*, and 9: Identification and Characterization of *H. influenzae*).

Personnel who are at risk of routine exposure to aerosolized *N. meningitidis* should strongly consider vaccination. Additional health and safety information can be found in Chapter 4: Biosafety. While laboratory-acquired infections with *S. pneumoniae* or *H. influenzae* are not as extensively reported, fatal infections with these bacteria can occur, and vaccination against these organisms may be recommended in some laboratories.

Because the primary purpose of this manual is to aid in the identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* from clinical specimens collected from suspected cases of bacterial meningitis, the methods described here will not allow for identification of other isolates that may be of clinical importance but are less likely encountered. Microbiologists should refer to clinical microbiology manuals, such as the American Society for Microbiology's *Manual of Clinical Microbiology*, for procedures to identify other bacteria.

I. Processing CSF specimens

A note about centrifugation: *g* (1 x gravity) represents relative centrifugal force (RCF), but the recommended centrifugation speed is often listed in protocols as revolutions per minute (RPM). RCF is dependent on the length of the radius of the rotor, thus the same RPM may not generate the same *g* force in another centrifuge. Therefore, RCF should be used to describe the centrifuge speed. If only RPM is given, RCF can be calculated using this formula:

$$\text{RCF} = 0.00001118 \times r \times \text{RPM}^2$$

r = radius of rotor in centimeters

Once the CSF arrives in the microbiology laboratory, the volume of CSF available for analysis should be noted. If < 1 ml of CSF is available, it should not be centrifuged; instead, the CSF should be plated directly onto a blood agar plate (BAP) and onto a chocolate agar plate (CAP) and also used for the Gram stain. If > 1 ml of CSF is available (i.e., if the specimen volume is sufficient for centrifugation), it must be centrifuged at a force sufficient to sediment the bacteria. Typically, centrifugation at 1000 x g for 10-15 minutes is sufficient to sediment bacteria.

After the specimen has been centrifuged, the supernatant should be drawn off with a Pasteur pipette and reserved if antigen detection by latex agglutination is planned. The sediment should be vigorously mixed (e.g., in a closed tube using a vortex machine). Once it has been well-mixed, one or two drops of sediment should be used to prepare the Gram stain and one drop should be used to streak the primary culture media.

A. Cytological examination of the CSF

Laboratory examination of the CSF is usually the first step to confirm the presence of bacterial meningitis. Note that cytological examination should precede centrifugation and heating of the CSF. Typical CSF abnormalities associated with bacterial meningitis include the following:

- Turbidity
- Increased opening pressure (>180 mm water)
- Pleocytosis (usually of polymorphonuclear (PMN) leukocytes); WBC counts >10 cells/mm³
- Decreased glucose concentration (<45 mg/dl)
- Increased protein concentration (>45 mg/dl)

Note: normal cytology of the CSF of an infant is 10-30 WBC/mm³ (50% PMNs).

B. Presumptive identification by Gram stain, latex agglutination, or rapid diagnostic test (RDT)

In combination with a clinical picture and CSF examination consistent with bacterial meningitis, a presumptive diagnosis of bacterial meningitis caused by *N. meningitidis*, *S. pneumoniae*, or *H. influenzae* can be made after performing a Gram stain of the CSF sediment or by detection of specific antigens in the CSF by a latex agglutination test or using RDTs. Positive results for any of these tests can rapidly provide evidence of infection, even if cultures fail to grow.

1. Performing a Gram stain

The Gram stain is an empirical method for differentiating bacterial species into two large groups based on the chemical and physical properties of their cell walls. Gram-positive bacteria retain the primary stain while gram-negative bacteria take the color of the counterstain. A Gram stain can also serve to assess the quality of a clinical specimen. The CSF should be properly centrifuged in order to obtain the sediment for the procedure. Proper smear preparation using the CSF sediment should produce a monolayer of organisms sufficiently dense for easy visualization but thin enough to reveal morphological characteristics. Clean, new glass slides should be used.

Positive and negative quality control (QC) strains should be tested along with the unknown specimens. In addition to known reference strains for *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*, other reference strains that may be used include *Staphylococcus aureus* for gram-positive cocci and *Escherichia coli* for gram-negative rods.

Gram stain procedure for CSF

1. Centrifuge the CSF for 10-15 minutes at 1000 x g, if > 1 ml is available (see above).
2. Divide a glass slide into two sections using a marker. Use one section for the unknown CSF and the other section for a known organism for QC.
3. Prepare a smear by placing 1-2 drops of the well-mixed CSF sediment on the slide, allowing the drop(s) to form one large slightly turbid, uniform suspension.
 - To prepare a smear using an isolate, add a small drop of sterile water or physiological saline to the slide and create a slightly turbid, uniform suspension of cells from an overnight culture.
4. Let the suspension air dry. The suspension **MUST** be completely dry before proceeding.
5. Fix the smear by the flooding the slide with 95% methanol for a minimum of 2 minutes (3). Rinse with distilled water. Shake off excess water.
 - If methanol is not available, heat-fix the smears by quickly passing the slide through a flame three times. Do not over-heat the slide as over-heating will cause significant distortion or destruction of the cells.
 - It is possible to use simple water (filet d'eau de robinet) if distilled water is not available for the entire Gram stain procedure.
6. Flood the slide with crystal violet ammonium oxalate for 1 minute to stain. Rinse with distilled water. Shake off excess water.
 - Avoid touching the slide with the tip of the reagent bottle or applying liquid directly onto the smear.
7. Flood the slide with Gram's iodine for 1 minute. The iodine acts as a mordant as it binds the alkaline crystal violet dye to the cell wall. Rinse with distilled water. Shake off excess water.
8. Decolorize with 95% ethanol until no more stain washes off (5-10 seconds may be enough). Rinse with distilled water. Shake off excess water.
 - It is essential to view decolorization closely: gram-positive bacteria can be made to appear gram-negative by over-decolorization and gram-negative bacteria can be made

to appear gram-positive by under decolorization.

9. Counterstain with safranin for 30 seconds or with carbol-fuchsin for 10-15 seconds. Rinse with distilled water. Shake off excess water.
10. Gently blot the slide using bibulous paper or a clean paper towel. Let air dry.
11. When dry, examine the stained smear under a microscope with 100X oil immersion objective.

Reading the Gram stain results (under microscopic examination):

- Gram-positive organisms will appear dark violet or purple.
- Gram-negative organisms will appear red or pink (from the counterstain).

N. meningitidis may occur intracellularly or extracellularly in PMN leukocytes and will appear as gram-negative, coffee-bean shaped diplococci.

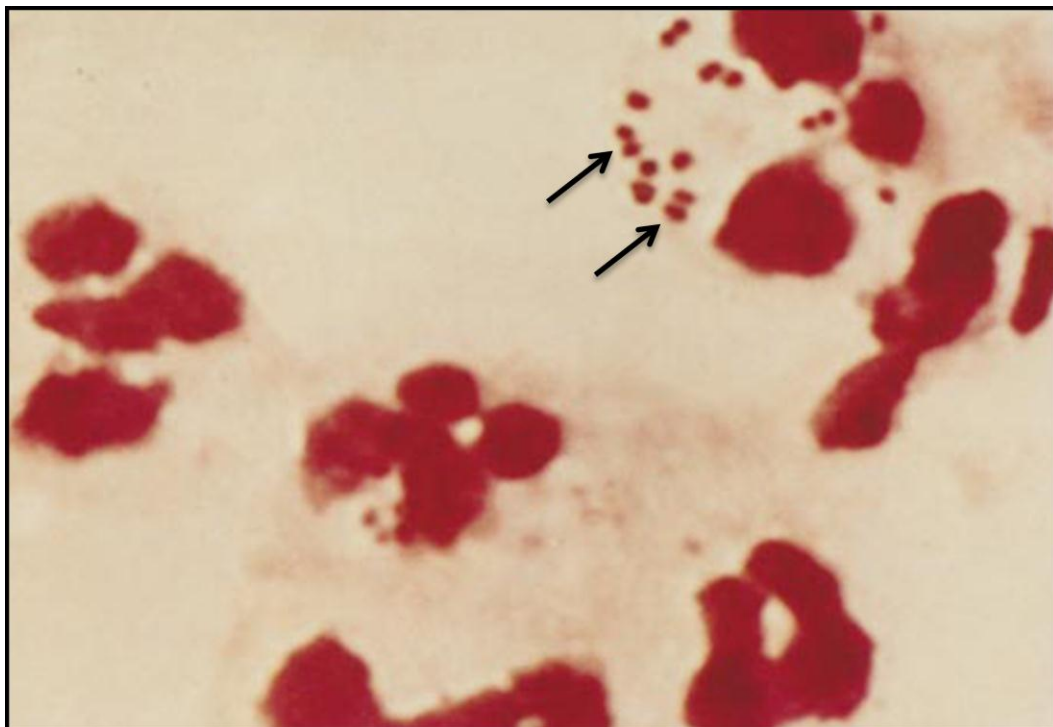


Figure 1. Gram stain of *N. meningitidis* in CSF with associated PMNs.

S. pneumoniae may occur intracellularly or extracellularly and will appear as gram-positive, lanceolate diplococci, sometimes occurring in short chains.

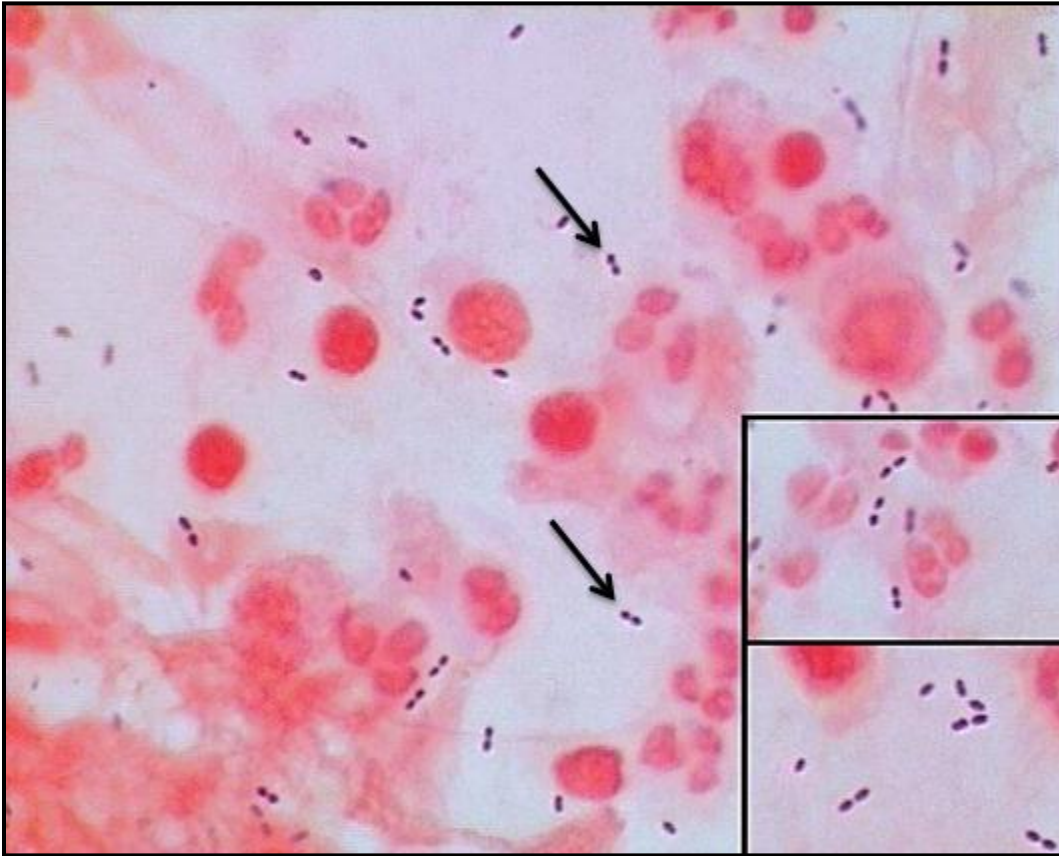


Figure 2. Gram stain of *S. pneumoniae* with WBCs

H. influenzae are small, pleomorphic gram-negative rods or coccobacilli with random arrangements.

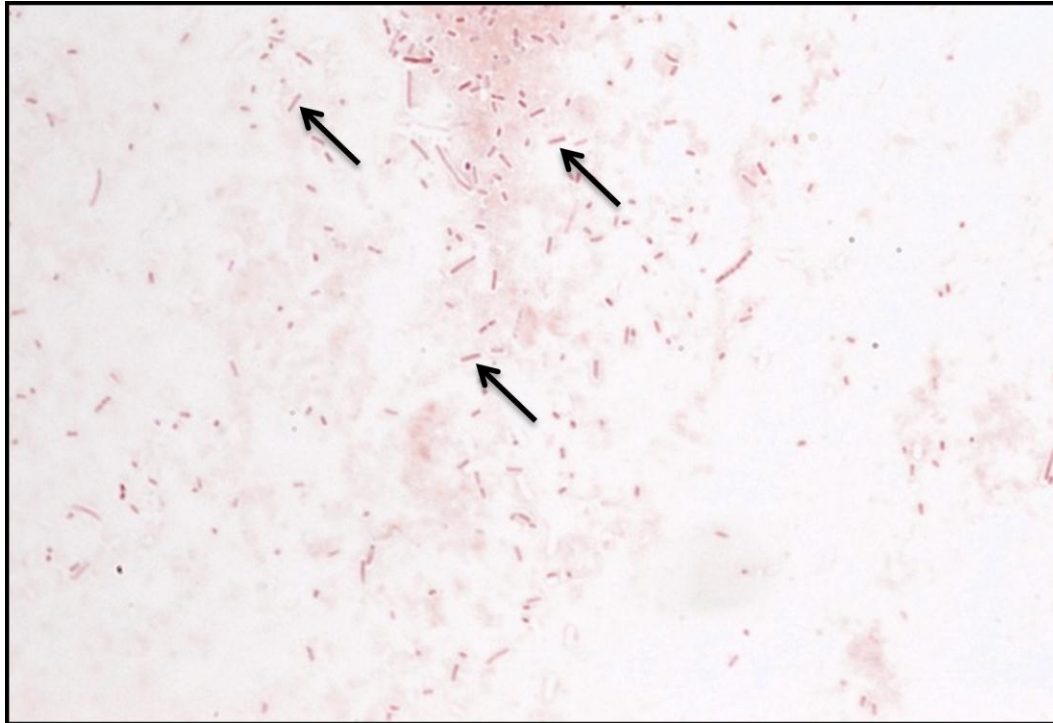


Figure 3. Gram stain of *H. influenzae*

2. Performing latex agglutination testing

Several commercial kits are available for latex agglutination testing. General recommendations and instructions for the detection of soluble bacterial antigens (capsular polysaccharide) are provided below, but the manufacturer's instructions included in the kit should be followed precisely when using these tests. For best results, the supernatant of the centrifuged CSF specimen should be tested as soon as possible. If immediate testing is not possible, the CSF specimen can be refrigerated (between 2-8°C) for several hours, or frozen at -20°C for longer periods. It is imperative that the kits be kept refrigerated before use, but never frozen, especially in tropical climates as the kits deteriorate at high temperatures which may make the test results unreliable before the expiration date of the kit.

Latex agglutination procedure for CSF

Follow the manufacturer's instructions on the package insert for the specific latex kit being used. General instructions are listed below:

1. Centrifuge the CSF for 10-15 minutes at 1000 x *g* and collect the supernatant.
 - The sediment should be used for Gram stain and primary culture.

2. Heat the CSF supernatant to be used for the test at 100°C for 3 minutes.
3. Shake the latex reagents gently until homogenous.
4. Place one drop of each latex reagent on a disposable card provided in the kit or a ringed glass slide.
5. Add 30-50 µl of the supernatant of the CSF to each latex reagent.
6. Rotate by hand for 2-10 minutes. If available, mechanical rotation at 100 rpm is recommended.
 - Avoid cross-contamination when mixing and dispensing reagents.
7. Examine the agglutination reactions under a bright light without magnification.

Reading the latex agglutination results

- Positive reaction: agglutination (or visible clumping) of the latex particles and slight clearing of the suspension occurs within 2-10 minutes (Figure 4).
- Negative reaction: the suspension remains homogenous and slightly milky in appearance.



Negative reaction

Positive reaction

Figure 4. Negative and positive latex agglutination reactions

3. Rapid diagnostic tests (RDTs)

A. RDT for meningococcal meningitis

RDTs have been developed for direct testing of CSF specimens without prior heat or centrifugation (2). The test is based on the principle of vertical flow immunochromatography in which gold particles and nitrocellulose membranes are coated with monoclonal antibodies to capture soluble serogroup-specific polysaccharide antigens in the CSF. The test consists of 2 duplex paper sticks (also called dipsticks), which together enable identification of four serogroups of *N. meningitidis* (A, C, W135, and Y). RDT1 tests for serogroups A and W135/Y and RDT2 tests for serogroups C and Y.

RDTs can be produced in large quantities, are relatively inexpensive, and remain stable for weeks in hot weather if protected from humidity; therefore, they are practical for immediate testing of specimens obtained during adverse conditions. Initial evaluation using RDTs on stored CSF from patients in Niger showed correct identification of the meningococcal serogroup 97% of the time (4). However, more recent studies under field conditions have shown similar specificity, but much lower sensitivity of 70% (6) and, in contrast, similar sensitivity but much lower specificity (5).

RDT procedure for CSF

Follow the manufacturer's instructions on the package insert. General instructions are listed below:

1. RDTs should be stored at 4°C in a moisture-proof bag until use.
2. Place the two dipsticks (RDT1 and RDT2) into two separate tubes (3 ml disposable plastic tubes are recommended) of 150-200 µl of CSF or a reference strain suspension in PBS, pH 7.2.
3. Record the chromatographic result on each strip after 10-15 minutes at room temperature (25°C).

Reading the RDT results

- Appearance of red lines on the dipsticks will indicate whether one of the four meningococcal serogroups has been detected in the CSF (Figure 5).
- The upper line on the dipstick is the positive control and should always be present.
- If the CSF is positive for one of the serogroups, a lower red line will also be present. The position of that red line indicates the specific serogroup based on the RDT that was tested. See Figure 5 for result combinations for each serogroup.
- A negative result consists of a single upper pink control line only.

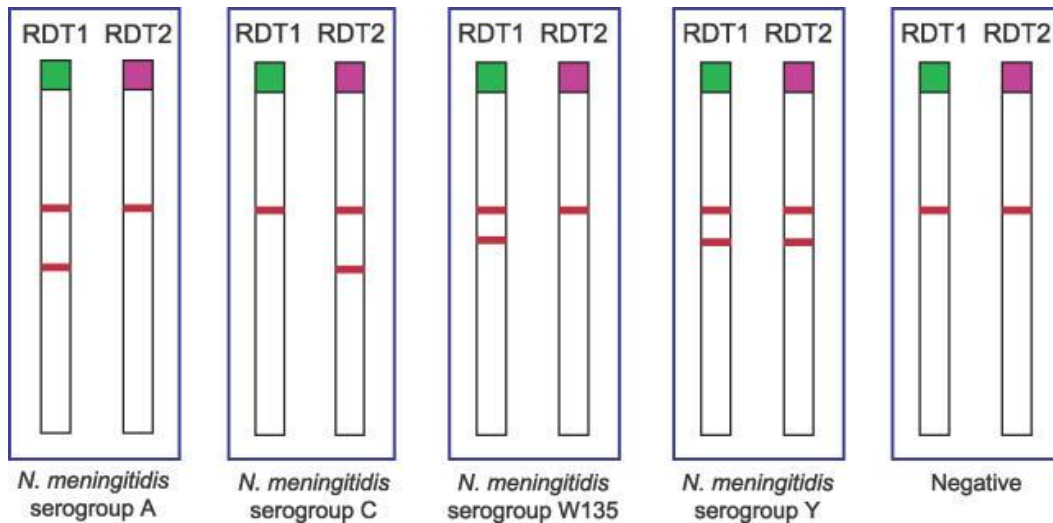


Figure 5. RDT results for *N. meningitidis* serogroups A, C, W135, and Y, as well as a negative control (2).

B. RDT for pneumococcal meningitis

RDT commercial kits are also available for *S. pneumoniae* detection by immunochromatography. The principle is the same as the one described above for *N.meningitidis*. Follow the manufacturer's instructions on the package insert.

II. Primary Culture

A. Selection of primary culture media

The best medium for growth of *S. pneumoniae* is a blood agar plate (BAP), which is a trypticase soy agar (TSA) plate containing 5% sheep blood. Human blood is NOT an acceptable substitute for the blood in the agar because the antibodies contained in human blood may inhibit bacterial growth. *S. pneumoniae* will also grow on a chocolate agar plate (CAP).

For *H. influenzae*, a CAP made with heat lysed blood or supplemented with hemin (X factor) and nicotinamide-adenine-dinucleotide (NAD; V factor) should be used. Growth of *H. influenzae* on a BAP may be achieved by adding a source of NAD, traditionally done by cross-streaking the inoculated medium with a *Staphylococcus aureus* or *Enterococcus* species strain. *H. influenzae* forms satellite colonies along the length of the staphylococcal or enterococcal growth. Additionally, applying filter paper (or disks) saturated with hemin and NAD to the surface of the BAP after the medium has been inoculated will produce a halo of growth around the strip or disk.

N. meningitidis grows on both BAP and CAP. Because *N. meningitidis* grows well in a humid atmosphere, if an infection with *N. meningitidis* is suspected, laboratorians may choose to add a shallow pan of water to the bottom of the incubator or add a dampened paper towel to the candle jar. The moisture source should be changed regularly to prevent contamination with molds.

Ordinarily, both BAP and CAP are used for subculture. If only one type of plate is available, a CAP should be used because it contains the hemin and NAD needed for *H. influenzae*, whereas a BAP does not.

If primary cultures appear to be contaminated, selective media may improve the isolation of these bacteria from specimens containing a mixed flora of bacteria and/or fungi. For primary isolation of *N. meningitidis*, a chocolate agar base containing vancomycin, colistin, nystatin, and trimethoprim can be used. For isolation of *S. pneumoniae*, tryptic soy agar with 5% sheep blood and either gentamicin, neomycin or sulfamethoxazole-trimethoprim (SXT) may be useful. For isolation of *H. influenzae*, chocolate agar with bacitracin can be used. Other antibiotic formulations are also available.

B. QC of primary culture media

All primary culture media should be tested for QC to ensure that the media will support the proper growth of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. One plate from each new lot of media received should be tested using an appropriate, well-characterized reference strain of *N. meningitidis*, *S. pneumoniae*, and/or *H. influenzae*. In addition, all media should be tested periodically (every 3 months) to ensure that it can support the growth of appropriate bacteria. One uninoculated plate from each new lot should also be tested in order to check for contamination of mold or other organisms in the laboratory and/or incubator. QC should be repeated on plates from a lot if they have been exposed to temperatures above 4°C or if there is reason to suspect that the plates have been contaminated since the initial QC was performed.

Procedure for QC of primary culture media

1. Inspect the media for any visible microbial contamination, discoloration, drying, deterioration, or other physical defects that may interfere with use.
2. Inoculate the media with pure colonies from 18-24 hour growth of a well-characterized reference strain. Streak for isolation and incubate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
3. Incubate an uninoculated plate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
4. Examine the inoculated and uninoculated plates after 18-24 hours.

Reading QC testing results

- Passing result: proper growth of the reference strain on appropriate media and no growth on uninoculated media.
- Failing result: no growth or poor growth of the reference strain on appropriate media and growth of organisms on the uninoculated media.

III. Inoculation of primary culture media from CSF specimens

A. Primary culture directly from CSF

1. If the CSF can be transported to a microbiology laboratory immediately (within 1 hour from the time of collection) for culture and analysis, inoculate 1-5 drops of CSF (depending on volume received in laboratory) directly onto both a BAP and CAP within 1 hour after collection.
 - If the CSF was centrifuged, use 1 drop of the well-mixed sediment for primary culture.
2. Using a sterile bacteriological loop, cross-streak the inoculum to obtain single, isolated colonies.
 - Disposable loops are preferred, but if using a wire loop, it must be sterilized prior to each step of the plate-streaking process.
 - BAP and CAP that have been properly streaked are shown in Figures 6, 7, and 8.
3. A back-up broth (e.g., brain-heart infusion broth with proper supplements) should be inoculated with some of the sediment pellet.
4. Agar plates and broth inoculated with the CSF sediment should be incubated for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

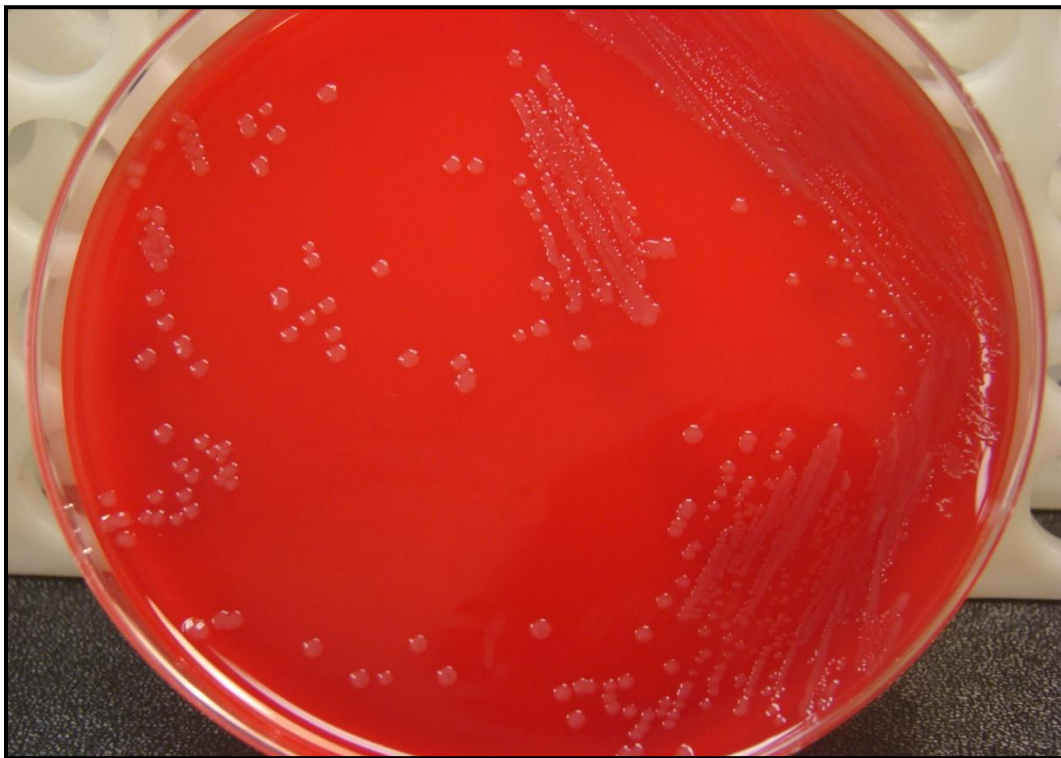


Figure 6. Proper streaking and growth of *N. meningitidis* on a BAP

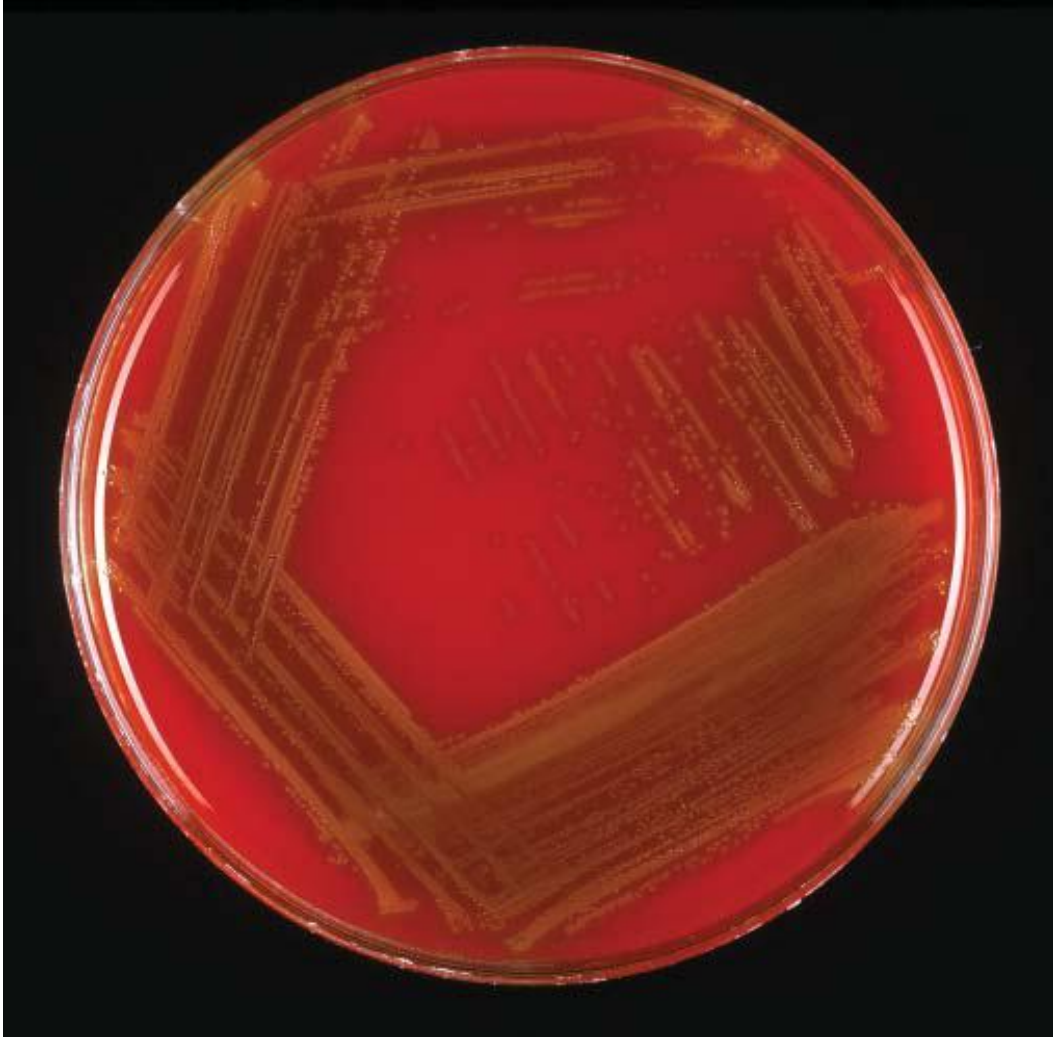


Figure 7. Proper streaking and growth of *S. pneumoniae* on a BAP

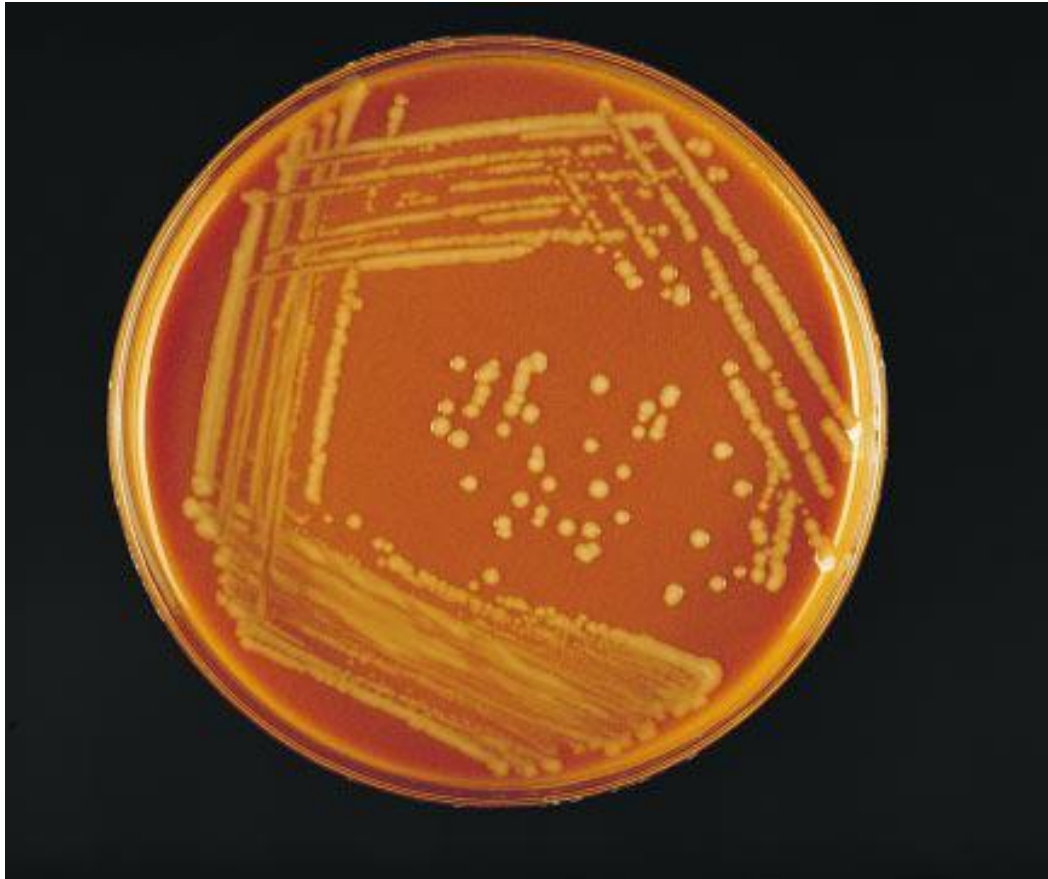


Figure 8. Proper streaking and growth of *H. influenzae* on a CAP

B. Primary culture from Trans-Isolate (T-I) medium

1. If the CSF cannot be transported to a microbiology laboratory immediately (within 1 hour from the time of collection) for culture and analysis, a bottle of T-I medium should be inoculated (1). To do this remove the aluminum cap with forceps, wipe the rubber stopper with a 70% alcohol swab (do not use povidone-iodine) and use a syringe to aseptically inoculate 0.5-1.0 ml of the CSF into the T-I medium for transport and growth of bacteria.
 - a. If the T-I medium cannot be transported to a microbiology laboratory the same day of inoculation, insert a venting needle (sterile cotton-plugged hypodermic needle) through the rubber stopper of the T-I bottle, which will encourage growth and survival of the bacteria.
 - Be sure that the venting needle does not touch the broth.
 - b. Incubate the inoculated T-I medium at 35-37°C with ~5% CO₂ (or in a candle-jar) overnight or until transport is possible. If transportation is delayed more the 4 days,

- remove the vented T-I bottle from the incubator or candle jar and place it at room temperature (25°C) until shipment.
- c. Remove the venting needle and wipe the rubber stopper with 70% alcohol before shipment. It is essential to avoid contamination when sampling the bottles to obtain specimens aseptically.
 - d. Once the T-I medium arrives in the microbiological laboratory, it can be cultured immediately.
2. If the T-I medium can be transported to a microbiology laboratory the same day of inoculation, do not vent the T-I bottle until it arrives in the receiving laboratory. Upon arrival, wipe the rubber stopper with 70% alcohol, insert a venting needle into the T-I bottle, incubate at 35-37°C with ~5% CO₂ (or in a candle-jar), and observe daily for turbidity in the liquid phase for up to 7 days.
 - Prior to subculture, remove the venting needle and wipe the rubber stopper with 70% alcohol.
 - Do not use povidone-iodine on the rubber stopper as it may be carried into the medium by the passing needle, thus inhibiting the growth of bacteria.
 3. After 18-24 hours of incubation at 35-37°C with ~5% CO₂ (or in a candle-jar) with a venting needle, use a sterile needle and syringe to transfer 50-100 µl of the liquid portion of the T-I medium onto both a BAP and CAP for primary culture.
 - Approximately 50-100 µl is used to streak each plate. To streak two plates, draw approximately 100-200 µl with the syringe at one time to minimize the possibility of contaminating the T-I medium.
 4. Streak the BAP and CAP for isolation, incubate the plates at 35-37°C with ~5% CO₂ (or in a candle-jar), and examine the plates daily for up to 72 hours.
 5. If no growth is observed, subculture the T-I medium again on day 4 and day 7.
 6. Isolates should always be inspected for purity of growth by looking at colony morphology before any testing is performed. If any contamination is seen, cultures should be re-streaked to ensure purity prior to testing.
 7. If the T-I medium appears to be contaminated, selective media may be used (see Section II.A.).

IV. Processing blood specimens

Laboratory personnel handling blood culture specimens must be able to isolate bacteria on appropriate primary culture media and properly subculture isolates to obtain pure cultures for testing.

A. Primary culture from a blood culture bottle

The blood culture bottle should be immediately inoculated (within 1 minute) after venipuncture to prevent the blood from clotting in the syringe. The inoculated blood culture bottle should be transported to a microbiology laboratory as soon as possible for incubation and subculture.

1. If transport to a microbiology laboratory is not possible the same day, place the blood culture bottle in an incubator at 35-37°C with ~5% CO₂ (or in a candle-jar) until transport is possible. Inoculated blood culture bottles should not be placed in the refrigerator.
2. If transport to a microbiology laboratory is feasible the same day, incubate the blood culture bottle at 35-37°C with ~5% CO₂ (or in a candle-jar).
3. Examine the blood culture bottle for turbidity at 14-17 hours and then every day for up to 7 days. Any turbidity or lysis of erythrocytes may be indicative of growth, and subcultures onto primary culture media should be made immediately.
 - Because *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* are fragile organisms, subcultures should be performed on day 4 and day 7 regardless of turbidity, as the absence of turbidity does not always correlate with the absence of bacterial growth.
4. Before subculture, swirl the blood culture bottle several times to mix the contents.
5. Disinfect the rubber septum of the blood culture bottle with a 70% alcohol swab.
 - Do not use povidone-iodine on the rubber septum as it may be carried into the medium by the passing needle, thus inhibiting the growth of bacteria.
 - Alternatively, if the blood culture bottle has a screw-cap, open the bottle and remove the fluid using sterile technique (i.e., flaming the bottle mouth upon opening and closing the cap).
6. Aspirate 1 ml with a sterile syringe and needle from the blood culture bottle and transfer 0.5 ml to a BAP and 0.5 ml to a CAP.
7. Streak the plates for isolation, incubate at 35-37°C with ~5% CO₂ (or in a candle-jar), and examine daily for up to 72 hours.
8. Isolates should always be inspected for purity of growth by looking at colony morphology before any testing is performed. If any contamination is seen, cultures should be re-streaked to ensure purity prior to testing.

9. Once pure bacterial growth has been confirmed by subculture from the blood culture bottle, the bottle should be disposed of according to proper safety procedures.

B. MacConkey agar media

If the microbiology laboratory has the resources to support the use of a third plate for subculture, MacConkey agar should be used, particularly if the specimen was obtained from a patient with fever of unknown origin, when typhoid fever (*S. typhi*) is suspected symptomatically, or if a Gram stain of blood culture broth reveals gram-negative bacilli. MacConkey agar is designed to grow gram-negative bacteria and stain them for lactose fermentation, in order to distinguish those that can ferment the sugar lactose (Lac+) from those that cannot (Lac-). It contains bile salts (to inhibit most gram-positive bacteria, except *Enterococcus* and some species of *Staphylococcus*, i.e., *Staphylococcus aureus*), crystal violet dye (which also inhibits certain gram-positive bacteria), neutral red dye, (which stains microbes fermenting lactose), lactose, and peptone.

1. Transfer approximately 0.5 ml of the blood culture broth onto MacConkey agar and streak for isolated colonies.
2. Incubate the MacConkey agar for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
3. Inspect plate for growth and identify the bacteria (Figure 9).



Figure 9. Growth of *Salmonella* ser. Typhi on MacConkey agar

V. Macroscopic examination of colonies

Presumptive identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* can be made on the basis of growth and colony morphology on a BAP and CAP and a Gram stain of the organisms (Table 1 and Figures 10 and 11).

Table 1. Presumptive identification of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* based on growth on primary culture media and Gram stain results

Growth on CAP	Growth on BAP	Gram stain	Presumptive ID
+	+	gram-negative diplococci	<i>N. meningitidis</i>
+	+	gram-positive diplococci	<i>S. pneumoniae</i>
+	-	gram-negative pleomorphic coccobacilli	<i>H. influenzae</i>



Figure 10. Growth of *N. meningitidis* on lower left and *S. pneumoniae* on upper left of a BAP



Figure 11. Growth of *N. meningitidis* on lower left, *S. pneumoniae* on top, and *H. influenzae* on lower right of a CAP

H. influenzae appear as large, colorless-to-grey, opaque colonies on a CAP (Figure 12). No hemolysis or discoloration of the medium is apparent. Encapsulated strains appear more mucoidal than non-encapsulated strains, which appear as smaller compact grey colonies. *H. influenzae* cannot grow on a BAP.

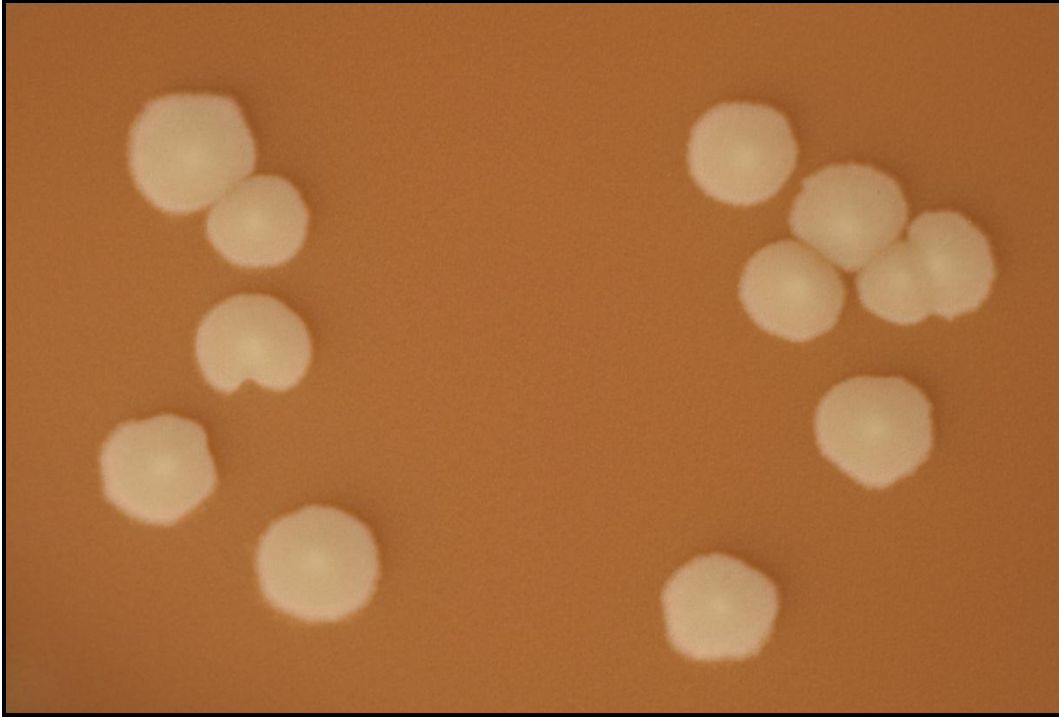


Figure 12. *H. influenzae* colonies on a CAP

Young colonies of *N. meningitidis* are round, smooth, moist, glistening, and convex, with a clearly defined edge on a BAP (Figure 13). Some colonies appear to coalesce with other nearby colonies. Actively growing colonies of *N. meningitidis* on a BAP are grey and unpigmented. Older cultures (> 24 hours) become more opaquely grey and sometimes cause the underlying agar to turn dark. On a CAP, *N. meningitidis* appear similar to *H. influenzae* (see description above). While *H. influenzae* produce a pungent indol smell that can differentiate it from *N. meningitidis*, plates should not be opened in order to smell the cultures.

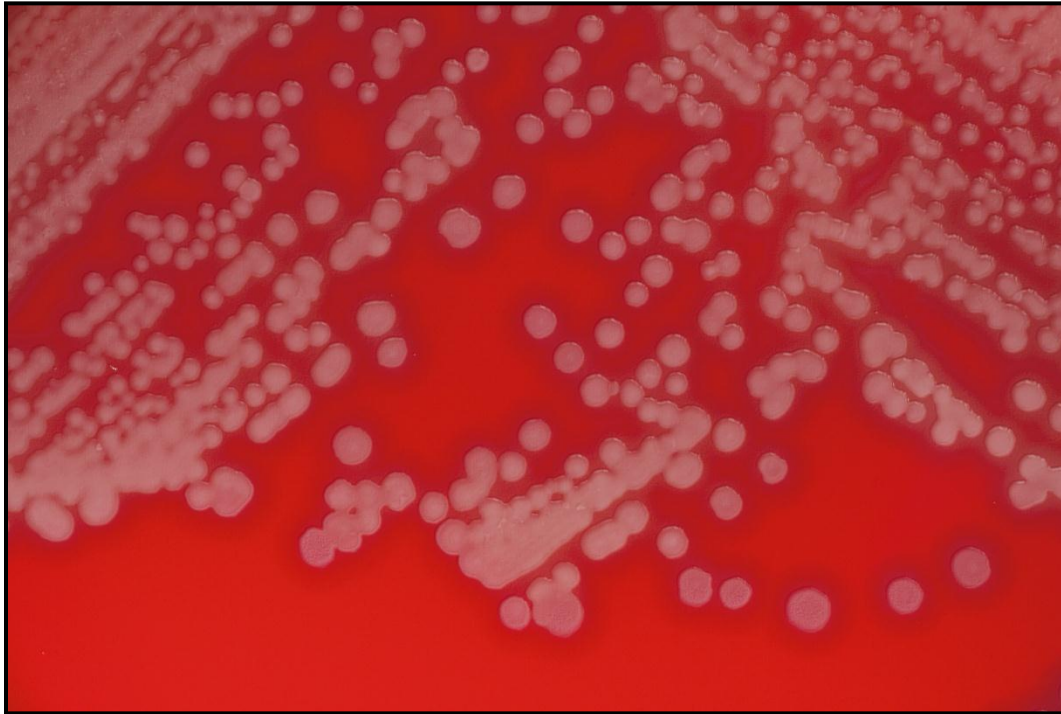


Figure 13. *N. meningitidis* colonies on a BAP

S. pneumoniae appear as small, grey, moist (sometimes mucoidal), watery colonies with a surrounding green zone of alpha-hemolysis on a BAP (Figure 14) and CAP. The degree of mucoidness of *S. pneumoniae* colonies is strain and serotype dependent and is also influenced by the freshness of the medium and the incubation atmosphere. Some serotypes appear more mucoidal than others; the fresher the medium, the more mucoidal the cultures appear. Young pneumococcal colonies appear raised, similar to viridans streptococci. Differentiating pneumococci from viridans streptococci on a CAP is difficult. However, once the pneumococcal culture ages 24-48 hours, the colonies become flattened, and the central portion becomes depressed, which does not occur with viridans streptococci (Figure 15). A microscope (30X-50X) or a 3X hand lens can also be a useful tool in differentiating pneumococci from alpha-hemolytic viridans streptococci.

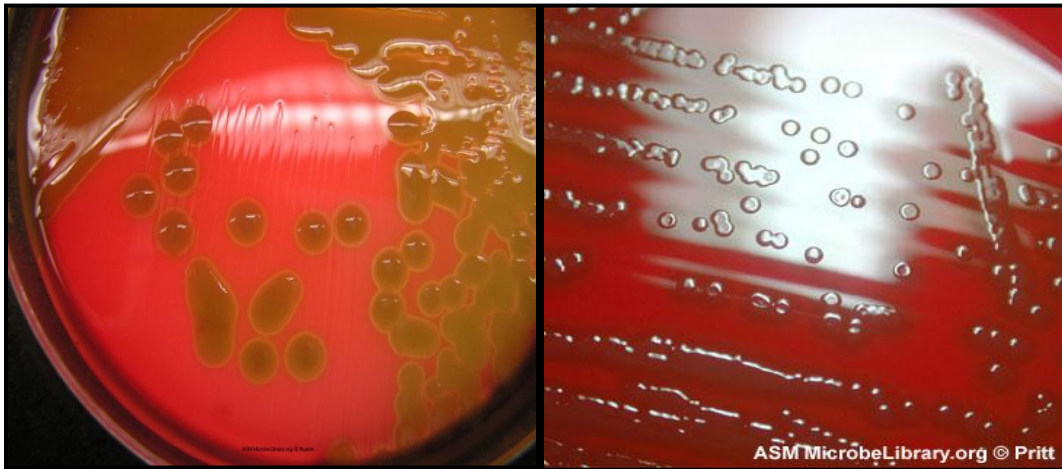


Figure 14. *S. pneumoniae* colonies on a BAP

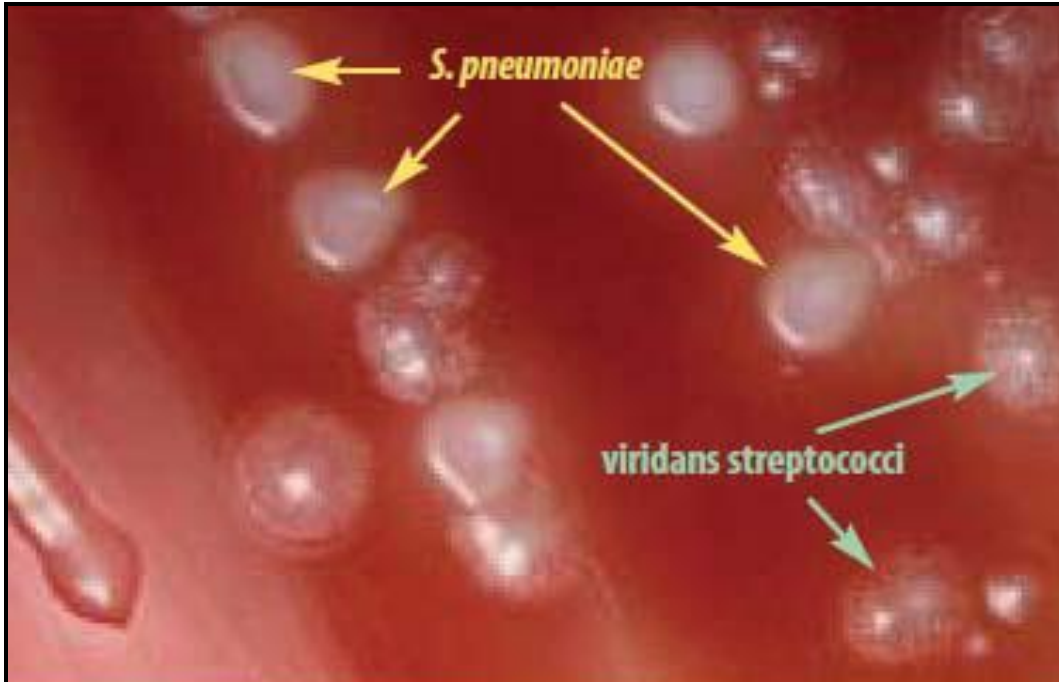


Figure 15. *S. pneumoniae* colonies have a flattened and depressed center after 24-48 hours of growth on a BAP, whereas the viridans streptococci retain a raised center

Other organisms that might appear on the culture plate along with *S. pneumoniae* are *Staphylococcus aureus*, *Staphylococcus epidermidis*, or another *Staphylococcus* species. Figure 16 shows the two different types of colonies growing on a BAP. The dull gray flat colony surrounded by a green zone of hemolysis is *S. pneumoniae* and the white colony with no hemolytic activity is *S. epidermidis*.



Figure 16. The small grey, flat colonies surrounded by a greenish zone of alpha-hemolysis are *S. pneumoniae* and the white colonies with no hemolytic activity are *S. epidermidis* (black arrow).

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CHAPTER 7

Identification and Characterization of *Neisseria meningitidis*

N. meningitidis are gram-negative, coffee-bean shaped diplococci that may occur intracellularly or extracellularly in PMN leukocytes. *N. meningitidis* is a fastidious organism, which grows best at 35-37°C with ~5% CO₂ (or in a candle-jar). It can grow on both a blood agar plate (BAP) and a chocolate agar plate (CAP). Colonies of *N. meningitidis* are grey and unpigmented on a BAP and appear round, smooth, moist, glistening, and convex, with a clearly defined edge. *N. meningitidis* appear as large, colorless-to-grey, opaque colonies on a CAP. Prior to identification and characterization testing procedures, isolates should always be inspected for purity of growth and a single colony should be re-streaked, when necessary, to obtain a pure culture. For the following identification and characterization procedures, testing should be performed on 18-24 hour growth from a BAP (Figure 1) or a CAP (Figure 2) at 35-37°C with ~5% CO₂ (or in a candle-jar).

The following tests are recommended to confirm the identity of cultures that morphologically appear to be *N. meningitidis* (Figure 3). *N. meningitidis* can be identified using Kovac's oxidase test and carbohydrate utilization. If the oxidase test is positive, carbohydrate utilization testing should be performed. If the carbohydrate utilization test indicates that the isolate may be *N. meningitidis*, serological tests to identify the serogroup should be performed. This sequence of testing is an efficient way to save costly antisera and time. Additional methods for identification and characterization of *N. meningitidis* using molecular tools are described in Chapter 10: PCR Methods and Chapter 12: Molecular Methods.

Biosafety Level 2 (BSL-2) practices are required for work involving isolates of *N. meningitidis*, as this organism presents a potential hazard to laboratory personnel and the surrounding working environment. Please refer to Chapter 4: Biosafety in order to follow the guidelines that have been established for laboratorians working in BSL-2 facilities as many of the tests described in this chapter require opening plates with live cultures and are often performed outside of a biosafety cabinet (BSC).

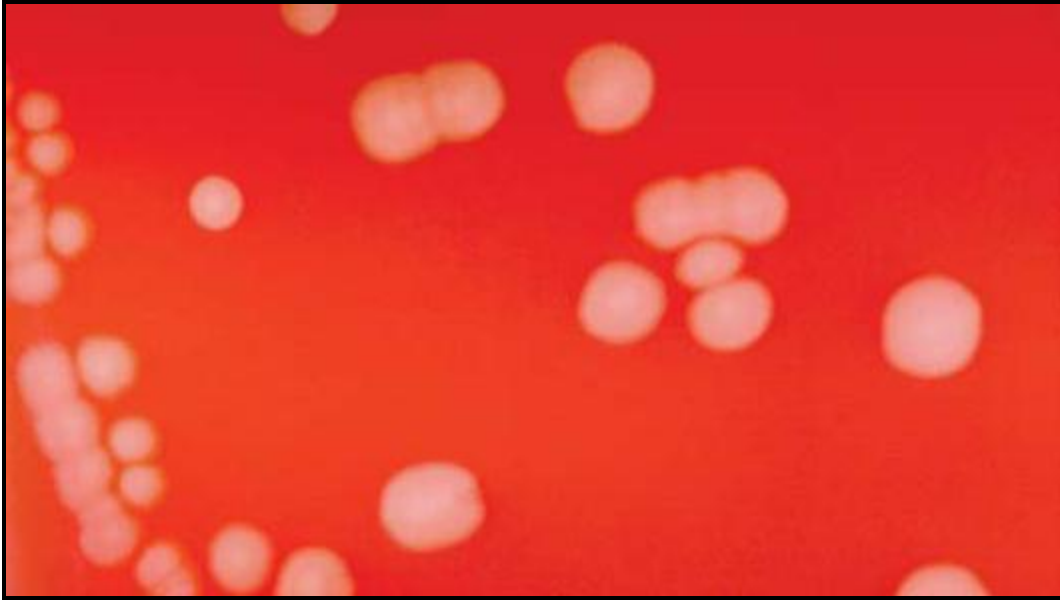


Figure 1. *N. meningitidis* colonies on a BAP

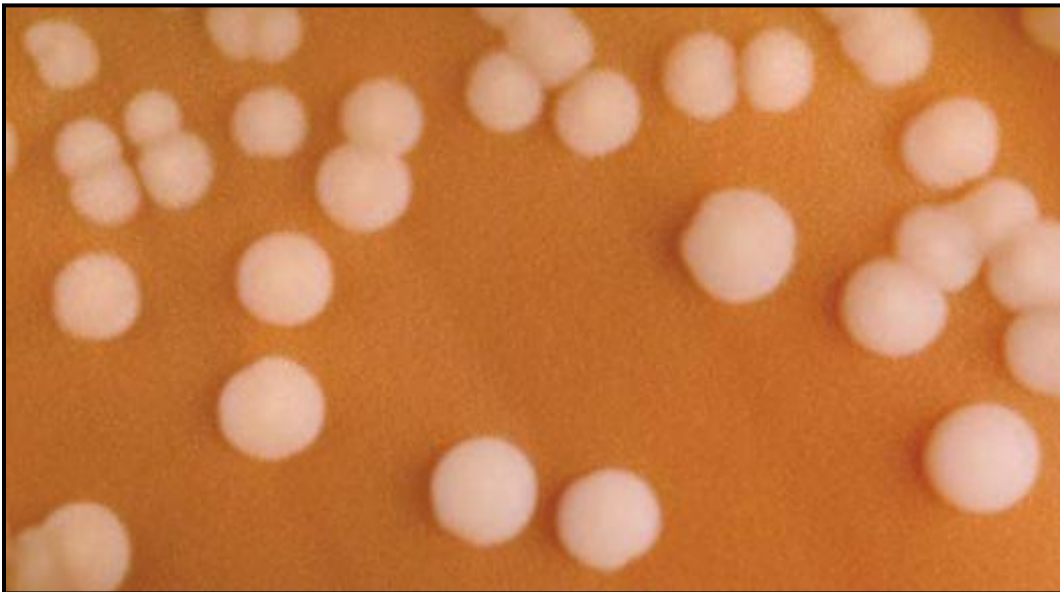


Figure 2. *N. meningitidis* colonies on a CAP

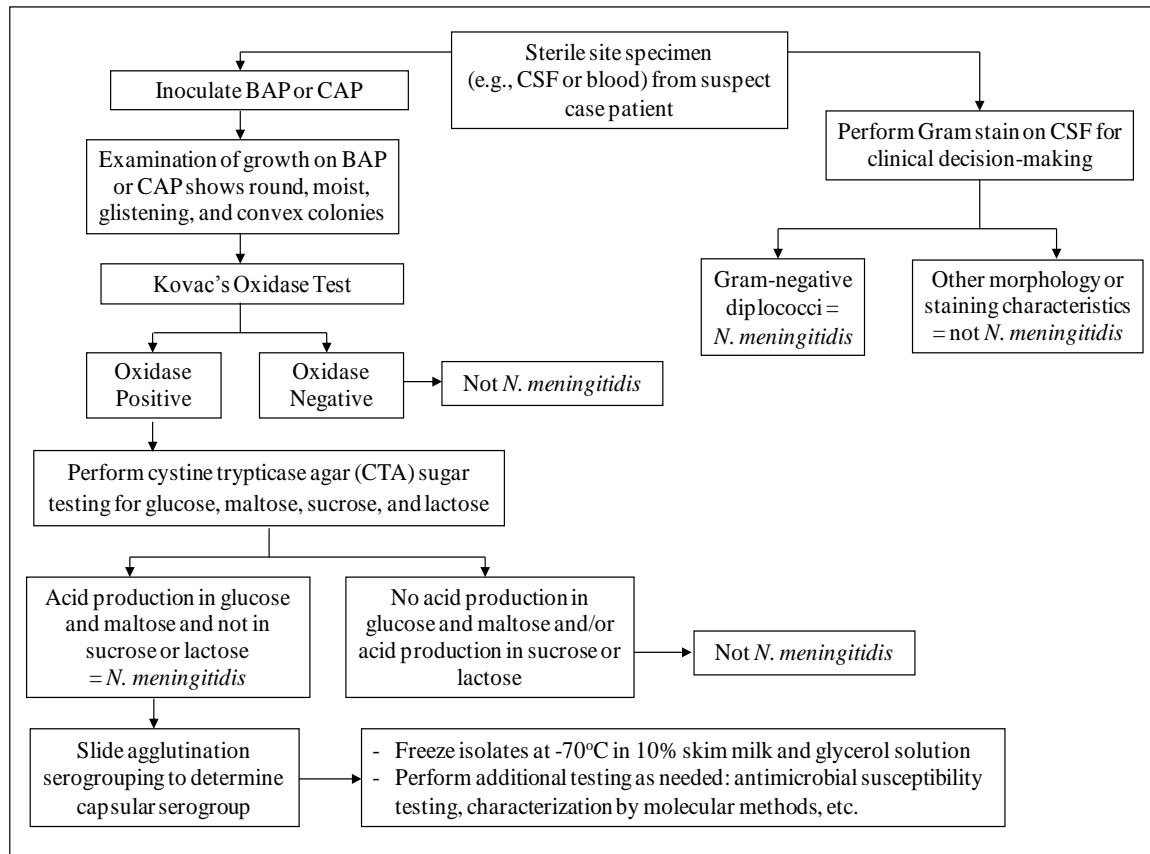


Figure 3. Flow chart for identification and characterization of a *N. meningitidis* isolate

I. Kovac's oxidase test

Kovac's oxidase test determines the presence of cytochrome oxidase. Kovac's oxidase reagent, tetramethyl-p-phenylenediamine dihydrochloride, is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain. This test aids in the recognition of *N. meningitidis*, but other members of the genus *Neisseria*, as well as unrelated bacterial species, may also give a positive reaction. Positive and negative quality control (QC) strains should be tested along with the unknown isolates to ensure that the oxidase reagent is working properly.

A. Preparation of 1% oxidase reagent from oxidase powder

To prevent deterioration of stock oxidase powder, the powder should be stored in a tightly sealed desiccator and kept in a cool, dark area. Kovac's oxidase reagent is intended only for *in vitro* diagnostic use. Avoid contact with the eyes and skin as it can cause irritation. In case of accidental contact, immediately flush eyes or skin with water for at least 15 minutes.

1. Prepare a 1.0% Kovac's oxidase reagent by dissolving 0.1 g of tetramethyl-p-phenylenediamine dihydrochloride into 10 ml of sterile distilled water.

2. Mix well and then let stand for 15 minutes.

- The solution should be made fresh daily and the unused portion should be discarded.
- Alternatively, the reagent could be dispensed into 1 ml aliquots and stored frozen at -20°C. The aliquots should be removed from the freezer and thawed before use. Discard the unused portion each day the reagent is thawed.

B. Performing Kovac's oxidase test

Filter paper method

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. On a nonporous surface (i.e., Petri dish or glass plate), wet a strip of filter paper with a few drops of Kovac's oxidase reagent.
3. Let the filter paper strip air dry before use.
4. Use a disposable plastic loop, a platinum inoculating loop, or a wooden applicator stick to pick a portion of a colony from overnight growth on the BAP and rub it onto the treated filter paper (Figure 4).
 - Do not use a nichrome loop, as it may produce a false-positive reaction.
5. Observe the filter paper for color change to purple.
6. Perform steps 3 and 4 with a positive and negative QC strain to ensure that the oxidase reagent is working properly.

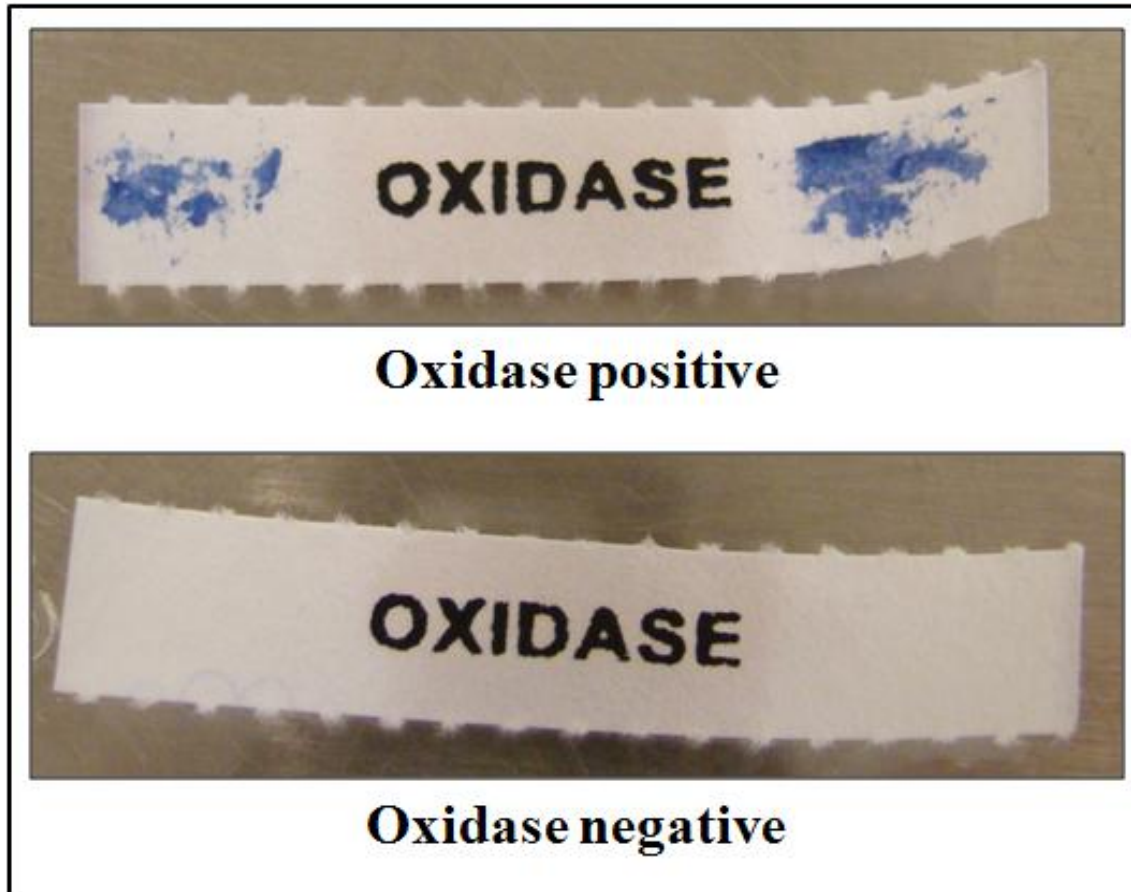


Figure 4. Kovac's oxidase test: a negative and positive reaction on filter paper

Plate method

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Dispense a few drops of Kovac's oxidase reagent directly on top of a few suspicious colonies growing on the 18-24 hour BAP.
 - Do not flood the entire plate as the bacteria exposed to the reagent are usually not viable for subculture.
3. Tilt the plate and observe colonies for a color change to purple.
4. Perform steps 1 and 2 with a positive and negative QC strain to ensure that the oxidase reagent is working properly.

C. Reading the oxidase test results

- Positive reactions will develop within 10 seconds in the form of a purple color where the bacteria were applied to the treated filter paper. Delayed reactions are unlikely with *N. meningitidis*.
- Negative reactions will not produce a color change on the treated filter paper.

II. Carbohydrate utilization by *N. meningitidis*: cystine trypticase agar (CTA) method

Carbohydrate utilization tests are used to validate the identification of a strain as *N. meningitidis*. For this procedure, 4 different carbohydrates (glucose [also called dextrose], maltose, lactose, and sucrose) are added to tubes containing a CTA base for a final concentration of 1%. A phenol red indicator is also included in the medium. It is a sensitive indicator that develops a yellow color in the presence of acid at a pH of 6.8 or less. A panel of four tubes, each containing a different carbohydrate, is used to test each isolate. *Neisseria* spp. produce acid from carbohydrates by oxidation, not fermentation. *N. meningitidis* oxidizes glucose and maltose, but not lactose or sucrose. While it is extremely rare, strains of *N. meningitidis* have been reported to either utilize glucose or maltose, but not both. Well-characterized QC strains should be tested along with the unknown isolates to ensure that the CTA sugars are working properly. *N. meningitidis* and/or *N. lactamica* isolates should be used to QC the CTA sugar media. Methods for preparation of the CTA sugar media are included in the Annex. CTA sugars should be stored at 4°C and warmed to room temperature (25°C) before use.

A. Performing CTA sugar testing

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Allow the 4 CTA sugars, glucose (also called dextrose), maltose, lactose, and sucrose, to warm to room temperature (25°C) and label the tubes with the lab ID.
3. Remove 3-5 colonies from overnight growth on the BAP using a 1 µl disposable loop.
4. Stab the CTA sugar several times into the upper 10 mm of medium. Approximately 8 stabs with the same loopful are sufficient.
 - Use a separate disposable loop for inoculating each carbohydrate to be tested.
5. Fasten the screw-cap of each tube loosely and place the tubes in a 35-37°C incubator without CO₂. Incubate the CTA sugars for at least 72 hours (and up to 5 days) before discarding them as negative.
6. Observe the CTA sugars for development of visible turbidity and color change to yellow.
7. Perform steps 1-5 using a *N. meningitidis*, *N. lactamica*, and a *N. sicca* QC strain to ensure that the CTA sugars are working properly.

B. Reading the CTA sugar results

Development of visible turbidity and a yellow color in the upper portion of the medium indicates growth of bacteria and production of acid and is interpreted as a positive test (Figure 5). Although reactions may occur as early as 24 hours after inoculation, some reactions are delayed and negative results should not be interpreted before 72 hours of incubation. A color change to yellow without turbidity is usually not a positive reaction. Carbohydrate utilization results for differentiating *N. meningitidis* from other *Neisseria* spp. and bacteria are listed in Table 1.



Figure 5. CTA sugar reactions for *N. meningitidis* with utilization of glucose (dextrose) and maltose, indicated by acid production (color change to yellow), and no utilization of lactose or sucrose

Table 1. Carbohydrate utilization by some *Neisseria* and *Moraxella* spp.

Organism	Acid Production from:			
	Glucose ¹	Maltose	Lactose	Sucrose
<i>Neisseria meningitidis</i>	+	+	-	-
<i>Neisseria lactamica</i>	+	+	+	-
<i>Neisseria gonorrhoeae</i>	+ ²	-	-	-
<i>Neisseria sicca</i>	+	+	-	+
<i>Moraxella catarrhalis</i>	-	-	-	-

¹ Glucose may also be referred to as dextrose.

² Some strains of *N. gonorrhoeae* are weak acid producers and may appear to be glucose negative in the CTA medium.

III. Identification of *N. meningitidis* serogroup

Twelve serogroups, based on the biochemical composition of capsular polysaccharides, are currently recognized: A, B, C, H, I, K, L, W135, X, Y, Z, and 29E (Z'). Serogroup D is no longer recognized as a serogroup. Serogroups A, B, C, W135 and Y are the 5 most common causes of bacterial meningitis. Serogroup A has been the most common cause of epidemics in Africa and Asia. Serogroups C, W135, and X have also been reported as causes of epidemics in several parts of Africa as well. Serogroup-specific antisera for these major serogroups are available commercially.

It is not always practical to test for all serogroups for which antisera are available in a laboratory. Testing algorithms may be set up in laboratories with previous knowledge of the predominance or lack of serogroups within that particular geographic region in order to test for the most common serogroups first. Modifications may be made to the testing algorithm for any laboratory based on information about current strains that are circulating in the region. For example, in Africa, testing with antisera for serogroups A and W135 (and X in some regions) as well as with a saline control to detect nonspecific autoagglutination should be adequate to characterize most case isolates. Strains reacting negatively with A, W135, and X antisera should then be tested with other available antisera, particularly C, Y, and B. Nearly all case isolates are serogroupable, if they are tested against a comprehensive panel of antisera and proper controls are used. In rare instances, an isolate that does not react with any of the serogroup-specific antisera or reacts with more than one serogroup-specific antisera is considered nongroupable (NG). Polyvalent antisera containing combinations of serogroups (e.g., A, B, and C or X, Y, W135, and Z) may also be used.

It is essential that reference laboratories have the capacity to isolate, identify, and characterize the serogroup of isolates of *N. meningitidis*. This valuable data provides laboratories and public health authorities with the tools to identify outbreaks controllable by vaccination campaigns, recognize serogroups causing sporadic disease, and detect emergence of new outbreak strains.

A. Slide agglutination serogrouping (SASG) test for serogrouping *N. meningitidis* isolates

Formalin-killed meningococcal suspensions should be used for SASG testing rather than saline suspensions of living organisms to maintain a safe working environment. A solution of 5% formalinized physiological saline is sufficient to kill the bacteria. However, formalin is a carcinogen and must be stored and handled with great care. Alternatively, work should be performed under a biosafety hood if formalin is not used. Antisera should be stored in the refrigerator at 4°C and warmed to room temperature (25°C) before use. It must be put back in the refrigerator as soon as testing is finished to prevent the loss of binding activity of the antibody.

B. Performing the SASG test

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).

2. Clean a glass slide with alcohol (optional if slides are pre-cleaned).
3. Divide the slide into equal sections (e.g., twelve 11 X 22 mm sections on a standard 50 X 75 mm slide) with a liquid impermeable pen or a wax pencil.
 - Each isolate will require as many sections on the slide as the individual serogroup-specific antisera that will be tested as well as a saline negative control.
4. In the lower portion of each of the sections of the glass slide described in step (2), add 10 μ l of the 5% formalinized saline with a micropipettor.
 - The instructions specify using a micropipettor with sterilized filtered tips to measure the 10 μ l of the 5% formalinized saline to suspend the bacteria. The micropipettor will transfer precise and equal measurements for a proper SASG reaction.
 - If a micropipettor and tips are not available, sterile, disposable 10 μ l inoculation loops can be used to transfer 10 μ l of the 5% formalinized saline, but often do not deliver accurate amounts (between 5-10 μ l).
5. Use a sterile, disposable 10 μ l inoculating loop to collect a few colonies from the surface of the overnight culture incubated on the BAP.
6. Suspend the bacteria in the 5% formalinized saline solution in the lower portion of each of the sections of the slide. The suspension should be moderately opaque (see saline control in Figure 6). Do not allow the cell suspension to dry before adding the antisera.
 - If the bacteria are difficult to suspend directly on the slide, make a moderately milky suspension (comparable to McFarland 6 standard) of the test culture in a small vial with 250 μ l of 5% formalinized saline and briefly vortex the suspension to mix and break up any pellets. Add 10 μ l of this suspension to the lower portion of the slide.
7. In the upper portion of each of the sections of the glass slide described in step (2), add 10 μ l of the serogroup-specific antisera to be tested as well as unformalinized saline or phosphate buffered saline (PBS) for a negative control with a micropipettor.
 - DO NOT use the dropper provided with the antisera because it usually delivers larger amounts than is necessary and can easily be contaminated.
 - If a micropipettor and tips are not available, sterile, disposable 10 μ l inoculation loops can be used to transfer 10 μ l of the antisera, but often do not deliver accurate amounts (between 5-10 μ l).
 - Dispose of the tip or loop used to transfer the antisera to the slide in a waste container after each use to avoid contamination of the antisera. If the source of antisera is contaminated, a new vial must be used.

8. Gently tilt the slide to mix the cell suspensions with the antisera in each section. Continue to gently rock the slide for 1 to 2 minutes to allow the lower and upper portions to completely blend. Do not use a circular motion while rocking, as it can cause the sections with different serogroup-specific antisera to run together and contaminate each other.
9. After 2 minutes, examine the SASG reactions under a bright light and over a black background. Use the rating system in Figure 6 to determine the intensity of the agglutination reaction in each section of the slide. Disregard any agglutination that occurs after the 2 minute time period.
10. Record the SASG results in the laboratory log book.

C. Reading the SASG results

1. Rating the intensity of the agglutination reaction

Agglutination occurs when the antisera bind to the bacterial cells causing the cells to agglutinate or clump together, thus making the cell suspension appear clearer. The intensity of the agglutination reaction may vary according to the density of the cell suspension or the antisera used. A description on the intensity ratings shown in Figure 6 are listed below.

- 4+ All of the cells agglutinate and the cell suspension appears clear
- 3+ 75% of the cells agglutinate and the cell suspension remains slightly cloudy
- 2+ 50% of the cells agglutinate and the cell suspension remains slightly cloudy
- 1+ 25% of the cells agglutinate and the cell suspension remains slightly cloudy
- +/- Less than 25% of the cells agglutinate and a fine granular matter occurs
- 0 No visible agglutination; the suspension remains cloudy and smooth

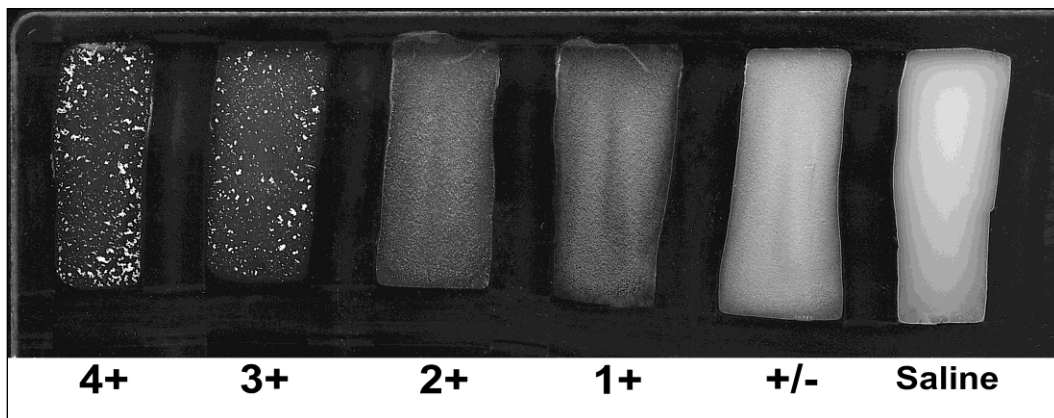


Figure 6. Rating the intensity of the agglutination reaction

2. Determining the serogroup

- A positive result is designated by a 3+ or 4+ (strong agglutination) within 1-2 minutes, except for serogroup B, which is considered positive with a rating of 2+ or greater.

- A negative result is designated by a 0 (saline), +/-, 1+ or 2+ (weak agglutination).
- The serogroup is determined when a positive result occurs with only one of the antisera and not with the saline.
- If a serogroup is not determined, the isolate is considered NG. The following result combinations are all reported as NG:
 - Agglutination in the saline, regardless of strong reactions with other antisera, characterizes the culture as autoagglutinating.
 - Agglutination with more than one serogroup-specific antisera in the absence of agglutination in saline characterizes the culture as polyagglutinating or cross-reactive.
 - No agglutination with any of the antisera or the saline characterizes the strain as non-reactive.

D. Troubleshooting procedures

N. meningitidis isolates are subject to variability (encapsulated vs. unencapsulated, small vs. large colonies, slow growers vs. fast growers, and heavy agglutinators vs. light agglutinators) and may be unclear or difficult to interpret. Some troubleshooting procedures are listed below:

1. Repeat the test directly on the slide using growth from another section of the same plate.
2. Make a cell suspension in a small tube and vortex if the result from SASG directly on the slide is unclear and repeat the test.
3. Add 20 µl of antisera directly to slide and then add a loop full of organism without diluting the specimen with 5% formalinized saline.
4. Subculture and retest fresh growth the following day.
5. If the original plate contains different size colonies, make a subculture for each type of colony and test both cultures the next day. The larger colonies usually indicate better capsule production and therefore better reactivity. However, the smaller colonies will occasionally give a better result.
 - If discrepancies are not immediately resolved, any subsequent SASG repeats should be used in conjunction with control strains.

E. Quality control (QC) of antisera for SASG testing

A set of reference strains for *N. meningitidis* serogroups A, B, C, W135, X, Y, Z, 29E (one per serogroup) and a nongroupable *N. meningitidis* strain should be used to QC the antisera before testing any unknown isolates. QC of the antisera should be:

- Performed for each new lot of antisera received in the laboratory.
- Performed biannually after initial QC testing.
- Repeated if a vial has been exposed to temperatures above 4°C or if there is reason to suspect that the vial has been contaminated since the initial QC was performed.

Follow the SASG testing procedure to QC each lot of antisera using all reference strains available in the laboratory. Record the results provided on the example QC sheet in Figure 7.

Reading the QC test results

Passing test:

- The antiserum must give 3+ or 4+ agglutination with homologous antigens within 1-2 minutes.
- The antiserum must not react with heterologous *N. meningitidis* serogroups, with the NG reference strain, or in saline.

Failing test:

- The antiserum agglutinates with one or more reference strains and/or with the NG reference strain and/or in saline.

	A (strain number)	B (strain number)	C (strain number)	W135 (strain number)	X (strain number)	Y (strain number)	Z (strain number)	29E (Z') (strain number)	NG (strain number)
A Lot#	++++	-	-	-	-	-	-	-	-
B Lot#	-	++++	-	-	-	-	-	-	-
C Lot#	-	-	++++	-	-	-	-	-	-
W135 Lot#	-	-	-	++++	-	-	-	-	-
X Lot#	-	-	-	-	++++	-	-	-	-
Y Lot#	-	-	-	-	-	++++	-	-	-
Z Lot#	-	-	-	-	-	-	++++	-	-
29E Lot#	-	-	-	-	-	-	-	++++	-
Saline	-	-	-	-	-	-	-	-	-

Figure 7. Example QC sheet for testing antisera against all *N. meningitidis* serogroups

IV. Commercial identification kits

Several commercial identification systems that use biochemical or enzymatic substrates are available for identification of *Neisseria* spp. These systems may occasionally require supplemental tests, and additional characteristics, such as microscopic and colony morphology, must be considered. Generally, each system is self-contained, but the addition of one or more reagents to complete certain reactions may be necessary. The manufacturer's instructions should be followed precisely when using these kits. For detailed instructions and use of appropriate control strains, consult the Clinical Microbiology Procedures Handbook (2).

V. Serotyping and serosubtyping of *N. meningitidis* with monoclonal antibodies (Mabs)

Differentiation and classification of bacterial strains at the sub-species level require methods that are highly reproducible, little affected by the experimental conditions, and give effective discrimination of epidemiologically unrelated strains. Further characterization of bacteria causing meningitis is especially important to document the spread of particularly pathogenic clones. Many immunoassays have been developed for serological characterization of

N. meningitidis antigens. Immunological detection of specific epitopes of two major outer-membrane proteins, PorB and PorA, is the basis for the serological classification system defining, respectively, the serotypes and serosubtypes of the species 1, 3-7). A simple, low cost method for serotyping and serosubtyping meningococci, dot-blotting with Mabs, is described below.

A. Preparation of whole-cell suspensions

- Whole-cell suspensions should not be used for PCR testing.
1. Grow the *N. meningitidis* isolate to be tested along with an appropriate reference isolate for QC on a BAP for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
 2. Collect 3-5 colonies using a sterile disposable loop or sterile swab and suspend in 1 ml of PBS (pH=7.2) with 0.02% sodium-azide.
 3. Inactivate the bacteria by heating the suspension at 60°C for 30 minutes.
 4. Adjust the optical density with PBS to 0.2 measured at 650 nm (equivalent to a McFarland 1.5).
 5. Maintain whole-cell suspensions at 4°C until use.

B. Performing the dot-blotting serotyping/serosubtyping test

1. Cut strips (0.5 x 10 cm) of nitrocellulose paper (pore 0.45 µm). Divide each strip into 10 sections of 1 cm in length using a wax pencil and align them on a glass or hard plastic plate.
 - Use gloves and/or forceps when handling nitrocellulose paper.
2. Label the strips with a permanent pen on the left section.
3. Dot 2 µl of the whole-cell suspension of a relevant reference isolate in the middle of the next marked section and 2 µl of the test isolates successively onto each of the next sections, as shown in Figure 8.
4. Dry the strips for 15 minutes or more.
5. Add 2 ml of the blocking buffer (PBS supplemented with 3% bovine serum albumin [BSA]) into each well of an 8-well incubation tray (8-well trays with lids are commercially available).
6. Transfer the strips to be tested with the same Mab to a well (maximum 4 strips per well). Mix gently for 30 minutes. The strips must be fully wetted by the blocking buffer.

- The minimum incubation volume of the blocking buffer for 1 strip is 0.5 ml. Up to 4 strips with about 40 dotted samples can be incubated in a total volume of 2 ml. Consider leaving an empty well in the incubation trays between each set of strips that are incubated with different Mabs.
7. Add the Mabs to the respective wells. Depending on the supplier of the Mab, the final dilution may range from 1:10 to 1:500,000.
 8. Place the lid on the incubation tray and incubate with gentle mixing overnight.
 9. Carefully remove the antibody solutions from the strips using a pipet tip connected to a suction pump or similar device. Rinse the tip with water between each set of strips that were incubated with different Mabs to prevent transfer of the Mabs.
 10. Rinse the strips 3 times with no more than 2 ml PBS directly in the wells. Remove the PBS in the same manner in which the antibody solutions were removed.
 11. Add 2 ml of PBS supplemented with 3% BSA with horseradish peroxidase-labelled rabbit anti-mouse antibody (typically used at a 1:2,000 dilution) and incubate for 2 hours with gentle mixing. Be sure that the strips are fully wetted.
 12. Rinse the strips in 2 ml of PBS for 5 minutes twice as in step 9.
 13. Wash the strips for several minutes in 0.05 M sodium acetate buffer, pH 5.0, and then discard the solution.
 14. Put the strips in a plastic box and add 10 ml of sodium acetate buffer with 0.4 ml of 3-amino-9-ethylcarbazole (AEC) in dimethylformamide (DMF). Mix for 2-3 minutes.
 15. Add 10 µl of H₂O₂ at 30% and stain each dot until distinct red dots are observed for the reference isolate (2-10 minutes).

Mab 1	Ref. A	Isol. 1	Isol. 2	Isol. 3	Isol. 4	Isol. 5	Isol. 6	Isol. 7	Isol. 8
Mab 2	Ref. B	Isol. 1	Isol. 2	Isol. 3	Isol. 4	Isol. 5	Isol. 6	Isol. 7	Isol. 8
Mab 3	Ref. C	Isol. 1	Isol. 2	Isol. 3	Isol. 4	Isol. 5	Isol. 6	Isol. 7	Isol. 8
Mab 4	Ref. D	Isol. 1	Isol. 2	Isol. 3	Isol. 4	Isol. 5	Isol. 6	Isol. 7	Isol. 8

Figure 8. Example of nitrocellulose paper strip for applying whole-cell suspensions

C. Reading the dot-blotting serotyping/serosubtyping test results

1. Rinse the strips under running water, align them on a glass plate, and absorb the excess water with filter paper or tilt the plate slightly to dry the strips at room temperature (25°C).
2. Grade the staining intensity of each dot (positive, weak, or negative) visually relative to the reference strain.

3. When dried, the strips can be taped onto a sheet of paper and kept in a plastic pocket protected from light.

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CHAPTER 8

Identification and Characterization of *Streptococcus pneumoniae*

S. pneumoniae may occur intracellularly or extracellularly as gram-positive lanceolate diplococci, but can also occur as single cocci or in short chains of cocci. *S. pneumoniae* is a fastidious bacterium, growing best at 35-37°C with ~5% CO₂ (or in a candle-jar). It is usually cultured on media that contain blood, but can also grow on a chocolate agar plate (CAP). On a blood agar plate (BAP), colonies of *S. pneumoniae* appear as small, grey, moist (sometimes mucoid), colonies and characteristically produce a zone of alpha-hemolysis (green) (Figure 1). The alpha-hemolytic property differentiates this organism from many species, but not from the commensal alpha-hemolytic (viridans) streptococci. Differentiating pneumococci from viridans streptococci is difficult as young pneumococcal colonies appear raised, similar to viridans streptococci. However, once the pneumococcal culture ages 24-48 hours, the colonies become flattened, and the central portion becomes depressed, which does not occur with viridans streptococci (Figure 2). A microscope (30-50X) or a 3X hand lens can also be a useful tool in differentiating pneumococci from viridans streptococci. Prior to identification and characterization testing procedures, isolates should always be inspected for purity of growth and a single colony should be re-streaked, when necessary, to obtain a pure culture. For the following identification and characterization procedures, it is essential to test alpha-hemolytic colonies that are less than a day old, typically grown overnight at 35-37°C with ~5% CO₂ (or in a candle-jar).

The following specialized tests are used to identify colonies on a BAP that resemble pneumococci (Figure 3). *S. pneumoniae* can be identified using Gram stain, catalase, and optochin tests simultaneously, with bile solubility as a confirmatory test. If these tests indicate that the isolate is *S. pneumoniae*, serological tests to identify the serotype can be performed. This sequence of testing is an efficient way to save costly serotyping reagents and time. Additional methods for identification and characterization of *S. pneumoniae* using molecular tools are described in Chapter 10: PCR Methods and Chapter 12: Molecular Methods. Additional protocols used for streptococcal species identification and updates to existing methods can be found at: <http://www.cdc.gov/ncidod/biotech/strep/strep-doc/index.htm>.

Biosafety Level 2 (BSL-2) practices are required for work involving isolates of *S. pneumoniae*, as this organism presents a potential hazard to laboratory personnel and the surrounding working environment. Please refer to Chapter 4: Biosafety in order to follow the guidelines that have been established for laboratorians working in BSL-2 facilities as many of the tests described in this chapter require opening plates with live cultures and are often performed outside of a biosafety cabinet (BSC).

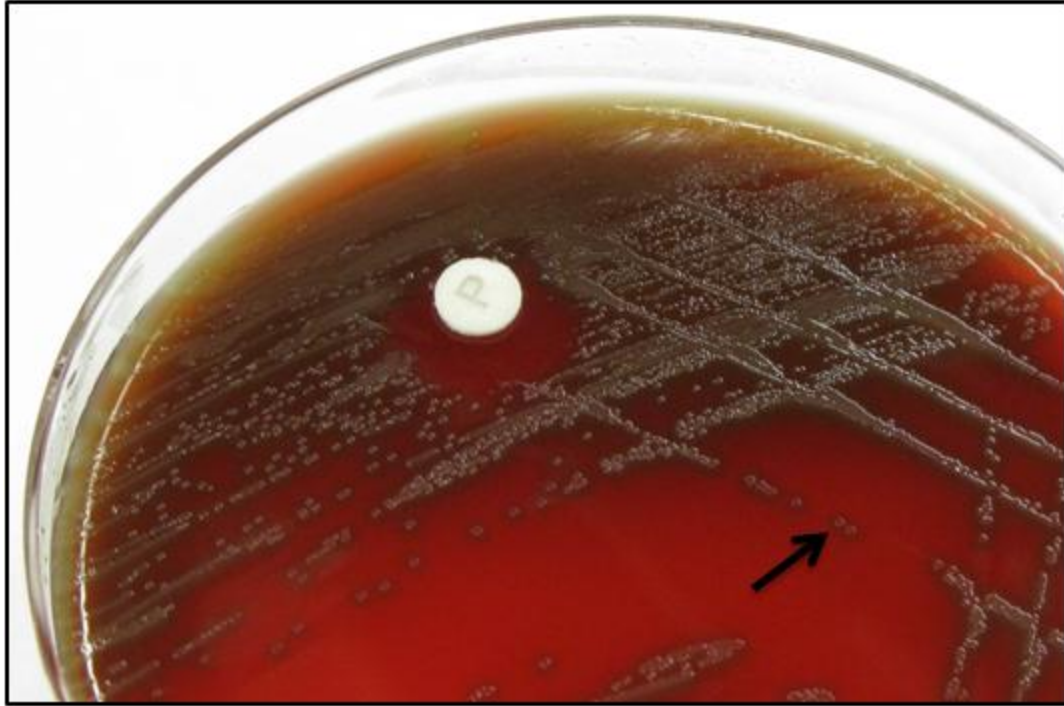


Figure 1. *S. pneumoniae* colonies with a surrounding green zone of alpha-hemolysis (black arrow) on a BAP

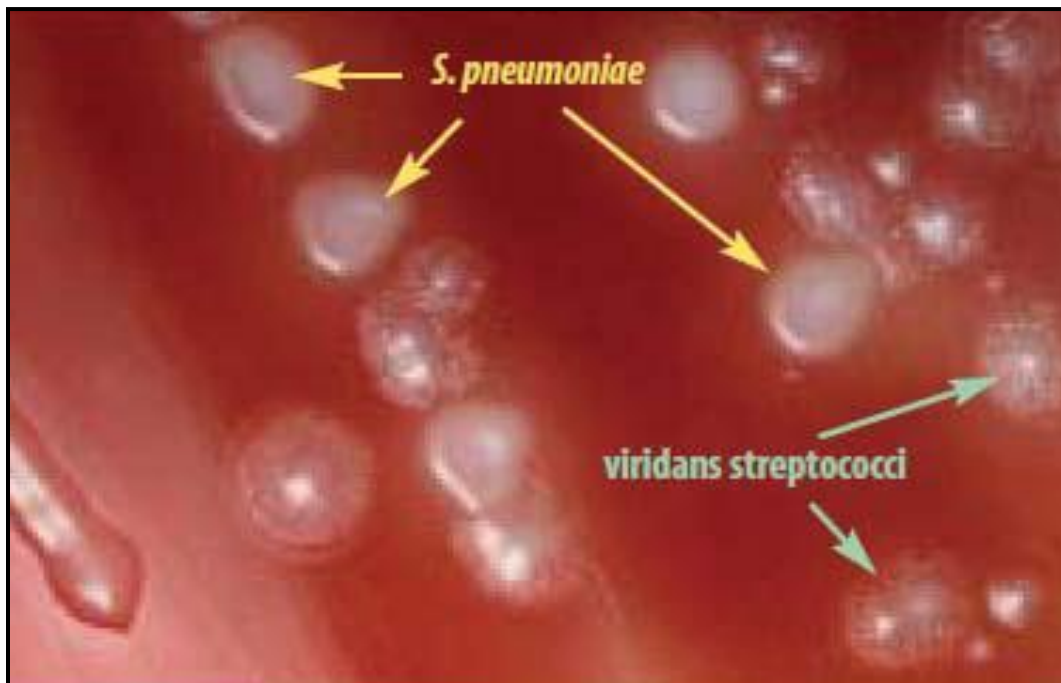


Figure 2. *S. pneumoniae* colonies have a flattened and depressed center after 24-48 hours of growth on a BAP, whereas the viridans streptococci retain a raised center

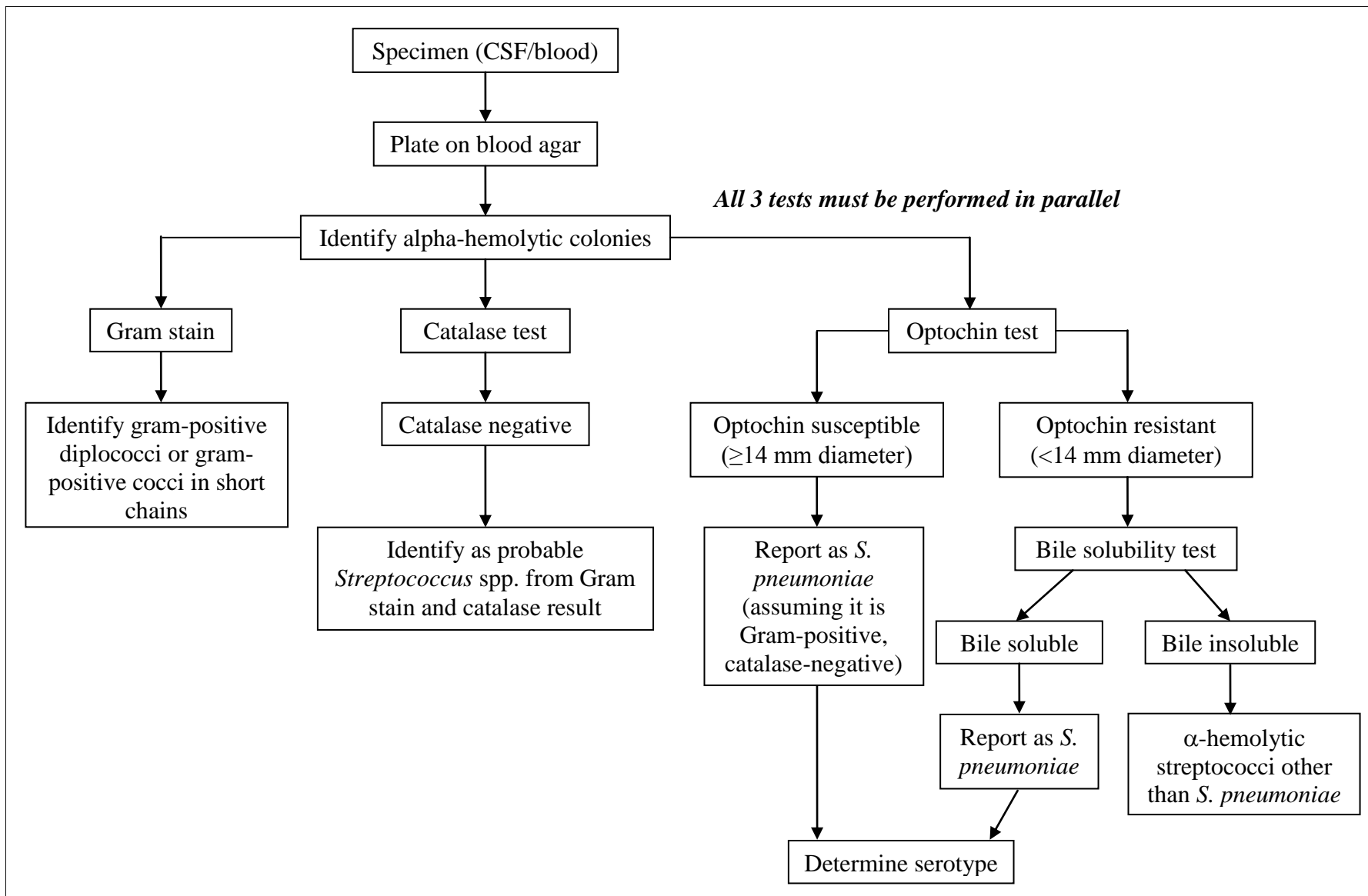


Figure 3. Flow chart for identification and characterization of a *S. pneumoniae* isolate

I. Catalase test

Catalase is the enzyme that breaks down hydrogen peroxide (H_2O_2) into H_2O and O_2 . The oxygen is given off as bubbles in the liquid. The catalase test is primarily used to differentiate between gram-positive cocci. Members of the genus *Staphylococcus* are catalase-positive, and members of the genera *Streptococcus* and *Enterococcus* are catalase-negative.

A. Performing the catalase test

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO_2 (or in a candle-jar).
2. From overnight growth on the BAP, use a disposable loop to carefully remove a colony and place it on a glass slide.
 - Do not transfer any of the blood agar to the slide as erythrocytes in the blood agar will cause a false-positive reaction.
3. Add 1.0 ml of 3% H_2O_2 to the slide and mix with the bacteria.
 - H_2O_2 can be obtained from a commercial drug store.
 - After initially opening, store H_2O_2 at 4°C in a tightly closed bottle as it will slowly lose potency once opened.
4. Observe the bacterial suspension on the slide immediately for vigorous bubbling.
5. It is essential to use a known positive and negative quality control (QC) strain. A *Staphylococcus* spp. strain can be used for a positive control and a known *S. pneumoniae* strain or any other streptococcal spp., i.e., *S. pyogenes* can be used for a negative control.

B. Reading the catalase test results

- The absence of bubbling from a transferred colony indicates a negative test.
- Any bubbling from a transferred colony indicates a positive test (Figure 4).

C. Troubleshooting

- False positives will result from transfer of red blood cells so take care when picking colonies from the BAP for this test.

D. Quality control

- It is essential to use a known positive and negative QC strain as described in the procedure. Opened bottles should be checked against a known catalase positive organism every 6 months.

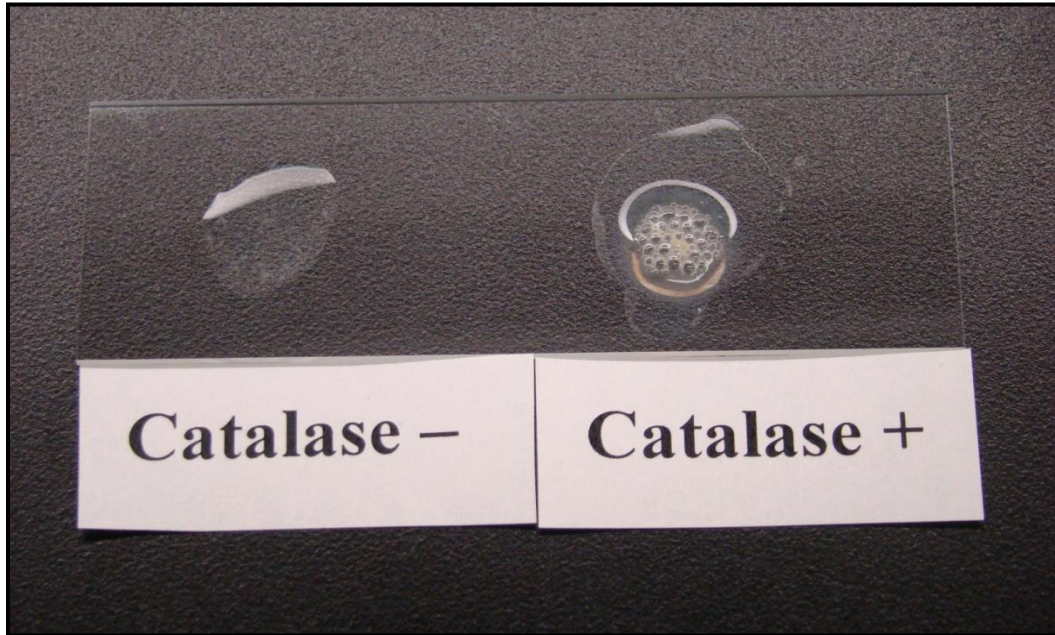


Figure 4. Negative and positive catalase test results. The absence of bubbling from a transferred colony indicates a negative test. All streptococci are catalase-negative.

II. Optochin test

S. pneumoniae strains are sensitive to the chemical optochin (ethylhydrocupreine hydrochloride). Optochin sensitivity allows for the presumptive identification of alpha-hemolytic streptococci as *S. pneumoniae*, although some pneumococcal strains are optochin-resistant. Other alpha-hemolytic streptococcal species are optochin-resistant.

A. Performing the optochin test

Optochin (P) disks (6 mm, 5 µg) can be obtained from a commercial vendor. Optochin disks are often called “P disks” and many commercial versions are labeled with a capital “P”. If a commercial source of P disks is not available, a 1:4000 solution of ethylhydrocupreine hydrochloride can be applied to sterile 6 mm filter paper disks.

1. Grow the strain(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Use a disposable loop to remove an isolated colony from the overnight culture on the BAP and streak onto one half of a BAP.

- Two different isolates can be tested on the same plate, but care must be taken to ensure that the cultures do not overlap.
3. Place a P disk within the streaked area of the plate and incubate the BAP overnight at 35-37°C with ~5% CO₂ (or in a candle-jar).
 4. Observe the growth on the BAP near the P disk and measure the zone of inhibition, if applicable.

B. Reading the optochin test results

- Using a 6 mm, 5 µg disk, a zone of inhibition of 14 mm or greater indicates sensitivity and allows for presumptive identification of pneumococci (Figure 5).
- Zones of inhibition should be measured from the top surface of the plate with the top removed.
- Use either calipers or a ruler with a handle attached for these measurements. Measure the diameter of the zone holding the ruler over the center of the surface of the disk when measuring the zone of inhibition. In the case of an isolate completely resistant to optochin, the diameter of the disk (6 mm) should be recorded.

C. Troubleshooting

- A smaller zone of inhibition (< 14 mm) or no zone of inhibition indicates that the bile solubility test is required. It is important to remember that pneumococci are sometimes optochin-resistant.

D. Quality control

- Each new lot of optochin disks should be tested with positive and negative controls. The growth of *S. pneumoniae* strain ATCC 49619 is inhibited by optochin and growth of *S. mitis* strain ATCC 49456 is not inhibited by optochin.



Figure 5. Optochin test for *S. pneumoniae* using optochin disks. The strain on the left is *S. mitis* and is resistant to optochin with no zone of inhibition. The strain on the right is susceptible to optochin and is *S. pneumoniae*.

III. Bile solubility test

The bile (sodium deoxycholate) solubility test distinguishes *S. pneumoniae* from all other alpha-hemolytic streptococci. *S. pneumoniae* is bile soluble whereas all other alpha-hemolytic streptococci are bile resistant. Sodium deoxycholate (2% in water) will lyse the pneumococcal cell wall.

A. Preparation of 2% sodium deoxycholate (bile salt) solution

1. Dissolve 2 g of sodium deoxycholate into 100 ml sterile distilled water.

B. Performing the bile solubility test

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Add bacterial growth from the overnight BAP to 1.0 ml of 0.85% saline to achieve turbidity in the range of a 0.5-1.0 McFarland standard.
3. Divide the cell suspension equally into 2 tubes (0.5 ml per tube).
4. Add 0.5 ml of 2% sodium deoxycholate (bile salts) to one tube. Add 0.5 ml of 0.85% saline to the other tube. Mix each tube well.
5. Incubate the tubes at 35-37°C in CO₂.
6. Vortex the tubes.
7. Observe the tubes for any clearing of turbidity after 10 minutes. Continue to incubate the tubes for up to 2 hours at 35-37°C in CO₂ if negative after 10 minutes. Observe again for clearing.

C. Reading the bile solubility test results

- A clearing of the turbidity in the bile tube but not in the saline control tube indicates a positive test (Figure 6).

D. Troubleshooting

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

E. Quality control

- Each new lot of sodium deoxycholate should be tested with positive and negative QC strains. *S. pneumoniae* strain ATCC 49619 can be used as a positive control and *S. mitis* strain ATCC 49456 can be used as a negative control.

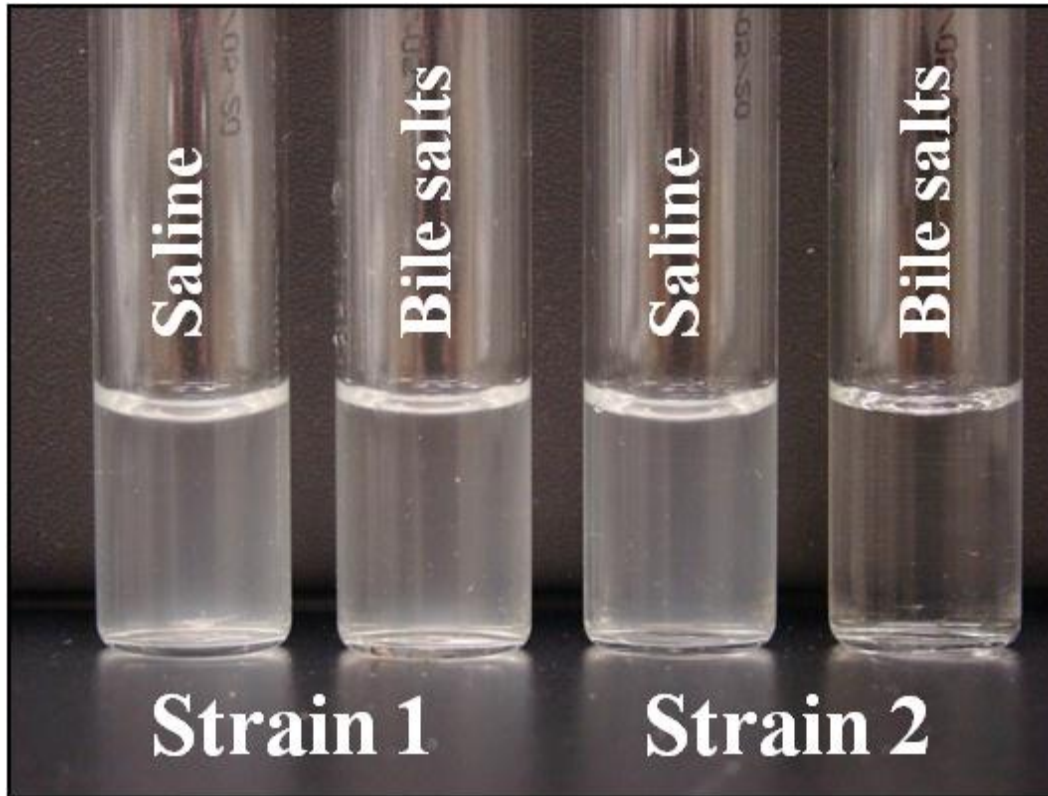


Figure 6. Results of the bile solubility test are shown for two different strains of bacteria. For strain 1, a slight decrease in turbidity is observed in the tube containing the bile salts (2nd from left), but the contents are almost as turbid as the control tube (far left); therefore, strain 1 is not *S. pneumoniae*. For strain 2, all turbidity in the tube containing the bile salts (far right) has cleared, indicating that the cells have lysed, in contrast to the control tube (2nd from right), which remains turbid; therefore, strain 2 is *S. pneumoniae*.

IV. Commercial test kits for identification

Several commercial identification systems that use slide agglutination tests are available for identification of colony growth from a BAP as *S. pneumoniae*. These identification tests use suspensions of latex beads with rabbit antibody specific for *S. pneumoniae* capsular antigens. Visible agglutination occurs when the *S. pneumoniae* capsular antigen reacts with the antibody-coated latex beads. The manufacturer's instructions should be followed precisely when using these kits. These kits should be regularly subjected to QC using a non-pneumococcal streptococcal species, since they can become cross-reactive with prolonged storage.

V. Determining *S. pneumoniae* capsular serotypes using serologic methods

Although serotyping of pneumococci is not usually necessary for a clinical response, capsular serotype determination is a critical component of successful pneumococcal disease surveillance efforts. Effective current multivalent vaccines target combinations of key serotypes. Determination of serotype distributions associated with disease in certain regions provides

information regarding the potential usefulness of applying existing vaccines and is also critical for assessing vaccine impact.

Serotype distribution can be determined by culture of the organism followed by serological determination of the capsular type by latex agglutination and the quellung reaction. Many laboratories have opted to use simpler and less expensive methods of deducing capsular serotypes through the use of specific PCR reactions (see Chapter 10: PCR Methods and <http://www.cdc.gov/ncidod/biotech/strep/pcr.htm> for specific PCR protocols).

A. Latex agglutination

The standard quellung reaction test for serotyping pneumococci can be labor-intensive and time-consuming, and requires a certain level of experience to be performed competently. An agglutination method using anti-rabbit IgG-coated latex particles sensitized to pooled and select individual serotype-specific antisera (PCV7 serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F) for serogrouping/serotyping *S. pneumoniae* has been developed and kits are commercially available. The latex agglutination method is simpler and faster than the quellung reaction, but is only intended for partial serotyping as it can only narrow the identification down to a group or pool of serotypes. Then the quellung reaction should be performed using individual serotype-specific antisera for each serotype in the group or pool to identify the serotype.

Performing latex agglutination testing

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. From overnight growth on a BAP, use a sterile loop to prepare a light to moderate cell suspension (approximately equal to a 0.5 McFarland density standard) in 0.5 ml of 0.85% saline.
3. On a glass slide or reaction card, add 10 µl (1 droplet) of the latex reagent and 10 µl of the cell suspension. Mix the two suspensions together.
4. After 10-30 seconds, observe the latex agglutination reaction at an angle with oblique lighting.

Reading the latex agglutination results

- A positive reaction is indicated by agglutination (cells clumping together) appearing within 5-10 seconds.
- A negative reaction is indicated by no agglutination appearing within 5-10 seconds.

Troubleshooting

- The latex agglutination reaction should be examined within 5-10 seconds. If the reaction time exceeds 30 seconds, false positive reactions may occur.

Quality control

- Each lot of latex suspension should be tested for positive agglutination reactions using *S. pneumoniae* reference strains with known capsular serotypes.

B. Quellung reaction

For proper quellung-based serotyping, a high quality microscope is required. A positive quellung or Neufeld reaction is the result of the binding of the capsular polysaccharide of pneumococci with type specific antibody contained in the typing antiserum. Pneumococcal typing sera are commercially available as pooled, group, or serotype-specific (see <http://www.ssi.dk/English.aspx>). It is recommended to initially test with pooled antisera in succession until a positive reaction is observed. Typing should then proceed by testing with individual group and serotype-specific antisera included in the antisera pool that gave a positive reaction to determine the serogroup and serotype. An antigen-antibody reaction causes a change in the refractive index of the capsule so that it appears “swollen” and more visible. After the addition of a counter stain (methylene blue), the pneumococcal cells stain dark blue and are 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 positive quellung reactions.

Performing the quellung reaction

1. Grow the isolate(s) to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. From overnight growth on the BAP, use a sterile loop to prepare a light to moderate cell suspension (approximately equal to a 0.5 McFarland density standard) in 0.5 ml of 0.85% saline.
 - Optimum quellung reactions can be observed when there are 25-50 cells visible in a microscopic field at 1000X magnification.
3. Dispense equal amounts of antiserum (5 µl) and methylene blue (5 µl) onto a microscope slide. Add approximately 0.2-1.0 µl of the diluted cell suspension and mix all three with a pipette tip.
4. Cover the suspension with a 22 mm² square cover-slip and incubate at room temperature (25°C) for 10-15 minutes.
 - Do not allow the fluid on the slide to dry.

5. Examine the slide at 1000X using an oil immersion lens.
6. Begin testing with pooled antisera. Once a positive reaction is obtained, proceed with individual group and serotype-specific antisera included in the pooled antisera that gave the positive reaction to determine the serogroup and serotype.

Reading the quellung reaction results

- A positive quellung reaction is observed when the capsule appears as a sharply demarcated halo around the dark blue stained cell (Figure 7).
- A negative quellung reaction is observed when there is no appearance of a clear, enlarged halo surrounding the stained cell.

Troubleshooting

- In some instances, observing a positive reaction can be difficult. Prepare and read all quellung reactions on the same day that the cell suspension is prepared.
- When reading the reactions, look for free floating single or paired cells.
- Agglutination (cells clumping together) is NOT a positive quellung reaction.
- If a quellung reaction is not observed in any of the antisera pools, the strain may be non-typeable, but identification of the strain as *S. pneumoniae* should be confirmed by optochin susceptibility and bile solubility testing.

Quality control

- Each lot of antisera received should be tested for positive quellung reactions using *S. pneumoniae* reference strains with known capsular serotypes.

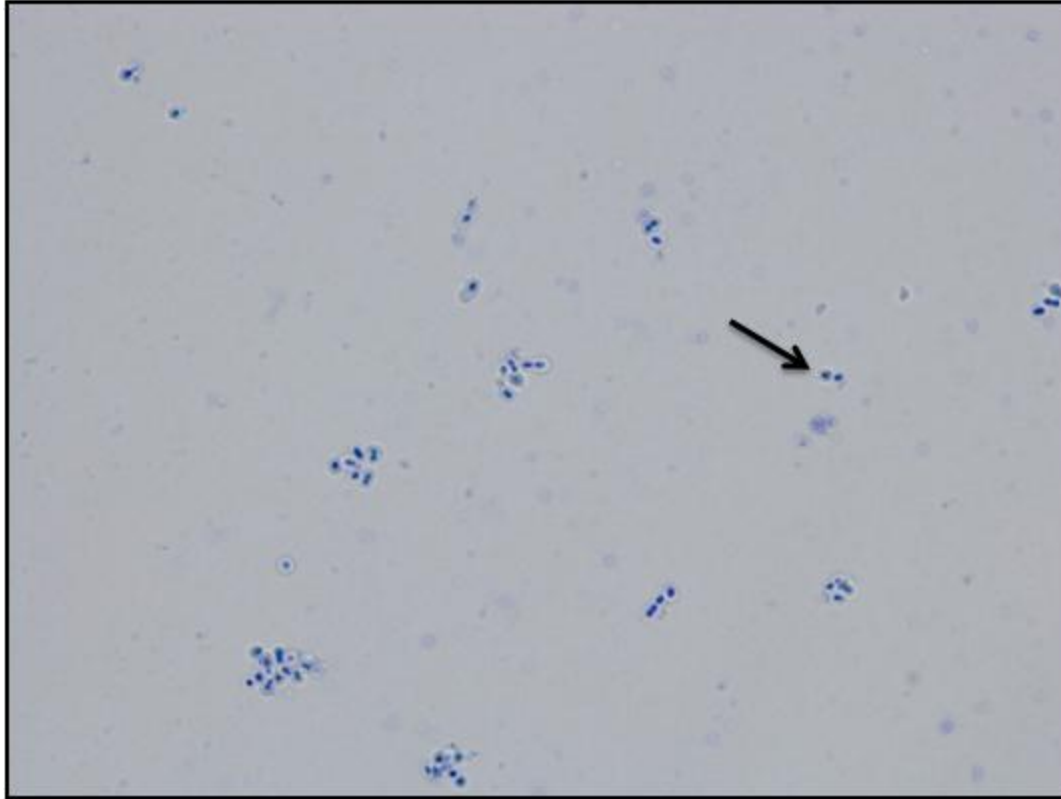


Figure 7. In a positive quellung reaction, the capsule appears as an enlarged clear halo surrounding the dark blue stained cell

VI. Determining *S. pneumoniae* capsular serotypes using PCR-based methods

The high cost of antisera, subjectivity in interpretation, and technical expertise requirements associated with these serologic methods have resulted in the development of PCR-based serotyping systems. PCR-based serotyping has the potential to overcome some of the difficulties associated with serologic testing and assays for direct detection of serotypes from clinical specimens are a valuable aid in surveillance, particularly in situations where culture is insensitive.

Conventional PCR-based methods have been developed to determine the serotypes of *S. pneumoniae* specimens. Conventional multiplex PCR assays are available for detecting 40 of the 93 *S. pneumoniae* serotypes. Schemes for testing based on the strains that are historically and/or currently circulating in specific regions are listed in Chapter 10: PCR Methods. Published real-time PCR serotyping assays are also available for many common pneumococcal serotypes.

VII. General methods for genotyping *S. pneumoniae*

The continued study of the “seroepidemiology” of pneumococcal disease and carriage isolates is important for understanding selective effects upon regional population structures of this species. Trends in pneumococcal carriage and disease epidemiology are influenced by selective factors in the environment, such as the use of antimicrobial drugs and the introduction of conjugate

vaccines. Understanding questions related to long-term effects of such pressures on the pneumococcal population require precise isolate characterization using molecular methods to characterize the strains at the genetic level. Chapter 12: Molecular Methods describes some of the most common typing methodologies used to differentiate *S. pneumoniae* and includes pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and the use of more variable loci such as the penicillin binding protein (*pbp*) genes and the pneumococcal surface protein (*pspA*) gene.

CHAPTER 9

Identification and Characterization of *Haemophilus influenzae*

H. influenzae are small, pleomorphic, gram-negative bacilli or coccobacilli with random arrangements. *H. influenzae* is a fastidious organism which grows best at 35-37°C with ~5% CO₂ (or in a candle-jar) and requires hemin (X factor) and nicotinamide-adenine-dinucleotide (NAD, also known as V factor) for growth. The standard medium used for growth of *H. influenzae* is a chocolate agar plate (CAP), which can be prepared with heat-lysed horse blood, a good source of both hemin and NAD, although sheep blood can also be used. Growth occurs on a CAP because NAD is released from the blood during the heating process of chocolate agar preparation (the heating process also inactivates growth inhibitors) and hemin is available from non-hemolyzed as well as hemolyzed blood cells. Alternatively, NAD can be included as a component of liquid *H. influenzae* growth media supplements, (available commercially or prepared in the laboratory), which are incorporated into the chocolate agar. *H. influenzae* appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on a CAP (Figure 1). Encapsulated strains appear more mucoidal than non-encapsulated strains, which appear as smaller, compact grey colonies. No hemolysis or discoloration of the CAP is apparent. While *H. influenzae* produce a pungent indol smell, plates should not be opened in order to smell the cultures. *H. influenzae* cannot grow on an unsupplemented BAP. Prior to identification and characterization testing procedures, isolates should always be inspected for purity of growth and a single colony should be re-streaked, when necessary, to obtain a pure culture. For the following identification and characterization procedures, testing should be performed on 18-24 hour growth from a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar) (Figure 2).

The following tests are recommended to confirm the identity of cultures that morphologically appear to be *H. influenzae* (Figure 3). *H. influenzae* can be identified using Kovac's oxidase test and determining the necessity of hemin and NAD as growth requirements. If the oxidase test is positive, hemin and NAD growth factor requirement testing should be performed. If the growth factor requirement test indicates that the isolate may be *H. influenzae*, serological tests to identify the serotype should be performed. This sequence of testing is an efficient way to save costly antisera and time. Additional methods for identification and characterization of *H. influenzae* using molecular tools are described in Chapter 10: PCR Methods and Chapter 12: Molecular Methods

Biosafety Level 2 (BSL-2) practices are required for work involving isolates of *H. influenzae*, as this organism presents a potential hazard to laboratory personnel and the surrounding working environment. Please refer to Chapter 4: Biosafety in order to follow the guidelines that have been established for laboratorians working in BSL-2 facilities as many of the tests described in this chapter require opening plates with live cultures and are often performed outside of a biosafety cabinet (BSC).



Figure 1. *H. influenzae* colonies on a CAP

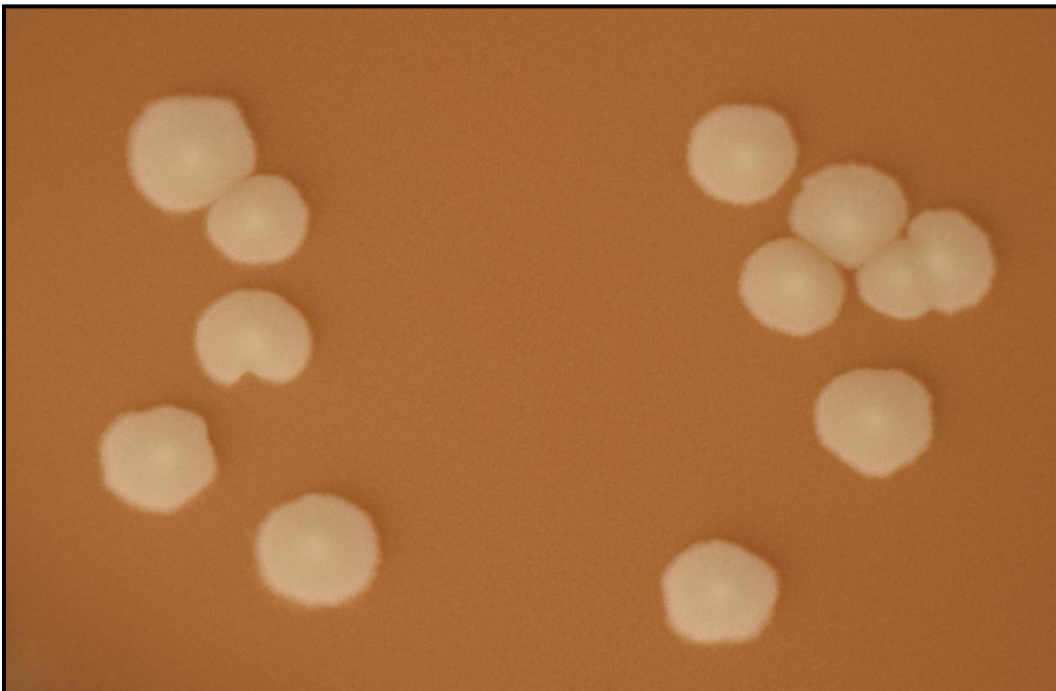


Figure 2. *H. influenzae* colonies on a CAP

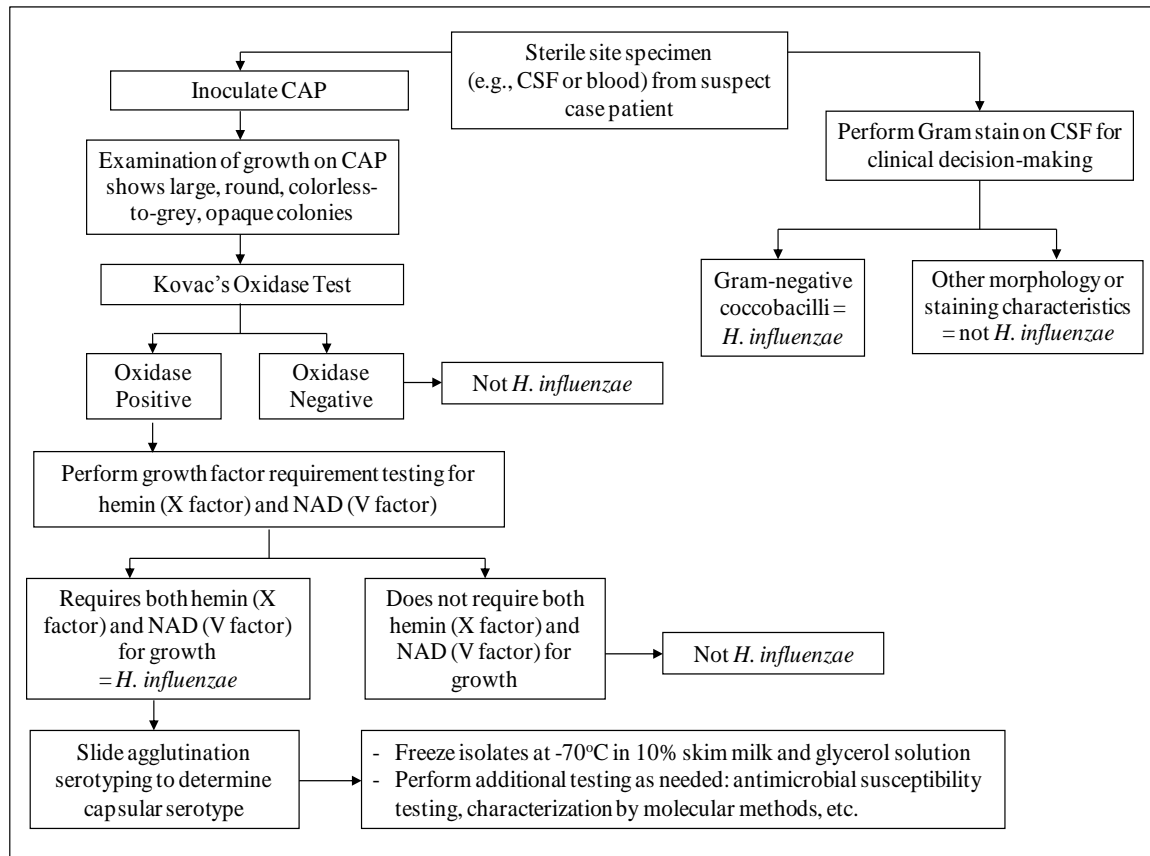


Figure 3. Flow chart for identification and characterization of a *H. influenzae* isolate

VI. Kovac's oxidase test

Kovac's oxidase test determines the presence of cytochrome oxidase. Kovac's oxidase reagent, tetramethyl-p-phenylenediamine dihydrochloride, is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain. This test aids in the recognition of *H. influenzae*, but other members of the genus *Haemophilus*, as well as unrelated bacterial species, may also give a positive reaction. Positive and negative quality control (QC) strains should be tested along with the unknown isolates to ensure that the oxidase reagent is working properly.

D. Preparation of 1% oxidase reagent from oxidase powder

To prevent deterioration of stock oxidase powder, the powder should be stored in a tightly sealed desiccator and kept in a cool, dark area. Kovac's oxidase reagent is intended only for *in vitro* diagnostic use. Avoid contact with the eyes and skin as it can cause irritation. In case of accidental contact, immediately flush eyes or skin with water for at least 15 minutes.

1. Prepare a 1.0% Kovac's oxidase reagent by dissolving 0.1 g of tetramethyl-p-phenylenediamine dihydrochloride into 10 ml of sterile distilled water.
2. Mix well and then let stand for 15 minutes.

- The solution should be made fresh daily and the unused portion should be discarded.
- Alternatively, the reagent could be dispensed into 1 ml aliquots and stored frozen at -20°C. The aliquots should be removed from the freezer and thawed before use. Discard the unused portion each day the reagent is thawed.

E. Performing Kovac's oxidase test

Filter paper method

1. Grow the isolate(s) to be tested for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. On a nonporous surface (i.e., Petri dish or glass plate), wet a strip of filter paper with a few drops of Kovac's oxidase reagent.
3. Let the filter paper strip air dry before use.
4. Use a disposable plastic loop, a platinum inoculating loop, or a wooden applicator stick to pick a portion of a colony from overnight growth on the CAP and rub it onto the treated filter paper (Figure 4).
 - Do not use a nichrome loop, as it may produce a false-positive reaction.
5. Observe the filter paper for color change to purple.
6. Perform steps 3 and 4 with a positive and negative QC strain to ensure that the oxidase reagent is working properly.

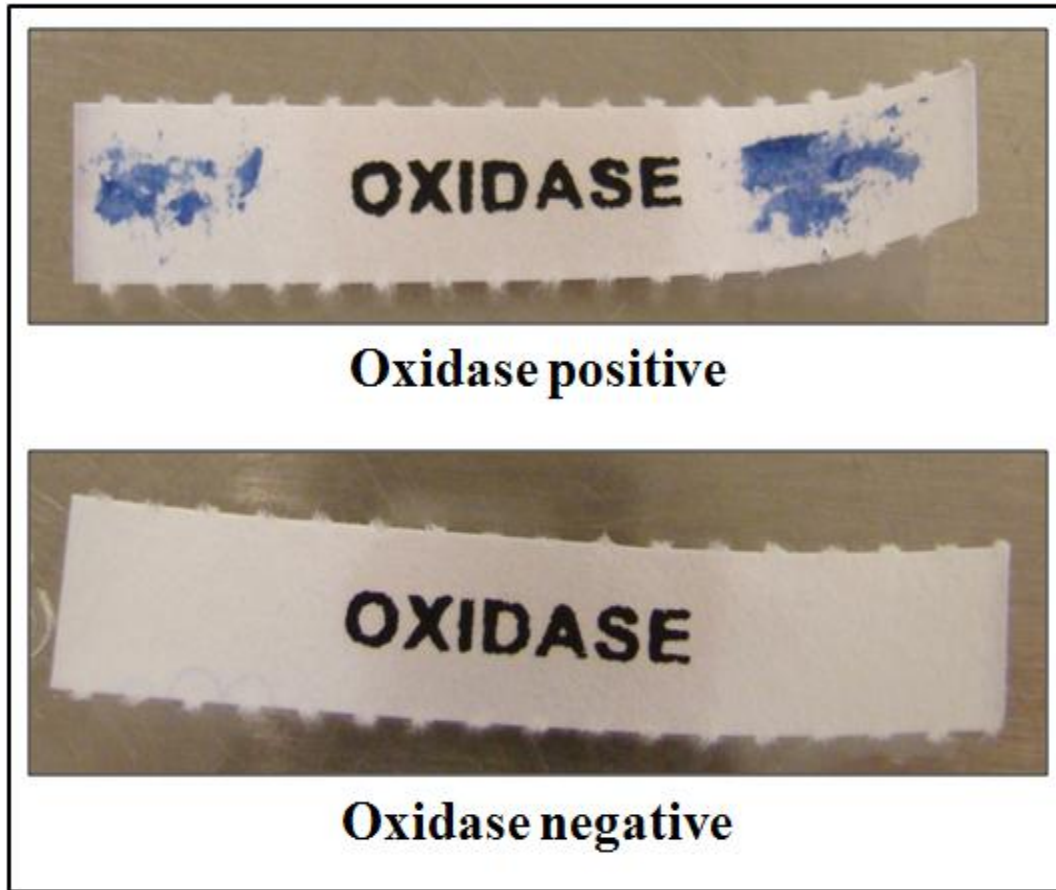


Figure 4. Kovac's oxidase test: a negative and positive reaction on filter paper

Plate method

1. Grow the isolate(s) to be tested for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Dispense a few drops of Kovac's oxidase reagent directly on top of a few suspicious colonies growing on the 18-24 hour CAP.
 - Do not flood the entire plate as the bacteria exposed to the reagent are usually not viable for subculture.
3. Tilt the plate and observe colonies for a color change to purple.
4. Perform steps 1 and 2 with a positive and negative QC strain to ensure that the oxidase reagent is working properly.

F. Reading the oxidase test results

- Positive reactions will develop within 10 seconds in the form of a purple color where the bacteria were applied to the treated filter paper. Delayed reactions are unlikely with *H. influenzae*.
- Negative reactions will not produce a color change on the treated filter paper.

VII. Identification of hemin and NAD as growth requirements

H. influenzae is a fastidious organism and can be identified on the basis of growth requirements for hemin and NAD. *H. influenzae* can be differentiated from most other species of *Haemophilus* by its specific requirement for both hemin and NAD for growth (Table 1). *H. haemolyticus* is the only other species requiring both hemin and NAD for growth; however, this species differs from *H. influenzae* by producing beta-hemolysis (clear) on horse or rabbit blood. For patients with bacterial meningitis, *H. influenzae* must be considered as the presumptive causative agent as opposed to *H. haemolyticus* when both hemin and NAD factors are required for growth. To differentiate between the two species, hemolysis must be checked on horse or rabbit blood agar (see Section II.B., *Haemophilus* ID Quad plate section below). *H. haemolyticus* usually causes hemolysis on these media, while *H. influenzae* does not. It has recently been reported that *H. haemolyticus* tend to rapidly lose their hemolytic property when passed *in vitro* (2). This has made the definitive identification of *H. influenzae* and *H. haemolyticus* using only biochemical tests very difficult and other methods, such as molecular testing, may be employed for differentiating between the two species.

Table 1. Identification of *Haemophilus* spp. by their growth requirements for hemin (X factor) and NAD (V factor) and β -hemolysis on horse blood agar

Organism	Requirement for hemin (X factor)	Requirement for NAD (V factor)	β -hemolysis on horse blood agar
<i>H. influenzae</i>	+	+	-
<i>H. parainfluenzae</i> ¹	-	+	-
<i>H. haemolyticus</i>	+	+	+
<i>H. parahaemolyticus</i>	-	+	+
<i>H. aphrophilus</i> ²	+	-	-
<i>H. paraphrophilus</i> ^{1,2}	-	+	-

¹ *H. parainfluenzae* is ornithine decarboxylase positive, whereas *H. paraphrophilus* is negative.

² Although their requirements for hemin and NAD factor differ from each other, *H. aphrophilus* and *H. paraphrophilus* have recently been reclassified as a single species with a new genus: *Aggregatibacter aphrophilus* (3).

A. Performing hemin and NAD growth factor requirement test using paper disks and/or strips

Growth factor requirements can be identified with paper disks and/or strips using the principles of agar diffusion.

Growth factor requirement procedure using paper disks and/or strips

1. Grow the isolate(s) to be tested for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Prepare a moderately heavy suspension of cells (comparable to a 1.0 McFarland standard) from overnight growth on the CAP in a suitable broth (trypticase soy, heart infusion, or peptone water) and mix well using a vortex.
 - Do not transfer any of the chocolate agar media from the plate to the cell suspension as even the smallest amount of agar will affect the test and may lead to misidentification of the bacteria.
3. Inoculate one half of a heart infusion or trypticase soy agar plate with 10 µl of the cell suspension using a sterile loop or swab and allow the suspension to dry.
 - Two different isolates can be tested on the same plate, but care must be taken to ensure that the cultures do not overlap.
4. Place paper disks or strips containing hemin, NAD, and hemin/NAD on the inoculated plate after the inoculum has dried.
 - When two bacterial strains are tested on the same plate (Figure 5), the disks must be placed in the exact manner shown, keeping the individual hemin and NAD disks separated by the one containing both factors and leaving as much space between the disks as possible.
5. Perform steps 1-4 using a *H. influenzae* and a different *Haemophilus* spp. QC strain to ensure that the hemin and NAD disks or strips are working properly.
6. Carefully invert the plate and incubate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
7. Observe growth around the paper disks or strips.

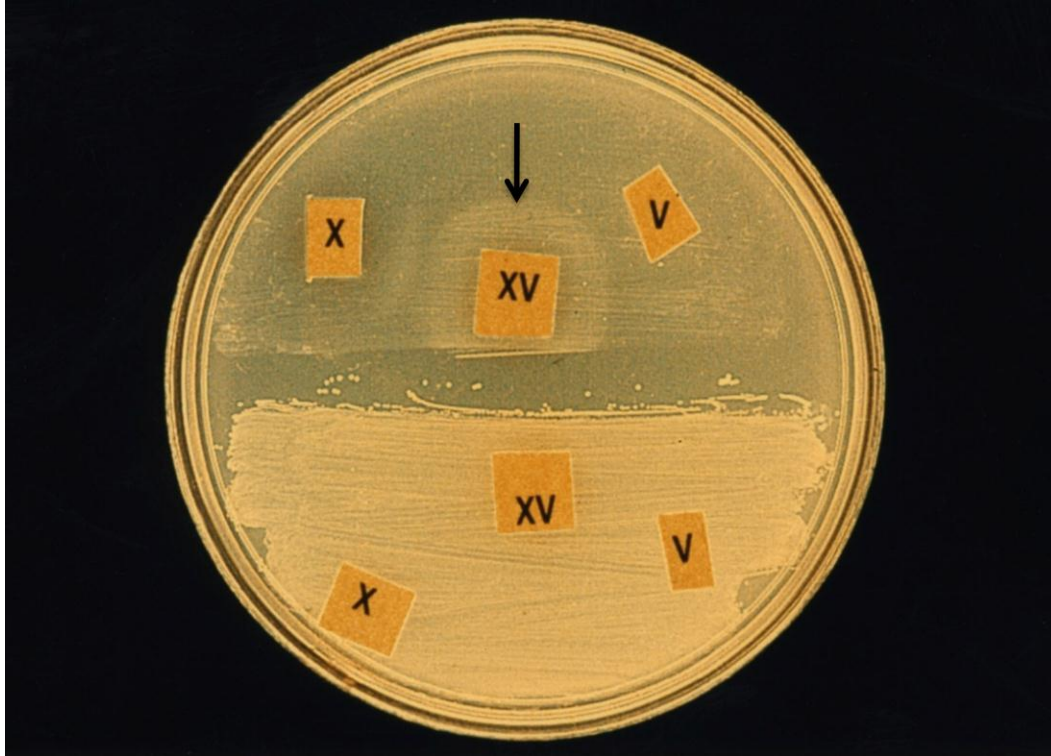


Figure 5. Identification of hemin (X factor) and NAD (V factor) as growth requirements using paper disks. The top strain is only growing around the disk containing both hemin and NAD (black arrow), and is presumptively identified as *H. influenzae*.

Reading the hemin and NAD paper disk and/or strip results

- *H. influenzae* will only grow around the paper disk containing both hemin and NAD, as shown in Figure 5 on the upper half of the plate (see black arrow).
 - *H. haemolyticus* will also only grow around the paper disk containing both hemin and NAD. To differentiate between the two species, hemolysis must be checked on horse or rabbit blood agar by inoculating the cell suspension mentioned above on heart infusion agar with 5% rabbit blood (or agar infusion base containing horse blood). Alternatively, a *Haemophilus* ID Quad plate can be used (see Section II.B. below).
- Other *Haemophilus* spp. will grow around the disk containing both hemin and NAD and either the individual hemin or the NAD disk.
- Alternatively, the porphyrin test (2) can be used. This determines the hemin requirement of the isolate while avoiding the problem of hemin carryover from primary culture media and hemin contamination of test media (3).

B. Performing hemin and NAD growth factor requirement test using *Haemophilus* ID Quad plates

Haemophilus ID Quad plates are another method for determining growth requirements of *Haemophilus* isolates (Figure 6). While they are more expensive than the paper disks or strips, they can test for β -hemolysis (clear) on horse blood and assist in differentiating *H. haemolyticus* from *H. influenzae*. The Quad plate is divided into four compartments. One quadrant includes medium containing hemin only (Figure 6, lower left). The second quadrant includes medium containing NAD only (Figure 6, upper left). The third quadrant contains medium that includes both hemin and NAD (Figure 6, upper right). The fourth quadrant contains heart infusion agar or blood agar base with 5% horse blood (Figure 6, lower right) for detecting hemolysis and differentiating *H. haemolyticus* from *H. influenzae*.

Growth factor requirement procedure using *Haemophilus* ID Quad plates

1. Grow the isolate(s) to be tested for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Prepare a suspension of cells (comparable to a 0.5 McFarland standard) from overnight growth of suspected *Haemophilus* on a CAP in trypticase soy broth or distilled water and mix well using a vortex.
3. Use a sterile, inoculating loop to streak one loopful of the cell suspension onto one quadrant of the Quad plate. Streak the entire quadrant, starting at the periphery of the plate and streaking toward the center of the plate.
 - Use a different inoculating loop to streak each of the other quadrants with the cell suspension.
 - Stab into the blood agar for detection of β -hemolysis (clear).
4. Incubate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
5. After incubation, examine the individual quadrants for growth and the quadrant with horse blood for hemolysis where the plate was stabbed with the loop (Figure 6).

Reading the *Haemophilus* ID Quad plate results

- *H. influenzae* will only grow on the quadrant containing both hemin and NAD and the quadrant containing horse blood. It will not hemolyze the horse blood cells.
- *H. haemolyticus* will only grow on the quadrant containing both hemin and NAD and the quadrant containing horse blood. It will hemolyze the horse blood cells.
 - *H. haemolyticus* may lose their hemolytic property when passed *in vitro*. This has made the definitive identification of *H. influenzae* and *H. haemolyticus* using only biochemical tests very difficult and other methods, such as molecular testing, may be employed for differentiating between the two species.

- An organism growing on either the hemin or the NAD quadrant is likely another *Haemophilus* species
- If growth occurs on every quadrant, the isolate is probably not a *Haemophilus* spp.
- *H. influenzae* may occasionally show slight growth in the quadrant containing NAD only.

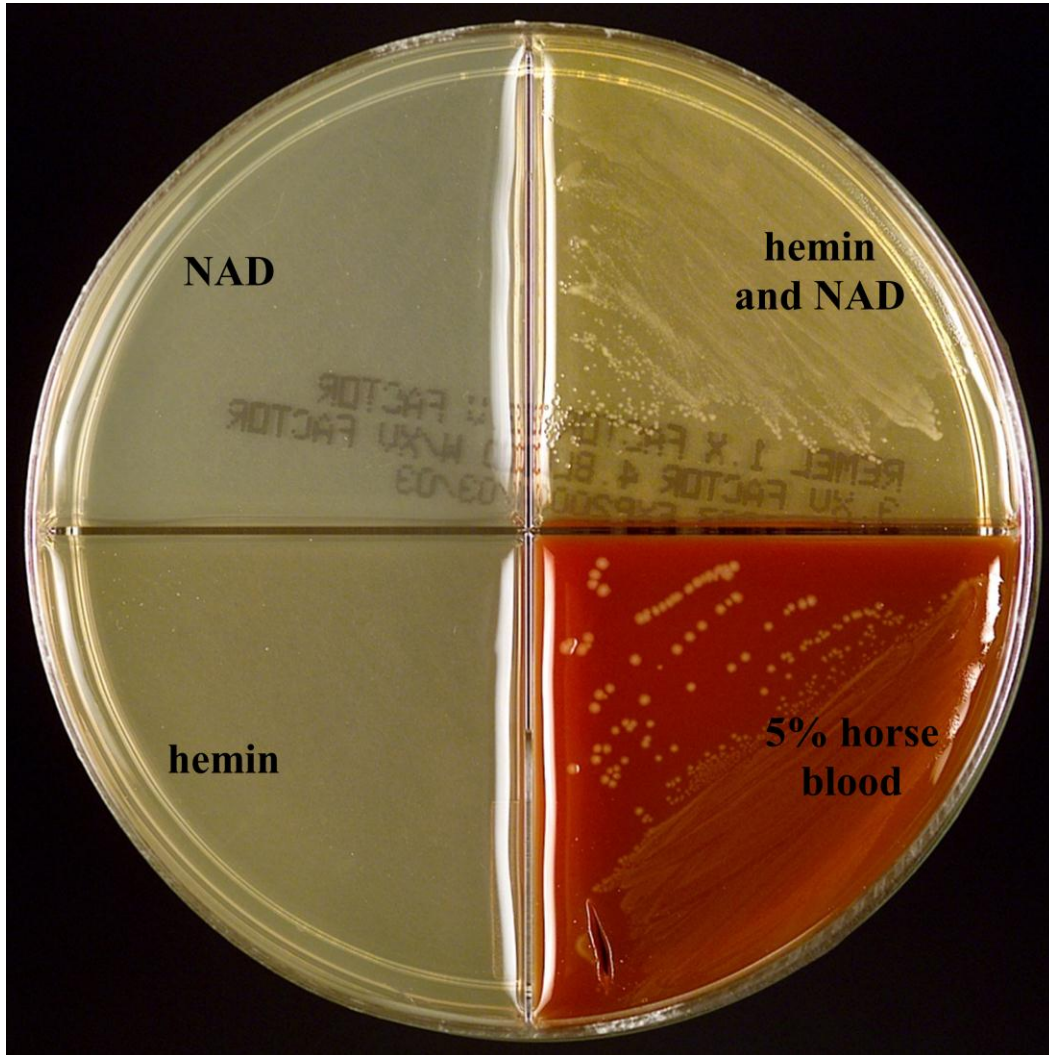


Figure 6. Growth pattern for *H. influenzae* on a *Haemophilus* ID Quad plate

Quality control of Quad plates

QC should be performed on each new lot of Quad plates before they are used for unknown isolates to ensure that they will support the proper growth of *Haemophilus* spp. Three plates from each new lot received should be tested using a well-characterized reference strain of *H. influenzae*, *H. haemolyticus*, and *H. parahaemolyticus*. One uninoculated plate from each new lot should also be tested in order to check for contamination of mold or other organisms in the laboratory and/or incubator. QC should be repeated on plates from a lot if they have been

exposed to temperatures above 4°C or if there is reason to suspect that the plates have been contaminated since the initial QC was performed.

Procedure for quality control of Quad plates

1. Examine the Quad plates for evidence of microbial contamination, discoloration, drying, deterioration, or other physical defects that may interfere with use. Note the firmness of the agar during the inoculation procedure.
2. Grow the reference strains to be tested for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
3. Inoculate the Quad plates using cell suspensions from the reference strains as described in the Quad plate procedure above.
4. Incubate the plates for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
5. As a negative control for contamination, incubate an uninoculated plate from each new lot for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
6. Examine the inoculated and uninoculated plates after 18-24 hours for proper growth of *Haemophilus* spp.

Reading the quality control test results

- *H. influenzae* will only grow on the quadrant containing both hemin and NAD and the quadrant containing horse blood. It will not hemolyze the horse blood cells.
- *H. haemolyticus* will only grow on the quadrant containing both hemin and NAD and the quadrant containing horse blood. It will hemolyze the horse blood cells.
- *H. parahaemolyticus* will grow on all quadrants except for the one containing only hemin. It will hemolyze the horse blood cells.
- Passing result: proper growth of the reference strain on appropriate quadrants and no growth on uninoculated media.
- Failing result: no growth or poor growth of the reference strain on appropriate media and/or growth of organisms on the uninoculated media.

VIII. Identification of *H. influenzae* serotype

Haemophilus influenzae can be encapsulated with one of six types of antigenically distinct capsules which can be serotyped using antisera to each capsule (serotypes a-f). *H. influenzae* may also be non-encapsulated and such strains that cannot be serotyped are called *H. influenzae* nontypeable (NT). Individual serotype-specific antisera for these major serotypes are available

commercially. A polyvalent antiserum that recognizes all 6 serotypes is also available commercially.

It is not always practical to test for all serotypes for which antisera are available in a laboratory. Testing algorithms may be set up in laboratories with previous knowledge of whether or not a *H. influenzae* serotype b (Hib) vaccination program has been implemented within that particular geographic region. Modifications may be made to the testing algorithm for any laboratory based on information regarding Hib vaccination status of the region.

It is essential that reference laboratories have the capacity to isolate, identify, and characterize the serotype of isolates of *H. influenzae*. This valuable data provides laboratories and public health authorities with the tools to identify outbreaks controllable by vaccination campaigns and recognize serotypes causing sporadic disease.

SAST test algorithm for areas without an established Hib vaccination program

If a Hib vaccination program has not been implemented in the country or region from which the isolate originated, it is likely that the *H. influenzae* isolate is serotype b and the isolate should first be tested for reactivity to serotype b antisera and a negative saline control. If the isolate reacts positively with the serotype b antiserum with no agglutination in saline, the isolate is identified as Hib. However, if the isolate is non-reactive with the serotype b antiserum, and if polyvalent antiserum is available, it should be tested with the polyvalent antiserum. If positive, the isolate should then be tested with the remaining monovalent antisera (a, c, d, e, and f) to determine the serotype. If negative for all monovalent antisera and positive for hemin and NAD growth requirements, then the isolate is considered NT.

SAST test algorithm for areas with an established Hib vaccination program

If the isolate is from a country or region with an established Hib vaccination program that has high Hib vaccine coverage, the isolate is likely to be NT or a serotype other than b. In this case, the isolate should first be tested with the polyvalent antiserum, if available, and a negative saline control. If positive for the polyvalent antiserum with no agglutination in the saline, the isolate should then be tested with the remaining monovalent antisera (a, b, c, d, e, and f) to determine the serotype. If the isolate is negative for the polyvalent antiserum and/or the monovalent antisera, and requires hemin and NAD for growth, then the isolate is considered NT.

A. Slide agglutination serotyping (SAST) test for serotyping *H. influenzae* isolates

Formalin-killed suspensions of *H. influenzae* should be used for SAST testing rather than saline suspensions of living organisms to maintain a safe working environment. A solution of 5% formalinized physiological saline is sufficient to kill the bacteria. However, formalin is a carcinogen and must be stored and handled with great care. Alternatively, work should be performed under a biosafety hood if formalin is not used. Antisera should be stored in the refrigerator at 4°C and warmed to room temperature (25°C) before use. It must be put back in the refrigerator as soon as testing is finished to prevent the loss of binding activity of the antibody.

B. Performing the SAST test

1. Grow the isolate(s) to be tested for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Clean a glass slide with alcohol (optional if slides are pre-cleaned).
3. Divide the slide into equal sections (e.g., twelve 11 X 22 mm sections on a standard 50 X 75 mm slide) with a liquid impermeable pen or a wax pencil.
 - Each isolate will require as many sections on the slide as antisera that will be tested (polyvalent and/or individual serotype-specific) as well as a saline negative control.
4. In the lower portion of each of the sections of the glass slide described in step (2), add 10 µl of the 5% formalinized saline with a micropipettor.
 - The instructions specify using a micropipettor with sterilized filtered tips to measure the 10 µl of the 5% formalinized saline to suspend the bacteria. The micropipettor will transfer precise and equal measurements for a proper SAST reaction.
 - If a micropipettor and tips are not available, sterile, disposable 10 µl inoculation loops can be used to transfer 10 µl of the 5% formalinized saline, but often do not deliver accurate amounts (between 5-10 µl).
5. Use a sterile, disposable 10 µl inoculating loop to collect a few colonies from the surface of the overnight culture incubated on the CAP.
6. Suspend the bacteria in the 5% formalinized saline solution in the lower portion of each of the sections of the slide. The suspension should be moderately opaque (see saline control in Figure 7). Do not allow the cell suspension to dry before adding the antisera.
 - If the bacteria are difficult to suspend directly on the slide, make a moderately milky suspension (comparable to McFarland 6.0 standard) of the test culture in a small vial with 250 µl of 5% formalinized saline and briefly vortex the suspension to mix and break up any pellets. Add 10 µl of this suspension to the lower portion of the slide.
7. In the upper portion of each of the sections of the glass slide described in step (2), add 10 µl of the polyvalent and/or serotype-specific antisera to be tested as well as unformalinized saline or phosphate buffered saline (PBS) for a negative control with a micropipettor.
 - DO NOT use the dropper provided with the antisera because it usually delivers larger amounts than is necessary and can easily be contaminated.
 - If a micropipettor and tips are not available, sterile, disposable 10 µl inoculation loops can be used to transfer 10 µl of the antisera, but often do not deliver accurate amounts (between 5-10 µl).

- Dispose of the tip or loop used to transfer the antisera to the slide in a waste container after each use to avoid contamination of the antisera. If the source of antisera is contaminated, a new vial must be used.
8. Gently tilt the slide to mix the cell suspensions with the antisera in each section. Continue to gently rock the slide for 1 to 2 minutes to allow the lower and upper portions to completely blend. Do not use a circular motion while rocking, as it can cause the sections with different serogroup-specific antisera to run together and contaminate each other.
 9. After 2 minutes, examine the SAST reactions under a bright light and over a black background. Use the rating system in Figure 7 to determine the intensity of the agglutination reaction in each section of the slide. Disregard any agglutination that occurs after the 2 minute time period.
 10. Record the SAST results in the laboratory log book.

C. Reading the SAST results

Rating the intensity of the agglutination reaction

Agglutination occurs when the antisera bind to the bacterial cells causing the cells to agglutinate or clump together, thus making the cell suspension appear clearer. The intensity of the agglutination reaction may vary according to the density of the cell suspension or the antisera used. A description on the intensity ratings shown in Figure 7 are listed below.

- 4+ All of the cells agglutinate and the cell suspension appears clear
- 3+ 75% of the cells agglutinate and the cell suspension remains slightly cloudy
- 2+ 50% of the cells agglutinate and the cell suspension remains slightly cloudy
- 1+ 25% of the cells agglutinate and the cell suspension remains slightly cloudy
- +/- Less than 25% of the cells agglutinate and a fine granular matter occurs
- 0 No visible agglutination; the suspension remains cloudy and smooth

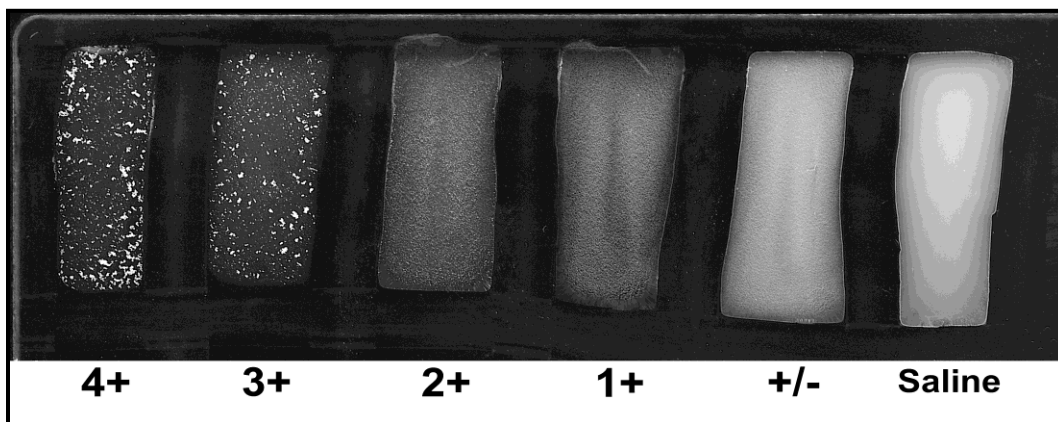


Figure 7. Rating the intensity of the agglutination reaction

Determining the serotype

- A positive result is designated by a 3+ or 4+ (strong agglutination) within 1-2 minutes.
- A negative result is designated by a 0 (saline), +/-, 1+ or 2+ (weak agglutination).
- The serotype is determined when a positive result occurs with the polyvalent antiserum and/or only one of the serotype-specific antisera and not with the saline.
- If a serotype is not determined, the isolate is considered NT. The following result combinations are all reported as NT:
 - Agglutination in the saline, regardless of strong reactions with the polyvalent or other serotype-specific antisera, characterizes the culture as autoagglutinating.
 - Agglutination with the polyvalent and/or more than one serotype-specific antisera in the absence of agglutination in saline characterizes the culture as polyagglutinating or cross-reactive.
 - No agglutination with the polyvalent or any of the serotype-specific antisera or the saline characterizes the strain as non-reactive.
 - Although rare, an isolate positive for the polyvalent antiserum, but negative for the serotype-specific antisera is considered NT.

D. Troubleshooting the SAST procedure

H. influenzae isolates are subject to variability (encapsulated vs. unencapsulated, small vs. large colonies, slow growers vs. fast growers, and heavy agglutinators vs. light agglutinators) and may be unclear or difficult to interpret. Some troubleshooting procedures are listed below:

1. Repeat the test directly on the slide using growth from another section of the same plate.
2. Make a cell suspension in a small tube and vortex if the result from SAST directly on the slide is unclear and repeat the test.
3. Add 20 μ l of antisera directly to slide and then add a loopful of organism without diluting the specimen with 5% formalinized saline.
4. Subculture and retest fresh growth the following day.
5. If the original plate contains different size colonies, make a subculture for each type of colony and test both cultures the next day. The larger colonies usually indicate better capsule production and therefore better reactivity. However, the smaller colonies will occasionally give a better result.

- If discrepancies are not immediately resolved, any subsequent SAST repeats should be used in conjunction with control strains.

E. Quality control of antisera for SAST testing

A set of reference strains for *H. influenzae* serotypes a, b, c, d, e, and f (one per serotype) and a nontypeable *H. influenzae* strain should be used to QC the antisera before testing any unknown isolates. QC of the antisera should be:

- Performed for each new lot of antisera received in the laboratory.
- Performed biannually after initial QC testing.
- Repeated if a vial has been exposed to temperatures above 4°C or if there is reason to suspect that the vial has been contaminated since the initial QC was performed.

Follow the SAST testing procedure to QC each lot of antisera using all reference strains available in the laboratory. Record the results provided on the example QC sheet in Figure 8.

Reading the quality control test results

Passing test:

- The antiserum must give 3+ or 4+ agglutination with homologous antigens within 1-2 minutes.
- The antiserum must not react with heterologous *H. influenzae* serotypes, with the NT reference strain, or in saline.

Failing test:

- The antiserum agglutinates with one or more reference strains and/or with the NT reference strain and/or in saline.

	a (strain number)	b (strain number)	c (strain number)	d (strain number)	e (strain number)	f (strain number)	NT (strain number)
a Lot#	++++	-	-	-	-	-	-
b Lot#	-	++++	-	-	-	-	-
c Lot#	-	-	++++	-	-	-	-
d Lot#	-	-	-	++++	-	-	-
e Lot#	-	-	-	-	++++	-	-
f Lot#	-	-	-	-	-	++++	-
Poly Lot#	++++	++++	++++	++++	++++	++++	-
Saline	-	-	-	-	-	-	-

Figure 8. Example QC sheet for testing antisera against all *H. influenzae* serotypes

IX. Commercial identification kits

Several commercial identification systems that use biochemical or enzymatic substrates are available for identification of *Haemophilus* spp. These systems may occasionally require supplemental tests, and additional characteristics, such as microscopic and colony morphology, must be considered. Generally, each system is self-contained, but the addition of one or more reagents to complete certain reactions may be necessary. The manufacturer's instructions should be followed precisely when using these kits. Some of the commercial identification kits also include biotyping of *H. influenzae*. For detailed instructions and use of appropriate control strains, consult the Clinical Microbiology Procedures Handbook (1).

References

1. Clinical Microbiology Procedures Handbook, 3rd edition. 2010. ASM. Washington, D.C. 2,540 pages.
2. **Murray, P. R., E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.).** 2007. Manual of Clinical Microbiology, 9th ed, vol. ASM Press, Washington, D. C.

3. **Norskov-Lauritsen, N., and M. Kilian.** 2006. Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include NAD factor-dependent and NAD factor-independent isolates. *International Journal of Systematic and Evolutionary Microbiology* **56**:2135-2146.

CHAPTER 10

PCR for Detection and Characterization of Bacterial Meningitis Pathogens: *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*

I. Overview of PCR technologies

In developing countries, the most commonly used approaches for detection and characterization of bacterial meningitis pathogens include culture, Gram stain, and latex agglutination. Although culture is considered the gold standard for case confirmation in clinics, the positive rate is relatively low due to suboptimal storage and transportation conditions, culture practice, and/or antibiotic treatment administered before the specimen is collected. While Gram staining is important, inexpensive, and should be performed whenever possible, it merely gives a clue as to the genus and species of the etiological agent. The reading of latex agglutination results is subjective and can be difficult to interpret, especially when a specimen's bacterial load is low. It is also not feasible to do quality control on latex agglutination. Culture should be kept as the gold standard as cultured bacteria are sources of data for antibiotic susceptibility, complete subtyping, the expression of antigens that are to be included in future vaccines, and pathophysiology of isolates. Specimens that do not yield any culture can still be analyzed by molecular methods (see below) that can be applied on DNA extracted from clinical specimens (typically, blood and CSF).

A. Polymerase chain reaction (PCR)

PCR was developed in the mid- to late 1980s (36, 42) and is considered one of the most important methodological inventions in molecular biology. It is designed to permit selective amplification of a specific target DNA sequence(s) within a heterogeneous collection of DNA sequences (e.g., total genomic DNA). In PCR, the DNA target is exponentially amplified through repeating three major steps: 1) denaturation of double-stranded DNA into single-stranded DNA; 2) annealing of primers to the complementary single-stranded target sequences; and 3) extension of the primers in the 5' to 3' direction by heat-stable DNA polymerase to produce double-stranded DNA molecules. The copy number of DNA molecules is doubled in each extension step, generating millions of copies of the original DNA molecules when PCR is completed. Because the method does not require live or intact cells, PCR is a valuable tool for detecting bacterial pathogenic agents from clinical specimens, where bacteria die or lyse easily due to inappropriate storage conditions or prior antibiotic treatment. PCR is now widely used in the diagnosis and surveillance of bacterial pathogens because of its high sensitivity and specificity and high throughput capabilities. It provides a complementary tool to classic phenotype-based methods such as culture, Gram stain, and latex agglutination and often enhances confirmatory results (3).

A number of conventional PCR assays have been developed for detection and subtyping of bacterial and viral pathogens. Conventional PCR detects products at the end point of DNA amplification by visualizing amplicons using agarose gel electrophoresis. Gel-based detection requires that tubes containing PCR amplicons be opened and manipulated, thereby greatly increasing the risk of contamination of laboratory space, equipment, and reagents with amplified

materials. Conventional PCR is also very time consuming and less sensitive and specific than a type of PCR called real-time PCR, so it is primarily used for typing assays employing purified cultures or clinical specimens that contain the organism in high density. The use of real-time PCR is rapidly expanding because it is easier to perform and, being a closed system, it reduces potential contamination problems inherent to conventional PCR. Many of the same precautions mentioned for conventional PCR assays also apply to real-time PCR assays.

B. Real-time PCR technology

Real-time PCR is also known as quantitative real-time polymerase chain reaction (Q-PCR/qPCR) or kinetic polymerase chain reaction, which combines amplification and detection in one step through the use of fluorescent dyes. There are two types of detection systems: non-specific and specific. Non-specific detection systems use a fluorescent dye that intercalates into any double-stranded DNA molecules and emits enhanced fluorescence. This detection system is relatively inexpensive but susceptible to false positivity. Specific detection systems rely on fluorescent resonance energy transfer probes that specifically recognize target sequences, thus making them the systems of choice for the molecular detection assays described here. Specific detection systems are more expensive than non-specific detection systems and require sophisticated probe designs. Three types of probes are currently in use, including: hydrolysis, hybridization, and hairpin probes (7, 13). A fluorescent signal is only generated if the probe interacts with its specific target and is subsequently hydrolyzed during amplification. The resulting increase in fluorescence is proportional to the amount of amplified PCR product in the reaction.

The first use of dual-labeled hydrolysis probes was reported in 1993 (30) and has been widely used in many laboratories since that time. A dual-labeled hydrolysis probe is an oligonucleotide (~17-35 bp long) labeled with a reporter fluorophore (usually a short wavelength colored dye) at the 5' end and a quencher fluorophore (usually a long wavelength colored dye) at the 3' end or at an internal thymine or "T" residue. Optimally, the quencher dye should be 7-15 base pairs from the reporter dye. When the probe is intact and excited by a light source, the fluorescence emission of the reporter (or donor) dye is absorbed by the quencher (or acceptor) dye as a result of the close proximity of the dyes. This process is also known as fluorescence resonance energy transfer (FRET). During PCR amplification, the probe anneals to an internal region of the target DNA template between the forward and reverse primer. When DNA polymerase catalyzes the extension of the primer and reaches the region where the probe is bound, the 5' exonuclease activity of the DNA polymerase cleaves the probe and releases the reporter from the quencher. This allows emission of the fluorescence from the reporter dye to be observed because it is no longer absorbed by the quencher which has diffused away (Figure 1). The increase in fluorescence is proportional to the amount of amplified PCR product in the reaction and is measured cumulatively over the course of the entire PCR run (7, 30).

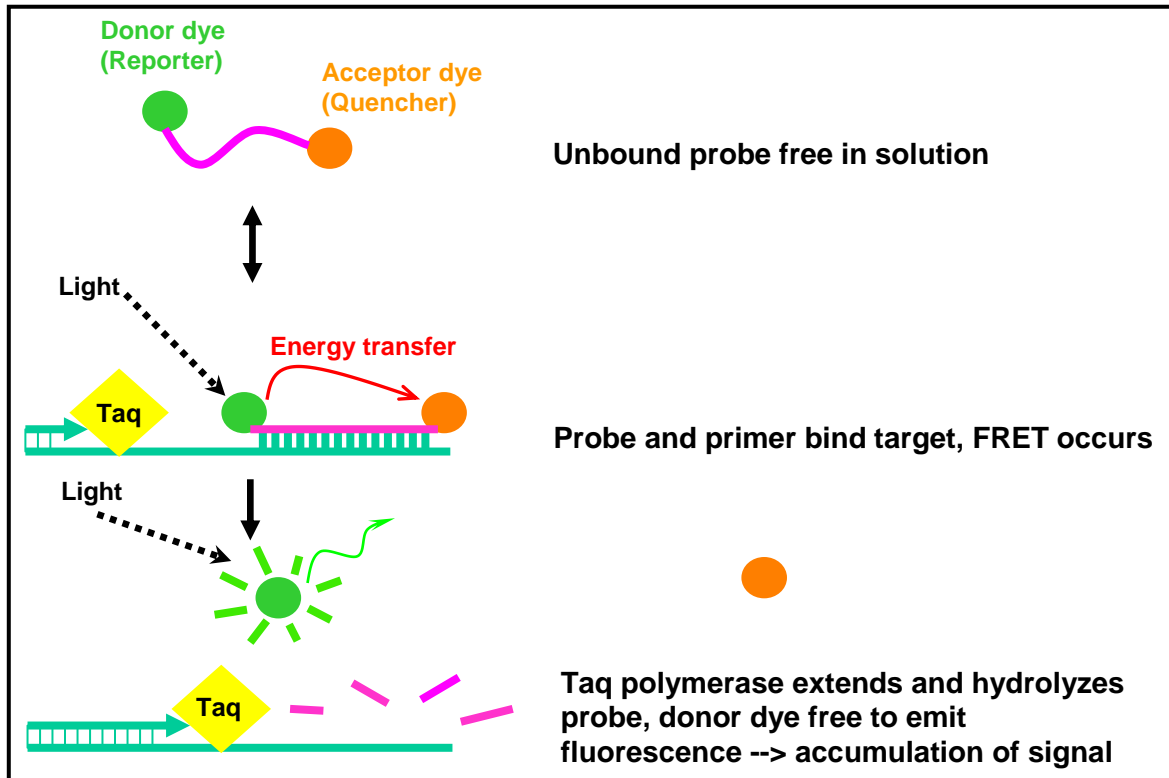


Figure 1. Chemistry of a dual-labeled hydrolysis probe

II. Target gene assays for detection and characterization of bacterial meningitis etiologies

Over the past several years, conventional and real-time PCR assays have been developed for detection of bacterial meningitis pathogens. Reliable assays have been extensively evaluated using invasive clinical isolates and/or clinical specimens from around the world (8, 12, 15, 17, 29, 35, 52, 60). In general, validated assays should have high sensitivity and specificity. They can be used as complementary approaches in bacterial disease diagnosis. The gene targets, DNA sequence of primers and probes, and the final concentration used in the PCR reactions described here to detect and characterize *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* are listed in Tables 2-5.

The PCR strategy typically employed to detect the causative agent in a suspected case of bacterial meningitis is to first run each of the species-specific assays concurrently on the DNA extracted from the clinical specimen or isolate. The appropriate serogroup/serotype specific assays should then be run on any positive specimens. See Section V below, “Workflow for detection of bacterial meningitis pathogens by PCR”, for more details.

A. Species-specific real-time PCR assays

PCR detection of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* can be achieved by amplification of several potential gene targets (8, 35, 53, 60). The following assays have been

developed and validated to be used on DNA extracted from clinical specimens (typically, blood and CSF) and bacterial isolates.

N. meningitidis

Two genes can be targeted in *N. meningitidis* species-specific assays, *ctrA* and *sodC*. The capsule transport to cell surface gene, *ctrA*, is highly conserved among isolates responsible for invasive meningococcal infections and has been used in both real-time and conventional PCR to detect *N. meningitidis* (35). It is a gene within the capsule locus (Figure 2). However, since at least 16% of carried meningococci lack *ctrA* (10, 14, 41), a real-time PCR assay to detect all meningococci, regardless of encapsulation status, was recently developed and validated (15). This assay targets the Cu, Zn superoxide dismutase gene, *sodC*, which is not genetically linked to the capsule locus. The *sodC* assay detects encapsulated meningococci, but it is also useful for detecting nongroupable meningococci that do not contain an intact *ctrA*, as will be recovered during carriage studies. For this reason, it is recommended that *sodC* be used for detection of *N. meningitidis*, if possible. *sodC* and *ctrA* primers and probes are listed in Table 2.

H. influenzae

The protein D encoding gene, *hpd*, encodes protein D, a highly conserved, surface-exposed lipoprotein that is present in all encapsulated and non-encapsulated *H. influenzae* (24, 45). The conserved nature of this gene and its presence in all strains of *H. influenzae* characterized to date make it a highly attractive gene target for the development of a *H. influenzae* species-specific real-time PCR assay. The recently developed and validated *hpd* real-time PCR assay is capable of detecting all six serotypes (a-f) and nontypeable (HiNT) *H. influenzae* with high sensitivity and specificity (60). Real-time PCR assays targeting *bexA* were developed and distributed because *bexA* is present in all six serotypes of *H. influenzae*. However, though sensitive for detection of Hib, it is less sensitive for Hia, Hic, and Hid, and does not detect Hie, Hif, or HiNT and should no longer be used. The primers and probes for the *hpd* assay are listed in Table 2.

S. pneumoniae

Both conventional and real-time PCR assays have been developed for the detection of *S. pneumoniae*, and target genes have included the pneumolysin (*ply*), autolysin (*lytA*), and pneumococcal surface adhesion (*psaA*) genes (8). However, false-positive results with *ply*-based PCR have been reported when applied to upper respiratory tract specimens. A suggested explanation for these false positives is the detection of non-pneumococcal alpha-hemolytic streptococci (37, 47), which are normally present in the respiratory flora (e.g., *Streptococcus mitis* group and *Streptococcus oralis*) which sometimes contain a *ply* gene (62). The PCR detection assay for *S. pneumoniae* using a specific segment of the autolysin gene (*lytA*) is recommended because it is highly conserved within the species and it has been shown that this assay best separates *S. pneumoniae* from the genotypically similar species *S. mitis*, *S. oralis*, and *S. pseudopneumoniae* (33). The real-time PCR assay *lytA* primers and probes that have been found to be extremely reliable for detection of *S. pneumoniae* are listed in Table 2. Due to recombination events that occur between pneumococci and closely related streptococci, there

will probably be rare false-positives or false-negatives for virtually any real-time assay for pneumococcal identification.

B. Serogroup/serotype-specific real-time PCR assays

The capsule gene loci of both *N. meningitidis* and *H. influenzae* have areas that are both unique and conserved within each serogroup (*N. meningitidis*) or serotype (*H. influenzae*) thus providing gene targets for the development of real-time PCR assays designed to identify each specific serogroup or serotype.

N. meningitidis

N. meningitidis is classified into 12 serogroups on the basis of the chemical composition and linkage type of saccharide subunits of the capsular polysaccharide that are expressed on the bacterial cell surface. Major disease-causing serogroups include A, B, C, Y, and W135, the latter four of which produce sialic acid containing capsular polysaccharides; whereas serogroup A produces a poly- α 1-6-linked N-acetylmannosamine 6-phosphate capsule (31). Outbreaks caused by serogroup X meningococci, which express poly- α 1-4-linked N-acetylglucosamine 1-phosphate capsule (6) have also been reported (2, 20). Serogroup D is no longer recognized as a serogroup of *N. meningitidis*.

As illustrated in Figure 2, the genetic organization of the capsule locus is conserved among the serogroups. The capsule expression genes are located in four operons: one that encodes capsule biosynthesis (called *syn* or *sia* genes, depending on which nomenclature system is used) and three that encode the capsule transport to the cell surface proteins (*ctr*). The gene products of the *ctr* operon share high similarity with the ATP-dependent transporters of the ABC family (19) and are highly conserved among the major disease-causing serogroups (1, 18, 39, 55) and serogroup X (56). Sensitive real-time PCR assays targeting *ctrA*, which is the first gene in the capsule transport operon, have been developed for detection of all encapsulated and some non-encapsulated (nongroupable) *N. meningitidis* (12, 35), though a more specific and sensitive assay using the *sodC* gene as a target has been developed. The genetic differences among the capsule biosynthesis operons of meningococcal serogroups have facilitated the development of real-time PCR assays targeting serogroup-specific genes for capsule biosynthesis to determine the capsule genotype of a meningococcal isolate (35).

The gene *sia* (for sialic acid biosynthesis (16, 22), also called *syn* for capsule biosynthesis (21, 51), are used for genotyping for serogroups B (*synD*), C (*synE*), Y (*synF*) and W135 (*synG*). The *sacB* gene is targeted for serogroup A and the *xcbA* gene, which most likely encodes the capsule polymerase, is targeted for serogroup X (2, 35). These target genes are depicted within the structure of the capsule gene complex for serogroups A, B, C, Y, W135, and X (Figure 2). The most current adapted primer and probe sequences for the serogrouping real-time PCR assays are listed in Table 3, though, periodically, the primers and probes are adapted as new information regarding probe chemistries and allelic variations become available.

There have been several different systems in place for naming the genes for meningococcal capsule biosynthesis. Some groups have called this operon *syn* for capsule biosynthesis (21, 51);

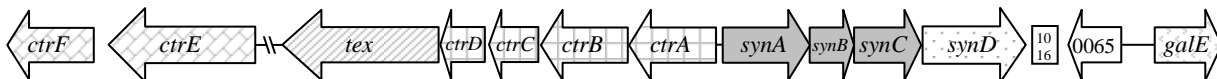
other groups have used the *sia* nomenclature for sialic acid biosynthesis (16, 22); still others have referred to them as *neu* genes based on homologies to *E. coli* K1 genes for *N*-acetylneuraminic acid biosynthesis (21). While the *siaD* genes of serogroups B, C, W135, and Y were initially thought to be alleles, more extensive sequencing analysis demonstrated that this is not so. The *synD* and *synE* genes of serogroups B and C, respectively, are alleles and encode capsular polysaccharide polymerases that catalyze different linkages of sialic acid monomers ($\alpha 2 \rightarrow 8$ linkage for serogroup B and $\alpha 2 \rightarrow 9$ linkage for serogroup C). However, the Y and W135 genes are over twice the size of the B and C genes and differ in nucleotide sequence (11, 50), though they are highly similar to each other. In addition, the polymerases for serogroups Y and W135 link heteropolymers of sialic acid plus either glucose or galactose, respectively. Thus, the capsular polysaccharide polymerase genes of serogroups Y and W135 are alleles. To continue to call all of these polymerase genes *siaD* would be a misnomer. For these reasons and for simplicity, this text will use the *synABCD/E/F/G* nomenclature listed above and in Table I.

Table 1. Serogroup capsule type and gene targets for genotyping real-time PCR assays

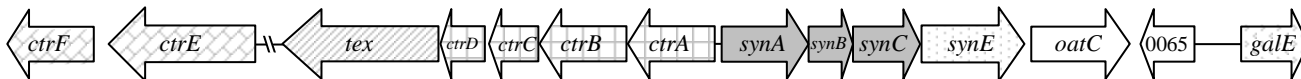
Sero-group	Capsule type	Gene Target Name	Alternate Gene Names	Ref
A	($\alpha 1 \rightarrow 6$)- <i>N</i> -acetyl-D-mannosamine-1-phosphate	<i>sacB</i>		(31)
B	($\alpha 2 \rightarrow 8$)- <i>N</i> -acetylneuraminic acid	<i>synD</i>	<i>siaD</i> <i>siaD</i> of B <i>siaD_B</i>	(5, 9, 23, 48, 61)
C	($\alpha 2 \rightarrow 9$)- <i>N</i> -acetylneuraminic acid	<i>synE</i>	<i>siaD</i> of C <i>siaD_C</i>	(5, 9, 50, 58, 61)
W135	6-D-Gal($\alpha 1 \rightarrow 4$)- <i>N</i> -acetylneuraminic acid($\alpha 2 \rightarrow 6$)	<i>synG</i>	<i>siaD</i> of W135 <i>siaD_W</i>	(4, 9, 14, 35, 49, 61)
X	($\alpha 1 \rightarrow 4$)- <i>N</i> -acetyl-D-glucosamine-1-phosphate	<i>xcbB</i>		(2, 6)
Y	6-D-Glc($\alpha 1 \rightarrow 4$)- <i>N</i> -acetylneuraminic acid($\alpha 2 \rightarrow 6$)	<i>synF</i>	<i>siaD</i> of Y <i>siaD_Y</i>	(4, 9, 14, 35, 49, 61)

For serogroups B, C, Y and W135, the first three genes of the *syn* operon encode functions for the synthesis of capsule polysaccharide precursors (32, 48, 51). The fourth gene product is a polymerase that catalyzes the formation of polymers with the serogroup-specific linkage. In serogroups B and C, the products of a four-gene operon (*synABC* plus the polysialyltransferase gene *synD* [Nmen B] or *synE* [Nmen C]) are responsible for biosynthesis of the sialic acid (also known as *N*-acetylneuraminic acid, NeuNAc, or NANA) homopolymer. Expression of the poly-*N*-acetylmannosamine-1-phosphate capsule polymer of serogroup A requires the *sacABCD* operon (formerly known as *mynABCD* (49), while expression of the poly-*N*-acetyl-D-glucosamine-1-phosphate capsule of serogroup X requires the *xcbABC* operon.

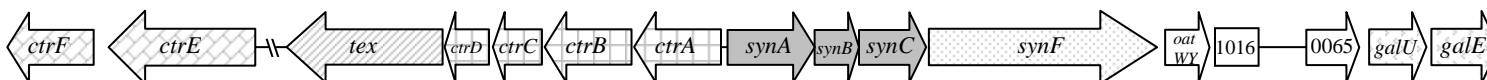
***N. meningitidis* Serogroup B [(α 2 \rightarrow 8)-*N*-acetylneuraminic acid]**



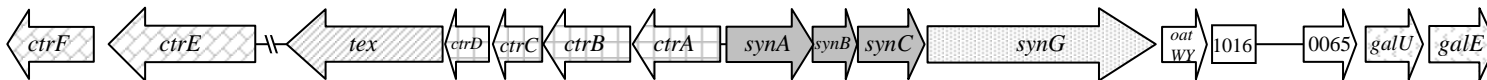
***N. meningitidis* Serogroup C [(α 2 \rightarrow 9)-*N*-acetylneuraminic acid]**



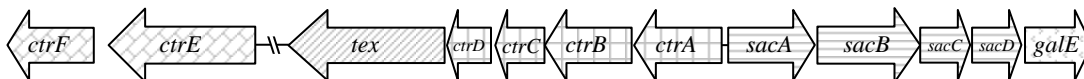
***N. meningitidis* Serogroup Y [6-D-Glc (α 1 \rightarrow 4)-*N*-acetylneuraminic acid (α 2 \rightarrow 6)]**



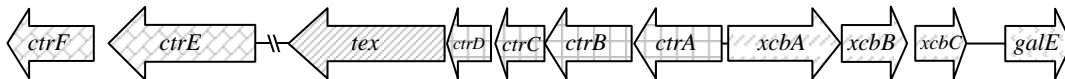
***N. meningitidis* Serogroup W135 [6-D-Gal (α 1 \rightarrow 4)-*N*-acetylneuraminic acid (α 2 \rightarrow 6)]**



***N. meningitidis* Serogroup A [(α 1 \rightarrow 6)-*N*-acetyl-D-mannosamine-1-phosphate]**



***N. meningitidis* Serogroup X [(α 1 \rightarrow 4)-*N*-acetyl-D-glucosamine-1-phosphate]**



N. gonorrhoeae*, *N. lactamica*, and some nongroupable *N. meningitidis



Figure 2. Genetic maps of the capsule gene complex (*cps*) of *N. meningitidis* (adapted from (14)). The *ctrABCD* operon encodes ATP-dependent export proteins (grid pattern). *synABC* (solid gray) *D/E/F/G* (dotted), *sacABCD* (horizontal striped), and *xcbABC* (dashed upward diagonal), encode the serogroup-specific enzymes for capsule polymer biosynthesis. *oatC* (serogroup C) and *oatWY* (serogroups W135 and Y), are co-transcribed with the *syn* operons and encode O-acetyltransferases (9). *lipA* and *lipB* code for proteins that were originally proposed to add a phospholipid-anchoring group onto the polysaccharide reducing end before transport (19). *ctrE* and *ctrF* (diagonal brick), formerly known as *lipA* and *lipB*, respectively, are involved in capsule transport (57). These gene products were once thought to be involved in post-polymerization modification. Many nongroupable, carried meningococci lack all or part of the capsule locus (10, 14); those lacking the entire locus have a genetic configuration at this position like those of *N. gonorrhoeae* and *N. lactamica*, which are not known to synthesize capsule.

Haemophilus influenzae

The capsule locus of all six serotypes of *H. influenzae* (Hi a, b, c, d, e, and f) consists of three regions encoding functions for capsule polysaccharide synthesis, modification, and translocation (Figures 3 and 4) (25, 43, 44). *bexDCBA* in the ATP-driven export region (also known as Region I) code for protein components of an ATP-driven polysaccharide export apparatus. *hcsA* and *hcsB* in post polymerization modification region (also known as Region III) share high similarity with *lipA* and *lipB* (recently renamed *ctrE* and *ctrF*), respectively, which are involved in modification and export of meningococcal capsule polysaccharide (43). Both regions are common to all serotypes. The same nomenclature is used for genes in the two regions for all six serotypes. The serotype-specific region (previously Region II) contains genes for capsule synthesis and is unique to each serotype. The serotype-specific genes are named *acs*, *bcs*, etc. for “a capsule synthesis”, “b capsule synthesis”, and so on.

The *cap* locus encodes functions for *H. influenzae* capsule synthesis. The genetic organization of the *cap* locus has been well characterized in Hib and Hif, which belong to two phylogenetic divisions that are defined by multilocus enzyme electrophoresis typing. The ATP-driven export region includes most of Hia and Hib strains, and all of Hic, Hid, and Hie strains. Strains from this region have at least one completed *cap* locus flanked by insertion sequence (IS) element *IS1016*, except Hie. The majority of Hib strains and some Hia strains from this region have the direct-repeat configuration with the second copy of the *bexA* gene partially deleted as illustrated in Figure 4. The truncated *cap* locus is not required for capsule synthesis (26-28). In Hif, Hib, and Hia strains of the serotype-specific region and some Hie strains of the ATP-driven export region, the *cap* locus is flanked by *sodC* and HI1637 (26, 44).

While serotype b causes the vast majority of *H. influenzae* disease in countries without a Hib vaccination program, serotypes a, c, d, e, and f, and nontypeable *H. influenzae* (NTHi), also contribute to case numbers (43, 59). As implementation of Hib vaccine becomes more widespread, it is important to monitor incoming specimens for both b and non-b serotypes of *H. influenzae*. Therefore, serotype-specific real-time PCR assays have been developed that target the serotype-specific region genes where possible, or the 5' end of *bexD*, which is less conserved among the serotypes compared with the other genes in the export region. The genes targeted for real-time PCR assays specific to each serotype are as follows: *acsB* (Hia), *bcsB* (Hib), *ccsD* (Hic), *dcsE* (Hid), *ecsH* (Hie), and *bexD* (Hif) (Figure 3). The primer and probe information for

each of these serotyping assays can be found in Table 4. Each of these assays has been shown to be highly specific and sensitive for their respective serotypes.

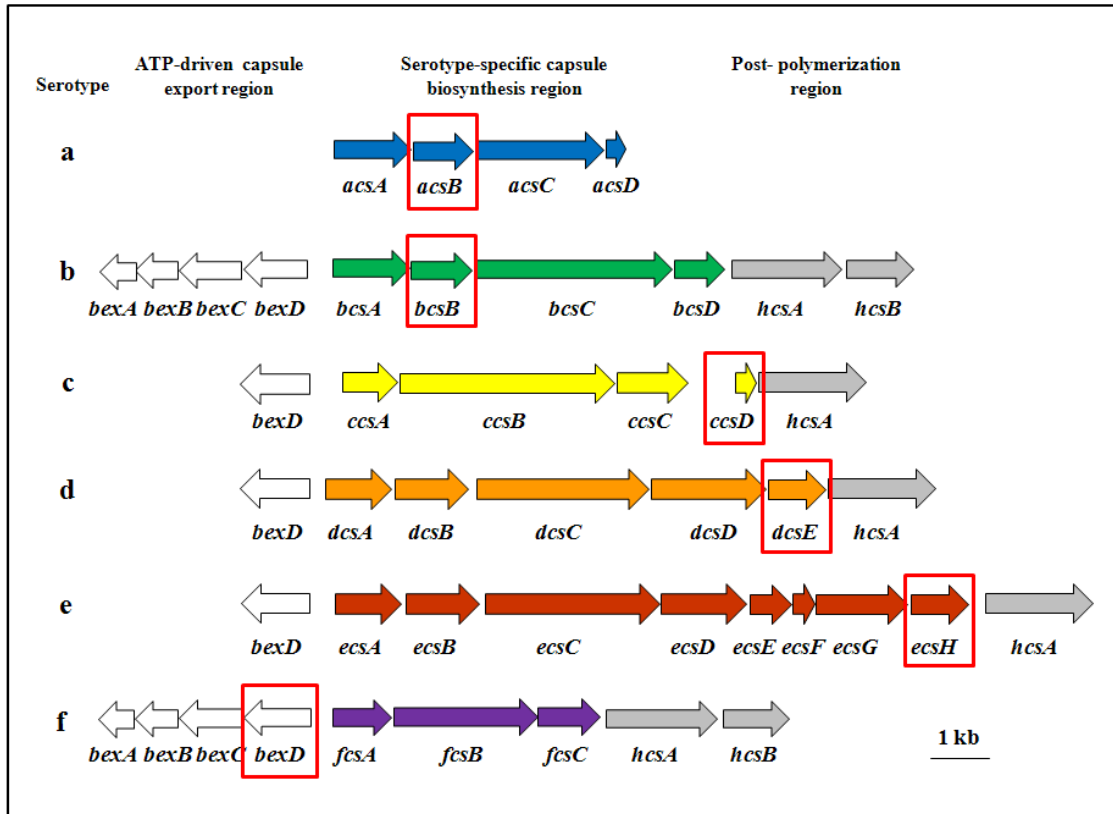


Figure 3. Capsule loci for *H. influenzae* serotypes a, b, c, d, e, and f, including the target genes for serotype-specific real-time PCR assays. The capsule locus of all six serotypes of *H. influenzae* (Hia-f) consists of three regions encoding functions for capsule polysaccharide synthesis, modification, and translocation. *bexDCBA* in the ATP-driven export region (white arrows) code for protein components of an ATP-driven polysaccharide export apparatus. *hcsA* and *hcsB* are in the post polymerization modification region (gray arrows) and may be involved in the modification and export of capsule polysaccharide. The serotype-specific region (colored arrows) contains genes for capsule synthesis and is unique to each serotype. The serotype-specific genes are named *acs*, *bcs*, etc. for “a capsule synthesis”, “b capsule synthesis”, and so on. With the exception of the Hif serotype-specific assay, the target genes for the serotype-specific assays can be found in this region and are highlighted by the red boxes.

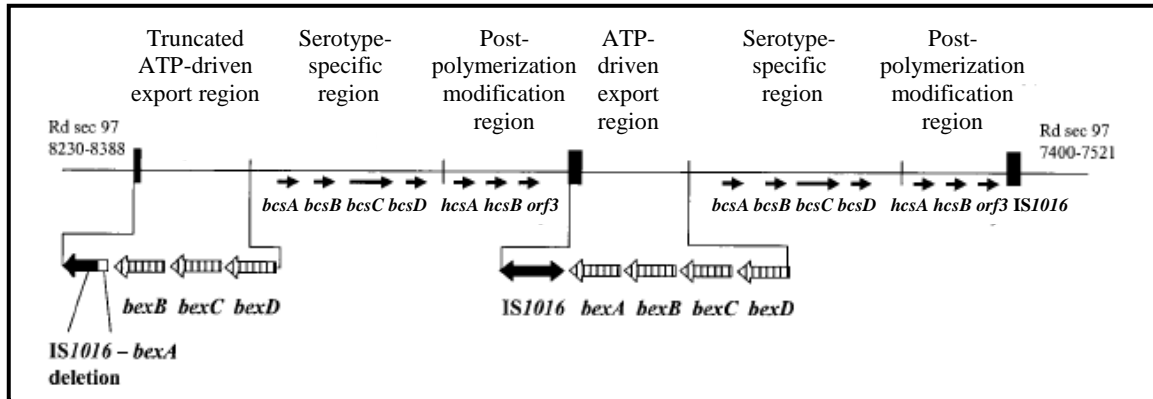


Figure 4. Genetic organization of the *cap* locus in Hib (adapted from (43)). Partially duplicated *cap* locus of Hib Hi 1007 showing the truncated ATP-driven export region with the 1.2-kb deletion between IS1016 and *bexA*.

C. *S. pneumoniae* serotyping PCR assays

S. pneumoniae can be further classified into at least 93 serotypes based on the immunochemistry of their capsular polysaccharides. The high cost of antisera, subjectivity in interpretation, need for a complete set of control strains, and technical expertise requirements associated with these serologic methods have resulted in the more recent development of PCR-based serotyping systems. PCR-serotyping has the potential to overcome some of the difficulties associated with serologic testing and the development of PCR-based assays for direct detection of serotypes from clinical specimens is a valuable aid in surveillance, particularly in situations where culture is insensitive. PCR assays (both conventional and real-time) for the detection of the more common serotypes are being developed and are discussed in more detail below (38, 54).

Multiplex conventional PCR assays for serotyping *S. pneumoniae*

A multiplex PCR-based serotyping scheme that includes 40 serotype specificities has been developed (38). This PCR approach has the potential to greatly reduce reliance upon conventional serotyping and provides serotype-determining potential to laboratories that lack type-specific antisera and other reagents needed for conventional serotyping, yet have the equipment necessary for DNA amplification and electrophoresis.

The multiplex approach uses 9 reactions to identify 40 serospecificities (Table 5 for primers, Tables 6-8 for schemes, and Figure 5 for PCR products) but also provides some flexibility that allows for altering combinations of serotypes included in each sequential reaction. These can be modified based on the most prevalent serotypes in any given geographic region but do require validation to ensure no cross reaction between serotype primer sets. Three such schemes based upon pneumococcal serotype prevalence in the USA, Africa, and Latin America have been designed (Tables 6-8). These schemes will continue to be refined as additional serotypes are added and primer sets updated to improve specificity and sensitivity. The most current methods are described at the CDC *Streptococcus* website and should be consulted on a regular basis (www.cdc.gov/ncidod/biotech/strep/PCR.htm).

Real-time PCR assays for serotyping *S. pneumoniae*

A number of real-time PCR assays for serotyping *S. pneumoniae* have been published in the literature and others are being developed (34, 40). These real-time assays are recommended for determining serotypes from clinical specimens when DNA may be present in low amounts and insufficient for conventional multiplex PCR serotyping. Please see the following website for an example: www.cdc.gov/ncidod/biotech/strep/PCR.htm.

D. Multiplex real-time PCR for pathogen detection

Real-time PCR allows for development of multiplex assays for detection of several genes in the same reaction by using specific probes with different fluorescent dye labels. Multiplex real-time PCR assays are available for detection of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* in a single reaction. In addition, assays for serogrouping *N. meningitidis* and serotyping *S. pneumoniae* using a multiplex approach have also been developed. However, they are not recommended for routine use. The multiplex approach requires careful optimization. Changes in DNA polymerase or PCR reagent concentrations may lead to loss of sensitivity. The assays need to be optimized and re-evaluated, which is a very time-consuming process and requires a complete set of reference strains.

III. Preparation of DNA template for PCR

A. General considerations

Both crude DNA preps (boiled cell suspension) and extracted genomic DNA can be used as template for PCR amplification. DNA extraction from clinical specimens or isolates should be performed in a separate room from the room used for PCR reaction assembly (prior to DNA addition). If separate rooms are not possible, separate laboratory benches should be used. The use of boiled extracts may limit the risk of contamination as it limits manipulation. Moreover, PCR on clinical specimens should be performed in a separate room from where bacteria are cultured with strict organization of the laboratory workflow. Use of a biological safety cabinet is necessary for infectious materials such as clinical isolates, blood, and CSF. Separate pipettes, laboratory coats, and gloves should be used for performing DNA extraction procedures. Signage indicating which spaces and equipment are DNA-free and which are used to prepare or manipulate DNA would be helpful. UV irradiation and decontamination of surfaces and equipment with 10% bleach followed by 70% ethanol should be performed after any manipulation of nucleic acids at the laboratory bench. Always use filter-barrier pipette tips and change and discard gloves frequently.

B. Equipment, consumables, and reagents for DNA extraction

Equipment

Microcentrifuge with refrigerating function

Water bath or dry block heater

Vortexer

Freezer

Refrigerator

pH meter
Balance
Stir plate

Consumables:

10% bleach (10:1, water: concentrated bleach) (make fresh weekly)
70% ethanol
1.5 ml microcentrifuge tubes (sterile, DNAase free, or PCR grade)
1 set of micropipettors (1-10 μ l, 2-20 μ l, 20-200 μ l, and 100-1000 μ l)
Pre-sterilized filter tips (10 μ l, 200 μ l, and 1000 μ l)

Reagents:

TE Buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA)
Tris buffer (10 mM Tris HCl, pH 8.0)
Lysozyme
Mutanolysin
Proteinase K (20 mg/ml)
Lysis buffer (4% SDS, 10 mM EDTA pH 8.0)
Digestion buffer
Phenol
Chloroform
Phenol: chloroform (1:1)
Commercial DNA extraction kits are available for culture and blood and body fluids
Commercial PCR Master Mix containing dNTPs and DNA polymerase is available

C. Preparing reagent solutions

EDTA, 0.1 M, pH 8.0 (100ml)

1. Dissolve 3.7 g EDTA in 70 ml distilled deionized H₂O (ddH₂O).
2. Adjust pH to 8.0 with 10 M NaOH.
3. Add ddH₂O to 100 ml.
4. Autoclave at 121°C for 20 minutes.
5. Store at room temperature.

Tris-HCl, 0.1 M, pH 8.0

1. Dissolve 1.2 g Tris base in 80 ml ddH₂O.
2. Adjust to pH 8.0 with concentrated HCl.
3. Mix and add ddH₂O to 100 ml.
4. Autoclave at 121°C for 20 minutes.
5. Store at room temperature.

Tris-HCl, 0.1 M, pH 7.6

1. Dissolve 1.2 g Tris base in 80 ml ddH₂O.
2. Adjust to pH 7.6 with concentrated HCl.
3. Mix and add ddH₂O to 100 ml.
4. Autoclave at 121°C for 20 minutes.

5. Store at room temperature.

20% Sodium dodecyl sulfate (SDS)*

1. Dissolve 20 g SDS in 100 ml sterile ddH₂O.
2. Place the container into a 50-60°C water bath to facilitate dissolving.
3. Avoid vigorous shaking that generates bubbles.
4. Store at room temperature.

*Eye and respiratory protection should be worn when weighing powdered SDS.

TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA)

1. Add 10 ml of 0.1 M Tris-HCl, pH 8.0.
2. Add 1 ml of 0.1 M EDTA, pH 8.0.
3. Add sterile ddH₂O to 100 ml and mix well.
4. Store at room temperature.

Tris-HCl buffer (10 mM, pH 8.0)

1. Add 10 ml of 0.1 M Tris-HCl.
2. Add 90 ml sterile ddH₂O and mix well.
3. Store at room temperature.

Lysis buffer (4% SDS, 10 mM EDTA pH 8.0)

1. Add 20 ml of 20% SDS.
2. Add 10 ml of 0.1 M EDTA pH 8.0.
3. Add sterile ddH₂O to 100 ml and mix well.
4. Store at room temperature.

Mutanolysin stock solution (2,500 U/ml)

1. Reconstitute the entire bottle of mutanolysin with sterile ddH₂O to produce a concentration of 2,500 U/ml.
2. Aliquot into sterile screw-top microcentrifuge tubes.
3. Store aliquots at -20°C.

Digestion buffer (0.04 g/ml lysozyme and 75 U/ml mutanolysin in TE buffer)

1. For 1 ml of TE buffer containing lysozyme and mutanolysin, add 40 mg lyophilized lysozyme to a 1.5 ml microcentrifuge tube.
2. Add 1 ml TE buffer.
3. Add 30 µl of a stock solution of mutanolysin at 2500 U/ml.
 - This solution should be prepared ≤ 15 minutes prior to use and should not be reused.

Proteinase K (20 mg/ml)

1. Dissolve 200 mg of proteinase K powder in 10 ml of sterile ddH₂O.
2. Aliquot 1 ml into microcentrifuge tubes.
3. Store at -20°C.

Hyaluronidase (30 mg/ml)

1. Dilute 100 mg in 3.3 ml sterile ddH₂O to make 30 mg/ml solution.

2. Dispense in 500 µl aliquots; store at -20°C.

Sodium acetate (3.0 M, pH 5.5)

1. Dissolve 40.8 g sodium acetate in 80 ml sterile ddH₂O.
2. Adjust pH to 5.5 with glacial acetic acid.
3. Add sterile ddH₂O to final volume of 100 ml.
4. Autoclave at 121°C for 20 minutes.
5. Store at room temperature.

Phenol: chloroform (1:1)

1. Melt solid or liquified phenol in a 68°C water bath. Liquified phenol should be stored at -20°C.
2. Mix equal volumes of phenol and chloroform.
3. Add an equal volume of 0.1 M Tris-HCl pH 7.6 to the phenol.
4. Mix for 15 minutes and place the bottle back to water bath to allow the phases to separate.
5. Remove the top aqueous layer as much as possible.
6. Repeat step b-d until the top aqueous layer reaches ~pH 7.6 (pH should be measured with pH paper. Do not use a pH meter.).
7. After the phenol is equilibrated, add an equal volume of 0.01 M Tris-HCl (pH 7.6).
8. Store in a dark glass bottle at 4°C up to 6 months.

D. Fast preparation of DNA template from clinical isolates

***N. meningitidis* and *H. influenzae* (gram-negative)**

1. Dispense 1.0 ml of 10 mM Tris (pH 8.0) buffer into 1.5 ml microcentrifuge tubes and label.
2. Harvest colonies from 18-24 hour pure cultures of *H. influenzae* or *N. meningitidis* using a sterile polyester or rayon-tipped swab and swirl the swab in the Tris buffer to make a turbid suspension (equivalent to a McFarland 3.0 standard). Be careful not to pick up pieces of agar on the swab.
3. Vortex briefly and boil cell suspension at 100°C for 10 minutes.
4. Proceed immediately with PCR or store at -20°C.

Fast DNA extraction protocol for *S. pneumoniae* (gram-positive)

1. Dispense 300 µl of 0.85% NaCl into 1.5 ml microcentrifuge tubes and label.
2. Harvest colonies (use 1 loopful of a 10 µl loop) from 18-24 hour pure cultures of *S. pneumoniae* using a sterile polyester or rayon-tipped swab and swirl the swab in the 0.85% NaCl to make a turbid suspension (equivalent to McFarland 3.0 standard). Be careful not to pick up pieces of agar on swab.

3. Vortex briefly and incubate at 70°C for 15 minutes.
4. Microcentrifuge at 12,000 x *g* for 2 minutes and remove the supernatant.
5. Re-suspend in 50 µl TE buffer (10 mM Tris-HCl, 100 µM EDTA, pH 8.0) and add 10 µl mutanolysin (3000 U/ml)* and 8 µl of hyaluronidase (30 mg/ml)**
6. Incubate at 37°C for 30 minutes up to 18 hours (overnight).
7. Heat-inactivate the enzymes in the suspension by boiling at 100°C for 10 minutes.
8. Microcentrifuge at 12,000 x *g* for 4 minutes and remove supernatant for use as DNA template.
9. Proceed immediately with PCR or store at -20°C.

*Mutanolysin (10,000 U). Dilute in 3.3 ml of TE buffer to make 3000 U/ml stock solution, store at -20°C as 500 µl aliquots.

**Hyaluronidase (100 mg). Dilute in 3.3 ml of TE buffer to make 30 mg/ml solution, store at -20°C as 500 µl aliquots.

E. Extracting genomic DNA from clinical isolates and specimens

Efficient extraction of the DNA template is a necessary step for any real-time PCR assay. The goal of DNA extraction is to lyse the bacterial cells in the specimens to maximize bacterial DNA yield and quality while removing any PCR inhibitors (i.e. salts, proteins), dissolve the DNA in a buffer compatible with the enzymes used in the next step and concentrating the DNA at the same time. When considering a DNA extraction method, it is important to select one that will produce an adequate DNA yield for detection by real-time PCR (dependant on the assay-specific lower limit of detection) without purifying potential PCR inhibitors as well. Things to consider are the type and volume of specimen, nucleic acid sought (DNA or RNA), concentration of the target DNA present in the specimen, impurities present that could act as PCR inhibitors, facilities/equipment available, and safety requirements. Generally, methods with fewer steps decrease chances of contamination and loss of DNA. Commercial methods are available for both cell lysis and purification and include silica membrane, spin column, and magnetic bead technology, in addition to biochemical and physical methods. In general, these methods produce adequate results as long as the protocol provided by the manufacturer is precisely followed.

1. Bacterial cell lysis

The first step in extracting and purifying bacterial DNA is to lyse the bacterial cell walls for maximum DNA yield. There are multiple ways to lyse bacterial cells, either physically or chemically, and this step can be optimized by considering the suspected bacteria and starting specimen material, as well as the materials available to each laboratory. Chemical or enzymatic based lysis methods are typically simpler to perform and can be more cost efficient. Both *N. meningitidis* and *H. influenzae* are Gram negative and can be effectively lysed using lysis buffer

containing protease such as Proteinase K along with a detergent. Incubation temperature and duration vary between organisms and specimen material. The optimal temperature range for Proteinase K activity is between 55-65°C. At temperatures above 65°C, the enzyme activity decreases. However, specimens incubated at 37°C can be left for longer incubation periods without affecting DNA quality. Specimens should be incubated until cells are completely lysed (when solution clears) and the time will vary between specimens. Once the bacteria are completely lysed one should proceed to the next step.

For optimal yields of *S. pneumoniae*, which is gram-positive, additional enzyme digestion with lysozyme and mutanolysin will help to degrade the higher content of peptidoglycan in the cell wall before being lysed with buffer. The temperature and length of the enzyme incubation will depend on the concentration and type of enzyme used, as well as the lysis buffer used. High temperature incubation and repeated freeze/thaw cycles are generally used with higher concentrations of cells, such as when extracting from cultures. Physical lysis can be performed using a liquid or pressure cell homogenizer, sonication, or shaking with glass beads, although some of these methods will require additional and sometimes costly equipment and they tend to shear the DNA in to smaller fragments.

Enzyme lysis for clinical specimens of unknown etiology or known gram-positive cell suspensions (*S. pneumoniae*)

1. Prepare digestion buffer (0.04 g/ml lysozyme and 75 U/ml mutanolysin in TE buffer).
 - This solution should be prepared \leq 15 minutes before use and not reused.
2. Add 100 μ l of digestion buffer to each microcentrifuge tube.
3. Add 200 μ l of bacterial cell suspension or clinical specimen to each microcentrifuge tube. Vortex and incubate at 37°C for 1 hour.
 - If specimen volume is less than 200 μ l, note the volume in lab records and add TE buffer to a total volume of 200 μ l. If the specimen tube appears to be empty, wash the sides of the tube with 200 μ l TE buffer.
4. Add 200 μ l of cell lysis buffer to each microcentrifuge tube.
5. Add 20 μ l of Proteinase K (20 mg/ml) and invert each tube until the phases are completely mixed.
6. Incubate at 37°C for 30 minutes to 1 hour.
7. Purify DNA before using in real-time PCR reactions.

Enzyme lysis for known gram-negative clinical specimens or cell suspensions (*N. meningitidis* and *H. influenzae*)

1. Prepare cell lysis buffer (4% SDS, 1 mM EDTA pH 8.0).
2. Add 200 μ l of lysis buffer to each microcentrifuge tube.
3. Add 200 μ l of bacterial cell suspension or clinical specimen (CSF, serum, or blood) to each microcentrifuge tube.
4. Add 20 μ l of Proteinase K (20 mg/ml) for a final concentration of 1 mg/ml and invert each tube until the phases are completely mixed.
5. Incubate at 37°C for 30 minutes to 1 hour.
6. Purify DNA before using in real-time PCR reactions.

2. Removal of eukaryotes from blood

If DNA is being extracted from clinical specimens such as blood, CSF, or serum, considerations should be taken to remove potential PCR inhibitors from the surrounding material before lysing the cells. If the starting specimen is blood, steps to remove erythrocytes can result in higher DNA yield with fewer PCR inhibitors. Hemoglobin is very inhibitory to DNA polymerases. Erythrocytes can be removed through the use of a hypotonic buffer or by using gradient centrifugation to create a buffy coat in which leukocytes are concentrated. Bacteria in large volumes of clinical specimens can be concentrated by centrifugation or antigen capture to increase yield.

Preparation of buffy coat

1. Centrifuge the blood specimen at 2,500 x g for 10 minutes at room temperature.
2. Three layers should be apparent after centrifugation. The top layer should be clear and contains plasma. The light tan middle layer is the buffy coat and contains concentrated leukocytes. The bottom red layer contains erythrocytes.

3. Purification of DNA

Purification of the extraction product is important to remove any residual material that could potentially inhibit real-time PCR. Purification can be performed by many commercially available extraction kits or with the use of organic solvents, such as the chloroform/phenol method. Some methods may purify RNA along with DNA and as RNA may inhibit some reactions, use of RNAase improves purity of DNA as well.

Phenol/Chloroform to remove cell debris and proteins

Phenol is a hazardous organic solvent and safety precautions should be taken when working with phenol. Always use suitable chemical protection gloves when handling phenol containing

solutions. Specific waste procedures may be required for the disposal of solutions containing phenol.

1. To a lysed specimen, add an equal volume of phenol: chloroform solution (1:1). Mix well by inversion or briefly vortex.
2. Centrifuge the tube at 16,000 x g for 15 minutes in a microcentrifuge.
3. Carefully remove the top aqueous layer from the bottom phenol layer and transfer to a new tube, being careful to avoid the interface.
4. Steps 1-3 can be repeated until an interface is no longer visible.
5. To remove all traces of phenol, add an equal volume of chloroform to the aqueous layer and centrifuge the tube at 16,000 x g for 15 minutes in a microcentrifuge.
6. Carefully remove the top aqueous layer from the bottom chloroform layer and transfer to a new tube, being careful to avoid the interface.
7. Steps 5-6 can be repeated until an interface is no longer visible.
8. Precipitate the DNA by ethanol or isopropanol.

Precipitation of DNA by ethanol or isopropanol

1. Add a 0.1 (1/10th) volume of 3.0 M sodium acetate (pH 5.5) to the aqueous phase and then 2 volumes of 95% ethanol. Incubate at -20°C overnight or for shorter periods at -80°C (e.g. 20-30 minutes). Proceed with step 3.
2. If isopropanol is used: Add a 0.1 volume of 3.0 M sodium acetate (pH 5.5) to the aqueous phase and then 0.6 volumes of 100% isopropanol. Incubate at -20°C for 2 hours or for shorter periods at -80°C (e.g., 10-20 minutes).
3. Centrifuge at 16,000 x g for 30 min at 4°C.
4. Recover the precipitated DNA by centrifuging the tube at 16,000 x g for 15 minutes at 4°C. Remove the aqueous phase with care.
5. Add 2 volumes (of original sample) of 75% (v/v) ethanol and leave at room temperature for 5-10 minutes to remove excess salt and traces of phenol and chloroform from the pellet.
6. Centrifuge at 16,000 x g for 5 minutes. Remove with care as much ethanol as possible from the microcentrifuge tube using a filtered pipette tip to avoid dislodging the pellet.
7. Dry the DNA pellet in air, in a desiccator, or in a 50°C oven for 5 minutes.

8. The dried DNA may be dissolved in sterile Tris buffer (10mM Tris-HCl, pH 8.0) and stored at 4°C for further manipulation or at -20°C for long-term storage.

Storage of DNA

Extracted and purified DNA should be stored in a designated elution buffer from a commercial kit or in Tris buffer (10 mM Tris-HCl, pH 8.0). Distilled water can also be used but these specimens may experience degradation from acid hydrolysis. DNA can be kept at 4°C for short periods of time and at -20°C for long-term storage.

F. Alternative protocol for genomic DNA extraction: Boom method

Reagent preparation

Extraction buffer L6

1. Add 120 g of guanidinium isothiocyanate (GuSCN) to 100 ml of 0.1 M Tris/HCl (pH 6.4) and 22 ml of 0.2 M EDTA (pH 8.0) and 2.6 g of Triton X-100.
2. Stir overnight in the dark to dissolve.
3. Store away from light for up to 1 month.
 - GuSCN is toxic and care should be taken when handling this substance.

Extraction buffer L2

1. Add 120 g of guanidinium isothiocyanate (GuSCN) to 100 ml of 0.1 M Tris/HCl (pH 6.4).
2. Stir overnight in the dark to dissolve.
3. Store away from light for 1 month.

Size fractionated silica

1. Add 60 g of silicon dioxide to 500 ml of distilled water in a graduated cylinder and leave at room temperature for 24 hours.
2. Remove and discard 430 ml of supernatant and re-suspend solids in 500 ml of ddH₂O.
3. Leave at room temperature for 5 hours and remove and discard 440 ml of supernatant.
4. Add 600 µl of concentrated HCl (pH 2.0), mix, and aliquot into 1.5 ml volumes.
5. Sterilize by autoclaving and store away from light for up to 6 months.

DNA isolation

1. Add 100 µl of specimen (bacterial suspension or clinical specimen) to 500 µl of L6 extraction buffer and 10 µl of size fractionated silica in a 1.5 ml microcentrifuge tube. For double volumes of specimen, double the amount of L6 extraction buffer and silica, as well as reagents in the wash steps (L2 extraction buffer, ethanol and acetone).
2. Vortex the tube for 10 seconds and incubate, with shaking, at room temperature for 15 minutes.
3. Centrifuge the tube for 15 seconds at 16,100 x g and dispose of the supernatant.

4. Wash the pellet two times with 500 μ l L2 extraction buffer, two times with 500 μ l of 70% ethanol and one time with 500 μ l of acetone. Centrifuge for 15 seconds at 16,100 x g after each wash and dispose of the supernatant following appropriate procedures for chemical waste.
5. To remove the acetone, place the tube with the lid open at 56°C in a dry heating block for 5 minutes.
6. Elute the nucleic acid from the silica by adding 30 μ l of distilled water, close the tube, vortex, and incubate at 56°C for 15 minutes.
7. Centrifuge the tube at 16,100 x g for 2 minutes and collect the supernatant, taking care not to include any silica. The extracted DNA can be stored at 4°C overnight or at -70°C for long-term storage.

III. Conventional PCR

A. General considerations

PCR is a very sensitive method for amplifying a specific DNA target, but also very susceptible to contamination with extraneous DNA. Extra precautions should be taken to minimize such cross-contamination. It is recommended to physically separate the different steps including PCR reaction assembly, addition of template DNA to the reaction wells, and agarose gel detection of PCR products. If separate rooms are not possible, separate laboratory benches should be used for these steps. Working in an unventilated still air biocontainment cabinet (sometimes called a PCR hood), are also suggested to minimize cross-contamination. Separate micropipettors, laboratory coats, and gloves should be used for reaction assembly. Signage indicating which spaces and equipment are DNA-free and which are used to prepare or manipulate DNA would be helpful. Decontamination of surfaces and equipment with 10% bleach followed by 70% ethanol should be performed after any manipulation of nucleic acids at the laboratory bench. Always use filter-barrier pipette tips and change and discard gloves frequently.

B. Equipment, consumables, and reagents

Equipment:

PCR thermocycler

Unventilated biocontainment cabinet or PCR hood

Freezer

Refrigerator

Electrophoresis tank

Power supply

Stir plate

Microwave oven

Gel viewing system

Gel documentation system

Consumables:

10% bleach (10:1, water: concentrated bleach) (make fresh weekly)
70% ethanol
1.5 mL microcentrifuge tubes (sterile DNase free or PCR grade)
96 well polypropylene plates, tube strips or individual
1 set of micropipettors (1-10 μ l, 2-20 μ l, 20-200 μ l, and 100-1000 μ l)
Pre-sterilized filter tips (10 μ l, 200 μ l, and 1000 μ l)
Optical caps

Optional:

Commercial DNA-removing surface decontaminant liquid
Cap installing tool

Reagents:

DNA polymerase
dNTPs
Primers
Positive and negative control DNA diluted to approximately 5 μ g/ml
PCR grade water
TAE or TBE buffer
Agarose powder (molecular biology grade)
DNA ladder
6x DNA loading dye
Ethidium bromide
Bromophenol blue
Xylene cyanol FF
Sucrose

C. Preparing reagent stock solution and primer working solution**EDTA, 0.5 M, pH 8.0 (100 ml)**

1. Dissolve 18.6 g EDTA in 70 ml ddH₂O.
2. Adjust pH to 8.0 with 10 M NaOH (~5 ml).
3. Add ddH₂O to 100 ml and mix well on a stir plate.
4. Store at room temperature.

Ethidium bromide (EtBr), 10 mg/ml

1. Dissolve 0.2 g ethidium bromide in 20 ml ddH₂O.
2. Mix well and store at 4°C in the dark in 1 ml aliquots.
3. Store at room temperature.

TAE (Tris/acetate/EDTA) electrophoresis buffer, 50 X stock solution*

1. To 750 ml of ddH₂O add:
 - 242 g Tris base
 - 57.1 ml of glacial acetic acid
 - 100 ml of 0.5 M EDTA pH 8.0

2. Add ddH₂O to 1000 ml and mix well on a stir plate.
 3. Store at room temperature.
- *TAE stock solution should be diluted to 1X in H₂O before use.

TBE (Tris/borate/EDTA) electrophoresis buffer, 10X stock solution*

1. To 900 ml of ddH₂O add:
 - 108 g Tris base (890 mM)
 - 55 g boric acid (890 mM)
 - 40 ml 0.5 M EDTA, pH 8.0 (20 mM)
 2. Add ddH₂O to 1000 ml and mix well on a stir plate.
 3. Store at room temperature.
- *TBE stock solution should be diluted to 0.5X in H₂O before use.

6X DNA loading dye I

1. 0.25% bromophenol blue
2. 0.25% xylene cyanol FF
3. 40% (w/v) sucrose in water
4. Store at 4°C.

6X DNA loading dye II

1. 0.25% bromophenol blue
2. 0.25% xylene cyanol FF
3. 30% glycerol in water
4. Store at 4°C.

6X DNA loading dye III

1. 0.25% bromophenol blue
2. 40% (w/v) sucrose in water
3. Store at 4°C.

2% agarose gel

1. Add 2 g of electrophoresis-grade agarose to 100 ml of 1X TAE or 0.5X TBE buffer in a 250 ml flask or bottle.
2. Melt the agarose in a microwave until the agarose is fully melted and the solution is clear. Swirl the flask a few times while microwaving to avoid boiling and spilling over.
3. Cool to 55-60°C and then add 5 µl EtBr for a final concentration of 0.5 µg/ml.
 - EtBr is a powerful carcinogen and must be handled with care.

Primer working stock solution

The primer working stock solution should be 20 µM.

D. Performing multiplex PCR for *S. pneumoniae* serotype deduction

PCR protocol

Prior to beginning the PCR, plan the experiment by filling out and printing a plate template worksheet. Also, be sure sufficient quantities of primer working solutions to be used are available.

1. Remove DNA templates and positive control DNAs from -20°C to the DNA addition area to allow them to thaw completely.
2. In the PCR reaction assembly area, gather reagents needed for the PCR reactions, including PCR master mix, primers, and PCR grade water. If the reagents are stored at -20°C , allow them to thaw completely and vortex or flick each tube before use.
3. Sequential multiplex PCR reactions, based on one of the described schemes, are prepared in standard 25 μl reaction volumes using primers and concentrations described in Table 5.

4. Each PCR reaction should contain:

200 μM (each) of deoxynucleoside triphosphates (dNTPs)
3.5 mM MgCl_2
2 Units *Taq* DNA polymerase
Forward primer (Table 5)
Reverse primer (Table 5)
2.5 μl of DNA template from isolates (use 5 μl for clinical specimens)
PCR-grade water to 25 μl

5. A master mix can be prepared, which includes all components listed above except DNA template. When calculating volumes of master mix reagents, remember to add enough master mix reagents for 2 extra reactions than the number of specimens there are to be tested to ensure there will be enough master mix.
6. Pipette 22.5 μl of this master mix into each appropriate well of 96-well plate, according to your plate template worksheet.
7. Cover the wells of the plate using cap strips. Spray down the clean workspace with 10% bleach (10:1 water: concentrated bleach), and wipe. Repeat with 70% alcohol. Remove laboratory coat and gloves. Put on a fresh pair of gloves. Carefully transport the 96-well plate to the DNA addition area.
8. Put on new laboratory coat and keep the same pair of gloves on. Remove the cap strips from the plate. Add 2.5 μl of template DNA to each appropriate well of 96-well plate, according to your plate template worksheet.
9. At least one negative and one positive control should be set up for each serotype per PCR run.
 - Negative control: add 2.5 μl DNA resolving buffer to a reaction well instead of DNA template.

- Positive control: add 2.5 μ l of DNA template that is known to contain the amplified sequence to a reaction well.
10. Cap columns of wells as you go. Use the roller tool to secure caps tightly.
 11. Wipe down the dirty workspace with 10% bleach, then 70% ethanol. Remove laboratory coat and gloves. If possible, quickly spin the plate at 1000 rpm to bring down any droplets. Transport plate directly to and place it in the PCR thermocycler.
 12. The following PCR conditions are used:
 - 1 cycle of 94°C for 4 minutes
 - 30 cycles of 94°C for 45 seconds; 54°C for 45 seconds and 72°C for 2 minutes
 - 1 cycle of 72°C for 2 minutes
 13. Current methodologies are detailed at the CDC website (<http://www.cdc.gov/ncidod/biotech/strep/pcr.htm>).

Analysis of PCR products on an agarose gel

PCR products (10 μ l) are run on 2% agarose gels to determine band sizes using positive controls. A positive control for each serotype and a 50 bp ladder molecular size marker should be included on each gel.

1. Melt the 2% agarose gel in a microwave oven. Cool the agar to approximately 55°C. Add ethidium bromide or other gel stain. Pour into a gel casting cassette, insert the comb, and allow time for hardening (~30 minutes).
2. Add 1X TAE or TBE buffer to the electrophoresis tank and properly place the gel cassette containing the solidified agarose gel into the tank.
3. Briefly spin the PCR plate or tubes at 500 x g to ensure all liquid is at the bottom.
4. Mix 10 μ l of PCR reaction with 2 μ l of 6X loading dye.
5. Pipette the DNA/loading dye mixtures into the wells. Load 5 μ l of DNA size markers in one of the wells.
6. Run the gel at 50-100 volts for 15-20 minutes or until the Bromophenol blue dye band is halfway down the gel. The dye runs at approximately the same rate as a 500 base-pair DNA fragment.
7. Visualize the gel under a UV light and print out or save the image, if possible.
8. Each reaction should give two bands, i.e., species-specific positive control (*cpsA*, although some are *cpsA* negative) and a serotype-specific band.
9. Store the remainder of the amplicon at -20°C, if necessary.

Interpretation

Band sizes on agarose gels must match those of positive controls before assigning a putative serotype (Figure 5). PCR reactions are setup sequentially and will include those serotypes most frequently determined for each of the schemes. For example, if a strain is negative for any of the serotypes included in reaction 1 then proceed to reaction 2 and so forth until a serotype is determined. If all reactions are completed for a specific scheme and no serotype bands were identified that matched any of the positive controls then the strain may be a nontypeable or one of the serotypes not yet included in the scheme and would need to be further typed using the Quellung reaction (see Chapter 8: Identification and Characterization of *Streptococcus pneumoniae*). Resolution of individual serotypes within some of the positive reactions could be applicable in some circumstances. For example, to further resolve a PCR positive reaction for 12F/12A/44/46 (reaction 3 in Table 6), perform a Quellung reaction using type-specific antisera to determine whether the strain has a 12F, 12A, 44, or 46 capsular type. It must be mentioned that for practical reasons this is usually not necessary. For example, serotype 12F is commonly detected in disease and carriage specimens, while serotypes 12A, 44, and 46 are extremely rare.

Helpful tips

The positive pneumococcal control band for *cpsA* can be negative in 1-2% of PCR-serotypeable isolates. This is most often encountered in serotypes 25 and 38, but has also rarely been found for serotypes 14 and 35A. Although a *cpsA* negative result is relatively rare, at present a negative *cpsA* does not necessarily equate to a non-serotypeable isolate or a pneumococcus-negative clinical specimen.

Quality control

A positive control for each serotype included in the PCR reaction(s) should be run on each gel to ensure that bands are assigned to the correct serotype.

Table 2. Primers and probes used for detection of bacterial meningitis pathogens

Target	Primer or Probe Name	Real-time Primers and Probes Nucleotide Sequence (5' to 3')	Working Stock Conc (µM)	Final Conc (nM)	Suggested Probe Modifications
<i>N. meningitidis</i>					
<i>ctrA</i>	F753	TGTGTTCCGCTATACGCCATT	3.75	300	
	R846	GCCATATTCACACGATATACC	11.25	900	
	Pb820i	AACCTTGAGCAA"TCATTTATCCTGACGTTCT	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6
<i>sodC</i>	F351	GCACACTTAGGTGATTTACCTGCAT	3.75	300	
	R478	CCACCCGTGTGGATCATAATAGA	7.5	600	
	Pb387	CATGATGGCACAGCAACAAATCCTGTTT	1.25	100	5' FAM, 3' BHQ1
<i>H. influenzae</i>					
<i>hpd</i>	hpdF822	GGTAAATATGCCGATGGTGTTG	1.25	100	
	hpdR952	TGCATCTTACGCACGGTGTA	3.75	300	
	Pb896i	TTGTGTACACTCCGT"TTGGTAAAAGAACTTGACAC	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6
<i>S. pneumoniae</i>					
<i>lytA</i>	F373	ACGCAATCTAGCAGATGAAGCA	2.5	200	
	R424	TCGTGCGTTTTAATCCAGCT	2.5	200	
	Pb400i	TGCCGAAAACGC"TTTGATACAGGGAG	2.5	200	5' FAM, BHQ1 on "T", 3' SpC6
Human DNA					
RNAaseP	RNAsePF	CCA AGT GTG AGG GCT GAA AAG	4	400	
	RNAsePR	TGT TGT GGC TGA TGA ACT ATA AAA GG	4	400	
	RNAsePPb	CCC CAG TCT CTG TCA GCA CTC CCT TC	1	100	5' FAM, 3' BHQ1

Table 3. Primers and probes used for detection of the serogroups of *N. meningitidis*

Target	Primer or Probe Name	Real-time Primers and Probes Nucleotide Sequence (5' to 3')	Working Stock Conc (µM)	Final Conc (nM)	Suggested Probe Modifications
Nm A	F2531	AAAATTCAATGGGTATATCACGAAGA	3.75	300	
<i>sacB</i>	R2624	ATATGGTGCAAGCTGGTTTCAATAG	11.25	900	
	Pb2591i	CTAAAAG"TT"AGGAAGGGCACTTTGTGGCATAAT	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6
Nm B	F737	GCTACCCCATTTTCAGATGATTTGT	3.75	300	
<i>synD</i>	R882	ACCAGCCGAGGGTTTATTCTAC	3.75	300	
	Pb839i	AAGAGATGGGYAACAAC"TT"ATGTAATGTCTTTATTT	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6
Nm C	F478	CCCTGAGTATGCGAAAAAATT	11.25	900	
<i>synE</i>	R551	TGCTAATCCCGCCTGAATG	3.75	300	
	Pb495i	TTTCAATGC"TT" AATGAATACCACCGTTTTTTTGC	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6
Nm W135	F857	TATTATGGAAGGCATGGTGTATG	1.25	100	
<i>synG</i>	R964	TTGCCATTCCAGAAATATCACC	11.25	900	
	Pb907i	AAATATGGAGCGAATGATTACAGTAACTATAATGAA	2.5	200	5' FAM, BHQ1 on "T", 3' SpC6
Nm X	F173	TGTCCCAACCGTTTATTGG	11.25	900	
<i>xcbB</i>	R237	TGCTGCTATCATAGCCGCC	11.25	900	
	Pb196	TGTTTGCCACATGAATGGCGG	1.25	100	5' FAM, 3' BHQ1
Nm Y	F787	TCCGAGCAGGAAATTTATGAGAATAC	11.25	900	
<i>synF</i>	R929	TTGCTAAAATCATTGCTCCATAT	7.5	600	
	Pb1099i	TATGGTG"TT"ACGATATCCCTATCCTTGCCTATAAT	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6

Table 4. Primers and probes used for detection of the serotypes of *H. influenzae*

Target	Primer or Probe Name	Real-time Primers and Probes Nucleotide Sequence (5' to 3')	Working Stock Conc (µM)	Final Conc (nM)	Suggested Probe Modifications
Hi a	F261	GGT CTG CGG TGT CCT GTG T	1.25	100	
acsB	R427	CCG GTC ATC TTT TAT GCT CCA A	3.75	300	
	Pb375i	TAA TTT TCT TGC "T"CA ATA CCG CCT TCC CA	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6
Hi b	F192	TGA TGC ATT GAA AGA AGG TGT AAT TT	3.75	300	
bcsB	R359	CCT GCG GTA ATA ACA TGA TCA TAA A	7.5	600	
	Pb244i	TGT CGT GCA G" T" A GCA AAC CGT AAC CTT ACT C	1.25	100	5' FAM, BHQ1 on "T", 3' SpC6
Hi c	F7667	CAT TGG TGA TGG TTC AGT TAT TGG	7.5	600	
ccsD	R7784	TAC AGC ATT CAG CAA TAA TGG G	3.75	300	
	Pb7726i	ATT GCA "T"CG CCG CAG GAG TTC CCG	2.5	200	5' FAM, BHQ1 on "T", 3' SpC6
Hi d	F2211	CCT AAA ATA CGG ACC TAG TGC AC	7.5	600	
dcsE	R2255	CCG ATG AGA CCA AGT ATG GTT A	1.25	100	
	Pb2221i	AAC GAG C" T" A GAG CTG GTG CTG AA	3.75	300	5' FAM, BHQ1 on "T", 3' SpC6
Hi e	F1523	ACT AAA ATA TGG CCC AAA CCC AC	7.5	600	
ecsH	R1589	CCG ATG AGC CCA AGT ATG ATG A	7.5	600	
	Pb1555i	AAC GAG CAA AAG CCG G" T" G CGG AT	2.5	200	5' FAM, BHQ1 on "T", 3' SpC6
Hi f	F7164	CCC TGA AAA GCG TTG ACT TTG	7.5	600	
bexD	R7313	CCA ACT TCA GGA CCA AGT CAT TC	3.75	300	
	Pb7242i	TGC TGC TAA C" T" C AGA TGC ATC AGC TCC TT	2.5	200	5' FAM, BHQ1 on "T", 3' SpC6

Table 5. List of primers used for pneumococcal serotype deduction (<http://www.cdc.gov/ncidod/biotech/strep/pcr.htm>). Please note that this website should be used as the primary source of primer sequences and protocols due to periodic introduced improvements.

*All serotypes that are co-detected are listed

Primers*	Primer sequence (5'-3')	Gene	Primer Concentration (μ M)	Product size (bp)
1-f	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA	<i>wzy</i>	0.3	280
1-r	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C		0.3	
2-f	TAT CCC AGT TCA ATA TTT CTC CAC TAC ACC	<i>wzy</i>	0.3	290
2-r	ACA CAA AAT ATA GGC AGA GAG AGA CTA CT		0.3	
3-f	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G	<i>galU</i>	0.3	371
3-r	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G		0.3	
4-f ^a	CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G	<i>wzy</i>	0.3	430
4-r	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G		0.3	
5-f	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG	<i>wzy</i>	0.3	362
5-r	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG		0.3	
6A/6B/6C/6D -f	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG	<i>wciP</i>	0.3	250
6A/6B/6C/6D -r	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA		0.3	
6C/6D -f	CAT TTT AGT GAA GTT GGC GGT GGA GTT	<i>wciNbeta</i>	0.5	727
6C/6D -r	AGC TTC GAA GCC CAT ACT CTT CAA TTA		0.5	
7C/(7B/40)-f	CTA TCT CAG TCA TCT ATT GTT AAA GTT TAC GAC GGG A	<i>wcwL</i>	0.3	260
7C/(7B/40)-r	GAA CAT AGA TGT TGA GAC ATC TTT TGT AAT TTC		0.3	
7F/7A-f	TCC AAA CTA TTA CAG TGG GAA TTA CGG	<i>wzy</i>	0.4	599
7F/7A-r	ATA GGA ATT GAG ATT GCC AAA GCG AC		0.4	

Primers*	Primer sequence (5'-3')	Gene	Primer Concentration (μM)	Product size (bp)
8-f	GAA GAA ACG AAA CTG TCA GAG CAT TTA CAT	<i>wzy</i>	0.2	201
8-r	CTA TAG ATA CTA GTA GAG CTG TTC TAG TCT		0.2	
9N/9L-f	GAA CTG AAT AAG TCA GAT TTA ATC AGC	<i>wzx</i>	0.5	516
9N/9L-r	ACC AAG ATC TGA CGG GCT AAT CAA T		0.5	
9V/9A-f	GGG TTC AAA G TC AGA CAG TG A ATC TTA A	<i>wzy</i>	0.5	816
9V/9A-r	CCA TGA ATG A AA TCA ACA TT G TCA GTA GC		0.5	
10A-f	GGT GTA GAT TTA CCA TTA GTG TCG GCA GAC	<i>wcrG</i>	0.5	628
10A-r	GAA TTT CTT CTT TAA GAT TCG GAT ATT TCT C		0.5	
10F/(10C/33C)- f	GGA GTT TAT CGG TAG TGC TCA TTT TAG CA	<i>wzx</i>	0.3	248
10F/(10C/33C)-r	CTA ACA AAT TCG CAA CAC GAG GCA ACA		0.3	
11A/11D-f	GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G	<i>wzy</i>	0.3	463
11A/11D-r	GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC		0.3	
12F/(12A/44/46)-f	GCA ACA AAC GGC GTG AAA GTA GTT G	<i>wzx</i>	0.5	376
12F/(12A/44/46)-r	CAA GAT GAA TAT CAC TAC CAA TAA CAA AAC		0.5	
13-f	TAC TAA GGT AAT CTC TGG AAA TCG AAA GG	<i>wzx</i>	0.4	655
13-r	CTC ATG CAT TTT ATT AAC CG C TTT TTG TTC		0.4	
14-f	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT	<i>wzy</i>	0.3	189
14-r	GCC AAT ACT TCT TAG TCT CTC AGA TGA AT		0.3	
15A/15F-f	ATT AGT ACA GCT GCT GGA ATA TCT CTT C	<i>wzy</i>	0.3	434
15A/15F-r	GAT CTA GTG AAC GTA CTA TTC CAA AC		0.3	

Primers*	Primer sequence (5'-3')	Gene	Primer Concentration (μM)	Product size (bp)
15B/15C-f	TTG GAA TTT TTT AAT TAG TGG CTT ACC TA	<i>wzy</i>	0.3	496
15B/15C-r	CAT CCG CTT ATT AAT TGA AGT AAT CTG AAC C		0.3	
16F-f,	GAA TTT TTC AGG CGT GGG TGT TAA AAG	<i>wzy</i>	0.4	717
16F-r	CAG CAT ATA GCA CCG CTA AGC AAA TA		0.4	
17F-f	TTC GTG ATG ATA ATT CCA ATG ATC AAA CAA GAG	<i>wciP</i>	0.5	693
17F-r	GAT GTA ACA AAT TTG TAG CGA CTA AGG TCT GC		0.5	
18/(18A/18B/18C/18F)-f	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC	<i>wzy</i>	0.3	573
18/(18A/18B/18C/18F)-r	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC		0.3	
19A-f	GAG AGA TTC ATA ATC TTG CAC TTA GCC A	<i>wzy</i>	0.3	566
19A-r	CAT AAT AGC TAC AAA TGA CTC ATC GCC		0.3	
19F-f	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C	<i>wzy</i>	0.5	304
19F-r	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG		0.5	
20-f	GAG CAA GAG TTT TTC ACC TGA CAG CGA GAA G	<i>wciL</i>	0.3	514
20-r	CTA AAT TCC TGT AAT TTA GCT AAA ACT CTT ATC		0.3	
21-f	CTA TGG TTA TTT CAA CTC AAT CGT CAC C	<i>wzx</i>	0.2	192
21-r	GGC AAA CTC AGA CAT AGT ATA GCA TAG		0.2	
22F/22A-f	GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC	<i>wcwV</i>	0.5	643
22F/22A-r	CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC		0.5	
23A-f	TAT TCT AGC AAG TGA CGA AGA TGC G	<i>wzy</i>	0.5	722
23A-r	CCA ACA TGC TTA AAA ACG CTG CTT TAC		0.5	

Primers*	Primer sequence (5'-3')	Gene	Primer Concentration (μM)	Product size (bp)
23B-f	CCA CAA TTA G CG CTA TAT TCA TTC AAT CG	<i>wzx</i>	0.2	199
23B-r	GTC CAC GCT GAA TAA AAT GAA GCT CCG		0.2	
23F-f ^a	GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC	<i>wzy</i>	0.5	384
23F-r	CAC AAC ACC TAA CAC TCG ATG GCT ATA TGA TTC		0.5	
24A/24B/24F-f	GCT CCC TGC TAT TGT AAT CTT TAA AGA G	<i>wzy</i>	0.2	99
24A/24B/24F-r	GTG TCT TTT ATT GAC TTT ATC ATA GGT CGG		0.2	
31-f	GGA AGT TTT CAA GGA TAT GAT AGT GGT GGT GC	<i>wzy</i>	0.5	701
31-r	CCG AAT AAT ATA TTC AAT ATA TTC CTA CTC		0.5	
33F/33A/37-f	GAA GGC AAT CAA TGT GAT TGT GTC GCG	<i>wzy</i>	0.3	338
33F/33A/37-r	CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C		0.3	
34-f	GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC	<i>wzy</i>	0.3	408
34-r	CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC		0.3	
35A/35C/42-f	ATT ACG ACT CCT TAT GTG ACG CGC ATA	<i>wzx</i>	0.3	280
35A/35C/42-r	CCA ATC CCA AGA TAT ATG CAA CTA GGT T		0.3	
35B-f	GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG	<i>wcrH</i>	0.5	677
35B-r	CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G		0.5	
35F/47F-f	GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCA A	<i>wzy</i>	0.3	517
35F/47F-r	GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC		0.3	
38/25F-f	CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG	<i>wzy</i>	0.3	574
38/25F-r	ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC		0.3	

Primers*	Primer sequence (5'-3')	Gene	Primer Concentration (μM)	Product size (bp)
39-f	TCA TTG TAT TAA CCC TAT GCT TTA TTG GTG	wzy	0.2	98
39-r	GAG TAT CTC CAT TGT ATT GAA ATC TAC CAA		0.2	
<i>cpsA</i> -f	GCA GTA CAG CAG TTT GTT GGA CTG ACC	wzg	0.1	160
<i>cpsA</i> -r	GAA TAT TTT CAT TAT CAG TCC CAG TC		0.1	

Table 6. PCR-serotyping scheme for USA based on current serotype prevalence

Reaction	Serotypes included
1	6A/6B/6C/6D, 3, 19A, 22F/22A, 16F
2	8, 33F/33A/37, 15A/15F, 7F/7A, 23A
3	19F, 12F/12A/44/46, 11A/11D, 38, 35B
4	24A/24B/24F, 7C/7B/40, 4, 18A/18B/18C/18F, 9V/9A
5	14, 1, 23F, 15B/15C, 10A
6	39, 10F/10C/33C, 5, 35F/47F, 17F
7	23B, 35A/35C/42, 34, 9N/9L, 31
8*	6A/6B/6C/6D, 6C/6D

*Only performed if PCR-positive for 6A/6B/6C/6D in reaction 1

Table 7. PCR-serotyping scheme for Africa based on current serotype prevalence

Reaction	Serotypes included
1	14, 1, 5, 4, 18A/18B/18C/18F
2	6A/6B/6C/6D, 19F, 23F, 38/25F, 9V/9A
3	7C/7B/40, 3, 15B/15C, 7F/7A, 17F
4	8, 12F/12A/44/46, 9L/9N, 22F/22A, 23A
5	24A/24B/24F, 2, 11A/11D, 19A, 16F
6	21, 33F/33A/37, 15A/15F, 35F/47F, 13
7	39, 23B, 35A/35C/42, 20, 35B
8	10F/10C/33C, 34, 10A, 31
9*	6A/6B/6C/6D, 6C/6D

*Only performed if PCR-positive for 6A/6B/6C/6D in reaction 2

Table 8. PCR-serotyping scheme for Latin America based on current serotype prevalence

Reaction	Serotypes included
1	14, 6A/6B/6C/6D, 23F, 19A, 9V/9A
2	19F, 3, 15B/15C, 18A/18B/18C/18F, 17F
3	1, 5, 9L/9N, 7F/7A, 16F
4	8, 2, 4, 20, 22F/22A
5	7C/7B/40, 12F/12A/44/46, 11A/11D, 10A, 23A
6	21, 33F/33A/37, 15A/15F, 35F/47F, 13
7	39, 23B, 35A/35C/42, 38/25F/25A, 35B
8	24A/24B/24F, 10F/10C/33C, 34, 31
9	6A/6B/6C/6D, 6C/6D

*Only performed if PCR-positive for 6A/6B/6C/6D in reaction 1

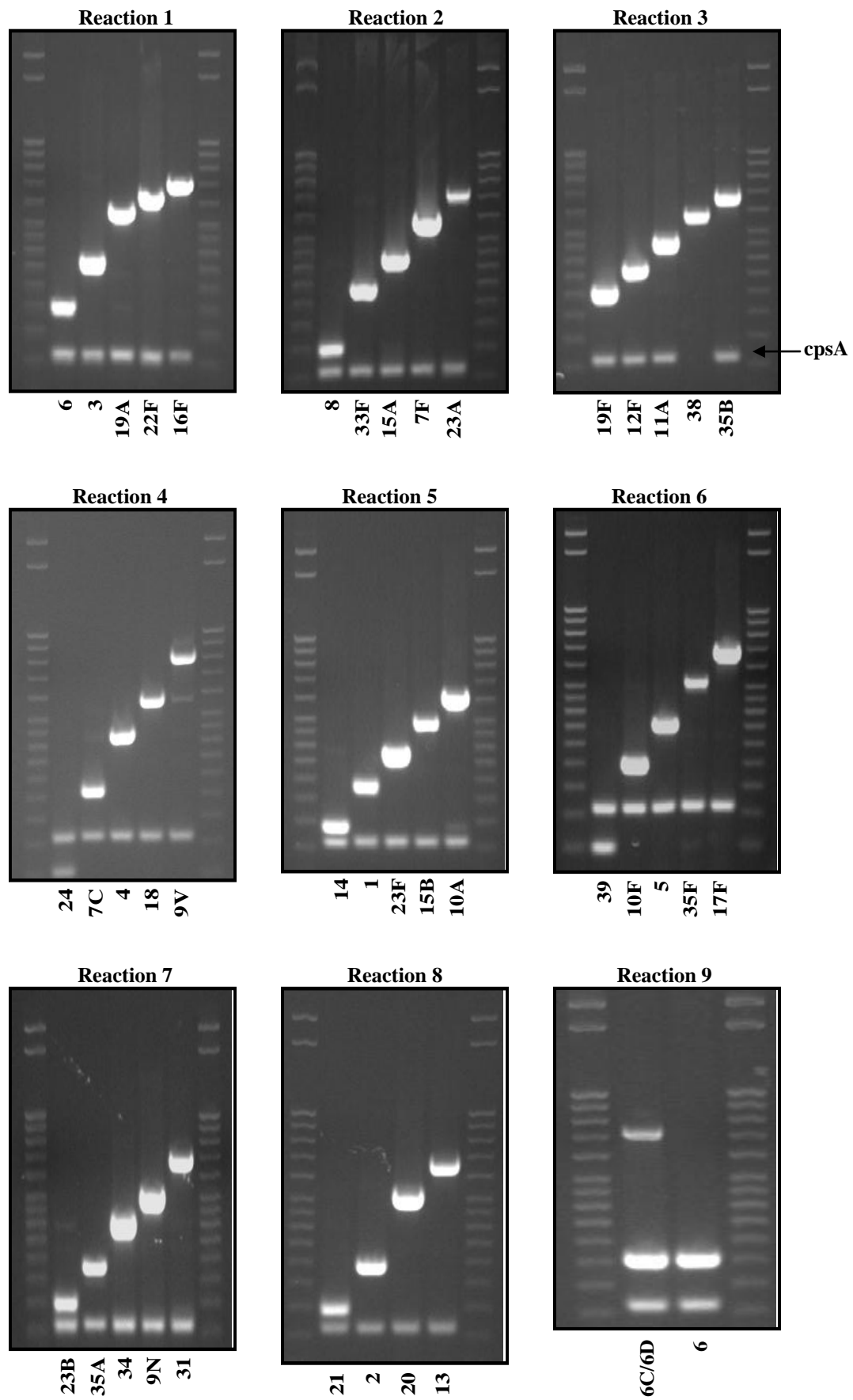


Figure 5. PCR products for USA serotyping scheme detailed in Table 6

IV. Real-time PCR

A. Workstation for real-time PCR reaction set-up

The extremely sensitive lower limit of detection of real-time PCR assays increases the chance of detection of cross-contamination with other DNA. Extra precautions should be taken to minimize such cross-contamination. It is strongly recommended that reaction assembly be performed in one room, which is designated a clean room, while DNA extraction from clinical specimens or isolates and addition of template DNA to the reaction wells should be performed in a separate room, which is designated a dirty room. If separate rooms are not possible, separate laboratory benches should be used for these two steps. Working in an unventilated biocontainment cabinet (sometimes called a PCR hood), is also suggested to minimize cross-contamination. Use of a biological safety cabinet is necessary for infectious materials but does not provide significant protection from amplicon cross-contamination. Separate micropipettors, laboratory coats, and gloves should be used for DNA extraction and reaction assembly. Signage indicating which spaces and equipment are DNA-free and which are used to prepare or manipulate DNA would be helpful. Decontamination of surfaces and equipment with 10% bleach followed by 70% ethanol should be done after any manipulation of nucleic acids at the laboratory bench. Always use filter-barrier pipette tips and change and discard gloves frequently. Even though real-time PCR is more expensive, it may be advisable to use given that it is a closed system and has much less potential for cross-contamination of the workspace.

B. Equipment for real-time PCR analysis

The main piece of equipment needed is the real-time PCR machine. There are more than 30 different real-time PCR thermocycler models that are manufactured by 14 companies (13). When choosing the machine to buy, be sure to consider the fluorescent filters that it will contain; the filters in the machine must be able to detect the wavelengths of light that will be emitted by the fluorophores conjugated to the probes. An accompanying desktop or laptop computer and appropriate software will be necessary to view and analyze the results that are generated by the machine. Given their replacement cost and sensitivity to damage by electrical surges, it is strongly recommended that this machine and computer be plugged directly into a battery backup with surge protection, to protect them from fluctuations in current, to protect your data, and to keep the reactions running in case power is lost.

C. Consumables and reagents

Consumables:

10% bleach (10:1, water: concentrated bleach) (make fresh weekly)
70% ethanol
1.5 ml microcentrifuge tubes (sterile, DNase free, or PCR grade)
96 well polypropylene plates, tube strips or individual PCR tubes
1 set of micropipettors (1-10 μ l, 2-20 μ l, 20-200 μ l, and 100-1000 μ l)
Pre-sterilized filter tips (10 μ l, 200 μ l, and 1000 μ l)
Optical caps

Optional:

Optical adhesive film
Commercial DNA-removing surface decontaminant liquid
Cap installing tool

Reagents:

TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
Tris buffer (10 mM Tris-HCl, pH 8.0)
Commercial PCR Master Mix (containing dNTPs, DNA polymerase, and reference dye)
Primers and dual-labeled hydrolysis probes (probes contain 5' fluorophore and 3' or internal quencher)
Positive control DNA diluted to about 5 µg/ml
PCR grade water

D. Preparing primer and probe working stocks

- The protocols, primers, and probes described in this chapter are adaptations of those previously described (8, 35, 60).

Table 2 lists the recommended nucleotide sequences, working concentrations, and suggested chemical modifications of the primers and probes for *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* species detection targeting the *ctrA* or *sodC*, *hpd*, and *lytA* genes, respectively. Primers and probes for the detection of *N. meningitidis* serogroups (Table 3) and *H. influenzae* serotypes (Table 4) are also given. Additional *S. pneumoniae* serotype assays are currently in development. A two-tiered approach for detecting and characterizing bacterial meningitis pathogens is recommended with the first tier being species-specific detection of *N. meningitidis*, *H. influenzae*, or *S. pneumoniae* with subsequent serogroup/serotype determination of any positive specimens (Figure 8).

Primers and probes must be diluted from concentrated stocks into working stocks. It is convenient to dilute working stocks to concentrations that will allow 2 µl of each primer and probe to be added to the master mix per reaction. The optimized working stock concentration of each primer and probe for the assays described here that will allow 2 µl of each to be added to the master mix is presented in the tables above.

To calculate the required dilution from concentrated stock to working stocks, use the formula: $(\text{Concentration}_1)(\text{Volume}_1) = (\text{Concentration}_2)(\text{Volume}_2)$. Example: A 500 µl working stock of the *sacB* forward primer is needed. For the purposes of this example, the *sacB* forward primer has a stock concentration of 220 µM. Table 3 shows that the working concentration of the *sacB* forward primer should be 3.75 µM; therefore the equation becomes:

$$(220 \mu\text{M is the given concentration of the concentrated stock})(x \mu\text{l of concentrated stock}) = (3.75 \mu\text{M is the desired concentration of the working stock})(500 \mu\text{l is the desired volume of working stock})$$

$$\text{Or: } (220 \mu\text{M})(x \mu\text{l}) / (3.75 \mu\text{M})(500 \mu\text{l})$$

Solve for x. $x = (3.75 \mu\text{M})(500 \mu\text{l}) / 220 \mu\text{M} = (1875 \mu\text{M}/\mu\text{l}) / 220 \mu\text{M} = 8.5 \mu\text{l}$ of concentrated stock.

Next, to calculate how much water to add to $x \mu\text{l}$ of concentrated stock calculated above:
 $500 \mu\text{l}$ total volume of working stock - $x \mu\text{l}$ of concentrated stock = $y \mu\text{l}$ of water to add

Therefore, using the example above: $500 \mu\text{l} - 8.5 \mu\text{l} = 491.5 \mu\text{l}$ of water.

Of this working stock of the primer, add $2 \mu\text{l}$ to the master mix per specimen to be tested.

When diluting primers and probes, it is important to use filter tips and to work in a clean space (i.e., free of template DNA) to avoid cross-contamination of these reagents. It is optimal to use a clean PCR cabinet or hood if one is available. Concentrated stocks of primers and probes should be stored at -20°C . If used on a regular basis, the working stocks can be stored at 4°C . Be sure to store probes in the dark, since they are light-sensitive (ideally, covered in aluminum foil and in a box). Concentrated stocks of probes are especially susceptible to degradation due to their fluorescent tags and should not be freeze-thawed more than 5 times. Primers should not be freeze-thawed more than 20 times (46). Therefore, it is recommended that the primer and probe stocks be aliquoted upon their arrival, before initially freezing them for longer-term storage.

The length of time for which primers and probes can be stored in this manner will vary. Performing positive controls with each reaction will help you to determine when your primers and probes need to be replaced.

E. Performing real-time PCR

General considerations

Before setting up the reactions, the real-time PCR machine should be turned on. For some machines, it can take about 20 minutes for the lamp to warm up.

The high sensitivity of real-time PCR significantly increases the risk for cross-contamination. Therefore, the following precautions are recommended:

- Separate rooms are suggested for handling DNA template (dirty room) and for handling other reagents (clean room) to avoid cross-contamination. Post signs to designate these areas.
- Always use barrier pipette tips.
- Always use clean laboratory coats. Use a different laboratory coat while in each of the separate rooms. Change gloves frequently, and be sure to wear fresh gloves when moving from the clean room to the dirty room.
- Use separate pipette sets in each room.

Assays are carried out in $25 \mu\text{l}$ reaction volumes, using a commercial PCR master mix according to the manufacturer's instructions. Per sample to be tested, each reaction mix contains: $2 \mu\text{l}$ of DNA sample, $2 \mu\text{l}$ of each primer, $2 \mu\text{l}$ of probe, $12.5 \mu\text{l}$ of master mix, and $4.5 \mu\text{l}$ of sterile,

PCR-grade water. Add enough reagents for 1-2 extra reactions than the number of specimens there are to be tested to ensure there will be enough mix.

Positive and negative controls are extremely important to ensure the laboratorian that contamination of reagents and workspace has not generated false positives and that the assay is detecting targets as expected. Non-template controls (NTCs) contain all reagents except for template DNA; instead, 2 µl of sterile, PCR-grade water should be added to the NTC reaction wells. It is advisable to add water used in the clean room to at least two NTC reaction wells and to add water used in the dirty room to at least two NTC reaction wells. If either NTC reaction generates an amplification curve that crosses the threshold, the water from that room should be discarded and replaced, and NTCs should be performed again to determine if other reagents are the source of contamination. An additional control for contamination at the DNA extraction step is the extraction of water, as suggested in the extraction protocol. If the extracted water negative control(s) generate an amplification curve that crosses the threshold, then contamination occurred during the DNA extraction process, and replacement of extraction reagents with new ones and cleaning of workspace and pipettes is recommended. Positive control reactions should be performed using DNA from known positive isolates. Dilute the positive control DNA in order to decrease the likelihood of contamination of the PCR workspace, thereby avoiding false positives. In summary, for each target gene to be detected (i.e., for each mix that is made up), the following controls should be run:

- No-template negative controls (NTCs), at least in duplicate
- Extracted-water negative controls for DNA prep equipment and reagent cross-contamination, in duplicate
- Positive control, using prepared DNA from a known isolate and running RNase P when using clinical specimens

A sample spreadsheet depicting the set-up of a 96-well real-time PCR plate is pictured below (Figure 6):

TaqMan Template - *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*

Date: _____ PCR #: _____ File name: _____

Note: Real-time PCR assays detecting: *N. men* (*sodC* or *ctrA*), *H. flu* (*hpd*), and *S. pneumo* (*lytA*)

	1	2	3	4	5	6	7	8	9	10	11	12		Rxn x 1 (μ l)	<i>sodC</i> , <i>hpd</i> , <i>lytA</i>
A	ntc-clean	unk-1	unk-3	unk-5	unk-7	unk-9	unk-11	unk-13	unk-15	unk-17	ntc-dirty	sodC +	Total needed		25
B	ntc-clean	unk-2	unk-4	unk-6	unk-8	unk-10	unk-12	unk-14	unk-16	Extracted H ₂ O	ntc-dirty		Mmix	12.5	312.5
C													H2O	4.5	112.5
D	ntc-clean	unk-1	unk-3	unk-5	unk-7	unk-9	unk-11	unk-13	unk-15	unk-17	ntc-dirty	hpd +	PrimerF	2	50
E	ntc-clean	unk-2	unk-4	unk-6	unk-8	unk-10	unk-12	unk-14	unk-16	Extracted H ₂ O	ntc-dirty		PrimerR	2	50
F													Probe	2	50
G	ntc-clean	unk-1	unk-3	unk-5	unk-7	unk-9	unk-11	unk-13	unk-15	unk-17	ntc-dirty	lytA +	Total mix	23	
H	ntc-clean	unk-2	unk-4	unk-6	unk-8	unk-10	unk-12	unk-14	unk-16	Extracted H ₂ O	ntc-dirty		DNA	2	

Controls: *N. men* (*sodC* or *ctrA*)

H. flu (*hpd*)

S. pneumo (*lytA*)

Cycling conditions	
50°C for 2 min	1 cycle
95°C for 10 min	1 cycle
95°C for 15 sec	50 cycles
60°C for 1 min	

Figure 6. Example PCR template sheet for the *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* species-specific real-time PCR assays. This example template should provide all the information needed for the laboratorian to set up the real-time PCR assay. The template should include: the assay(s) being run, date performed, PCR machine used, file name, cycle conditions, strain DNA to use as positive controls, and any notes to assist the laboratorian. On the layout, each of the wells to be used on the 96-well plate should be clearly labeled with either the specimen name or type of control. In this case, three assays are being run on same plate and each assay is color-coded: *sodC* (or *ctrA*) for *N. meningitidis* detection in pale red; *hpd* for *H. influenzae* detection in green, and *lytA* for *S. pneumoniae* detection in blue. The non-template controls (ntc) for both the clean and dirty rooms are done in duplicate for each assay type (more can be added if the user deems it necessary). One positive control well should be used for each assay type and is labeled *sodC* +, *hpd* +, and *lytA* +. The water extraction to control for contamination during DNA extraction is labeled Extracted H₂O. The 17 unknown specimens are labeled unk-1 to unk-17. The panel to the right depicts the amount of reagent needed for 1 reaction and also the amount needed to make a master mix of reagents for each assay being run. In this example, enough Master Mix (Mmix), which is the commercial mix of DNA polymerase, dNTPs, and buffer to be used, H₂O, forward and reverse primers, and probe should be made for 25 reactions. The number 25 was calculated from the number of wells needed for each assay plus an additional 2 wells to ensure that the user has enough master mix.

Real-time PCR protocol

- Prior to beginning, plan the experiment by filling out and printing a PCR template worksheet (see Figure 6 for an example). Also, be sure sufficient quantities of working stocks of primers and probes to be used are available.
1. Turn on the real-time PCR machine and make sure lamp is warming up.
 2. Remove DNA preps and positive control DNA from -20°C to the dirty room/hood to thaw.
 3. In the clean room/hood, gather reagents: commercial PCR master mix, primers, probes, and PCR grade water. If they are used infrequently enough and are therefore stored at -20°C, allow working stocks of primers and probes to thaw completely before use.

Vortex or flick each tube before using. Assemble one master mix per primer and probe set to be used. For each extraction to be tested, the master mix should contain:

12.5 µl of master mix
 4.5 µl of sterile, PCR-grade water
 2 µl of forward primer
 2 µl of reverse primer
2 µl of probe

23 µl total before adding 2 µl DNA

When calculating volumes of master mix reagents, add enough master mix reagents for 2 extra reactions to ensure there will be enough mix.

4. Pipette 23 μ l of this master mix into each appropriate well of a 96-well plate, according to the plate template worksheet. Add 2 μ l of PCR-grade water to the clean NTC wells and then cap only that row. Ensure that the lid is flush with the plate to avoid evaporation from wells during PCR.
 - Incorrectly capped or uncapped wells, if run in the machine, will lead to reaction failures and could contaminate the machine. If this happens, wipe down the interior of the machine with 70% isopropanol.
5. If available, cover plate with one-time use adhesive film. Wipe down the clean workspace with 10% bleach (10:1 water: concentrated bleach), then 70% ethanol and turn on UV light for 1 hour, if available. Remove laboratory coat and gloves. Put on a fresh pair of gloves. Carefully transport the plate to the dirty room/hood.
6. Place the plate in the dirty room/hood. Put on a new laboratory coat and keep the same pair of gloves on. If one was used, remove adhesive cover from plate.
7. According to your template worksheet, add 2 μ l to the appropriate well of the following in this order:
 - Template DNA
 - Extracted water controls
 - Dirty NTCs
 - Positive control DNA
8. Cap columns of wells as you go, capping NTC control wells last. Use the roller tool to secure caps tightly.
9. Wipe down the workspace with 10% bleach, then 70% ethanol and turn on the UV light for 1 hour, if available. Remove laboratory coat and gloves and discard the gloves.
10. If possible, spin the plate at 500 x *g* for a few seconds to bring down any droplets and to mix. Transport the plate directly to and place it in the real-time PCR machine.
11. Follow the instructions for machine operation that were provided by the manufacturer. Make sure that the machine is set to read the fluorescence of the reference dye contained in the commercial master mix, which is often ROX, in addition to the dye conjugated to each probe used (e.g., FAM, HEX, CY5). The cycle parameters suggested for the primers and probes given in the table are:

1 cycle of 50°C for 2 minutes

1 cycle of 95°C for 10 minutes

50 cycles of 95°C for 15 seconds + 60°C for 1 minute

12. Turn off the machine lamp when the assay is complete.

F. Data analysis

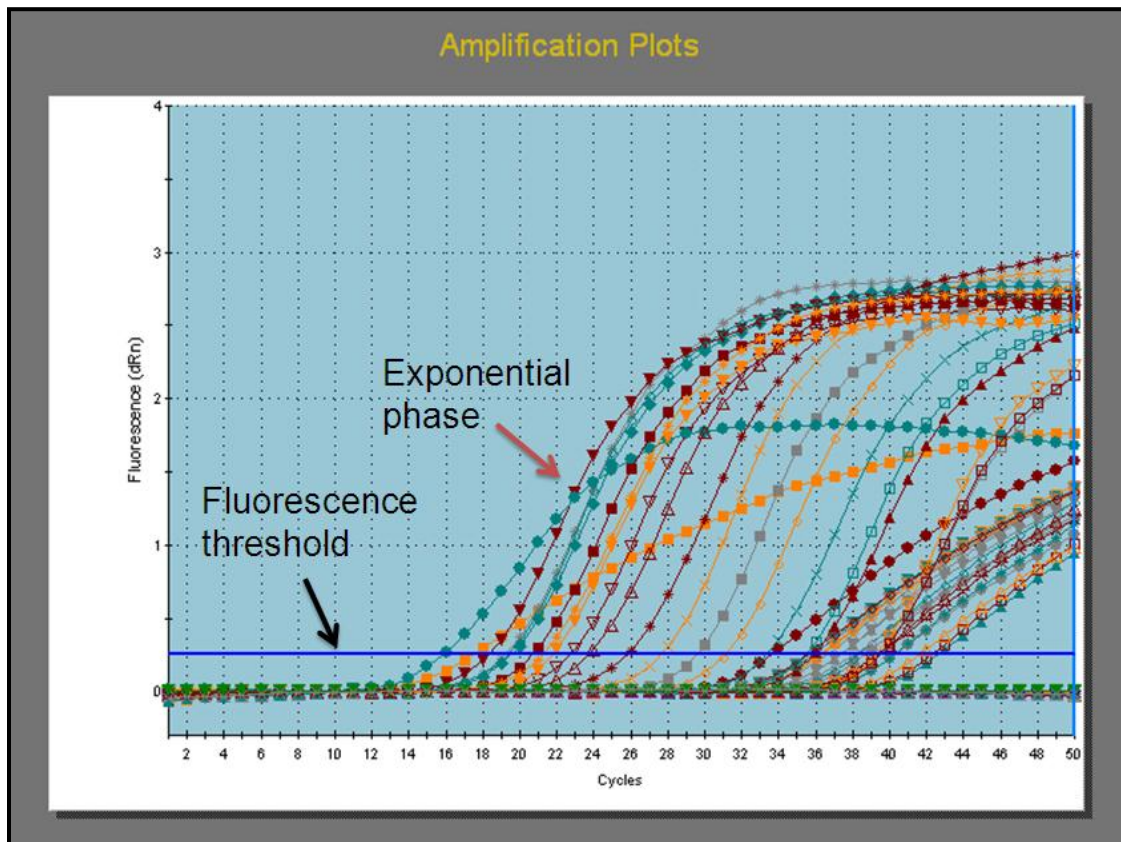


Figure 7. Amplification plot generated by a real-time PCR assay. This is a graph showing a plot of amplification cycle numbers on the X axis versus fluorescence units (dRn) on the Y axis for each reaction. dRn is the baseline subtracted fluorescent reading normalized to the reference dye. The green line of the fluorescence threshold is highlighted by the black arrow and the exponential phase is highlighted by the red arrow.

The readout of the data generated by real-time PCR machines will come in two main formats: amplification plots (Figure 7) and plate sample values. The curves that are generated should be sigmoidal in shape, ideally plateauing as the last cycle is approached, indicating complete use of reactants. The cycle number at which the fluorescence curve for each sample crosses the fluorescence threshold (green line in the above graph, which is generated automatically by the data analysis software) is referred to as the cycle threshold value, or C_t value. The plate sample values format of data readout is simply a listing of the C_t value generated by each reaction. The fluorescence threshold should be set higher than the negative controls and negative specimens and should be within the start of the exponential phase (see Figure 7). In an optimal PCR reaction running at 100% efficiency, exact doubling of the PCR product occurs at every cycle during the exponential phase. The laboratorian should carefully examine each curve and document all C_t values obtained. If possible, it is recommended that the electronic data files be

saved for each run of the real-time PCR machine, including amplification plots for future reference.

As with any experiment, results for the unknowns cannot be evaluated without first assessing the results for the controls. NTCs and negative controls should give “No Ct” and should produce amplification curves that are straight lines near zero. Positive controls should give C_t values less than 35, and the amplifications plots of the positive controls and any unknown specimens that generate curves should all be sigmoidal. The commercial master mix used may contain a reference dye, which is often ROX. If the real-time PCR machine is working properly, the amplification plot and plate sample value for ROX in each reaction should resemble those of the NTCs and negative controls. If these criteria are not met, it will be difficult to interpret the results of the unknown samples. If this is the case, refer to the troubleshooting section below.

When determining whether to call an unknown specimen positive or negative for an etiology, the following cutoff values are used in the Meningitis Laboratory at CDC:

Positive = $C_t \leq 35$

Negative = $C_t > 40$

Equivocal = C_t 36-40

- C_t values ≤ 35 are considered positive and C_t values > 40 are considered negative. Those with C_t values between 36 and 40 are considered equivocal and should be retested after diluting the template DNA 1:4 and 1:10 in PCR-grade water to reduce any inhibitors that may be interfering with the reaction. If the C_t value decreases to ≤ 35 , the specimen should be considered positive.
- The amplification plots should be analyzed to ensure they are smooth and sigmoidal in shape. If a plot is lacking these characteristics, it should be considered negative or retested.
- Other troubleshooting suggestions are described below.
- Labs that are starting real-time PCR programs should try to pair with a reference lab to help with QA/QC.

Commonly Used Terms

Reporter dye/fluorophore: Used to monitor PCR product accumulation.

Quencher dye: Absorbs the light energy of the excited-state reporter dye.

Reference dye: A dye, commonly ROX, which fluoresces at a constant level during the reaction. It is used to normalize the fluorescent signal of the reporter dye. Some systems do not use a reference dye.

C_t or cycle threshold: The PCR cycle number at which fluorescence measured by the instrument is at a statistically significant level above background. The C_t is inversely proportional to the log of the initial copy number.

Exponential phase: Phase at which exact doubling of the PCR product is accumulating at every cycle, assuming 100 % reaction efficiency. Exponential amplification occurs because all of the reagents are fresh and available and the kinetics of the reaction push the reaction to favor doubling of amplicon.

R: Raw fluorescence reading in arbitrary units

R_n: Fluorescent reading normalized to the reference dye

dR_n, ΔR_n: Baseline subtracted fluorescent reading normalized to the reference dye

NTC (non-template control): A sample type containing all the reaction components except the DNA template. Instead of template DNA, 2 μl of sterile, PCR-grade water is added to the NTC reaction wells. Water added in the clean room is called “clean NTC” and water added in the dirty room is called “dirty NTC”.

Amplification plot: Graph showing a plot of amplification cycle numbers on the X axis versus fluorescence units on the Y axis for each reaction

V. Workflow for detection of bacterial meningitis pathogens by PCR

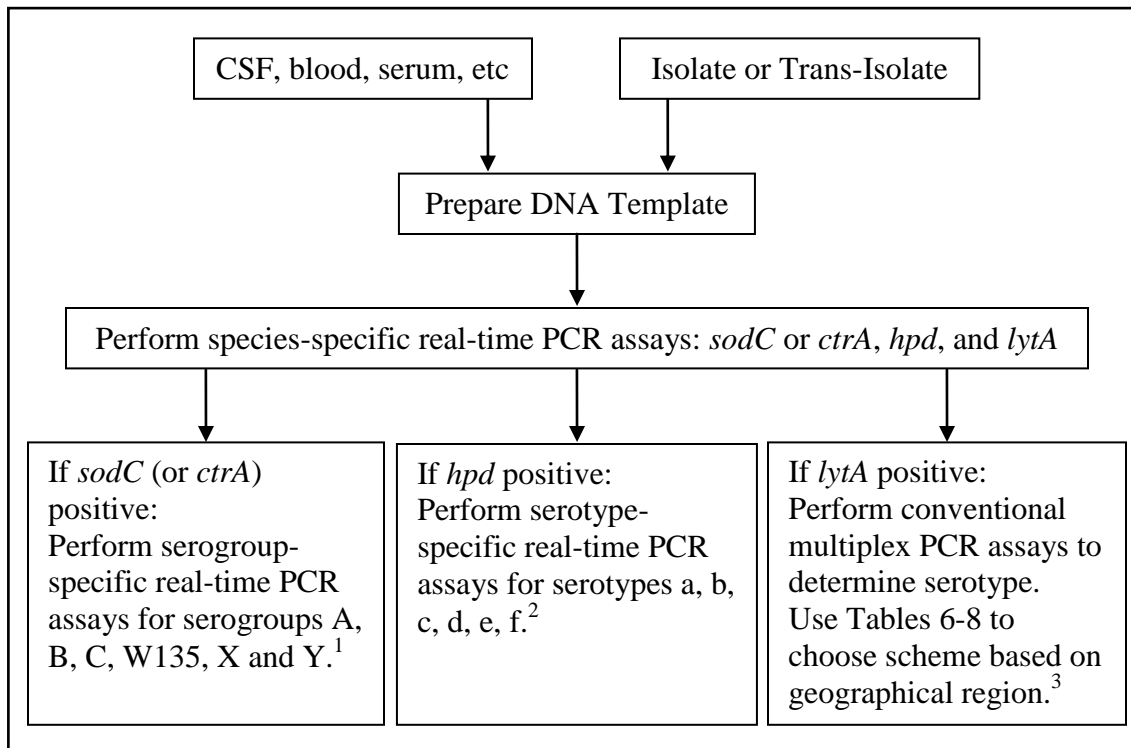


Figure 8. Workflow for detection and characterization of bacterial meningitis pathogens by PCR. Once DNA template is prepared from either a clinical specimen, an isolate, or from sampling a bottle of inoculated Tran-Isolate medium, the species-specific real-time PCR assays should be run. Any reactions positive for *N. meningitis*, *H. influenzae*, or *S. pneumoniae* should be further characterized using the appropriate serogrouping or serotyping PCR assay.

¹ It is not always practical to test for all serogroups for which assays are available in a laboratory. Testing algorithms may be set up in laboratories with previous knowledge of the predominance or lack of serogroups within that particular geographic region to test for the most common serogroups first. Modifications may be made to the testing algorithm for any laboratory based on information about current strains that are circulating in the region. For example, in Africa, testing to detect serogroups A and W135 (and X in some regions) should be adequate to characterize most specimens. Specimens reacting negatively in the A and W135 assays should then be tested using the other available assays, particularly C, Y, X and B. Nearly all invasive specimens are serogroupable, if they are tested against a comprehensive panel of assays and proper controls are used.

² It is not always practical to test for all serotypes for which assays are available in a laboratory. If a Hib vaccination program does not exist or is fairly new, testing all *hpd* positive specimens for Hib first may be adequate to characterize most specimens.

³ For bacterial isolates confirmed as *S. pneumoniae* by microbiological methods, conventional multiplex PCR serotyping is used. For clinical specimens that are *lytA*-positive by real-time PCR and have C_t values ≤ 30 , conventional multiplex PCR serotyping can be used. If C_t values are >30 , then a real-time PCR serotyping approach is recommended for increased sensitivity.

VI. Troubleshooting

Cross-contamination is the number one problem in most laboratories. Due to the sensitivity of real-time PCR, contamination is always a concern. If cross-contamination is suspected because there are C_t counts in known negatives or NTCs, consider:

- Are separate rooms, laboratory coats, gloves, and pipettes being used for master mix preparation versus template addition?
- Are sterile barrier-filtered tips being used?
- Are bleach and ethanol being used to clean work areas?
- Reaction tubes should not be re-opened and should be immediately discarded.
- Reaction tubes should be protected during transport from the clean room to the dirty room.
- Consumable reagents (tips, plates, caps, adhesive films, etc.) should not be reused.
- The amount of positive control DNA should be limited.

If there are high or no C_t counts in known positives, consider:

- Is specimen handling questioned? Was there sufficient specimen volume?
 - Clinical specimens should ideally be shipped on dry ice and frozen at -70°C .
 - The sample volume should ideally be at least 200 μl for optimal yields.
- Was the DNA extraction process performed correctly?
 - If mutanolysin and lysozyme are not used, DNA from gram-positive bacteria may not be efficiently extracted and thus will not be detectable.
 - Could any of the extraction reagents, supplies, or workspaces be contaminated?
- Are poor DNA integrity and/or inhibitors suspected?
 - Dilute the template DNA 1:4 and 1:10 and repeating the reaction. If C_t values decrease compared to undiluted, this can be indicative of an inhibitor in the DNA preparation. The specimen should be considered positive if the C_t count is less than 35 upon repeat.
 - Buffers containing phosphate such as phosphate buffered saline should be avoided as phosphate is a strong inhibitor of PCR.
 - If possible, consider sending the specimen or extraction to a reference laboratory.
 - Perform human RNAaseP assay to determine if extensive DNA degradation had occurred.
 - A dilution of the DNA preparation should also be assayed for RNAaseP to determine if inhibitors are present.
- Did a power failure or electrical current surge affect your results?
 - It is strongly recommended that the real-time PCR machine and associated computer be plugged directly into a surge protector with a battery back-up.

If problems persist, contact an experienced reference laboratory for assistance.
 Recommended for further reading: (7, 46).

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CHAPTER 11

Antimicrobial Susceptibility Testing of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*

Each laboratory must decide their own level of susceptibility testing to provide the essential data for public health decision making relevant to that laboratory's situation. *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* have all been associated with treatment or chemoprophylaxis failures due to strains resistant to or with reduced susceptibility to the antimicrobials used. In addition to monitoring for clinical or chemoprophylactic failures, surveillance of the antibiotic susceptibility patterns in circulating strains of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* is part of monitoring the emergence and spread of strains with reduced susceptibility to antimicrobials. In order for a laboratory to successfully undertake isolation, identification, and antimicrobial susceptibility testing responsibilities, it must participate in on-going investments in materials, supplies, media, reagents, and quality control, along with periodic training of personnel and quality assessment or proficiency testing. Any deviations from antimicrobial susceptibility testing methods as described in the following pages may invalidate the test results, especially for fastidious organisms such as *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*.

Antimicrobial susceptibility test methods must be performed as described according to internationally recognized clinical guidelines such as those provided by the Clinical and Laboratory Standards Institute (CLSI) (formerly known as National Committee on Clinical Laboratory Standards – NCCLS) (<http://www.clsi.org/>), which is an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis. The Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (<http://www.sfm-microbiologie.org/>) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (<http://www.eucast.org/>), whose main objectives are to harmonize breakpoints for antimicrobial agents in Europe, to act as the breakpoint committee for the European Medicines Agency (EMA) during the registration of new antimicrobial agents, and to also provide internationally recognized clinical guidelines. The overarching goals of these committees are to provide meaningful guidelines for clinical and epidemiological interpretation of results.

There are a variety of methods by which one can determine the antimicrobial susceptibility of a bacterial pathogen, commonly including disk diffusion, agar dilution or broth microdilution, and antimicrobial gradient strip diffusion (14). The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by CLSI and others. If performed precisely according to the following protocol, this method will yield data that can reliably predict the *in vivo* effectiveness of the drug in question. Although disk diffusion will provide information for most antimicrobial agents regarding interpretation of a strain as susceptible, intermediate, or resistant, it does not provide accurate information about the minimal inhibitory concentration (MIC). In addition, disk diffusion does not produce reliable results with some antibiotic/organism combinations, such as for penicillin G in *N. meningitidis* and *S. pneumoniae*. Therefore, this laboratory manual also recommends use of antimicrobial gradient strip diffusion to gather data about the MIC of antimicrobial agents.

Antimicrobial gradient strips are an antimicrobial susceptibility testing method that is as technically simple to perform as disk diffusion and produces semi-quantitative results that are measured in micrograms per milliliter ($\mu\text{g}/\text{ml}$). It is drug-specific, consists of a thin plastic antibiotic gradient strip that is applied to an inoculated agar plate, and is convenient in that it applies the principles of agar diffusion to perform semi-quantitative testing. The continuous concentration gradient of stabilized, dried antibiotic is equivalent to 15 two-fold dilutions by a conventional reference MIC procedure as suggested by CLSI. Antimicrobial gradient test strips have been compared and evaluated beside both the agar and broth dilution susceptibility testing methods recommended by CLSI. Authoritative reports indicate that an ~85-100% correlation exists between the accepted conventional MIC determinations and the MIC determined by the test strip procedure for a variety of organism-drug combinations (2, 3, 9, 10). Some studies have cited gradient test strip MICs as approximately one dilution higher than MICs determined by standard dilution methods.

MIC testing can also be done by dilution; but because agar dilution and broth microdilution are expensive and technically complex, this manual recommends that countries that do not currently do MIC testing by dilution methods should utilize a reference laboratory rather than developing the assay in-country. Alternatively, if resources are available, laboratories may purchase commercially available, frozen MIC panels and follow the manufacturer's instructions to carry out the MIC test. It is important to note that the accuracy and reproducibility of these tests are dependent on following standard quality control/quality assurance (QC/QA) testing procedures and conditions in laboratories on an on-going basis.

This guide describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results for *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* put forth by CLSI, CA-SFM, and EUCAST. In multiple instances, the zone diameter and MIC interpretative standards differ for the same antimicrobial between CLSI, CA-SFM, and EUCAST. These differences arise for many reasons, including: different databases of susceptibility data, differences in interpretation of that data, differences in both antimicrobials and dosages used in different parts of the world, and public health policies. The interpretive standards put forth by all 3 organizations are to be treated as guidelines and may be modified to meet the needs of the region. It is incumbent on the laboratory and public health system to remain alert for clinical treatment failures and trends of decreasing susceptibility to antimicrobials, regardless of which set of interpretive standards are utilized.

I. Antimicrobial susceptibility program recommendations

Antimicrobial susceptibility testing is a resource-intensive activity requiring a significant amount of labor, well-trained technicians, and quality control processes that must be maintained. Each laboratory considering starting a testing program should perform a cost-benefit analysis to determine the amount of testing that can be done without adversely affecting other laboratory functions. While the optimal testing situation would be to perform susceptibility testing on all incoming isolates, that is unlikely to be practical or economical. Susceptibility testing of a subset of both endemic and epidemic isolates (i.e., every 10th isolate) would provide useful data. During an epidemic caused by a clonal strain, testing every 25th isolate may be sufficient. These numbers are arbitrary and may have to be revised as the epidemiologic situation changes. If an

isolate is found to be resistant to a given antimicrobial, it would be prudent to test more isolates epidemiologically associated with the resistant isolate.

It is imperative that monitoring for clinical and/or chemoprophylaxis failures be performed regardless of the amount of susceptibility testing being performed. A communication network should be set up to allow clinicians to notify public health officials of the potential treatment failure and to ship specimens from suspected treatment or chemoprophylaxis failures to the reference laboratory for susceptibility testing. A mechanism must also exist to allow clinicians and public health officials to receive the susceptibility data in a timely fashion. In addition, a communication network should include links to pharmacies and pharmacists to monitor for changes in prescription practices and antibiotic usage. Changes may reflect treatment and/or chemoprophylaxis failures and may warrant further investigation.

II. Quality control for antimicrobial susceptibility testing of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*

In order to ensure the validity and accuracy of the results obtained by susceptibility testing, it is vital that a quality control (QC) system be in place in the laboratory. The goals of QC are to verify the repeatability and accuracy of the susceptibility test being used, the performance of reagents used in the tests, and the performance of the laboratorians performing the tests and reading the results. Therefore, it is vital to include control organisms with known zone diameters or MIC ranges to the antibiotics being tested. CLSI, CA-SFM, and EUCAST have recommended strains that are to be used as quality controls for antimicrobial susceptibility tests. See Tables 1-5 for strains and limits for both disk diffusion and MIC determination recommended by CLSI, CA-SFM, and EUCAST. A laboratory should choose which QC strain(s) to use based on the antimicrobials to be tested for susceptibility.

If QC testing of antimicrobial tests are performed daily for 20 or 30 days for each strain and antimicrobial agent combination with no more than 1 out of 20 tests outside of control limits (see Tables 1-5), then the tests can be performed once per week. Alternatively, if testing is done less frequently, then QC testing should be performed with every group of tests. They should also be done with each new batch of antimicrobial susceptibility test medium and every time a new lot of disks or gradient strips are used. Note that CLSI QC and breakpoint guidelines can be found in the document: Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement (5).

A. Corrective action for out of range quality control results

Adapted from:

1. CLSI. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Tenth Edition. CLSI document M2-A10. Wayne, PA: Clinical and Laboratory Standards Institute; 2009, p 27-33.
2. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Eighth Edition. CLSI document M07-A8. Wayne, PA: Clinical and Laboratory Standards Institute; 2009, p 32-40.

QC results periodically will be out of the normal range. If zone diameters or MICs produced by the control strains are out of the expected ranges, the laboratorian should consider the following possible sources of error:

- **Antimicrobial susceptibility tests are affected by variations in media, inoculum size or growth phase, incubation time, temperature, and other environmental factors.** The medium used may be a source of error if it fails to conform to CLSI, CA-SFM, or EUCAST recommended guidelines. For example, agar containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing the zones of growth inhibition to be smaller or less distinct. Organisms may appear to be resistant to these drugs when in fact they are not. QC/QA guidelines for preparation of the media must be closely followed.
- **If the depth of the agar in the plate is not uniformly 3-4 mm,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
- **If the pH of the test medium is not between 7.2 and 7.4,** the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected. Note: do not attempt to adjust the pH of the Mueller-Hinton agar test medium if it is outside the range.

Table 1. CLSI recommendations for acceptable limits for quality control strains used to monitor accuracy of Kirby-Bauer disk diffusion

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CLSI document M100-S21; 2011, pp 114-117.

Antimicrobial Agent	Disk Content	<i>E. coli</i> ¹ ATCC 25922 ²	<i>H. influenzae</i> ³ ATCC 49427	<i>S. pneumoniae</i> ⁴ ATCC 49619
zone diameter, nearest whole mm				
Ampicillin	10 µg	16-22	13-21	30-36
Ceftriaxone	30 µg	29-35	31-39	30-35
Ciprofloxacin	5 µg	30-40	34-42	- ⁵
Erythromycin	15 µg	-	-	25-30
Penicillin	10 units	-	-	24-30
Rifampicin	5 µg	8-10	22-30	25-30
Trimethoprim-sulfamethoxazole (cotrimoxazole) ⁶	1.25/23.75 µg	23-29	24-32	20-28

¹ Values are valid for testing this QC strain on Mueller-Hinton agar without blood or other supplements.

² ATCC: American Type Culture Collection, Manassas, Virginia, USA. <http://www.atcc.org/>.

³ Values are valid for testing this QC strain on *Haemophilus* test medium incubated in 5% CO₂ for 16-18 hr at 35°C.

⁴ Values are valid for testing this QC strain on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood incubated in 5% CO₂ for 16-18 hr at 35°C.

⁵ - = no data available.

⁶ Trimethoprim-sulfamethoxazole is a combination of two drugs used in treatment (cotrimoxazole). The 1:20 ratio is that at which the greatest synergy in treatment has been demonstrated in serum. The disks are impregnated with 1.25 µg trimethoprim and 23.75 µg sulfamethoxazole to mimic the 1:20 ratio.

Table 2. CLSI recommendations for acceptable limits for QC strains used to monitor accuracy of minimal inhibitory concentrations (MIC)

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CLSI document M100-S21; 2011, pp 122-125.

Antimicrobial Agent	<i>E. coli</i> ¹ ATCC 25922	<i>H. influenzae</i> ² ATCC 49427	<i>S. pneumoniae</i> ³ ATCC 49619
	(µg/ml)		
Ampicillin	2-8	2-8	0.06-0.25
Ceftriaxone	0.03-0.12	0.06-0.25	0.03-0.12
Ciprofloxacin	0.004-0.015 ⁴	0.004-0.03	- ⁵
Erythromycin	-	-	0.03-0.12
Penicillin	-	-	0.25-1
Rifampicin	4-16	0.25-1	0.015-0.06
Tetracycline	0.5-2	4-32	0.06-0.5
Trimethoprim-sulfamethoxazole (cotrimoxazole) ⁶	≤0.5/9.5	0.03/0.59-0.25/4.75	0.12/2.4-1/19

¹ Values are valid for testing this QC strain on cation-adjusted Mueller-Hinton broth (CAMHB) without blood or other supplements.

² Values are valid for testing this QC strain on *Haemophilus* test medium broth incubated in ambient air for 20-24 hr at 35°C.

³ Values are valid for testing this QC strain on CAMHB with lysed horse blood (2.5-5.0% v/v) incubated in 5% CO₂ or ambient air for 20-24 hr at 35°C. Note that for *S. pneumoniae* that is not ATCC 49619, the plates should be incubated in ambient air.

⁴ QC limits are the same for ciprofloxacin if *E. coli* ATCC 25922 is tested in CAMHB with lysed horse blood (2.5-5.0% v/v).

⁵ - = no data available.

⁶ Trimethoprim-sulfamethoxazole is a combination of two drugs used in treatment (cotrimoxazole). The greatest synergy in serum has been found when the two drugs are at a 1:20 ratio, thus the breakpoints are given at a 1/20 ratio of trimethoprim/sulfamethoxazole; i.e., ≤0.5 µg/ml trimethoprim/9.5 µg/ml sulfamethoxazole.

Table 3. CA-SFM recommendations for acceptable limits for QC strains used to monitor accuracy of Kirby-Bauer disk diffusion and to monitor accuracy of MIC

Kirby-Bauer disk diffusion QC limits are given for *E. coli* CIP 7624 and *S. aureus* CIP 7625 and MIC QC limits are given for *S. pneumoniae* CIP 107808. Many antibiotics listed in this chapter do not have QC limits set by CA-SFM.

Comite de L'Antibiogramme de la Société Française de Microbiologie, Société Française de Microbiologie, Recommendations 2010, p8. More information available at http://www.sfm-microbiologie.org/UserFiles/file/casfm_2010.pdf.

Antimicrobial Agent	Disk Content	<i>E. coli</i> ¹ CIP 7624 ²	<i>Staphylococcus aureus</i> ¹ CIP 7625 ³	<i>S. pneumoniae</i> ⁴ CIP 104485
		zone diameter, nearest whole mm		(µg/ml)
Cefotaxime	30 µg	32.5-37.5	- ⁵	0.03-0.25
Ciprofloxacin	5 µg	31-38	-	-
Erythromycin	15 units	-	26.5-31.5	-
Penicillin G	6 µg (10 units)	-	31-38.5	0.125-0.5
Rifampicin	30 µg	-	34.0-39.0	-
Trimethoprim-sulfamethoxazole (cotrimoxazole) ⁶	1.25/23.75 µg	25.5-30.5	28.0-32.5	-

¹ Values are valid for testing this QC strain on Mueller-Hinton agar, McFarland 0.5, incubated in ambient air for 20-24 h at 35-37°C.

² CIP: Collection de Bactéries de l' Institut Pasteur, Paris, France <http://www.crbip.pasteur.fr/>. This strain is equivalent to *E. coli* ATCC 25922.

³ CIP: This strain is equivalent to *S. aureus* ATCC 25923.

⁴ Values are valid for testing this QC strain on Mueller-Hinton agar supplemented with 5% sheep blood, McFarland 0.5, incubated in ambient air for 18-24 h at 35-37°C. For cotrimoxazole, use Mueller-Hinton agar supplement with 5% haemolysed horse blood.

⁵ - = no data available.

⁶ Trimethoprim-sulfamethoxazole is a combination of two drugs used in treatment (cotrimoxazole). The 1:20 ratio is that at which the greatest synergy in treatment has been demonstrated in serum. The disks are impregnated with 1.25 µg trimethoprim and 23.75 µg sulfamethoxazole to mimic the 1:20 ratio.

Table 4. EUCAST recommendations for acceptable limits for QC strains used to monitor accuracy of Kirby-Bauer disk diffusion

Data is from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website, <http://www.eucast.org>, version 1.3, December 2010. More information can be found at this website.

Antimicrobial Agent	Disk Content	<i>E. coli</i> ¹ ATCC 25922 ²	<i>H. influenzae</i> ³ ATCC 9334 ⁴	<i>S. pneumoniae</i> ⁵ ATCC 49619 ⁶
zone diameter, nearest whole mm				
Ampicillin	2 µg	- ⁷	19-25	25-31
Ampicillin	10 µg	16-22	-	-
Azithromycin	15 µg	-	-	-
Benzylpenicillin	1 unit	-	-	15-21
Cefotaxime	5 µg	25-31	29-35	28-34
Ceftriaxone	30 µg	29-35	33-41	32-38
Chloramphenicol	30 µg	21-27	30-38	24-30
Ciprofloxacin	5 µg	30-40	31-39	22-28
Clindamycin	2 µg	-	-	22-28
Erythromycin	15 µg	-	12-18	26-32
Levofloxacin	5 µg	29-37	32-38	21-27
Meropenem	10 µg	28-34	28-34	30-38
Nalidixic acid	30 µg	22-28	27-33	-
Rifampicin	5 µg	-	20-26	26-32
Telithromycin	15 µg	-	15-21	27-33
Tetracycline	30 µg	18-25	28-34	28-34
Trimethoprim-sulfamethoxazole (cotrimoxazole) ⁸	1.25/23.75 µg	23-29	26-34	20-26

¹ Values are valid for testing this QC strain on Mueller-Hinton agar, McFarland 0.5, air, 35±1°C, 18±2 hours.

² Equivalent *E. coli* QC strains are: NCTC 12241, CIP 7624, DSM 1103, and CCUG 17620
 NCTC: National Collection of Type Cultures, PHLS Central Public Health Laboratory, London, U.K. <http://www.hpacultures.org.uk/collections/nctc.jsp>
 CIP : Collection de Bactéries de l' Institut Pasteur, Paris, France <http://www.crbip.pasteur.fr/>
 DSM: DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany <http://www.dsmz.de/>
 CCUG: Culture Collection, University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden <http://www.ccug.se/>

³ Values are valid for testing this quality control strain on Mueller-Hinton agar with 5% horse blood and 20 µg/ml NAD, McFarland 0.5, 5% CO₂, 35±1°C, 18±2 hours.

⁴ Equivalent *H. influenzae* QC strains are: NCTC 8468, CIP 54.94, and CCUG 23946.

- ⁵ Values are valid for testing this QC strain on Mueller-Hinton agar with 5% horse blood and 20 µg/ml β-NAD, McFarland 0.5, 5% CO₂, 35±1°C, 18±2 hours.
- ⁶ Equivalent *S. pneumoniae* QC strains are: NCTC 12977, CIP 104340, DSM 11967, and CCUG 33638.
- ⁷ - = no data available.
- ⁸ Trimethoprim-sulfamethoxazole is a combination of two drugs used in treatment (cotrimoxazole). The 1:20 ratio is that at which the greatest synergy in treatment has been demonstrated in serum. The disks are impregnated with 1.25 µg trimethoprim and 23.75 µg sulfamethoxazole to mimic the 1:20 ratio.

Table 5. EUCAST recommendations for acceptable limits for QC strains used to monitor accuracy of MIC*

Data is from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website, <http://www.eucast.org>, version 1.3, December 2010. More information can be found at this website.

*EUCAST does not yet have established MIC QC limits for *H. influenzae* ATCC 9334

Antimicrobial Agent	<i>E. coli</i> ¹ ATCC 25922 ²	<i>S. pneumoniae</i> ³ ATCC 49619 ⁴
	(µg/ml)	
Ampicillin	2-8	0.064-0.25
Azithromycin	- ⁵	0.064-0.25
Benzylpenicillin	-	-0.25-1
Cefotaxime	0.032-0.125	0.032-0.125
Ceftriaxone	0.032-0.125	0.032-0.125
Chloramphenicol	2-8	2-8
Ciprofloxacin	0.004-0.016	-
Clindamycin	-	0.032-0.125
Erythromycin	-	0.032-0.125
Levofloxacin	0.008-0.064	0.5-2
Meropenem	0.008-0.064	0.064-0.25
Nalidixic acid	1-4	-
Rifampicin	-	0.016-0.064
Telithromycin		0.004-0.032
Tetracycline	-	0.125-0.5
Trimethoprim-sulfamethoxazole (cotrimoxazole) ⁶	≤0.5/9.5 ⁷	0.125/2.4-1/19

¹ Values are valid for testing this QC strain on Mueller-Hinton agar, McFarland 0.5, air, 35±1°C, 18±2 hours.

² Equivalent *E. coli* QC strains are: NCTC 12241, CIP 76.24, DSM 1103, and CCUG 17620.

³ Values are valid for testing this QC strain on Mueller-Hinton agar with 5% horse blood and 20 µg/ml β-NAD, McFarland 0.5, 5% CO₂, 35±1°C, 18±2 hours.

⁴ Equivalent *S. pneumoniae* QC strains are: NCTC 12977, CIP 104340, DSM 11967, and CCUG 33638.

⁵ - = no data available.

⁶ Trimethoprim-sulfamethoxazole is a combination of two drugs used in treatment (cotrimoxazole). The greatest synergy in serum has been found when the two drugs are at a 1:20 ratio, thus the breakpoints are given at a 1/20 ratio of trimethoprim/sulfamethoxazole; i.e., ≤0.12 µg/ml trimethoprim/2.4 µg/ml sulfamethoxazole.

⁷ Target set by International Standard ISO 20776-1: 2006. No range available from EUCAST.

- **If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the 0.5 McFarland turbidity standard, the antimicrobial susceptibility test results will be affected.** For instance, a resistant organism could appear to be susceptible if too few bacteria are used in the inoculum. Also, even if the isolates are susceptible, when colonies from blood agar medium are used to prepare a suspension by the direct inoculum method, trimethoprim or sulfonamide antagonists may be carried over and produce a haze of growth inside the zones of inhibition surrounding trimethoprim-sulfamethoxazole (cotrimoxazole) disks. In addition, only cultures in growth phase, i.e., cultures grown within 20-24 hours, should be used.

If the out of range result is due to an obvious reason such as use of the wrong disk or gradient strip, use of the wrong control strain, obvious contamination of the strain, or use of the wrong incubation temperatures or conditions, then document the reason and retest the strain on the day the error is observed. If the repeated result is within range, no further corrective action is required.

If no obvious reason for the out of range result is apparent, immediate corrective action is required. Test the out of range antimicrobial agent/organism combination on the day the error is observed and monitor for a total of five consecutive test days and document all results. If the results of the tests from all five days are within acceptable range, no additional corrective action is necessary. However, if any of the five results remain out of range, then additional corrective action is required. In the interim, daily control tests must be performed until the problem is solved.

If immediate corrective action does not solve the problem, then other common sources of error need to be investigated to verify that:

- Results were measured and transcribed correctly.
- The turbidity standard had not expired, was stored properly, met performance requirements, and was adequately mixed prior to use.
- All materials, including disks and gradient test strips, used were within their expiration dates and were stored and used at the proper temperature.
- The incubator was at the proper temperature and atmosphere.
- Other equipment used such as pipettors were functioning properly and measuring accurately.
- The control strain had not changed and was not contaminated.
- Inoculum suspensions were prepared and adjusted correctly.
- The inoculum was used within 15 minutes of preparation.
- Inoculum for the test was prepared from a plate incubated for the correct length of time and was not more than 24 hours old.

If necessary, obtain a new QC strain from either freezer storage or from a reliable source. New lots of materials may be necessary, as well. It may be helpful to exchange quality control strains and materials with another laboratory using the same method to verify results. Until the problem is resolved, it may be necessary to use an alternative test method, if one is available.

III. Antimicrobial susceptibility testing of *N. meningitidis*

N. meningitidis does not commonly show widespread resistance to many antimicrobial agents, though resistance to sulfonamides has become quite common (8). *N. meningitidis* with reduced susceptibility to penicillin is common in many areas of the world, though the clinical significance of this resistance has not yet been established (10, 13, 22). In addition, resistance to rifampicin has been reported and such strains have resulted in prophylaxis and treatment failures (16, 19, 21). Recently, sporadic resistance to ciprofloxacin, an antibiotic commonly used for chemoprophylaxis of non-pregnant adults in many countries, has been reported throughout the world, including Europe, South America, Australia, Asia, and North America (1, 4, 6, 18, 20, 23). Third generation cephalosporins such as ceftriaxone tend to be the empiric drugs of choice for primary treatment in most areas of the world but ceftriaxone non-susceptible isolates have been reported in India (12).

Susceptibility testing of *N. meningitidis* is difficult as a consensus has not been reached regarding the best techniques for *N. meningitidis* susceptibility testing or their standardization. The guidelines presented here must be adapted to each laboratory's needs, capabilities, and capacities as well as to the overarching public health requirements. This manual contains suggested antibiotics to screen for reduced susceptibility and guidelines on how to both perform and interpret susceptibility tests, but they should be modified and interpreted to meet the needs of each country, region, and laboratory. CLSI, CA-SFM, EUCAST, and other international organizations are working together to attempt to reach consensus recommendations.

A. Selection of antibiotics to screen for susceptibility

Selecting the antibiotics to screen for susceptibility is largely driven by the geographical regions in which the isolates originate and the capacity of the laboratory. It is recommended to routinely determine the susceptibility of circulating strains of *N. meningitidis* against the antimicrobials used for primary treatment and chemoprophylaxis in the geographical region from which the specimens originate. It may not be necessary for the laboratory to perform susceptibility testing on all routine surveillance isolates; a random representative subset can be tested. Furthermore, during an outbreak it is not necessary to perform susceptibility testing on all isolates. Laboratories could also consider periodic, non-routine surveillance for characteristics such as β -lactamase production in penicillin or cephem class resistant strains, and ceftriaxone and chloramphenicol resistance (if not already routinely tested), for example. These data would help provide information to public health agencies and international reference laboratories regarding the emergence of new *N. meningitidis* strains with reduced susceptibility to antimicrobials of clinical and public health concern.

B. Biosafety precautions

Several fatal laboratory-acquired meningococcal disease cases have been reported, thus biosafety should be a top priority (17). Biosafety Level 2 (BSL2) practices are recommended for *N. meningitidis* in most countries. Whenever possible, procedures likely to generate aerosols should be performed in a biological safety cabinet. Consider vaccination of laboratorians that work with

invasive meningococcal isolates, if possible, although current vaccines are not protective against all serogroups.

C. Antimicrobial susceptibility testing of *N. meningitidis* by Kirby-Bauer disk diffusion

Kirby-Bauer disk diffusion is the least expensive screen for antimicrobial susceptibility testing, but results can be difficult to interpret. These tests can be useful for screening isolates to categorize them as susceptible, intermediate, resistant, or non-susceptible for several antimicrobials. However, since this test does not determine MICs, it is not useful for detecting subtle trends of decreasing susceptibility. Kirby-Bauer disk diffusion tests do not produce reliable results for ampicillin and penicillin and false intermediate, resistant, or non-susceptible results are seen with *N. meningitidis*. False intermediate or resistant results are not unusual when testing the susceptibility of an isolate of *N. meningitidis* to ciprofloxacin with a 5 µg ciprofloxacin disk. Thus, results demonstrating an isolate with reduced susceptibility should be verified using a MIC test.

Either 150-mm or 100-mm plates can be used for Kirby-Bauer disk diffusion depending on the number of antimicrobial agents to be tested per isolate. CLSI guidelines state that no more than two disks can be used on a 100-mm plate and up to five disks can be used on a 150-mm plate (Figure 1). Mueller-Hinton with 5% sheep blood agar with a depth of 4 mm is used when testing *N. meningitidis* isolates using this method.

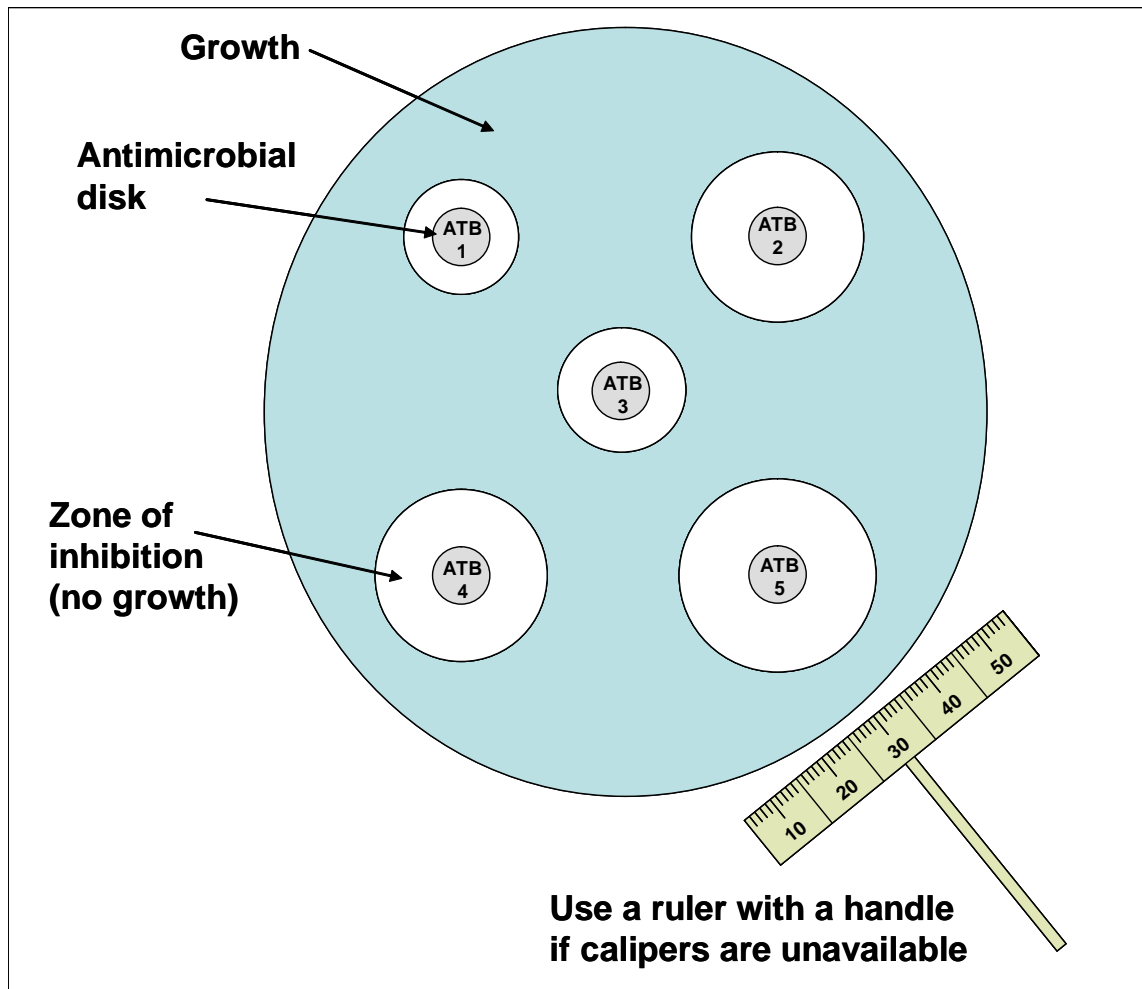


Figure 1. The antimicrobial susceptibility disk diffusion test: approximate disk placement and measurement of inhibition zone diameters.
 ATB1 = antibiotic 1, ATB2 = antibiotic 2, etc

Isolates to be tested should be subcultured onto a chocolate agar plate and incubated in a CO₂-enhanced atmosphere (5% CO₂ in a CO₂-incubator or candle-extinction jar) at 35±2°C for 20-24 hours prior to testing. If the organism has been frozen, it should be subcultured twice when it is removed from the freezer before proceeding with susceptibility testing.

Remove agar plates from the refrigerator and allow them to come to room temperature (25°C) before inoculating. Warm the cation-adjusted Mueller-Hinton broth (CAMHB) to 35°C before using. Allow the antibiotic disks that will be used in the batch of testing to warm to room temperature (25°C).

1. Using a sterile cotton-tip applicator, touch the surface of one to four morphologically identical, isolated colonies. Immerse the applicator into a tube containing sterile CAMHB. Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix the cells using a vortex to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. Adjust the

turbidity of the inoculum to that of a 0.5 McFarland turbidity standard (approximately 1 to 4×10^8 CFU/ml). Preparation of a McFarland turbidity standard is described in the Annex. If the turbidity of the inoculum is greater than the standard, dilute it with CAMHB to equal the turbidity of the standard. This suspension must be used within 15 minutes.

- Perform regular colony counts to verify that the density of the inoculum suspension is correct. For example, dilute the suspension 1:100 and subculture 10 μ l onto the recommended media. An acceptable inoculum should give approximately 100-500 colonies. It is not necessary to perform colony counts on every isolate tested.
2. Immerse a sterile cotton-tipped swab into the adjusted inoculum. Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a blood agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure even distribution of the inoculum and confluent growth of the bacteria. Use a single swab of inoculum and do not return the swab to the broth after each rotation.
 3. Allow the inoculum to dry on the surface of the plate (which should take approximately 5-10 minutes). Be sure the plate is entirely dry before proceeding, but do not exceed 15 minutes.
 4. When the surface of the inoculated plate is dry and the disks are at room temperature, place the disks onto the agar with an applicator or sterile forceps. Make sure that the disks are spaced enough distance apart on the agar so the zones of inhibition do not overlap (Figure 1). Press down on the disks to ensure complete contact with the agar surface. Alternatively, a mechanical disk dispenser can be used. Once applied, it is important to not move the antibiotic disks as the antibiotic will begin to diffuse immediately upon contact with the plate.
 5. Incubate the plates in an inverted position in a 5% CO₂ atmosphere or candle jar for 20-24 hours at 35 \pm 2°C.
 6. After overnight incubation, measure the diameter of each zone of inhibition with a ruler or calipers (Figure 2). Measurements should be performed in a biosafety cabinet, if possible. The zones of inhibition on the media containing blood are measured from the top surface of the plate with the top removed. Use either calipers or a ruler with a handle attached for these measurements, holding the ruler over the center of the surface of the disk when measuring the inhibition zone (Figures 1 and 2).
 - Care should be taken not to touch the disk or surface of the agar. Decontaminate the ruler occasionally to prevent transmission of the bacteria. In all measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony of the unaided eye. Record the results to the nearest millimeter (mm).
 7. Interpret the antimicrobial susceptibility of the strain being tested (and check that results for the QC strains are within the acceptable control range) by comparing the results to the CLSI

(Table 6) or CA-SFM (Table 7) standard zone sizes. See Figure 3 for a sample worksheet for recording antimicrobial susceptibility test results for *N. meningitidis*.

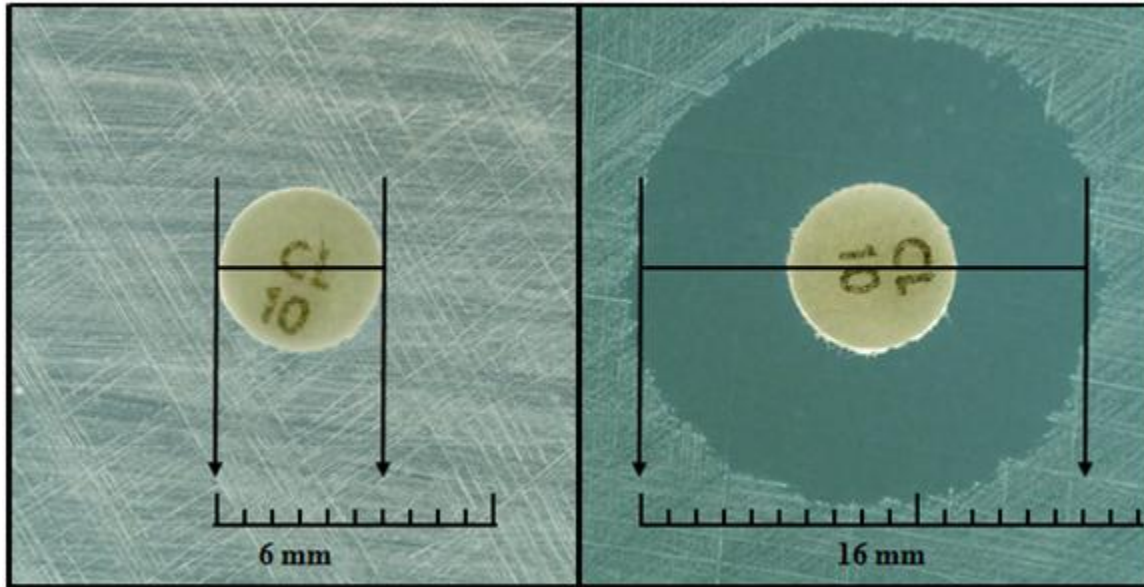


Figure 2. Images showing proper measurement of zone diameter. In the case of an isolate completely resistant to the antimicrobial, simply measure the diameter of the disk: 6 mm (left). When there is a zone of inhibition, measure the diameter as shown: 16 mm (right).

Date of Testing: ____ / ____ / ____						
Test performed by: _____			Interpretation of susceptibility: S= susceptible I= intermediate R= resistant			
Specimen number	Meningitis Isolate?	Organism	Antibiotic #1	Antibiotic #2	Antibiotic #3	Antibiotic #4
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
			mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R
QC strain	N/A	QC strain	mm µg/ml Yes No	mm µg/ml Yes No	mm µg/ml Yes No	mm µg/ml Yes No

Figure 3. Sample sheet for recording data and quality control information. **Note:** After 20-24 hours of incubation, check the results for the QC strain(s) against the standard acceptable ranges; if they are within control limits, continue reading results for the test isolate. Record disk diffusion results in mm and MIC results in µg/ml. Inhibition zone ranges and breakpoints for interpretation of results may be found in Tables 1-5, depending on the QC strains used and the guidelines being followed.

Table 6. Kirby-Bauer disk diffusion zone diameter interpretive standards and MIC interpretive standards for *N. meningitidis* as recommended by CLSI

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CLSI document M100-S21; 2011, pp 108-110.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, nearest whole mm			MIC Interpretive Standard (µg/ml)		
		S	I	R	S	I	R
PENICILLINS							
Penicillin ¹		- ²	-	-	≤ 0.06	0.12-0.25	≥ 0.5
CEPHEMS							
Ceftriaxone ³	30 µg	≥ 34	-	-	≤ 0.12	-	-
MACROLIDES							
Azithromycin ^{3,4,5}	15 µg	≥ 20	-	-	≤ 2	-	-
FLUOROQUINOLONES							
Ciprofloxacin ⁴	5 µg	≥ 35	33-34	≤ 32	≤ 0.03	0.06	≥ 0.12
PHENICOLS							
Chloramphenicol ⁶	30 µg	≥ 26	20-25	≤ 19	≤ 2	4	≥ 8
ANSAMYCINS							
Rifampicin ³	5 µg	≥ 25	20-24	≤ 19	≤ 0.5	1	≥ 2

¹ Disk diffusion tests with penicillin for *N. meningitidis* are unreliable. MIC tests should be used for this organism.

² - = no data available.

³ For some antimicrobial agents, the absence or rare occurrence of resistant strains precludes defining any results categories other than “susceptible”.

⁴ May be appropriate only for prophylaxis of meningococcal case contacts. These breakpoints do not apply to therapy of patients with invasive meningococcal disease. Recently, it has been suggested that screening for reduced susceptibility to ciprofloxacin using a 30 µg nalidixic acid disk is useful for detecting meningococcal quinolone-resistant strains associated with *gyrA* mutations (7).

⁵ Interpretive criteria were developed initially using MICs determined by incubation in ambient air for the pharmacodynamic calculations.

⁶ Not routinely reported on isolates from the urinary tract.

Table 7. Kirby-Bauer disk diffusion zone diameter interpretive standards and MIC interpretive standards for *N. meningitidis* as recommended by CA-SFM

Comite de L'Antibiogramme de la Société Française de Microbiologie, Société Française de Microbiologie, Recommendations 2010, p 44. More information available at: http://www.sfm-microbiologie.org/UserFiles/file/casfm_2010.pdf.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, nearest whole mm		MIC Interpretive Standard (µg/ml)	
		S	R	S	R
PENICILLINS					
Penicillin G ¹	- ²	-	-	≤ 0.06	> 0.25
Amoxicillin ¹	-	-	-	≤ 0.12	> 1
Oxacillin ¹	5 µg	≥ 18	-	-	-
CEPHEMS					
Cefotaxime ²	30 µg	-	-	≤ 0.12	-
Ceftriaxone ²	30 µg	-	-	≤ 0.12	-
PHENICOLS					
Chloramphenicol	30 µg	≥ 30	-	≤ 2	> 4
ANSAMYCINS					
Rifampicin ³	30 µg	≥ 30	-	≤ 0.25	-
FLUOROQUINOLONES					
Ciprofloxacin	-	-	-	≤ 0.03	> 0.06

¹ Disk diffusion tests with amoxicillin and penicillin G for *N. meningitidis* are unreliable. Testing can be performed using 5 µg/ml oxacillin disks. Diameters of ≥ 18 mm are sensitive to penicillin G, but the MICs against penicillin G and/or amoxicillin should be determined for those diameters of < 18 mm.

² - = no data available.

³ Used only for prophylaxis.

Table 8. MIC interpretive standards for *N. meningitidis* as recommended by EUCAST

Data is from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website. <http://www.eucast.org>, version 1.3, December 2010.

EUCAST does not yet have interpretive guidelines for Kirby-Bauer disk diffusion for *N. meningitidis*.

Antimicrobial Agent/Class	MIC ($\mu\text{g/ml}$) Interpretive Standard	
	S	R
PENICILLINS		
Benzylpenicillin	≤ 0.064	> 0.25
Ampicillin	≤ 0.125	> 1.0
CEPHEMS		
Cefotaxime ¹	≤ 0.125	> 0.125
Ceftriaxone ¹	≤ 0.125	> 0.125
CARBAPENEMS		
Meropenem ^{1,2}	≤ 0.25	> 0.25
ANSAMYCINS		
Rifampicin	≤ 0.25	> 0.25
TETRACYCLINES		
Tetracycline ³	≤ 1.0	> 2.0
FLUOROQUINOLONES		
Ciprofloxacin ⁴	≤ 0.032	> 0.064
PHENICOLS		
Chloramphenicol	≤ 2.0	> 4.0

¹ Strains with MIC values above the susceptible breakpoint are very rare or not yet reported. The identification and antimicrobial susceptibility tests on any such isolate must be repeated and if the result is confirmed, the isolate should be sent to a reference laboratory. Until there is evidence regarding clinical response for confirmed isolates with MIC above the current resistant breakpoint they should be reported resistant.

² These breakpoints relate to meningitis only.

³ Tetracycline can be used to determine minocycline susceptibility for prophylaxis of *N. meningitidis* infections.

⁴ Breakpoints apply only to use in the prophylaxis of meningococcal disease.

D. Minimal inhibitory concentration testing of *N. meningitidis* by antimicrobial gradient strips

Either 150-mm or 100-mm plates can be used for the gradient strip testing, depending on the number of antimicrobial agents to be tested per isolate. Two different gradient strips can be placed in opposite gradient directions on a 100-mm plate (Figure 4). Although one manufacturer states that up to six gradient strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five gradient strips be used on a 150-mm plate, with the end with the lowest concentration of antibiotic placed towards the center of the plate (Figure 5). Depending on the bacteria/antimicrobial combinations, five strips on a 150-mm plate may lead to overlapping ellipses. If this occurs, testing should be repeated using four strips per plate.

Isolates to be tested should be subcultured onto a chocolate agar plate and incubated in a CO₂-enhanced atmosphere (5% CO₂ in a CO₂-incubator or candle-extinction jar) at 35±2°C for 20-24 hours prior to testing. If the organism has been frozen, it should be subcultured twice when it is removed from the freezer before proceeding with susceptibility testing.

Mueller-Hinton agar with 5% sheep blood is used when testing *N. meningitidis* isolates with gradient strips. Warm the cation-adjusted Mueller-Hinton broth (CAMHB) to 35°C before using. Allow the gradient strips that will be used in the batch of testing to warm to room temperature (25°C). It is recommended to follow the directions on the package insert included with the gradient strips.

1. Using a sterile cotton-tip applicator, touch the surface of one to four morphologically identical, isolated colonies. Immerse the applicator into a tube containing sterile CAMHB. Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix the cells using a vortex to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard (approximately 1 to 4 ×10⁸ CFU/ml). Preparation of a McFarland turbidity standard is described in the Annex. If the turbidity of the inoculum is greater than the standard, dilute it with CAMHB to equal the turbidity of the standard. This suspension must be used within 15 minutes.
 - Perform regular colony counts to verify that the density of the inoculum suspension is correct. For example, dilute the suspension 1:100 and subculture 10 µl onto the recommended media. An acceptable inoculum should give approximately 100-500 colonies. It is not necessary to perform colony counts on every isolate tested.
2. Immerse a sterile cotton-tipped swab into the adjusted inoculum. Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a 15x150-mm Mueller-Hinton agar with 5% sheep blood plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure even distribution of the inoculum and confluent growth of the bacteria. Use a single swab of inoculum and do not return the swab to the broth after each rotation.

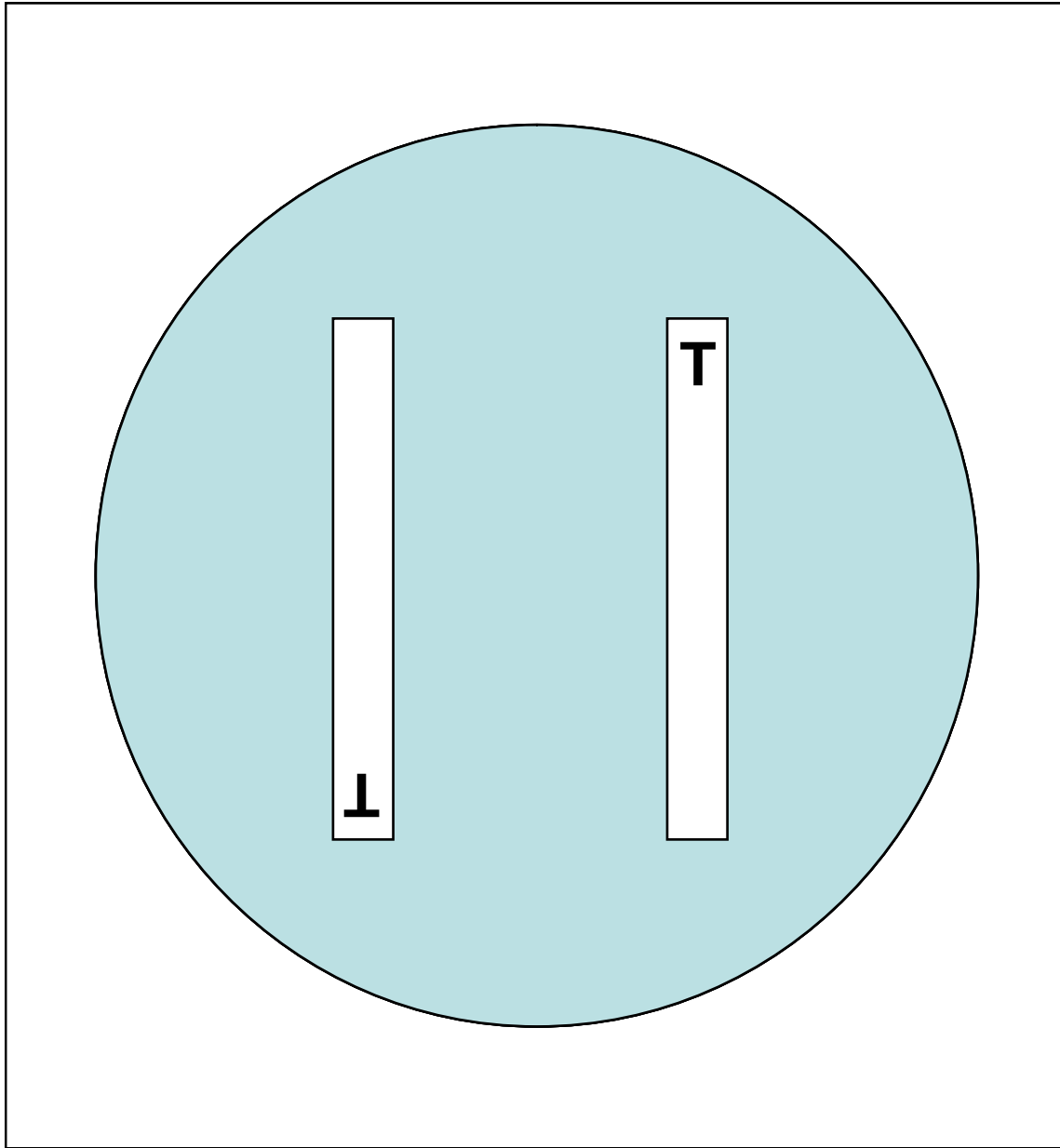


Figure 4. Placement of gradient strips on a 100-mm agar plate. Gradient strips are placed in opposite orientation. “T” represents the top of the gradient strip.

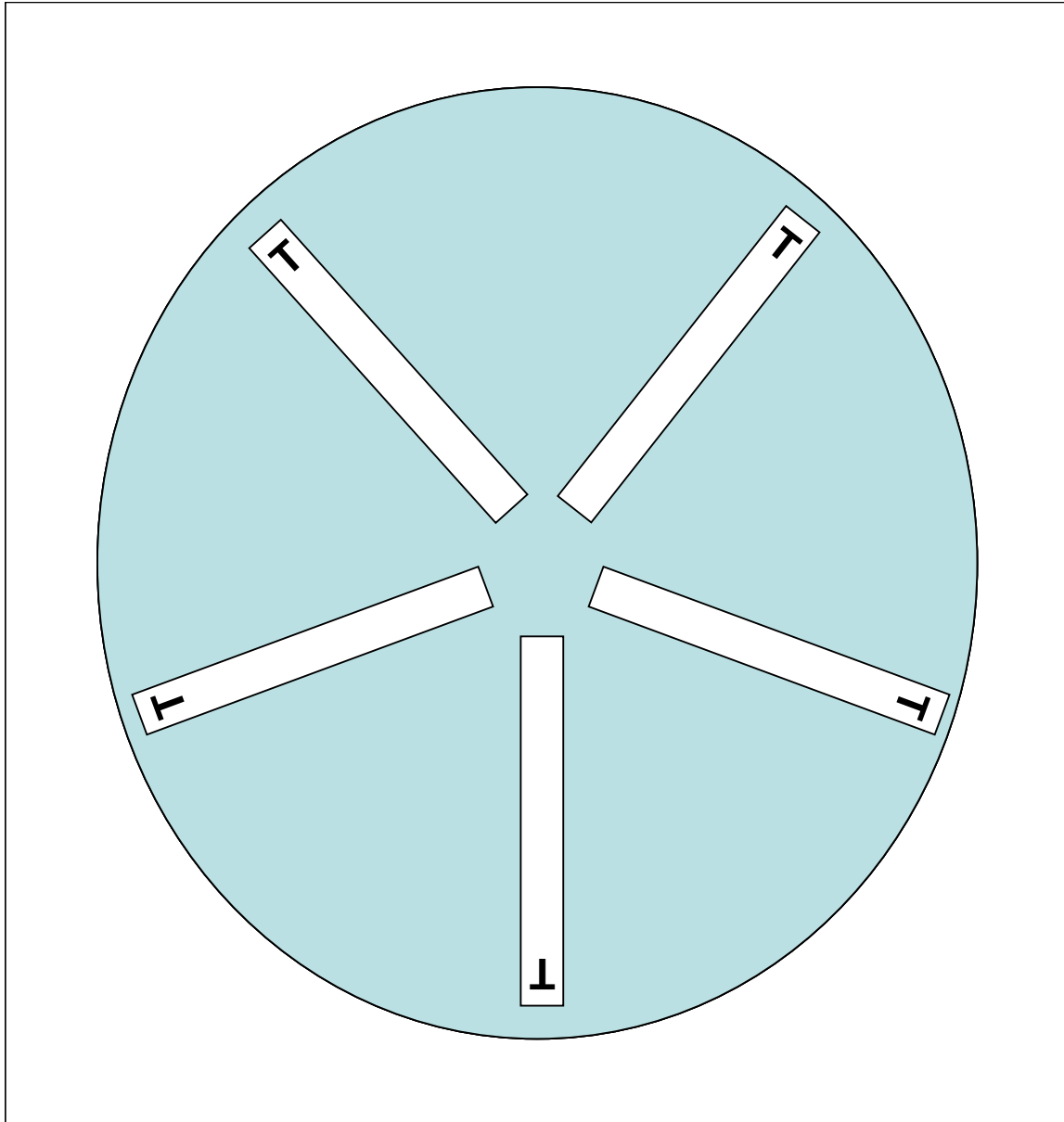


Figure 5. Placement of gradient strips on a 150-mm agar plate. Area of lowest antibiotic concentration should be oriented towards the center of the plate. “T” represents the top of the gradient strip.

3. Allow the inoculum to dry on the surface of the plate (which should take approximately 5-10 minutes). Be sure the plate is entirely dry before proceeding.
4. When the surface of the inoculated plate is dry and the gradient strips are at room temperature, place the antimicrobial gradient strips onto the agar with an applicator or sterile forceps. Make sure that the printed MIC values are facing upward (i.e., that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar). Alternatively, robotic gradient strip applicators are available from some manufacturers. Once applied, it is important to not move the antimicrobial gradient strips as the antibiotic diffuses into the agar immediately upon contact.
 - Return the antimicrobial gradient strips that will not be used in this batch of testing to the -20°C freezer (some strips can be stored at 4°C. Follow the manufacturer's instructions).
5. Incubate the plates in an inverted position in a 5% CO₂ atmosphere for 18–22 hours at 37°C. A candle-extinction jar may be used if a CO₂-incubator is not available. Because *N. meningitidis* grows well in a humid atmosphere, laboratorians may choose to add a shallow pan of sterile water to the bottom of the incubator or add a dampened paper towel to the candle-extinction jar.
6. After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the test can be read (see below). QC results must be reviewed before reading and interpreting the MIC.

Reading and interpreting the gradient strips

Read the MIC at the point where the zone of inhibition intersects the MIC scale on the strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read at the point of complete inhibition of all growth including hazes. A reading guide for the gradient strips, which shows organism related effects, drug-related effects, resistance mechanism-related effects, and technical and handling effects can be found at: <http://www.abbiotest.com/pdf/pi/75002206.pdf>.

Record the QC results first. If zones produced by the QC strain are out of the expected ranges (see Tables 2, 3, and 5, depending on the strains and guidelines being used), the laboratorian should consider possible sources of error. Because antimicrobial susceptibility test results can be affected by many factors not necessarily associated with the actual susceptibility of the organism (e.g., inoculum size, growth phase, agar depth, storage, time, and others), QC practices must be followed carefully (see Section II above). If all antimicrobial agents are in the control range, read the test MICs. Note any unusual observations such as a zone of incomplete killing (trailing endpoints) or single colonies growing within the ellipse.

The gradation marks on the gradient strip correspond to the standard 2-fold dilution concentrations for the agar dilution method, but also include increments between those standard values. The standard values are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and

the next-higher standard value (i.e., the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of an isolate to penicillin, an MIC recorded from the gradations on the gradient strip might be 0.094 µg/ml; however, the reported MIC would be 0.125 µg/ml.

The manufacturer of the gradient strips recommends following the MIC breakpoints developed for agar and broth microdilution. The interpretive standards and MIC breakpoints recommend by CLSI (Table 6), CA-SFM (Table 7), and EUCAST (Table 8) are given.

IV. Antimicrobial susceptibility testing of *H. influenzae*

This laboratory manual describes susceptibility testing of *H. influenzae* by the disk diffusion method and by the antibiotic gradient strip testing method. Although disk diffusion will provide information as to whether a strain is susceptible, intermediate, or resistant, the gradient strip method provides more detailed information about the minimal inhibitory concentration (MIC) of an antimicrobial agent. In addition, testing for strains employing a β-lactamase will be described.

The β-lactamase usually observed circulating in *H. influenzae* is a TEM-type β-lactamase, which inactivates some antibiotics belonging to the β-lactam family such as penicillin, ampicillin, and amoxicillin, but, fortunately, does not inactivate third generation cephalosporins such as ceftriaxone or cefotaxime. This β-lactamase is inhibited by clavulanic acid. However, as clavulanic acid does not cross the blood-brain barrier well, the association of amoxicillin and clavulanic acid must not be employed for the treatment of a meningitis due to *H. influenzae*. In this case, ceftriaxone or cefotaxime have to be recommended. Resistance due to a reduction of affinity to the Penicillin Binding Protein (PBP) is rare but has been reported (3).

A. Testing *H. influenzae* for β-lactamase production

A rapid β-lactamase test may yield clinically relevant information earlier than the results of antimicrobial susceptibility testing, so it should be performed as soon as a *H. influenzae* is identified.

1. **Nitrocefin-based tests are the preferred method.** The reagent is composed of paper disks impregnated with chromogenic cephalosporin, which releases a red compound on hydrolysis by a β-lactamase.

Performing the test: The disks can be stored in their cartridge at 2-8°C until the expiration date. After opening the cartridge, disks can be stored for 2 months at 2-8°C.

- a. Allow the tube containing the cartridge to come to room temperature (25°C).
- b. Moisten a disk with sterile distilled water.
- c. Collect a few isolated colonies of the strains to be tested and spread them over the surface of the disk.

Reading and interpretation

The appearance of a red color reveals a positive reaction.

The reaction is negative if no color has appeared after 30 minutes.

Quality control strains

<i>Staphylococcus aureus</i>	ATCC 29213	result	+
<i>Enterococcus faecalis</i>	ATCC 29212	result	-

2. **A modified acidometric agar plate method can test *H. influenzae* for the presence of β -lactamase activity** (11, 15). Penicillin and phenol are combined in a non-nutrient plate; the pH indicator detects increased acidity resulting from the cleavage of the β -lactam ring of penicillin that yields penicilloic acid and leads to a color change in the agar.
 - a. Using a loop, harvest several isolated colonies and place in a spot on the plate (penicillin + phenol).
 - b. Apply known β -lactamase positive and negative control strains.
 - c. Incubate in ambient air at 37°C for 15 min.
 - d. Observe the plate for color change in the agar surrounding each discretely spaced colony. The agar surrounding the positive control strain should be yellow, whereas the agar surrounding the negative control strain should not exhibit any change in color.

Interpretation of the results

A positive β -lactamase test predicts that the *H. influenzae* isolate is resistant to penicillin, ampicillin, and amoxicillin. In cases of meningitis, ceftriaxone or cefotaxime should be recommended to treat the patient. If results of susceptibility testing indicate resistance to a penicillin-class antibiotic and the isolate is negative for β -lactamase, those results do not rule out resistance due to other mechanisms, such as reduction of affinity in the PBP. Reduction of affinity in PBP is unusual in *H. influenzae* and the presence of such a mechanism can be detected using a 30 μ g cefalotin disk (zone diameter < 17 mm) or a 2 μ g ampicillin disk (zone diameter < 20 mm). In these cases, activity of third generation cephalosporins is only weakly affected.

B. Antimicrobial susceptibility testing of *H. influenzae* by Kirby-Bauer disk diffusion

The antibiotic susceptibility testing of *H. influenzae* using the disk diffusion method will provide information as to whether a strain is susceptible, intermediate, or resistant to an antimicrobial. Dilution methods or antimicrobial gradient strips can be used to accurately determine the MICs to antimicrobials, but are not necessarily better than disk diffusion in providing reliable information about determining whether the isolate is susceptible, intermediate, or resistant to an

antimicrobial. Laboratories must use standardized procedures to guarantee the accuracy and reproducibility of antibiotic susceptibility testing.

Media and disks for antimicrobial susceptibility testing

For *H. influenzae*, antimicrobial susceptibility can be determined using the disk diffusion method. The method presented in this chapter is a modification of the Kirby-Bauer technique that has been standardized by CLSI. If performed precisely according to the following protocol, this method will provide data that can reliably predict the in vivo effectiveness of the drug in question. The accuracy and reproducibility of this test is dependent on the consistent use of a standard set of procedures in the laboratory.

The optimal medium is Haemophilus test medium (HTM). The Mueller-Hinton agar used to make HTM should be thymidine free to obtain consistent results if susceptibility to cotrimoxazole is to be tested. HTM medium consists of the following ingredients: Mueller-Hinton agar supplemented with 15 µg/ml NAD, 15 µg/ml bovine hemin, and 5 mg/ml yeast extract. The pH is adjusted to 7.2 to 7.4. Recommended agents tested are ampicillin, ceftriaxone and/or cefotaxime, and chloramphenicol, which are antibiotics commonly used for the treatment of meningitis.

The 10 µg-ampicillin disk predicts both intrinsic (PBP-mediated) and β-lactamase mediated penicillin and ampicillin resistance of *H. influenzae*. A 30 µg-chloramphenicol disk is used for predicting resistance to chloramphenicol, and a 30 µg-ceftriaxone and/or cefotaxime disk is used for predicting susceptibility to these antibiotics. The zone diameter sizes can only be properly interpreted when HTM is used. The results have to be compared to standards that have been validated, such as those recommended by CLSI (Table 9), CA-SFM (Table 10), and EUCAST (Table 11).

Quality control

QC tests should be performed once per week if susceptibility tests are performed daily or with every group of tests when testing is done less frequently than every day. QC tests have to be done for each new batch of test medium or new lot of disks. If the results found for the control strain are accurate, the procedure is assumed to be correct. If this is not the case, the tests can be affected by variation in media, inoculum size, incubation time, temperature, the depth of the agar in the plate (uniformly 3-4 mm), the pH (between 7.2-7.4), disk potency, the purity of the culture for inoculum, or if the concentration of bacteria does not approximate the 0.5 McFarland turbidity standard. CLSI, CA-SFM, and EUCAST list recommended QC strains and test limits (Tables 1, 3, and 4). A laboratory should choose which QC strain(s) to use based on the antimicrobials to be tested for susceptibility.

Table 9. Kirby-Bauer disk diffusion zone diameter interpretive standards and MIC interpretive standards for *H. influenzae* as recommended by CLSI

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CLSI document M100-S21; 2011, pp 88-91.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, Nearest Whole mm			MIC Interpretive Standard (µg/ml)		
		S	I	R	S	I	R
PENICILLINS							
Ampicillin ¹	10 µg	≥ 22	19-21	≤ 18	≤ 1	2	≥ 4
CEPHEMS							
Cefotaxime ²	30 µg	≥ 26	-	-	≤ 2	-	-
Ceftriaxone ²	30 µg	≥ 26	-	-	≤ 2	-	-
PHENICOLS							
Chloramphenicol	30 µg	≥ 29	26-28	≤ 25	≤ 2	4	≥ 8

¹ In most cases, a direct β-lactamase test can provide a rapid means of detecting ampicillin and amoxicillin resistance. The majority of isolates of *H. influenzae* that are resistant to ampicillin and amoxicillin produce a TEM-type β-lactamase.

² For some antimicrobial agents, the absence or rare occurrence of resistant strains precludes defining any results categories other than “susceptible”.

Table 10. Kirby-Bauer disk diffusion zone diameter and MIC interpretive standards for *H. influenzae* as recommended by CA-SFM

Comite de L'Antibiogramme de la Société Française de Microbiologie, Société Française de Microbiologie, Recommendations 2010, pp 42-43. More information available at http://www.sfm-microbiologie.org/UserFiles/file/casfm_2010.pdf.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, Nearest Whole mm		MIC (µg/ml) Interpretive Standard	
		S	R	S	R
PENICILLINS					
Ampicillin ^{1,2}	2 µg	≥ 20	< 20	≤ 1	>1
CEPHEMS					
Ceftriaxone or Cefotaxime ³	-	-	-	≤ 0.12	-
PHENICOLS					
Chloramphenicol	30 µg	≥ 30	< 26	≤ 1	> 2

¹ A positive direct chromogenic β-lactamase test predicts resistance to penicillin, ampicillin, and amoxicillin.

² β-lactamase-negative, ampicillin-resistant (BLNAR) strains are rare, but detection of decreased susceptibility to beta-lactams in BLNAR strains is possible using a 2 µg ampicillin disk (diameter < 20 mm) or a 30 µg cephalothin disk (diameter < 17 mm).

³ Neither clinical failure nor resistance has been reported for these antimicrobial agents, thus the criteria for interpretative breakpoints has not been established for any category other than susceptible.

Table 11. Kirby-Bauer Disk Diffusion Zone Diameter and MIC Interpretive Standards for *H. influenzae* as Recommended by EUCAST

Data from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website. <http://www.eucast.org>. Version 1.3, December, 2010.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, Nearest Whole mm		MIC ($\mu\text{g/mL}$) Interpretive Standard	
		S	R	S	R
PENICILLINS					
Ampicillin ^{1,2}	2 μg	≥ 16	< 16	≤ 1	>1
CEPHEMS					
Cefotaxime ³	5 μg	≥ 22	< 22	≤ 0.125	-
Ceftriaxone ³	30 μg	≥ 27	< 27	≤ 0.125	-
PHENICOLS					
Chloramphenicol	30 μg	≥ 30	< 26	≤ 1	> 2

¹ Report β -lactamase positive strains resistant to penicillins without β -lactamase inhibitors.

² Breakpoints relate only to β -lactamase negative strains. Strains may be resistant to penicillins, aminopenicillins, cephalosporins and/or carbapenems due to changes in penicillin binding proteins (BLNAR, β -lactamase negative ampicillin resistant) and a few strains have both resistance mechanisms (BLPACR, β -lactamase positive, amoxicillin/clavulanate resistant).

³ Neither clinical failure nor resistance has been reported for these antimicrobial agents, thus the criteria for interpretative breakpoints has not been established for any category other than susceptible.

Antimicrobial susceptibility testing procedure of *H. influenzae* by Kirby-Bauer disk diffusion

Either 150-mm or 100-mm plates can be used for Kirby-Bauer disk diffusion depending on the number of antimicrobial agents to be tested per isolate. CLSI guidelines state that no more than two disks can be used on a 100-mm plate and up to five disks can be used on a 150-mm plate (Figure 1).

Isolates to be tested should be subcultured onto a supplemented chocolate agar plate and incubated in a CO₂-enhanced atmosphere (5% CO₂ in a CO₂-incubator or candle-extinction jar) at 35±2°C for 20-24 hours prior to testing. If the organism has been frozen, it should be subcultured twice when it is removed from the freezer before proceeding with susceptibility testing.

Remove agar plates from the refrigerator and allow them to come to room temperature (25°C) before inoculating. If HTM broth is to be used to make the 0.5 McFarland, warm it to 35°C before using. Allow the antibiotic disks that will be used in the batch of testing to warm to room temperature (25°C).

1. Using a sterile cotton-tip applicator, touch the surface of one to four morphologically identical, isolated colonies. Immerse the applicator into a tube containing sterile HTM broth or saline. Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix the cells using a vortex to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard (approximately 1 to 4 x 10⁸ CFU/ml). Preparation of a McFarland turbidity standard is described in the Annex. If the turbidity of the inoculum is greater than the standard, dilute it with HTM broth or saline to equal the turbidity of the standard. This suspension must be used within 15 minutes.
 - Perform regular colony counts to verify that the density of the inoculum suspension is correct. For example, dilute the suspension 1:100 and subculture 10 µl onto the recommended media. An acceptable inoculum should give approximately 100-500 colonies. It is not necessary to perform colony counts on every isolate tested.
2. Immerse a sterile cotton-tipped swab into the adjusted inoculum. Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a HTM plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure even distribution of the inoculum and confluent growth of the bacteria. Use a single swab of inoculum and do not return the swab to the broth after each rotation.
3. Allow the inoculum to dry on the surface of the plate (which should take approximately 5-10 minutes). Be sure the plate is entirely dry before proceeding, but do not exceed 15 minutes.
4. When the surface of the inoculated plate is dry and the disks are at room temperature, place the disks onto the agar with an applicator or sterile forceps. Make sure that the disks are

spaced enough distance apart on the agar so the zones of inhibition do not overlap (Figure 1). Press down on the disks to ensure complete contact with the agar surface. Alternatively, a mechanical disk dispenser can be used. Once applied, it is important to not move the antibiotic disks as the antibiotic will begin to diffuse immediately upon contact with the plate.

5. Incubate the plates in an inverted position in a 5% CO₂ atmosphere or candle jar for 20–24 hours at 35±2°C.
6. After overnight incubation, measure the diameter of each zone of inhibition with a ruler or calipers (Figure 2). Measurements should be performed in a biosafety cabinet, if possible. The zones of inhibition on the media are measured by holding the Petri dish a few inches above a black, nonreflective background illuminated with reflected light. Use either calipers or a ruler with a handle attached for these measurements, holding the ruler over the center of the surface of the disk when measuring the inhibition zone (Figures 1 and 2).
 - Care should be taken not to touch the disk or surface of the agar. Decontaminate the ruler occasionally to prevent transmission of the bacteria. In all measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony of the unaided eye. Record the results to the nearest millimeter (mm).
7. Interpret the antimicrobial susceptibility of the strain being tested (and check that results for the QC strains are within the acceptable control range) by comparing the results to the CLSI (Table 9), CA-SFM (Table 10), or EUCAST (Table 11) standard zone sizes. See Figure 3 for a sample worksheet for recording antimicrobial susceptibility test results for *H. influenzae*.

C. Minimal inhibitory concentration testing of *H. influenzae* by antimicrobial gradient strips

In reference laboratories, it is necessary to precisely monitor for any changes in the MICs of the isolates. It can be done either by an antibiotic dilution method or using antimicrobial gradient strips. The gradient strip method is convenient and reliable, giving 85-100% correlation with the dilution method. Gradient strips must be stored at -20°C or 4°C (follow the manufacturer's recommendations). Either 150-mm or 100-mm plates can be used for the gradient strip testing, depending on the number of antimicrobial agents to be tested per isolate. Two different gradient strips can be placed in opposite gradient directions on a 100-mm plate (Figure 4). Although one manufacturer states that up to six gradient strips can be used on a 150-mm plate, this laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five gradient strips be used on a 150-mm plate, with the end with the lowest concentration of antibiotic placed towards the center of the plate (Figure 5). Depending on the bacteria/antimicrobial combinations, five strips on a 150 mm plate may lead to overlapping ellipses. If this occurs, testing should be repeated using four strips per plate.

Isolates to be tested should be subcultured onto a supplemented chocolate agar plate and incubated in a CO₂-enhanced atmosphere (5% CO₂ in a CO₂-incubator or candle-extinction jar) at 35±2°C for 20-24 hours prior to testing. If the organism has been frozen, it should be

subcultured twice when it is removed from the freezer before proceeding with susceptibility testing.

Remove agar plates from the refrigerator and allow them to come to room temperature (25°C) before inoculating. If HTM broth is to be used to make the 0.5 McFarland, warm it to 35°C before using. Allow the gradient strips that will be used in the batch of testing to warm to room temperature (25°C). It is recommended to follow the directions on the package insert included with the gradient strips

1. Using a sterile cotton-tip applicator, touch the surface of one to four morphologically identical, isolated colonies. Immerse the applicator into a tube containing sterile HTM broth or saline. Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix the cells using a vortex to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard (approximately 1 to 4×10^8 CFU/ml). Preparation of a McFarland turbidity standard is described in the Annex. If the turbidity of the inoculum is greater than the standard, dilute it with HTM broth or saline to equal the turbidity of the standard. This suspension must be used within 15 minutes.
 - Perform regular colony counts to verify that the density of the inoculum suspension is correct. For example, dilute the suspension 1:100 and subculture 10 µl onto the recommended media. An acceptable inoculum should give approximately 100-500 colonies. It is not necessary to perform colony counts on every isolate tested.
2. Immerse a sterile cotton-tipped swab into the adjusted inoculum. Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a HTM plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure even distribution of the inoculum and confluent growth of the bacteria. Use a single swab of inoculum and do not return the swab to the broth after each rotation.
3. Allow the inoculum to dry on the surface of the plate (which should take approximately 5-10 minutes). Be sure the plate is entirely dry before proceeding, but do not exceed 15 minutes.
4. When the surface of the inoculated plate is dry and the gradient strips are at room temperature, place the antimicrobial gradient strips onto the agar with an applicator or sterile forceps. Make sure that the printed MIC values are facing upward (i.e., that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar). Alternatively, robotic gradient strip applicators are available from some manufacturers. Once applied, it is important to not move the antimicrobial gradient strips as the antibiotic diffuses into the agar immediately upon contact.
 - Return the antimicrobial gradient strips that will not be used in this batch of testing to the -20°C freezer (some strips can be stored at 4°C. Follow the manufacturer's instructions).

5. Incubate the plates in an inverted position in a 5% CO₂ atmosphere or candle jar for 20–24 hours at 35±2°C.
6. After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the test can be read (see below). QC results must be reviewed before reading and interpreting the MIC.

Reading and interpreting the gradient strips

Read the MIC at the point where the zone of inhibition intersects the MIC scale on the strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read at the point of complete inhibition of all growth including hazes. A reading guide for the gradient strips, which shows organism related effects, drug-related effects, resistance mechanism-related effects, and technical and handling effects can be found at: <http://www.abbiotest.com/pdf/pi/75002206.pdf>.

Record the QC results first. If zones produced by the control strain are out of the expected ranges (see Tables 2, 3, and 5, depending on the strains and guidelines being used), the laboratorian should consider possible sources of error. Because antimicrobial susceptibility test results can be affected by many factors not necessarily associated with the actual susceptibility of the organism (e.g., inoculum size, growth phase, agar depth, storage, time, and others), QC practices must be followed carefully (see Section II above). If all antimicrobial agents are in the control range, read the test MICs. Note any unusual observations such as a zone of incomplete killing (trailing endpoints) or single colonies growing within the ellipse.

The gradation marks on the gradient strip correspond to the standard 2-fold dilution concentrations for the agar dilution method, but also include increments between those standard values. The standard values are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (i.e., the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of an isolate to penicillin, an MIC recorded from the gradations on the gradient strip might be 0.094 µg/ml; however, the reported MIC would be 0.125 µg/ml.

The manufacturer of the gradient strips recommends following the MIC breakpoints developed for agar and broth microdilution. The interpretive standards and MIC breakpoints recommend by CLSI (Table 9), CA-SFM (Table 10), and EUCAST (Table 11) are given.

V. Antimicrobial susceptibility testing of *S. pneumoniae*

This section describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results for *S. pneumoniae* by the disk diffusion method and the antimicrobial gradient strip method. Although disk diffusion will provide information for most antimicrobial agents regarding interpretation of a strain as susceptible, intermediate, or resistant, the antimicrobial gradient strip test provides general information about the MIC of antibiotic.

The accuracy and reproducibility of this test are dependent on following a standard set of procedures and conditions in laboratories on an everyday basis.

Quality control

Quality control tests must be performed as part of the normal laboratory routine. To verify that antimicrobial susceptibility test results are accurate, at least one control organism should be included with each test or new set of testing conditions. CLSI, CA-SFM, and EUCAST list recommended QC strains and test limits (Table 1-5). A laboratory should choose which QC strain(s) to use based on the antimicrobials to be tested for susceptibility. Further information about trouble-shooting out of range quality control results can be found in Section II above.

A. Antimicrobial susceptibility testing of *S. pneumoniae* by Kirby-Bauer disk diffusion

Mueller-Hinton agar medium supplemented with 5% sheep blood is recommended for determining the antimicrobial susceptibility of *S. pneumoniae* specimens by disk diffusion. The agar plates should have a uniform depth of 3-4 mm. Prepare the inoculum for antimicrobial susceptibility testing of *S. pneumoniae* from fresh pure cultures of *S. pneumoniae* (grown overnight on blood or chocolate agar). Prepare cell suspensions of the bacteria to be tested in sterile physiological saline or Mueller-Hinton broth. A cell suspension equal to a density of a 0.5 McFarland turbidity standard is used for the inoculum (approximately 1 to 4×10^8 CFU/ml). Preparation of a McFarland turbidity standard is described in the Annex.

1. Suspend viable colonies from an overnight sheep blood or chocolate agar plate in a tube of broth or saline to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard; be careful not to form froth or bubbles in the suspension when mixing the cells with the broth.
2. Compare the density of the suspension to the 0.5 McFarland turbidity standard by holding the suspension and McFarland turbidity standard in front of a light against a white background with contrasting black lines. If the density is too heavy, the suspension should be diluted with saline or broth (whichever was used to make the suspension). If the density is not sufficient, additional bacteria should be added to the suspension. This suspension should be used within 15 minutes.
 - Perform regular colony counts to verify that the density of the inoculum suspension is correct. For example, dilute the suspension 1:100 and subculture 10 μ l onto the recommended media. An acceptable inoculum should give approximately 100-500 colonies. It is not necessary to perform colony counts on every isolate tested.
3. When the proper density is achieved, dip a cotton swab into the bacterial suspension. Lift it out of the broth and remove excess fluid by pressing and rotating the swab against the wall of the tube.
4. Use the swab to inoculate the entire surface of the supplemented Mueller-Hinton agar plate three times, rotating the plate 60 degrees between each inoculation. Use the same swab with

each rotated streak, but do not re-dip the swab in the inoculum (i.e., the bacterial cell suspension).

5. Allow the inoculum to dry before placing the disks on the plates. Drying usually takes only a few minutes and should take no longer than 15 minutes. If drying takes longer than 15 minutes, use a smaller volume of inoculum in the future by pressing more liquid out of the swab.
6. After the plate is dry, place the antimicrobial disks on the plates (Figure 1). Use sterile forceps to place the disks on the Mueller-Hinton agar and tap them gently to ensure they adhere to the agar. Alternatively, a mechanical disk dispenser can be used. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.
7. Incubate the plates in an inverted position in a 5% CO₂ atmosphere for 20-24 hours at 37°C. A candle-extinction jar may be used if a CO₂-incubator is not available.
 - If this is a new batch of Mueller-Hinton agar, the antimicrobial disks are new, or it is an otherwise appropriate time to perform QC, follow steps 1 through 7 above and run parallel tests on the reference strain(s). Appropriate disk diffusion zone sizes for the reference QC strains are included in Tables 1, 3 and 4.
8. After overnight incubation, measure the diameter of each zone of inhibition with a ruler or calipers. The zones of inhibition are measured from the top surface of the plate with the top removed. Use either calipers or a ruler with a handle attached for these measurements, holding the ruler over the center of the surface of the disk when measuring the inhibition zone (Figure 1).
 - Care should be taken not to touch the disk or surface of the agar. Decontaminate the ruler occasionally to prevent transmission of the bacteria. In all measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony. Record the results in millimeters (mm). Figure 3 provides a sample form for recording results.
9. Interpret the antimicrobial susceptibility of the strain being tested (and check that results for the QC strain(s) are within the acceptable control range) by comparing the results to the CLSI (Table 12), CA-SFM (Table 13), or EUCAST (Table 14) standard zone sizes.

B. Minimal inhibitory concentration testing of *S. pneumoniae* by antimicrobial gradient strips

For *S. pneumoniae*, disk diffusion testing indicates whether an organism is susceptible or resistant to an antimicrobial for most agents. However, disk diffusion testing for pneumococcal isolates using oxacillin (a penicillin family antibiotic) is not sufficient to distinguish between complete and intermediate resistance. For surveillance purposes, a laboratory may want to quantify the results of the oxacillin disk diffusion test by performing MIC testing of penicillin or any other β -lactam antibiotic that would be used for treatment. As mentioned earlier in this

manual, MIC testing by dilution can be expensive and challenging, and because of the technical complexity required for these tests, countries that do not currently do MIC testing by dilution should utilize an international reference laboratory rather than developing the assay in-country. In countries where MIC testing is done at more than one laboratory,

Table 12. Kirby-Bauer disk diffusion zone diameter interpretive standards and MIC interpretive standards for *S. pneumoniae* as recommended by CLSI

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CLSI document M100-S21; 2011, pp 96-99.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, Nearest Whole mm			MIC Interpretive Standard (µg/ml)		
		S	I	R	S	I	R
PENICILLINS							
Penicillin ^{1,2,3} parenteral (meningitis)	1 µg ⁴ oxacillin	≥ 20	-	-	≤ 0.06	-	≥ 0.12
CEPHEMS							
Cefotaxime ^{2,3,5,6} (meningitis)	-	-	-	-	≤ 0.5	1	≥ 2
MACROLIDES							
Erythromycin ⁶	15 µg	≥ 21	16-20	≤ 15	≤ 0.25	0.5	≥ 1
FOLATE PATHWAY INHIBITORS ^{8,9}							
Trimethoprim-sulfamethoxazole (co-trimoxazole)	1.25/23.75µg	≥ 19	16-18	≤ 15	≤ 0.5/9.5	1/19-2/38	≥ 4/76
TETRACYCLINES							
Tetracycline ¹⁰	30 µg	≥ 23	19-22	≤ 18	≤ 2	4	≥ 8
FLUOROQUINOLONES							
Levofloxacin	5 µg	≥ 17	14-16	≤ 13	≤ 2	4	≥ 8
LINCOSAMIDES							
Clindamycin	2 µg	≥ 19	16-18	≤ 15	≤ 0.25	0.5	≥ 1
KETOLIDES							
Telithromycin	15 µg	≥ 19	16-18	≤ 15	≤ 1	2	≥ 4

- ¹ Rx: Use of penicillin in meningitis requires therapy with maximum doses of intravenous penicillin (e.g., at least 3 million units every four hours in adults with normal renal function).
- ² Penicillin, cefotaxime, and ceftriaxone should be tested by a reliable MIC method and reported routinely with CSF isolates of *S. pneumoniae*.
- ³ For CSF isolates, report only meningitis interpretations.
- ⁴ Isolates of pneumococci with oxacillin zone sizes of ≥ 20 mm are susceptible ($\text{MIC} \leq 0.06 \mu\text{g/ml}$) to penicillin. Penicillin and cefotaxime, ceftriaxone, or meropenem MICs should be determined for those isolates with oxacillin zone diameters of ≤ 19 mm, because zones of ≤ 19 mm occur with penicillin-resistant, intermediate, or certain susceptible strains. For isolates with oxacillin zones ≤ 19 mm, do not report penicillin as resistant without performing a penicillin MIC test.
- ⁵ Rx: Use of cefotaxime or ceftriaxone in meningitis requires therapy with maximum doses.
- ⁶ Breakpoints for isolates from cases of meningitis are identical to ceftriaxone.
- ⁷ Susceptibility and resistance to azithromycin, clarithromycin, and dirithromycin can be predicted by using erythromycin.
- ⁸ The Mueller-Hinton agar used for this test should be thymidine free to obtain accurate results.
- ⁹ Trimethoprim-sulfamethoxazole is a combination of two drugs used in treatment (cotrimoxazole). The 1:20 ratio is that at which the greatest synergy in treatment has been demonstrated in serum. The disks are impregnated with 1.25 μg trimethoprim and 23.75 μg sulfamethoxazole. The MIC breakpoints mimic the 1/20 ratio.
- ¹⁰ Organisms susceptible to tetracycline are also considered susceptible to doxycycline and minocycline.

Table 13. Kirby-Bauer disk diffusion zone diameter and MIC interpretive standards for *S. pneumoniae* as recommended by CA-SFM

Comite de L'Antibiogramme de la Société Française de Microbiologie, Société Française de Microbiologie, Recommendations 2010, pp 38-39. More information available at http://www.sfm-microbiologie.org/UserFiles/file/casfm_2010.pdf.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, Nearest Whole mm		MIC Interpretive Standard (µg/ml)	
		S	R	S	R
PENICILLINS					
Penicillin G ^{1,2,3}	5 µg Oxacillin	-	-	≤ 0.06	> 2
CEPHEMS					
Cefotaxime ^{1,2,3}		-	-	≤ 0.5	> 2
Ceftriaxone ^{1,2,3}		-	-	≤ 0.5	> 2
MACROLIDES					
Erythromycin ⁴	15 IU	≥ 26	< 24	≤ 0.25	> 0.5
FOLATE PATHWAY INHIBITORS ^{5,6,7}					
Trimethoprim-sulfamethoxazole	1.25/23.75 µg	≥ 16	< 10	≤ 2/38	>8/152
TETRACYCLINES					
Tetracycline ⁸	30 IU	≥ 23	< 21	≤ 1	> 2
FLUOROQUINOLONES					
Levofloxacin ⁹	5 µg	≥ 17	< 17	≤ 2	> 2
LINCOSAMIDES					
Clindamycin	15 µg	≥ 21	< 17	≤ 0.5	> 0.5
KETOLIDES					
Telithromycin ¹⁰	15 µg	≥ 24	< 21	≤ 0.25	> 0.5

¹ Disk diffusion tests with penicillin G for *S. pneumoniae* are unreliable, but testing can be performed using 5 µg/ml oxacillin (OXA-5) disks according to the following criteria: Diameters of ≥ 26 mm using OXA-5 disks are sensitive to penicillin G and other β-lactams. Diameters of < 26 mm using OXA-5 disks have reduced susceptibility.

- ² This test cannot assess the level of resistance to penicillin G or other β -lactams. Using disks of other β -lactam antibiotics cannot determine the level of resistance to these β -lactams.
- ³ In cases of severe infection, clinical failure, or any strain with reduced susceptibility, it is necessary to determine the MIC to penicillin G and at least one of β -lactam with pharmacodynamic properties which are consistent with therapeutic efficacy (amoxicillin, cefotaxime, or ceftriaxone). Strains categorized as intermediate (MIC of $> 0.064 \mu\text{g/ml}$ to $>2 \mu\text{g/ml}$) or even with low level resistance should be considered resistant in the case of meningitis, but sensitive to high doses in the case of respiratory infections.
- ⁴ Interpretation valid for azithromycin, clarithromycin, dirithromycin, and roxithromycin.
- ⁵ Trimethoprim-sulfamethoxazole testing predicts susceptibility and resistance to trimethoprim-sulfamethoxazole and sulfonamides.
- ⁶ The Mueller-Hinton agar used for this test should be thymidine free to obtain accurate results.
- ⁷ Trimethoprim-sulfamethoxazole is a combination of two drugs used in treatment (co-trimoxazole). The 1:20 ratio is that at which the greatest synergy in treatment has been demonstrated in serum. The disks are impregnated with $1.25 \mu\text{g}$ trimethoprim and $23.75 \mu\text{g}$ sulfamethoxazole to mimic the 1:20 ratio. The MIC breakpoints mimic the 1/20 ratio; i.e., $\leq 2 \mu\text{g/ml}$ trimethoprim/ $38 \mu\text{g/ml}$ sulfamethoxazole.
- ⁸ Interpretations valid for other tetracyclines.
- ⁹ The screening of pneumococci with reduced susceptibility to fluoroquinolones is realized by measuring sensitivity to norfloxacin. If the diameter around the disk of norfloxacin ($5 \mu\text{g}$) is less than 10 mm and/or if the MIC is $> 16 \mu\text{g/ml}$, there is a high risk of in vivo selection of mutants resistant to fluoroquinolones and clinical failure.
- ¹⁰ Resistance to telithromycin must be verified by retesting in the absence of CO_2 .

Table 14. Kirby-Bauer disk diffusion zone diameter and MIC interpretive standards for *S. pneumoniae* as recommended by EUCAST

Data is from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website, <http://www.eucast.org>, version 1.3, December, 2010.

Antimicrobial Agent/Class	Disk Content	Zone Diameter, Nearest Whole mm		MIC Interpretive Standard (µg/ml)	
		S	R	S	R
PENICILLINS¹					
Oxacillin (screen) ^{1,2}	1 µg	≥ 20	< 20	-	-
Benzylpenicillin ^{1,2,3}		-	-	≤ 0.064	> 2
CEPHEMS					
Cefotaxime ^{4,5}		-	-	≤ 0.5	> 2
Ceftriaxone ^{4,5}		-	-	≤ 0.5	> 2
MACROLIDES					
Erythromycin ⁶	15 µg	≥ 22	< 19	≤ 0.25	> 0.5
FOLATE PATHWAY INHIBITORS					
Trimethoprim-sulfamethoxazole (cotrimoxazole) ⁷	1.25/23.75 µg	≥ 18	< 15	≤ 1	> 2
TETRACYCLINES					
Tetracycline ⁸	30 µg	≥ 23	< 20	≤ 1	> 2
FLUOROQUINOLONES⁹					
Levofloxacin ^{9,10}	5 µg	≥ 19	< 19	≤ 2	> 2
LINCOSAMIDES					
Clindamycin ¹¹	2 µg	≥ 19	< 19	≤ 0.5	> 0.5
KETOLIDES					
Telithromycin ¹⁰	15 µg	≥ 25	< 22	≤ 0.25	> 0.5

- ¹ Most MIC values for penicillin, ampicillin, amoxicillin, and piperacillin (with or without a β -lactamase inhibitor) differ by no more than one dilution step and isolates fully susceptible to benzylpenicillin (MIC ≤ 0.064 $\mu\text{g/ml}$; susceptible by oxacillin disk screen, see note 2) can be reported susceptible to β -lactam agents that have been given breakpoints.
- ² Screen for β -lactam resistance with the oxacillin 1 μg disk. Isolates categorized as susceptible can be reported susceptible to benzylpenicillin, phenoxymethylpenicillin, and aminopenicillins (with or without β -lactamase inhibitor) irrespective of clinical indication. Isolates categorized as oxacillin resistant can be reported resistant to benzylpenicillin and phenoxymethylpenicillin in meningitis. For other β -lactams, determine the MIC of the agent considered for clinical use.
- ³ In meningitis, only isolates with MIC ≤ 0.064 $\mu\text{g/ml}$ (susceptible by oxacillin disk screen, see note 2) should be categorized susceptible to benzylpenicillin, otherwise report resistant. For indications other than meningitis and pneumonia, use breakpoints of 0.064 and 2 $\mu\text{g/ml}$.
- ⁴ Strains with MIC values above the susceptible breakpoint are very rare or not yet reported. The identification and antimicrobial susceptibility tests on any such isolate must be repeated and if the result is confirmed the isolate sent to a reference laboratory. Until there is evidence regarding clinical response for confirmed isolates with MIC above the current resistant breakpoint, they should be reported resistant.
- ⁵ Screen for β -lactam resistance with the oxacillin 1 μg disk. Isolates categorized as susceptible can be reported susceptible to cefepime, cefotaxime, cefpodoxime, ceftriaxone and cefuroxime, and cefuroxime axetil. Isolates categorized as oxacillin resistant should be tested with an MIC method with the agent considered for clinical use.
- ⁶ Erythromycin can be used to determine susceptibility to azithromycin, clarithromycin, and roxithromycin.
- ⁷ Trimethoprim-sulfamethoxazole in the ratio 1:19. Breakpoints are expressed as the trimethoprim concentration.
- ⁸ Isolates susceptible to tetracycline are also susceptible to doxycycline and minocycline. Some isolates resistant to tetracycline may be susceptible to minocycline and/or doxycycline.
- ⁹ The norfloxacin disk diffusion test can be used to screen for fluoroquinolone resistance. Isolates categorized as susceptible can be reported susceptible to levofloxacin and moxifloxacin and intermediate to ciprofloxacin and ofloxacin. Isolates categorized as resistant should be tested for susceptibility to individual agents.
- ¹⁰ The breakpoints for levofloxacin relate to high dose therapy.
- ¹¹ Inducible clindamycin resistance can be detected only in the presence of a macrolide antibiotic. In disk diffusion tests, look for apparent antagonism of clindamycin by erythromycin (D-test).

standardization and QC should be conducted at each laboratory in accordance with the standardized guidelines presented in this manual.

Methods for preservation and storage of isolates and detailed methods for transport of isolates according to international regulations are presented in Chapter 14: Storage and Shipping of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*.

With increasing antimicrobial resistance testing being performed outside of international reference laboratories, antimicrobial gradient test strips serve as an assessment method that is both convenient and reliable. They require less technical expertise than MIC testing by dilution methods, but give comparable results. Store the antimicrobial gradient test strips as recommended by the manufacturer, usually at 4°C or in a freezer at -20°C.

Although this manual serves as a general guide for the use of antimicrobial gradient strips, always follow the manufacturer's directions, as certain antibiotic-bacteria ("drug-bug") combinations have special testing requirements.

This laboratory manual therefore suggests that Mueller-Hinton agar with 5% sheep blood should be used when performing antimicrobial susceptibility testing of *S. pneumoniae* with the antimicrobial test strips (except when testing for susceptibility to trimethoprim-sulfamethoxazole (cotrimoxazole), in which case horse blood should be used in place of sheep blood). Either 100-mm or 150-mm plates can be used, depending on the number of antimicrobial test strips used per sample (Figures 4 and 5). Two different antimicrobial test strips can be placed in opposite gradient directions on a 100-mm plate. This laboratory manual suggests that in order to avoid overlapping zones of inhibition of growth, not more than five antimicrobial test strips be used on a 150-mm plate. Depending on the bacteria/antimicrobial combinations, five strips on a 150 mm plate may lead to overlapping ellipses. If this occurs, redo the testing using four strips per plate.

Isolates to be tested should be subcultured onto a blood agar plate and incubated in a CO₂-enhanced atmosphere (5% CO₂ in a CO₂-incubator or candle-extinction jar) at 35±2°C for 20-24 hours prior to testing. If the organism has been frozen, it should be subcultured twice when it is removed from the freezer before proceeding with susceptibility testing.

Warm the cation-adjusted Mueller-Hinton broth (CAMHB) to 35°C before using. Allow the gradient strips that will be used in the batch of testing to warm to room temperature (25°C). It is recommended to follow the directions on the package insert included with the gradient strips.

1. Using a sterile cotton-tip applicator, touch the surface of one to four morphologically identical, isolated colonies. Immerse the applicator into a tube containing sterile CAMHB. Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix the cells using a vortex to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard (approximately 1 to 4×10^8 CFU/ml). Preparation of a McFarland turbidity standard is described in the Annex. If the turbidity of the inoculum is greater than the standard, dilute it with CAMHB to equal the turbidity of the standard. This suspension must be used within 15 minutes.

- Perform regular colony counts to verify that the density of the inoculum suspension is correct. For example, dilute the suspension 1:100 and subculture 10 µl onto the recommended media. An acceptable inoculum should give approximately 100-500 colonies. It is not necessary to perform colony counts on every isolate tested.
2. Allow the plate to dry for up to 15 minutes. Be sure the plate is entirely dry before proceeding. While the plate is drying, remove the antimicrobial test strips from cold storage (4°C or -20°C, depending on the manufacturer's recommendations), and allow the strips that will be used in the batch of testing to warm to room temperature (25°C). Return the strips that will not be used in this batch of testing to cold storage.
 3. Place the antimicrobial test strips onto the dried, inoculated agar plate with an applicator or sterile forceps (Figures 4 and 5). Make sure that the printed MIC values are facing upward (i.e., that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar). Alternatively, robotic gradient strip applicators are available from some manufacturers. Once applied, do not move the antimicrobial gradient strips as the drug diffuses into the agar immediately on contact.
 4. Incubate the plates in an inverted position in a CO₂-enriched atmosphere (2-5% CO₂) for 20–24 hours at 37°C. A candle-extinction jar may be used if a CO₂ incubator is not available.
 - Always follow the manufacturer's instructions included with each package of strips, because incubation conditions may vary by organism-antimicrobial (or “drug-bug”) combination.
 5. After incubation, there will be an ellipse of bacterial growth formed on the plate around the strip and the test strip can be read. It is important to review QC results before reading and interpreting the antimicrobial test strips MIC.

Reading and interpreting the gradient strips

Read the MIC at the point where the zone of inhibition intersects the MIC scale on the strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read at the point of complete inhibition of all growth including hazes. A reading guide for the gradient strips, which shows organism related effects, drug-related effects, resistance mechanism-related effects, and technical and handling effects can be found at: <http://www.abbiodisk.com/pdf/pi/75002206.pdf>.

Record the QC results first. If zones produced by the control strain are out of the expected ranges (see Tables 2, 3, and 5, depending on the strains and guidelines being used), the laboratorian should consider possible sources of error. Because antimicrobial susceptibility test results can be affected by many factors not necessarily associated with the actual susceptibility of the organism (e.g., inoculum size, growth phase, agar depth, storage, time, and others), QC practices must be followed carefully (see Section II above). If all antimicrobial agents are in the

control range, read the test MICs. Note any unusual observations such as a zone of incomplete killing (trailing endpoints) or single colonies growing within the ellipse.

The gradation marks on the gradient strip correspond to the standard 2-fold dilution concentrations for the agar dilution method, but also include increments between those standard values. The standard values are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that both the actual reading of the value from the strip and the next-higher standard value (i.e., the value to be used for interpretation) be included in the laboratory records for testing of the strain. For example, if testing susceptibility of an isolate to penicillin, an MIC recorded from the gradations on the gradient strip might be 0.094 µg/ml; however, the reported MIC would be 0.125 µg/ml.

The manufacturer of the gradient strips recommends following the MIC breakpoints developed for agar and broth microdilution. Interpretive breakpoints for *S. pneumoniae* antimicrobial combinations from CLSI (Table 12), CA-SFM (Table 13), and EUCAST (Table 14) are shown.

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CHAPTER 12

Characterization of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* by Molecular Typing Methods

I. Introduction

A number of DNA-based molecular typing methods have been used in epidemiological investigations of bacterial pathogens and studying bacterial population genetics and evolution. Some methods are suitable for characterizing strains that cause localized outbreaks; others are particularly useful for determining the long-term relationships of strains and their population structures (1). For local outbreak investigation, molecular typing methods with a high degree of resolution are necessary to detect subtle differences in what most likely are genetically related strains. These assays are also useful for detecting changes in molecular type during prolonged outbreaks. Highly discriminatory assays typically index genomic variations that accumulate rapidly and can be used to detect microvariations that can be used to identify strains circulating in a specific geographic area. The methods that have been used for this purpose including ribotyping, random amplified polymorphic DNA, fluorescent amplified-fragment length polymorphism, restriction fragment length polymorphism (RFLP), multiple-locus variable-number tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE). The high resolution of these assays will sometimes resolve subtle differences that MLST cannot, thus they are particularly useful for studying local epidemiology or tracing the spread of highly related strains during outbreaks/clusters of cases (1, 7, 15).

PFGE is one of the most widely used molecular typing methods because of the resolving power of the technique. In brief, PFGE uses a restriction enzyme to cut genomic DNA infrequently at a specific sequence to produce a number of fragments that are then size-fractionated on an agarose gel. The resultant banding patterns are analyzed and compared to other isolates. During an outbreak or a cluster of cases, PFGE is used in combination with epidemiologic information and other typing techniques to help identify outbreak isolates and determine the relationships among the isolates. A promising technique with similar discriminatory capabilities that is gaining acceptance is multiple-locus variable-number tandem repeat analysis (MLVA). In MLVA, the variability in the numbers of short tandem repeat sequences that are found in specific areas of the genome are utilized to create DNA fingerprints for epidemiological studies. Once the appropriate loci are established, MLVA yields clustering similar to PFGE (13) and the use of highly variable-number tandem repeats results in a high degree of differentiation with suitable resolution to discriminate between strains in an outbreak or cluster (24, 34).

Tracking the population biology of bacterial organisms on a global or even national scale requires a molecular typing method based on slowly accumulating selectively neutral genetic variations that will still distinguish between genotypes yet can identify clonal groups. One of the most common selectively neutral areas of a genome are housekeeping genes encoding proteins involved in the metabolism of the organism. The first molecular typing method to utilize this attribute was multilocus enzyme electrophoresis (MLEE). MLEE analyses the electrophoretic mobilities of metabolic housekeeping enzymes on a starch gel and equates changes in mobility of each enzyme with allelic variants of each loci (1, 7). In order to obtain high resolution, 20 or

more loci are analyzed for each isolate. The genetic variation leading to the changes necessary to cause mobility shifts are thought to be selectively neutral, thus the electrophoretic type of a bacterial clone should be relatively stable over time (7). MLEE was instrumental as a typing method in early global epidemiology studies and population biology analysis, but the method is technically demanding, and because MLEE is gel-based, results between laboratories are very difficult to compare.

In 1998, Maiden et al developed a DNA-based method of molecular typing *N. meningitidis* that retained the concepts utilized by MLEE of analyzing housekeeping genes not under any known immunologic selection. MLST defines strains by their allelic profiles determined from the nucleotide sequences of internal fragments of seven housekeeping genes rather than by shifting electrophoretic mobility patterns of the enzymes they encode (15). This approach has been adapted for use in typing many types of bacteria, including *H. influenzae*, and *S. pneumoniae*, and is now the most widely used method for molecular typing. While MLST is more expensive to perform, it has the following advantages over MLEE:

- Directly measures genetic variations, and therefore resolves more alleles per locus
- Is readily scalable and adaptable for a high throughput format
- Sequence data is reproducible and can be objectively compared between laboratories
- Sequence analysis can be performed remotely and the results returned via the internet
- Sequence data can be uploaded to a centralized database and is accessible to all scientists via the internet to produce a powerful tool for global epidemiology
- Information can be obtained from PCR amplification from clinical material

MLST is not suitable to use to characterize potential differences in strains within an outbreak as its power to resolve small evolutionary differences is too low. Therefore, to provide further discriminatory power, MLST data can be combined with PFGE and sequence data from other more variable loci under positive selective pressure, such as PorA, PorB, FetA, and fHbp in *N. meningitidis*, and the penicillin binding proteins (PBPs) and PspA in *S. pneumoniae*. It is this variability that makes these proteins excellent markers for short-term epidemiology investigations. Sequence data for these markers is also valuable for assessing the allelic distribution of these vaccine candidate proteins that can aid in rational vaccine design.

A. Multilocus sequence typing (MLST) and typing of other variable genes of *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*

The PCR primers and protocols included here are used at CDC and work well. However, many laboratories use different PCR reaction protocols, chemistries, buffers, primer concentrations and even different equipment for PCR amplification and DNA sequencing. Other protocols work well and it is up to the discretion of each laboratory to discern the optimal reagents and assay conditions for their laboratory, including appropriate validation and quality control.

The primers listed in this manual have been designed and optimized over time as more sequence data has become available. By comparing DNA sequences for a given area of interest from dozens to hundreds of different strains of *N. meningitidis*, *H. influenzae*, or *S. pneumoniae*, small areas of homology can be detected that provide an area from which PCR and DNA sequencing

primers can be designed that have the greatest likelihood of working. In some instances, a perfect consensus cannot be found which will react with all strains. In these cases, degenerate primers are designed that have a mix of nucleotides at a certain position. For example, when either a “C” or a “T” nucleotide can occupy a position, the International Union of Pure and Applied Chemistry (IUPAC), designates it as “Y”. For a complete list of IUPAC nucleotide abbreviations, see: <http://www.chem.qmul.ac.uk/iupac/>.

B. Preparation of DNA template

For each of the assays described below a preparation of DNA must be extracted. A pure culture of each isolate is grown on trypticase soy agar plates supplemented with 5% sheep blood for *N. meningitidis* and *S. pneumoniae*, or chocolate agar plates supplemented with hemin and NAD for *H. influenzae* in a humidified incubator for 18-24 hours at 37°C with 5% CO₂. Fast DNA extraction protocols for *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* can be found in Appendix 1 at the end of this chapter or <http://www.cdc.gov/ncidod/biotech/strep/pcr.htm>. Additional methods for DNA extraction that will provide purified DNA can be found in Chapter 10: PCR Methods or commercially available DNA extraction kits can be used.

II. Multilocus Sequence Typing (MLST)

A. Overview and MLST schemes for *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*

The housekeeping genes selected for MLST are not closely linked in bacterial genomes and have conserved sequence regions that are sufficient to design PCR primers that will amplify all or nearly all of the isolates. The number of housekeeping genes to be examined reflects an optimal balance between resolution of genotypes and laboratory workload. The internal fragments are of a length to provide sufficient variation for identification of different alleles and for each strand to be accurately sequenced using only a single primer for each direction. Sequences that differ by even a single nucleotide are considered unique and no weight is given to the number of nucleotide changes in a given allele. Each unique allele is assigned a number in order of discovery and each isolate can be characterized by its multilocus genotypic or allelic profile, designated as sequence type (ST), which is the combination of its alleles over the seven genetic loci. STs can be further grouped into clonal complexes (CC), which are defined in the *Neisseria* MLST profile database as a group of STs that share at least four of the seven loci in common with a central ST (30).

MLST schemes have been developed for *N. meningitidis* (15) (Table 1), *H. influenzae* (20) (Table 2), *S. pneumoniae* (7) (Table 3) and many others (see <http://www.mlst.net/>). Each scheme uses defined regions of seven housekeeping genes. A general protocol for performing MLST and analyzing the data is given below.

Table 1. *N. meningitidis* MLST scheme, including gene locus, amplicon length, and trimmed length of sequence used for allelic determination

Housekeeping genes	Gene locus	Trimmed length
Putative ABC transporter	<i>abcZ</i>	433
Adenylate kinase	<i>adk</i>	465
Shikimate dehydrogenase	<i>aroE</i>	490
Fumurate dehydrogenase	<i>fumC</i>	465
Glucose-6-phosphate dehydrogenase	<i>gdh</i>	501
Pyruvate dehydrogenase subunit	<i>pdhC</i>	480
Phosphoglucomutase	<i>pgm</i>	450

Table 2. *H. influenzae* MLST scheme, including gene locus, amplicon length, and trimmed length of sequence used for allelic determination

Housekeeping genes	Gene locus	Trimmed length
Adenylate kinase	<i>adk</i>	477
ATP synthase F1 subunit gamma	<i>atpG</i>	447
Fumarate reductase iron-sulfur protein	<i>frdB</i>	489
Fuculokinase	<i>fucK</i>	345
Malate dehydrogenase	<i>mdh</i>	405
Glucose-6-phosphate isomerase	<i>pgi</i>	468
RecA protein	<i>recA</i>	426

Table 3. *S. pneumoniae* MLST scheme, including gene locus, amplicon length, and trimmed length of sequence used for allelic determination

Housekeeping genes	Gene locus	Trimmed length
Shikimate dehydrogenase	<i>aroE</i>	405
Glucose-6-phosphate dehydrogenase	<i>gdh</i>	460
Glucose kinase	<i>gki</i>	483
Transketolase	<i>recP</i>	450
Signal peptidase I	<i>spi</i>	474
Xanthine phosphoribosyltransferase	<i>xpt</i>	486
D-alanine-D-alanine ligase	<i>ddl</i>	441

Table 4. MLST amplification primers for *N. meningitidis*

Gene locus	Primer name	Forward primer (5'-3')	Primer name	Reverse primer (5'-3')
<i>abcZ</i>	abcZ-P1C	TGTTCCGCTTCGACTGCCAAC	abcZ-P2C	TCCCCGTCGTAAAAACAATC
<i>adk</i>	adk-P1B	CCAAGCCGTGTAGAATCGTAAACC	adk-P2B	TGCCCAATGCGCCCAATAC
<i>aroE</i>	aroE-P1B	TTTGAAACAGGCGGTTGCGG	aroE-P2B	CAGCGGTAATCCAGTGCGAC
<i>fumC</i>	fumC-P1B	TCCCCGCCGTAAAAGCCCTG	fumC-P2B	GCCCGTCAGCAAGCCCAAC
<i>gdh</i>	gdh-P1B	CTGCCCCCGGGGTTTTTCATCT	gdh-P2B	TGTTGCGCGTTATTTCAAAGAAGG
<i>pdhC</i>	pdhC-P2B	CCGGCCGTACGACGCTGAAC	pdhC-P2B	GATGTGCGAATGGGGCAAACA
<i>pgm</i>	pgm-P1	CTTCAAAGCCTACGACATCCG	pgm-P2	CGGATTGCTTTCGATGACGGC

Table 5. MLST amplification primers for *H. influenzae*

Gene locus	Primer name	Forward primer (5'-3')	Primer name	Reverse primer (5'-3')
<i>adk</i>	adk-up	GGTGCACCGGGTGCAGGTAA	adk-dn	CCTAAAGATTTTATCTAACTC
<i>atpG</i>	atpG-up	ATGGCAGGTGCAAAGAGAT	atpG-dn	TTGTACAACAGGCTTTTGCG
<i>frdB</i>	frdB-up	CTTATCGTTGGTCTTGCCGT	frdB-dn	TTGGCACTTTCCACTTTTCC
<i>fucK</i>	fucK-up	ACCACTTTCGGCGTGGATGG	fucK-dn	AAGATTTCCCAGGTGCCAGA
<i>mdh</i>	mdh-up	TCATTGTATGATATTGCCCC	mdh-dn	ACTTCTGTACCTGCATTTTG
<i>pgi</i>	pgi-up	GGTGAAAAAATCAATCGTAC	pgi-dn	ATTGAAAGACCAATAGCTGA
<i>recA</i>	recA-up	ATGGCAACTCAAGAAGAAAA	recA-dn	TTACCAAACATCACGCCTAT

Table 6. MLST amplification primers for *S. pneumoniae*

Gene locus	Primer name	Forward primer (5'-3') ^{1,2}	Primer name	Reverse primer (5'-3') ^{1,2}
<i>aroE</i> ³	aroE-fwd	TCCTATTAAGCATCTATTTCTCCCTTC	aroE-rev	ACAGGAGAGGATTGGCCATCCATGCCACACTG
<i>gdh</i> ⁴	gdh-up	ATGGACAAACCAGCNAGYTT	gdh-dn	GCTTGAGGTCCCATRCTNCC
<i>gki</i> ⁴	gki-up	GGCATTGGAATGGGATCACC	gki-dn	TCTCCCGCAGCTGACAC
<i>recP</i> ³	recP-fwd	GAATGTGTGATTCAATAATCACCTCAAATAGAAGG	recP-rev	TGCTGTTTCGATAGCAGCATGGATGGCTTCC
<i>spi</i> ³	spi-fwd	CGCTTAGAAAGGTAAGTTATGAATTT	spi-rev	GAAGAGGCTGAGATTGGTGATTCTCGGCC
<i>xpt</i> ³	xpt-fwd	TTAACTTTTAGACTTTAGGAGGTCTTATG	xpt-rev	CGGCTGCTTGCGAGTGTTTTTCTTGAG
<i>ddl</i> ³	ddl-fwd	TAAAATCACGACTAAGCGTGTCTGG	ddl-rev	AAGTAGTGGGTACATAGACCACTGGG

¹IUPAC nucleotide designations: R = A or G; Y = C or T; N = A, T, G, or C.

²In this instance, R = equal mixture of A and G; W = equal mixture of A and T; etc.

³The primers for *aroE*, *recP*, *spi*, *xpt*, and *ddl* listed at <http://spneumoniae.mlst.net/misc/info.asp#experimental> are too close to the target sequences for accurate sequencing results using a capillary sequencer, thus alternative primers that lie about 40 bases further upstream and downstream of the target sequences are listed above. They can also be found at <http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm>.

⁴Primer sequence taken from: <http://spneumoniae.mlst.net/misc/info.asp#experimental>.

B. Primers used for PCR amplification

The primers used for PCR amplification for each scheme are shown in Table 4 (*N. meningitidis*), Table 5 (*H. influenzae*), and Table 6 (*S. pneumoniae*). In addition, the primers currently in use are listed in the MLST web pages for the schemes, <http://pubmlst.org/neisseria/> look under “information”, and <http://haemophilus.mlst.net/>, look under “Organism Specific Information” and go to page 2. Note that for *S. pneumoniae*, the primers listed are from two different sources. The original MLST primers for *S. pneumoniae* can be found at <http://spneumoniae.mlst.net/misc/info.asp#experimental>. However, it has been found that these primers for *aroE*, *recP*, *spi*, *xpt*, and *ddl* are too close to the target sequences for accurate sequencing results using a capillary sequencer, thus alternative primers that lie about 40 bases further upstream and downstream of the target sequences were designed and can be found at <http://www.cdc.gov/ncidod/biotech/strep/alt-MLST-primers.htm>.

C. PCR reaction setup and cycling conditions

The PCR conditions to be used for each scheme are given below (Tables 7 and 8). PCR set up and cycling conditions for each assay can also be found at <http://pubmlst.org/neisseria/> look under “information”, and <http://haemophilus.mlst.net/> or <http://spneumoniae.mlst.net/>, look under “Organism Specific Information”.

Reactions are performed in 50 µl volumes for *N. meningitidis* and *H. influenzae*, and in 25 µl volumes for *S. pneumoniae* in either in 8-well tube strips or in 96-well plates. If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA. When setting up the PCR reactions keep the reagents from warming up to room temperature by keeping them on ice. This is especially important for the *Taq* DNA polymerase.

Table 7. MLST PCR amplification reaction set-up for *N. meningitidis* and *H. influenzae*

Reagent	Volume (µl)*	Comments
PCR grade water	40.5	
10X buffer	5.0	
10 mM dNTPs	1.0	200 µM final concentration
20 µM forward primer	1.0	0.4 µM final concentration
20 µM reverse primer	1.0	0.4 µM final concentration
DNA polymerase	0.5	
Template DNA	1.0	
Final volume	50.0	

* Some laboratories scale back the reaction volumes to 25 µl. Adjust each component of the reaction accordingly if 25 µl volumes are desired.

Table 8. MLST PCR amplification reaction set-up for *S. pneumoniae*

Reagent	Volume (μ l)	Comments
PCR grade water	18.0 - 19.5	
10X buffer	2.5	
10 mM dNTPs	0.5	200 μ M final concentration
20 μ M forward primer	0.5	0.2 μ M final concentration
20 μ M reverse primer	0.5	0.2 μ M final concentration
DNA polymerase	0.5	
Template DNA	1 – 2.5	
Final volume	25.0	

Once the PCR reactions are set-up, immediately place them in the PCR machine and run. The cycling conditions for MLST differ slightly for each organism (Table 9, *N. meningitidis*, except for *pgm*; Table 10, *N. meningitidis*, *pgm*; Table 11, *H. influenzae*; and Table 12, *S. pneumoniae*). Note that the annealing temperature of the reactions may need to be optimized when adapting the protocols for use in your laboratory.

Table 9. PCR cycling conditions for *N. meningitidis* MLST amplifications, except for *pgm*

1x (94°C, 4 min)
35x (94°C, 1 min); (55°C, 1 min); (72°C, 1 min)
1x (72°C, 5 min)
1x (4°C, ∞)

Table 10. PCR cycling conditions for *pgm* gene for *N. meningitidis* MLST amplifications

1x (95°C, 5 min)
30x (94°C, 1 min); (65°C, 1 min)*; (72°C, 1 min) *Decrease the annealing temperature 0.5°C per cycle
10x (94°C, 1 min); (50°C, 1 min); (72°C, 2 min)
1x (72°C, 5 min)
1x (4°C, ∞)
1x (72°C, 2 min)
1x (4°C, ∞)

Table 11. PCR cycling conditions for *H. influenzae* MLST amplifications

1x (95°C, 4 min)
30x (95°C, 30 sec); (55°C, 30 sec)*; (72°C, 1 min) *Use 50°C for <i>adk</i> and <i>frdB</i> genes
1x (72°C, 10 min)
1x (4°C, ∞)

Table 12. PCR cycling conditions for *S. pneumoniae* MLST amplifications

1x (94°C, 5 min)
10x (94°C, 15 sec); (54°C, 30 sec); (72°C, 45 sec)
20x (94°C, 15 sec); (54°C, 30 sec); (72°C, 45 sec)* *Add 10 sec to the extension per cycle
1x (72°C, 10 min)
1x (4°C, ∞)

D. Analysis of PCR products on an agarose gel

It is useful to check for a successful PCR amplification before moving onto reaction clean-up and sequencing. The protocol for analyzing the amplified PCR products can be found in Appendix 2 at the end of this chapter.

E. Nucleotide sequencing of MLST PCR products

To perform DNA nucleotide sequencing, the DNA amplicons must be purified by the method of choice before sequencing reactions can be performed. These include gel filtration columns, a solid phase reversible immobilization magnetic bead-based system, or PEG₈₀₀₀/2.5M NaCl precipitation. Commercial kits for gel filtration columns are available from several companies and a protocol for PEG precipitation can be found at <http://pubmlst.org/neisseria/> under “information” then “PCR protocol”.

The PCR primers and protocols included here are those used at CDC and are optimized for these laboratories. Other protocols work well and it is up to the discretion of each laboratory to discern the optimal reagents and assay conditions for their laboratory, including appropriate validation and quality control. For a commonly used protocol, see: <http://pubmlst.org/neisseria/> under “information” then “sequencing protocol (microtiter plates)”.

Sequencing reactions for all assays described here should be carried out on both strands. Sequencing each strand provides greater confidence in the base calls versus having base calls made using a single data point.

F. Sequencing primers for *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* MLST

For *H. influenzae* and *S. pneumoniae*, the same primers used for PCR amplification are used for sequencing reactions. The sequencing primers used for *N. meningitidis* MLST are in Table 13.

Table 13. MLST sequencing primers for *N. meningitidis*

Gene locus	Primer name	Forward primer (5'-3')^{1,2}	Primer name	Reverse primer (5'-3')^{1,2}
<i>abcZ</i>	abcZ-S1A	AATCGTTTATGTACCGCAGR	abcZ-S2	GAGAACGAGCCGGGATAGGA
<i>adk</i>	adk-S1A	AGGCWGGCACGCCCTTGG	adk-S2	CAATACTTCGGCTTTCACGG
<i>aroE</i>	aroE-S1A	TCGGTCAAYACGCTGRTK	aroE-S2	ATGATGTTGCCGTACACATA
<i>fumC</i>	fumC-S1	TCCGGCTTGCCGTTTGTGTCAG	fumC-S2	TTGTAGGCGGTTTTGGCGAC
<i>gdh</i>	gdh-S3	CCTTGGCAAAGAAAGCCTGC	gdh-S4C	RCGCACGGATTCATRYGG
<i>pdhC</i>	pdhC-S1	TCTACTACATCACCCCTGATG	pdhC-S2	ATCGGCTTTGATGCCGTATTT
<i>pgm</i>	pgm-S1	CGGCGATGCCGACCGCTTGG	pgm-S2A	GGTGATGATTTTCGGTYGCRCC

¹IUPAC nucleotide designations: R = A or G; W = T or A; K = T or G; Y = C or T; N = A, T, G, or C.

²In this instance, R = equal mixture of A and G; W = equal mixture of A and T; etc.

G. Sequencing PCR setup and cycling conditions

Reactions are performed in 20 μl volumes either in 8-well tube strips or in 96-well plates. Some laboratories perform sequencing reactions in 10 μl volumes. Adjust each component of the reaction accordingly if 10 μl volumes are desired. If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA. The setup in Table 14 will work for sequencing MLST amplicons from *N. meningitidis*, *H. influenzae*, and *S. pneumoniae*.

Table 14. MLST sequencing PCR reaction set-up

Reagent	Volume (μl)	Comments
PCR grade water	11.0	
5X buffer	4.0	
Terminator nucleotides and polymerase*	1.0	200 μM final concentration
3.2 μM primer	1.0	0.16 μM final concentration
Purified amplicon DNA	3.0	
Final volume	20.0	

*Note: chemistries can vary, thus optimize the reactions for your particular system. Primer concentrations and PCR set up parameters may vary depending on the type of enzyme, chemistry, and protocols used in individual laboratories. Each laboratory should optimize the protocols.

Once the reactions are setup, follow the cycling conditions in Table 15.

Table 15. Cycling conditions for sequencing PCR

25x (95°C, 10 sec); (52°C, 5 sec); (60°C, 4 min)
1x (4°C, ∞)

H. Purification of the sequencing reaction products

Before the reaction products can be resolved on an automated DNA sequencer, the products must be purified to remove unincorporated fluorescent dyes, buffer, and unused deoxyribonucleotide triphosphates. This can be achieved using a variety of commercially available kits that utilize gel filtration or a solid phase reversible immobilization magnetic bead-based system to purify the products. Conversely, the reaction products can be purified by precipitation with ethanol and 3M sodium acetate, pH 4.6 (see <http://pubmlst.org/neisseria/> and click on “information” then “sequencing protocol (microtiter plates)”). Typically, the reaction products will be purified and dried down in a 96-well plate and reconstituted with formamide, EDTA, or water before electrophoresis. Each laboratory should follow the manufacturer’s protocol for the automatic sequencer system being employed.

See the Analysis of Sequence Data and Allele Determination section below for further information about data analysis.

III. *porA* and *porB* typing

A. Overview

The antigenic diversity expressed by the five major classes of outer membrane proteins (OMP) on the surface of *N. meningitidis* have been utilized to develop isolate subtyping and characterization schemes and to develop vaccines. The largest of the OMPs expressed by most meningococcal isolates is the PorA or class 1 protein. The PorA protein is a transmembrane protein predicted to have 8 loops exposed on the surface of the organism. These loops contain two hypervariable regions, VR1 (loop 1) and VR2 (loop 4), and two semivariable regions, SV1 (loop 5) and SV2 (loop 6). Sequence analysis of SV1 and SV2 demonstrated that sequence differences in these regions were too limited to generate subtype differences between strains, thus PorA typing is based on the VR1 and VR2 hypervariable sequences, which have greater resolution (16, 19).

Additionally, all *N. meningitidis* express PorB which is expressed as 2 alternate alleles that were previously called class 2 and 3 proteins. These have been named PorB2 and PorB3, respectively, and are mutually exclusive, though hybrids do exist. PorB has four hypervariable loop regions referred to as VR1, on loop I; VR2, on loop V; VR3, on loop VI; and VR4, on loop VII (32). Historically, serotyping is based on the reactivity of specific antisera to the variable regions of PorB. The antigenically important variable epitopes that are targeted by serological typing reagents reside in the surface-exposed loops of PorA and PorB (29, 31). In addition, these regions are targeted by the host immune response and are thus under intensive selective pressure. Subtyping isolates based on sequencing of the *porA* and *porB* genes and translation of the DNA into their respective amino acids for typing has been helpful in characterizing isolates in clusters or outbreaks and for determining differences between isolates. Note that the *porA* gene is deleted in some isolates, though this is a rare occurrence (32).

B. Primers used for PCR amplification

The primers used for PCR amplification of the *porA* and *porB* genes of *N. meningitidis* are shown in Table 16. Primer set 1 should be used first when amplifying the *porA* gene. If PCR amplification fails using primer set 1; alternative primer sets 2 and/or 3 should be used.

C. PCR reaction setup and cycling conditions

Reactions are performed in 50 µl volumes either in 8-well tube strips or in 96-well plates (Table 17). If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA.

Table 16. PCR primers for *porA* and *porB* amplification

Gene	Set	Forward primer 5'-3'	Reverse primer 5'-3'	¹ GenBank Accession #	² Amplicon size (nts)
<i>porA</i>	1	P14 GGGTGTGTTGCCCGATGTTTTTAGG	P22 TTAGAATTTGTGGCGCAAACCGAC	X12899	1236
<i>porA</i>	2 ³	P21 CTGTACGGCGAAATCAAAGCCGGCGT	P22 TTAGAATTTGTGGCGCAAACCGAC	EF564254	1115
<i>porA</i>	3 ³	U23 GTGTTTGCCCGATGTTTTAGGT	L24 TGCTGTCTTTATTGCCGTTTTTCT	X12899	1368
<i>porB</i>	1	PB-A1 TAAATGCAAAGCTAAGCGGCTTG	PB-A2 TTTGTTGATACCAATCTTTTCAG	EU301792	1755

¹The GenBank Accession number given may not be that of the strain actually used to design the PCR primers, but it is a strain that contains the primer sequences.

²These are the predicted amplicon sizes for the strain with the given GenBank Accession number. Because of the variable nature of these genes, the amplicon in other strains may differ from the size listed. *porA* is deleted in some strains, though deletions are rare.

³Primers sets 2 and 3 are alternate primer sets that can be used when the *porA* gene does not amplify with primer set 1.

Table 17. *porA* and *porB* PCR amplification reaction set-up

Reagent	Volume (μ l)*	Comments
PCR grade water	40.5	
10X buffer	5.0	
10 mM dNTPs	1.0	200 μ M final concentration
20 μ M forward primer	1.0	0.4 μ M final concentration
20 μ M reverse primer	1.0	0.4 μ M final concentration
DNA polymerase	0.5	
Template DNA	1.0	
Final volume	50.0	

* Some laboratories scale back the reaction volumes to 25 μ l. Adjust each component of the reaction accordingly if 25 μ l volumes are desired.

Once the PCR reactions are set-up, immediately place them in the PCR machine and run. The cycling conditions for *porA* and *porB* differ slightly for each assay (Table 18 for *porA* and Table 19 for *porB*). Note that the annealing temperature of the reactions may need to be optimized when adapting the protocols for use in your laboratory.

Table 18. Cycling conditions for *porA* amplification

1x (95°C, 5 min)
30x (95°C, 1 min); (60°C, 30 sec); (72°C, 2 min)
1x (72°C, 5 min)
4°C, ∞

Table 19. Cycling conditions for *porB* amplification

1x (94°C, 5 min)
35x (94°C, 1 min); (60°C, 30 sec); (72°C, 1 min)
1x (72°C, 5 min)
4°C, ∞

D. Analysis of PCR products on an agarose gel

It is useful to check for a successful PCR amplification before moving onto reaction clean-up and sequencing. The protocol analyzing the amplified PCR products can be found in Appendix 2 at the end of this chapter.

E. Nucleotide sequencing of *porA* and *porB* PCR products

To perform DNA nucleotide sequencing, the DNA amplicons must be purified by the method of choice before sequencing reactions can be performed. These include gel filtration columns, a solid phase reversible immobilization magnetic bead-based system, or PEG₈₀₀₀/2.5M NaCl

precipitation. Commercial kits for gel filtration columns are available from several companies and a protocol for PEG precipitation can be found at <http://pubmlst.org/neisseria/> under “information” then “PCR protocol”.

The PCR primers and protocols included here are those used at CDC and are optimized for these laboratories. Other protocols work well and it is up to the discretion of each laboratory to discern the optimal reagents and assay conditions for their laboratory, including appropriate validation and quality control. For a commonly used protocol, see: <http://pubmlst.org/neisseria/> under “information” then “sequencing protocol (microtiter plates)”.

Sequencing reactions for all assays described here should be carried out on both strands. Sequencing each strand provides greater confidence in the base calls versus having base calls made using a single data point.

F. Sequencing primers for *porA* and *porB* typing

The primers used to sequence the *porA* (Table 20) and *porB* (Table 21) amplicons are shown. VR1 of *porA* can be determined by using primers U86 and R435 and VR2 can be determined by using F435 and R773. To determine the class of the PorB protein, all seven of the primers listed in the chart for *porB* must be used.

Table 20. Sequencing primers for *porA*

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
<i>porA</i> VR1	U86 GCCCTCGTATTGTCCGCACTG	R435 TTGCTGTCCCAAGGATCAATGGC
<i>porA</i> VR2	F435 GCCATTAATCCTTGGGACAGCAA	R773 GGCATAGTTCCTGGGCAAACCGCCAT

Table 21. Sequencing primers for *porB*

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
<i>porB</i>	PB-S1 GCAGCCCTTCCTGTTGCAGC	PB-S2 TTGCAGATTAGAATTTTGTG
<i>porB</i>	8U TCCGTACGCTACGATTCTCC	8L GGAGAATCGTAGCGTACGGA
<i>porB</i>	244U CGCCCCGCGTTTCTTACG	244L CGTAAGAAACGCGGGGCG
<i>porB</i>		PB260 AGTGCGTTTGGAGAAGTCGT

G. Sequencing PCR setup and cycling conditions

Reactions are performed in 20 µl volumes either in 8-well tube strips or in 96-well plates. Some laboratories perform sequencing reactions in 10 µl volumes. Adjust each component of the reaction accordingly if 10 µl volumes are desired. If doing many reactions, it is useful to prepare

a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA. The sequencing PCR setup and cycling conditions are shown below in Tables 22 and 23, respectively.

Table 22. *porA* and *porB* sequencing PCR reaction set-up

Reagent	Volume (μ l)	Comments
PCR grade water	11.0	
5X buffer	4.0	
Terminator nucleotides and polymerase*	1.0	200 μ M final concentration
3.2 μ M primer	1.0	0.16 μ M final concentration
Purified amplicon DNA	3.0	
Final volume	20.0	

*Note: chemistries can vary, thus optimize the reactions for your particular system. Primer concentrations and PCR set up parameters may vary depending on the type of enzyme, chemistry, and protocols used in individual laboratories. Each laboratory should optimize the protocols.

Once the reactions are setup, follow the cycling conditions in Table 23.

Table 23. Cycling conditions for sequencing PCR

25x (95°C, 10 sec); (52°C, 5 sec); (60°C, 4 min)
1x (4°C, ∞)

H. Purification of the sequencing reaction products

Before the reaction products can be resolved on an automated DNA sequencer, the products must be purified to remove unincorporated fluorescent dyes, buffer, and unused deoxyribonucleotide triphosphates. This can be achieved using a variety of commercially available kits that utilize gel filtration or a solid phase reversible immobilization magnetic bead-based system to purify the products. Conversely, the reaction products can be purified by precipitation with ethanol and 3M sodium acetate, pH 4.6 (see <http://pubmlst.org/neisseria/> and click on “information” and “sequencing protocol (microtiter plates)”). Typically, the reaction products will be purified and dried down in a 96-well plate and reconstituted with formamide, EDTA, or water before electrophoresis. Each laboratory should follow the manufacturer’s protocol for the automatic sequencer system being employed.

See the Analysis of Sequence Data and Allele Determination section below for further information about data analysis.

IV. *fetA* typing

A. Overview

FetA (ferric enterobactin transport), formerly FrpB (iron-repressed protein B) is a 76 kDa iron-regulated OMP that is expressed to a high level during iron limitation (6). Modeling of the structure of FetA predicts 13 surface-exposed loops. Anti-FetA antibodies to the most variable of these loops have bactericidal properties (29). FetA has been proposed as a potential vaccine candidate. However, this region is highly variable which limits the potential of this antigen to be the sole component of a vaccine. Obtaining the amino acid sequence of this variable region by sequencing the DNA of this region of the *fetA* gene is useful in characterizing clones emerging or circulating in local populations (28). Note that the *fetA* gene is deleted in some isolates, though this is a rare occurrence (3, 17).

B. Primers used for PCR amplification

The primers used for PCR amplification of the *fetA* gene of *N. meningitidis* are shown in Table 24.

C. PCR reaction setup and cycling conditions

Reactions are performed in 50 µl volumes either in 8-well tube strips or in 96-well plates (Table 25). If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA.

Once the PCR reactions are set-up, immediately place them in the PCR machine and run. The cycling conditions for *fetA* are shown in Table 26. Note that the annealing temperature of the reactions may need to be optimized when adapting the protocols for use in your laboratory.

D. Analysis of PCR products on an agarose gel

It is useful to check for a successful PCR amplification before moving onto reaction clean-up and sequencing. The protocol analyzing the amplified PCR products can be found in Appendix 2 at the end of this chapter.

E. Nucleotide sequencing of *fetA* PCR products

To perform DNA nucleotide sequencing, the DNA amplicons must be purified by the method of choice before sequencing reactions can be performed. These include gel filtration columns, a solid phase reversible immobilization magnetic bead-based system, or PEG₈₀₀₀/2.5M NaCl precipitation. Commercial kits for gel filtration columns are available from several companies and a protocol for PEG precipitation can be found at <http://pubmlst.org/neisseria/> under “information” then “PCR protocol”.

The PCR primers and protocols included here are those used at CDC and are optimized for these laboratories. Other protocols work well and it is up to the discretion of each laboratory to discern the optimal reagents and assay conditions for their laboratory, including appropriate validation and quality control. For a commonly used protocol, see: <http://pubmlst.org/neisseria/> under “information” then “sequencing protocol (microtiter plates)”.

Sequencing reactions for all assays described here should be carried out on both strands. Sequencing each strand provides greater confidence in the base calls versus having base calls made using a single data point.

Table 24. PCR primers for *fetA* amplification

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	¹ GenBank Accession #	² Amplicon size (nts)
<i>fetA</i>	S1 CGGCGCAAGCGTATTCGG	S8 CGCGCCCAATTCGTAACCGTG	AF439258	1189

¹The GenBank Accession number given may not be that of the strain actually used to design the PCR primers, but it is a strain that contains the primer sequences.

²These are the predicted amplicon sizes for the strain with the given GenBank Accession number. Because of the variable nature of these genes, the amplicon in other strains may differ from the size listed. *fetA* is deleted in some strains, though it is rare (3, 17).

Table 25. *fetA* PCR amplification reaction set-up

Reagent	Volume (μl)*	Comments
PCR grade water	40.5	
10X buffer	5.0	
10 mM dNTPs	1.0	200 μM final concentration
20 μM S1	1.0	0.4 μM final concentration
20 μM S8	1.0	0.4 μM final concentration
DNA polymerase	0.5	
Template DNA	1.0	
Final volume	50.0	

* Some laboratories scale back the reaction volumes to 25 μl. Adjust each component of the reaction accordingly if 25 μl volumes are desired.

Table 26. Cycling conditions for *fetA* amplification

1x (95°C, 5 min)
40x (95°C, 1 min); (55°C, 1 min); (72°C, 2.5 min)
1x (72°C, 7 min)
4°C, ∞

F. Sequencing primers for *fetA* typing

The primers used to sequence the *fetA* amplicon are shown in Table 27.

Table 27. Sequencing primers for *fetA*

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
<i>fetA</i>	S12 TTCAACTTCGACAGCCGCCTT	S15 TTGCAGCGCGTTCR*TACAGGCG

G. Sequencing PCR setup and cycling conditions

Reactions are performed in 20 μ l volumes either in 8-well tube strips or in 96-well plates. Some laboratories perform sequencing reactions in 10 μ l volumes. Adjust each component of the reaction accordingly if 10 μ l volumes are desired. If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA. The sequencing PCR setup and cycling conditions are shown below in Tables 28 and 29, respectively.

Table 28. *fetA* sequencing PCR reaction set-up

Reagent	Volume (μ l)	Comments
PCR grade water	11.0	
5X buffer	4.0	
Terminator nucleotides and polymerase*	1.0	200 μ M final concentration
3.2 μ M primer	1.0	0.16 μ M final concentration
Purified amplicon DNA	3.0	
Final volume	20.0	

*Note: chemistries can vary, thus optimize the reactions for your particular system. Primer concentrations and PCR set up parameters may vary depending on the type of enzyme, chemistry, and protocols used in individual laboratories. Each laboratory should optimize the protocols.

Once the reactions are setup, follow the cycling conditions in Table 29.

Table 29. Cycling conditions for sequencing PCR

25x (95°C, 10 sec); (52°C, 5 sec); (60°C, 4 min)
1x (4°C, ∞)

H. Purification of the sequencing reaction products

Before the reaction products can be resolved on an automated DNA sequencer, the products must be purified to remove unincorporated fluorescent dyes, buffer, and unused deoxyribonucleotide triphosphates. This can be achieved using a variety of commercially available kits that utilize gel filtration or a solid phase reversible immobilization magnetic bead-based system to purify the

products. Conversely, the reaction products can be purified by precipitation with ethanol and 3M sodium acetate, pH 4.6 (see <http://pubmlst.org/neisseria/> and click on “information” then “sequencing protocol (microtiter plates)”). Typically, the reaction products will be purified and dried down in a 96-well plate and reconstituted with formamide, EDTA, or water before electrophoresis. It is recommended that each laboratory follow the manufacturer’s protocol for the automatic sequencer system being employed.

See the Analysis of Sequence Data and Allele Determination section below for further information about data analysis.

V. *fHbp* typing

A. Overview

Factor H binding protein (fHbp), also referred to as Genome-derived Neisserial Antigen 1870 (GNA1870) or lipoprotein 2086 (LP2086 protein), is a ~28 KD surface exposed protein that binds to human factor H, a negative regulator of the alternative pathway of complement activation. fHbp is widely distributed in *N. meningitidis*. Recruitment of factor H to the surface of *N. meningitidis* facilitates bacterial escape from the host innate immune system and promotes bacterial survival in the host (23). fHbp also induces bactericidal activity against *N. meningitidis* strains expressing this protein; thus it is a viable vaccine candidate protein and component vaccines including fHbp are currently under evaluation (9, 22). Analysis of the amino acid sequences from the mature form of fHbp, which does not include the leader peptide, reveals two distinct groups, subfamily A and B (22). However, DNA sequence analysis demonstrates three variant groups: group 1 (corresponding to subfamily B) and group 2 and 3 (together corresponding to subfamily A) (18). The data collected to date indicate some correlation of fHbp alleles with MLST or serogroups (18). Continuing determination of fHbp sequence diversity provides valuable information for the evaluation of potential vaccine efficacy and coverage as well as characterizing circulating populations of *N. meningitidis*.

B. Primers used for PCR amplification

The primers used for PCR amplification of the *fHbp* gene of *N. meningitidis* are shown in Table 30.

C. PCR reaction setup and cycling conditions

Reactions are performed in 50 µl volumes either in 8-well tube strips or in 96-well plates (Table 31). If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA.

Once the PCR reactions are set-up, immediately place them in the PCR machine and run. The cycling conditions for *fHbp* are shown in Table 32. Note that the annealing temperature of the reactions may need to be optimized when adapting the protocols for use in your laboratory.

Table 30. PCR primers for *fHbp* amplification

Gene	³ Forward primer 5'-3'	³ Reverse primer 5'-3'	¹ GenBank Accession #	² Amplicon size (nts)
<i>fHbp</i>	CDC3UNI GTCCGAACGGTAAATTATYGTG	CDC5UNI CTATTCTGVGTATGACTAG	FM999788	895

¹The GenBank Accession number given may not be that of the strain actually used to design the PCR primers, but it is a strain that contains the primer sequences.

²These are the predicted amplicon sizes for the strain with the given GenBank Accession number. Because of the variable nature of these genes, the amplicon in other strains may differ from the size listed.

³IUCAC designations: Y = C or T; V = C, G, or T.

Table 31. *fHbp* PCR amplification reaction set-up

Reagent	Volume (µl)	Comments
PCR grade water	31.5	
10X buffer	5.0	
10 mM dNTPs	1.0	200 µM final concentration
10 µM CDC3UNI	4.0	0.8 µM final concentration
10 µM CDC5UNI	6.0	1.2 µM final concentration
DNA polymerase	0.5	
Template DNA	2.0	
Final volume	50.0	

* Some laboratories scale back the reaction volumes to 25 µl. Adjust each component of the reaction accordingly if 25 µl volumes are desired.

Table 32. Cycling conditions for *fHbp* amplification

1x (94°C, 5 min)
30x (95°C, 15 sec); (50°C, 15 sec); (72°C, 1.5 min)
1x (72°C, 5 min)
4°C, ∞

D. Analysis of PCR products on an agarose gel

It is useful to check for a successful PCR amplification before moving onto reaction clean-up and sequencing. The protocol analyzing the amplified PCR products can be found in Appendix 2 at the end of this chapter.

E. Nucleotide sequencing of *fHbp* PCR products

To perform DNA nucleotide sequencing, the DNA amplicons must be purified by the method of choice before sequencing reactions can be performed. These include gel filtration columns, a solid phase reversible immobilization magnetic bead-based system, or PEG₈₀₀₀/2.5M NaCl precipitation. Commercial kits for gel filtration columns are available from several companies and a protocol for PEG precipitation can be found at <http://pubmlst.org/neisseria/> under “information” then “PCR protocol”.

The PCR primers and protocols included here are those used at CDC and are optimized for these laboratories. Other protocols work well and it is up to the discretion of each laboratory to discern the optimal reagents and assay conditions for their laboratory, including appropriate validation and quality control. For a commonly used protocol, see: <http://pubmlst.org/neisseria/> under “information” then “sequencing protocol (microtiter plates)”.

Sequencing reactions for all assays described here should be carried out on both strands. Sequencing each strand provides greater confidence in the base calls versus having base calls made using a single data point.

F. Sequencing primers for *fHbp* typing

The primers used to sequence the *fHbp* amplicon are the same as those used for amplification, but should be used at a concentration of 3.2 μ M.

G. Sequencing PCR setup and cycling conditions

Reactions are performed in 20 μ l volumes either in 8-well tube strips or in 96-well plates. However, some laboratories perform sequencing reactions in 10 μ l volumes. Adjust each component of the reaction accordingly if 10 μ l volumes are desired. If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA. The sequencing PCR setup and cycling conditions are shown below in Tables 33 and 34, respectively.

Table 33. *fHbp* sequencing PCR reaction set-up

Reagent	Volume (μ l)	Comments
PCR grade water	11.0	
5X buffer	4.0	
Terminator nucleotides and polymerase*	1.0	200 μ M final concentration
3.2 μ M primer	1.0	0.16 μ M final concentration
Purified amplicon DNA	3.0	
Final volume	20.0	

*Note: chemistries can vary, thus optimize the reactions for your particular system. Primer concentrations and PCR set up parameters may vary depending on the type of enzyme, chemistry, and protocols used in individual laboratories. Each laboratory should optimize the protocols.

Once the reactions are setup, follow the cycling conditions in Table 34.

Table 34. Cycling conditions for sequencing PCR

25x (95°C, 10 sec); (52°C, 5 sec); (60°C, 4 min)
1x (4°C, ∞)

H. Purification of the sequencing reaction products

Before the reaction products can be resolved on an automated DNA sequencer, the products must be purified to remove unincorporated fluorescent dyes, buffer, and unused deoxyribonucleotide triphosphates. This can be achieved using a variety of commercially available kits that utilize gel filtration or a solid phase reversible immobilization magnetic bead-based system to purify the products. Conversely, the reaction products can be purified by precipitation with ethanol and 3M sodium acetate, pH 4.6 (see <http://pubmlst.org/neisseria/> and click on “information” and “sequencing protocol (microtiter plates)”). Typically, the reaction products will be purified and dried down in a 96-well plate and reconstituted with formamide, EDTA, or water before electrophoresis. It is recommended that each laboratory follow the manufacturer’s protocol for the automatic sequencer system being employed.

See the Analysis of Sequence Data and Allele Determination section below for further information about data analysis.

VI. Penicillin-binding proteins (PBPs)

Alterations in PBPs are the major mechanism of resistance to penicillins and cephalosporins in *S. pneumoniae*. These bacteria possess several high-molecular-weight PBPs, and most decreased susceptibility and resistance is associated with alterations in PBP1a, PBP2b, and PBP2x. Remarkably, the vast majority of naturally occurring isolates displaying penicillin MICs ≥ 0.25 ug/ml are characterized by carrying mosaic alleles of these genes that contain segments of closely related non-pneumococcal species. Sequence differences in the genes encoding these

PBPs have been exploited to subtype β -lactam-resistant *S. pneumoniae* isolates. These methods include restriction fragment length polymorphisms (RFLPs) analysis of PCR products amplified from *pbp1a*, *pbp2b* and *pbp2x*, or comparisons of sequences of the amplified products to determine amino acid changes in these genes (5, 21, 26)

A. Primers used for PCR amplification

PCR reactions for *pbp1a*, *pbp2b* and *pbp2x* are carried out in 3 separate reactions using the primers listed in Table 35.

Table 35. Primers for amplification and sequencing of *pbp1a*, *pbp2b* and *pbp2x*

Primer	Sequence (5'-3')	Gene	Product size	Reference
pn1af	GGC ATT CGA TTT GAT TCG CTT CTA TCA T	<i>pbp1a</i>	2.4kb	(8)
pn1ar	CTG AGA AGA TGT CTT CTC AGG CTT TTG			
1a-S1	AAG CTC AAA AAC ATC TGT GGG	<i>pbp1a</i>	Sequencing	(2)
1a-S2	TAC TCC ACT CTA CAA CTG GG			
1a-S3	CCA ACA AAC ATT TCA TCT GGA GC			
pbp2bf	GAT CCT CTA AAT GAT TCT CAG GTG GCT GT	<i>pbp2b</i>	1.5kb	(8)
pbp2bR	GTC AAT TAG CTT AGC AAT AGG TGT TGG AT			
2b-S1	TTG CTG AAA AGT TAT TTC AAT TC	<i>pbp2b</i>	Sequencing	(2)
2b-S2	ATT GTC TTC CAA GGT TCA GCT			
pbp2xf	CGT GGG ACT ATT TAT GAC CGA AAT GGA G	<i>pbp2x</i>	2.1kb	(8)
pbp2xr2	GGC GAA TTC CAG CAC TGA TGG AAA TAA			
2x-S1	GGA ACA GAA CAA GTT TCC CAA C	<i>pbp2x</i>	Sequencing	(2)
2x-S2	GAT GCC ACG ATT CGA GAT TGG G			
2x-S3	TTT ACA GCT ATT GCT ATT GAT GG			

B. PCR reaction setup and cycling conditions

PCR reactions are set up as shown in Table 36.

Table 36. *pbp1a*, *pbp2b* and *pbp2x* PCR amplification reaction set-up for *S. pneumoniae*

Reagent	Volume (μl)	Comments
PCR grade water	22.45	
10X buffer	3.0	
10 mM dNTPs	0.75	250 μM final concentration
10 μM forward primer	0.9	0.3 μM final concentration
10 μM reverse primer	0.9	0.3 μM final concentration
<i>Taq</i> DNA polymerase	1.0	1 unit <i>Taq</i> polymerase
Template DNA	1.0	
Final volume	30.0	

Once the PCR reactions are set-up, immediately place them in the PCR machine and run. The cycling conditions are shown in Table 37. Note that the annealing temperature of the reactions may need to be optimized when adapting the protocols for use in your laboratory.

Table 37. Cycling conditions for *pbp1a*, *pbp2b* and *pbp2x* PCR amplification

1x (93°C, 5 min)
30x (93°C, 1 min); (52°C, 1 min); (72°C, 2 min)
1x (72°C, 2 min)
4°C, ∞

C. Analysis of PCR products on an agarose gel

To check for successful PCR amplification, run 5 μl of the end-products on a 1% agarose gel (see protocol in Appendix 2 at the end of this chapter). Store the remainder at -20°C or proceed to RFLP analyses and/or PCR cleanup and DNA sequencing.

D. RFLP analyses

PBP-gene amplicons are subjected to *HaeIII* plus *RsaI* digestion by the addition of 3U of the respective enzymes to 5 μl of unpurified PCR product, followed by 1-4 hr of incubation at 37°C (8). Fragments are then separated on a 2% agarose gel (see Appendix 2 at the end of the chapter, but add 2 g of agarose to 100 ml sterile distilled H₂O instead of 1 g) and should include a DNA ladder (1kb) on each gel to allow for comparison of fingerprints. After electrophoresis, the gel is visualized under UV and the image captured using a camera.

E. DNA sequencing

Sequencing of PCR products can also be setup to determine specific DNA sequences and amino acid changes that may be present in *pbp1a*, *pbp2b* and *pbp2x* genes. To perform DNA nucleotide sequencing, the DNA amplicons must be purified by the method of choice before sequencing reactions can be performed. These include gel filtration columns, a solid phase reversible immobilization magnetic bead-based system, or PEG₈₀₀₀/2.5M NaCl precipitation. Commercial kits for gel filtration columns are available from several companies and a protocol for PEG precipitation can be found at <http://pubmlst.org/neisseria/> under “information” then “PCR protocol”. Various chemistries and platforms are available for sequencing and many laboratories outsource their sequencing to commercial companies.

F. Sequencing primers for *pbp1a*, *pbp2b* and *pbp2x*

The primers used to sequence the *pbp1a*, *pbp2b* and *pbp2x* amplicons are the same as those used for amplification of the genes as well as the additional primers listed in Table 35, but should be used at a final concentration of 0.3 μ M.

G. Sequencing PCR setup and cycling conditions

Reactions are performed in 20 μ l volumes either in 8-well tube strips or in 96-well plates. However, some laboratories perform sequencing reactions in 10 μ l volumes. Adjust each component of the reaction accordingly if 10 μ l volumes are desired. If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA. The sequencing PCR setup and cycling conditions are shown below in Tables 38 and 39, respectively.

Table 38. *pbp1a*, *pbp2b* and *pbp2x* sequencing PCR reaction set-up

Reagent	Volume (μ l)	Comments
PCR grade water	11.0	
5X buffer	4.0	
Terminator nucleotides and polymerase*	1.0	200 μ M final concentration
3.2 μ M primer	1.0	0.3 μ M final concentration
Purified amplicon DNA	3.0	
Final volume	20.0	

*Note: chemistries can vary, thus optimize the reactions for your particular system. Primer concentrations and PCR set up parameters may vary depending on the type of enzyme, chemistry, and protocols used in individual laboratories. Each laboratory should optimize the protocols.

Once the reactions are setup, follow the cycling conditions in Table 39.

Table 39. Cycling conditions for sequencing PCR

25x (95°C, 10 sec); (52°C, 5 sec); (60°C, 4 min)
1x (4°C, ∞)

H. Interpretation

After PCR and RFLP analysis the patterns can be visually compared or compared by using an RFLP analyses program to determine clusters of related *pbp* patterns. For sequence analysis of the *pbp* genes, the nucleotide and derived amino acid sequence data for strains are compared to the corresponding sequence data for the β -lactam susceptible laboratory isolate R6 (sequence available at GenBank accession numbers: *pbp1a* M90527; *pbp2b* X16022; *pbp2x* X16367) using DNA sequence alignment software. Mutations at positions in or close to each of the three (SXXK, SXN, and KXG) conserved motifs for PBP2b, PBP2x and PBP1a can then be identified and compared.

VII. Pneumococcal surface protein A (PspA)

PspA is a surface protein and virulence factor found on all isolates of *S. pneumoniae* and is highly immunogenic. The *pspA* gene is variable at the nucleotide level, and the amino acid similarity of the surface-exposed N-terminal region of PspA proteins can be as little as 40%. Based on nucleotide and amino acid identity, *pspA* genes and encoded PspA proteins are classified together into six clades, subdivided into three families: family 1 (clades 1 and 2), family 2 (clades 3, 4, and 5), and the rarely observed family 3 (clade 6) (11). The extent of cross-reactivity of PspA clades follows roughly the degree of amino acid sequence homology and is maximal within strains of the same PspA family. The family can be recognized serologically, but the clade must be identified by the sequence. Studies have demonstrated that PspA family and clade distribution are independent of serotype, age, and clinical origin of the isolates, but are highly associated with genotype as determined by PFGE and/or MLST (33).

A PCR assay has been developed using primers specific for families 1, 2, and 3. Most isolates are either in family 1 or 2, thus two PCR reactions using primers family1- and 2-specific should be performed on each isolate. If both of these are negative, a PCR reaction using the primers specific for the rarely seen family 3 should be performed. Clade determination requires obtaining the DNA sequence of the allele, thus another PCR amplification reaction is required using primers that will amplify all three families.

A. Primers used for PCR amplification

Primers specific for family 1 are LSM12 and SKH63 and for family 2 are LSM12 and SKH52 (33). A test for PspA family 3 can also be run using primers SKH41 and SKH42 (10) (Table 40). Isolates that are negative in all PCR reactions are classified as nontypeable (10).

If the isolate is positive for one of the families, the clade in which the isolate belongs can be identified only through obtaining the DNA sequence of the allele. To do this, perform another

PCR amplification using primers LSM12 and SKH2, which will amplify all 3 families. This amplicon will then need to be sequenced (see below).

Table 40. Primers for amplification of *pspA* families¹

Primer	Sequence (5'-3')	Reference
LSM12	CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT	(33)
SKH63	TTTCTGGCTCATYAACTGCTTTC	
SKH52	TGGGGGTGGAGTTTCTTCTTCATCT	
SKH2	CCACATACCGTTTTCTTGTTCAGCC	
SKH41	CGCACAGACTTAACAGATGAAC	(10)
SKH42	CTTGTCCATCAACTTCATCC	

¹IUPAC designations: Y = C.

B. PCR reaction setup and cycling conditions

PCR reactions are set up as shown in Table 41.

Table 41. *pspA* PCR amplification reaction set-up for *S. pneumoniae*

Reagent	Volume (µl)	Comments
PCR grade water	21.45	
10X buffer	3.0	
10 mM dNTPs	0.75	250 µM final concentration
10 µM forward primer	0.9	0.3 µM final concentration
10 µM reverse primer	0.9	0.3 µM final concentration
<i>Taq</i> DNA polymerase	2.0	2 units <i>Taq</i> polymerase
Template DNA	1.0	
Final volume	30.0	

Once the PCR reactions are set-up, immediately place them in the PCR machine and run. The cycling conditions are shown in Table 42. Note that the annealing temperature of the reactions may need to be optimized when adapting the protocols for use in your laboratory.

Table 42. Cycling conditions for *pspA* PCR amplification

1x (95°C, 5 min)
30x (95°C, 1 min); (55°C, 1 min); (72°C, 3 min)
1x (72°C, 10 min)
4°C, ∞

C. Analysis of PCR products on an agarose gel

To check for successful PCR amplification and to determine to which family the allele belongs, run 5 µl of the end-products on a 1% agarose gel (see protocol in Appendix 2 at the end of the chapter). The remainder should be stored at -20°C or PCR cleanup and DNA sequencing can be performed immediately after successful PCR amplification has been confirmed.

D. Interpretation

The family 1-, 2- and 3-specific primers will produce PCR products that are approximately 1,000 bp for family 1, 1,200 bp for family 2 and 770 bp for family 3 and can be used to assign strains into *pspA* families.

E. DNA Sequencing to determine clade

The amplicon derived from the PCR amplification using primers LSM12 and SKH2 can be sequenced to determine the clade in which the isolate belongs. To perform DNA nucleotide sequencing, the DNA amplicons must be purified either by gel filtration columns, a solid phase reversible immobilization magnetic bead-based system, or PEG₈₀₀₀/2.5M NaCl precipitation before sequencing reactions can be performed. Commercial kits for gel filtration columns are available from several companies and a protocol for PEG precipitation can be found at <http://pubmlst.org/neisseria/> under “information” then “PCR protocol”. Various chemistries and platforms are available for sequencing and many laboratories outsource their sequencing to commercial companies.

F. Sequencing primers

Sequence using the LSM12 and SKH2 primers, but use them at a final concentration of 0.3 µM.

G. Sequencing PCR setup and cycling conditions

Reactions are performed in 20 µl volumes either in 8-well tube strips or in 96-well plates. However, some laboratories perform sequencing reactions in 10 µl volumes. Adjust each component of the reaction accordingly if 10 µl volumes are desired. If doing many reactions, it is useful to prepare a master mix of reagents equal to the number of reactions plus one for each locus with all components except for the DNA. The sequencing PCR setup and cycling conditions are shown below in Tables 43 and 44, respectively.

Table 43. Clade sequencing PCR reaction set-up

Reagent	Volume (μ l)	Comments
PCR grade water	11.0	
5X buffer	4.0	
Terminator nucleotides and polymerase*	1.0	200 μ M final concentration
100 nM primer	1.0	5 nM final concentration
Purified amplicon DNA	3.0	
Final volume	20.0	

*Note: chemistries can vary, thus optimize the reactions for your particular system. Primer concentrations and PCR set up parameters may vary depending on the type of enzyme, chemistry, and protocols used in individual laboratories. Each laboratory should optimize the protocols.

Once the reactions are setup, follow the cycling conditions in Table 44.

Table 44. Cycling conditions for sequencing PCR

25x (95°C, 10 sec); (52°C, 5 sec); (60°C, 4 min)
1x (4°C, ∞)

H. Interpretation

The DNA sequences generated are used to determine the amino acid sequence searched against the sequence database by using BLAST software (www.ncbi.nlm.nih.gov/BLAST). To further classify the strains, the diversity based on *pspA* clades is determined by comparing the amino acid sequences found for strains tested with those amino acid sequences of the clade-defining region of 24 invasive reference sequences retrieved from GenBank [Accession numbers AF071802 to AF071809 (Clade 1), AF07810 to AF071814, M74122 (Clade 2), AF071816 to AF071818 (Clades 3), AF071821, AF071824, AF071826, U89711 (Clade 4), AF071820 (Clade 5), and AF071823 (Clade 6)] (33). PspA proteins in the same clade share similar sequences and any variation of sequences within clades is restricted to single-amino-acid substitutions. Clade type can also be established by determining clusters using the DNA sequences to generate a dendrogram with software using Pearson's coefficient and the hierarchical unweighted pair group method (UPGMA). Proteins within the same clade are greater than 90% identical in sequence.

I. Quality control

Controls for PspA family 1 (Spain¹⁴-ST-18) and PspA family 2 (Spain^{23F}-ST-81) (PMEN clones http://www.sph.emory.edu/PMEN/pmen_table1.html) can be run in each PCR reaction set.

VIII. Analysis of sequence data and allele determination

After the sequence reaction products have been resolved and the data obtained from the sequencer, the trace files must be analyzed for incorrect base calls and the complementary strands must be aligned. For MLST, the alleles must be aligned and trimmed so that they correspond exactly to the regions that are used to define the alleles. For the OMPs, the sequence is translated so that the allele types or clade determination based on the amino acid sequence can be made. In addition the nucleotide changes in the domains typically responsible for penicillin resistance in the *pbp1a*, *pbp2b* and *pbp2x* genes in *S. pneumoniae* need to be determined. Various computer packages are suitable for assembling, aligning and editing the trace files from the automated DNA sequencer to create consensus sequence files suitable for these analyses.

A. MLST sequence analysis

Once consensus sequences are available, they can be uploaded to websites for allelic analysis and sequence type designations. For *N. meningitidis* MLST go to <http://pubmlst.org/neisseria/> and look under “Access main databases”, for *H. influenzae* MLST go to <http://haemophilus.mlst.net/> and use “locus query” to obtain allele types and “profile query” to obtain a sequence type, and for *S. pneumoniae* MLST go to <http://spneumoniae.mlst.net/> and use “locus query” to obtain allele types and “profile query” to obtain sequence types. Consensus sequences of each gene fragment are compared with those in the databases. The software checks that the sequences are the correct length and that they do not contain any undetermined characters. Options are available to identify the allele at a single locus, to enter an allele profile, to find isolates in the database that match or nearly match an allele profile, or to browse the database. Consensus sequences not represented in the database can be submitted as a new allele. The database curator evaluates the trace files of the sequence before assigning a number to the new allele and including it in the database. In addition, the databases have links to various programs for further data analyses.

Freeware programs that provide the capability of assembly, aligning, editing, producing a consensus sequence, and also provide MLST types and clonal complex types in one package are the Sequence Type Analysis and Retrieval System (STARS) and the Meningococcus Genome Informatics Platform (MGIP). In addition, both systems are expandable to include other species and loci. However, STARS is Linux-based, requires technical expertise to program for expansion and no longer has technical support. MGIP is a web-based interface requiring only a computer and an internet connection that allows the user to upload to the website sequence trace files and a worksheet template (<http://mgip.biology.gatech.edu/home.php>). Analyzed sequence data, including ST and CC designations, will be returned to the user within minutes of submission (12). Furthermore, MGIP will also perform OMP analysis and is expandable to include any loci the user wishes to add. At this writing, MGIP is only available for *N. meningitidis* loci, but is being expanded to include *S. pneumoniae* and *H. influenzae*.

B. Analyzing a set of sequence data using MGIP

MGIP does not require a user to register to use the website. However, if accessing the website functions as a public user any data uploaded is available for anyone to see and download. It is

thus advisable to register, which only requires a username, password, first and last name, email address, and institution.

MGIP requires two files to be uploaded in order to analyze data: 1) A zip file or compressed archive of the sequencing trace files; and 2) A spreadsheet template file in the CSV (comma separated values) format that identifies the name of the reaction, the type of assay (MLST, *porA*, etc), and primer in each well of the sequencing plate. Note that multiple typing schemes can be analyzed on a single plate.

1. Compressing (or zipping) the trace files

Uploading is done by the set, which is all of the trace file data from a 96-well plate. Do not rename the trace files. MGIP depends on the coordinates in each filename to map each trace file back to the spreadsheet. Thus, if you must rename the trace filenames, preserve the coordinates (e.g., instead of the filename 1433_B01.ab1, you can rename the file to 999_aroE_B01.ab1). In order to upload a data set:

- a. Put all of the sequencing files associated with the data set to be analyzed into a single folder.
- b. Zip the folder. In Windows, right click the folder, select "Send To" and then "Compressed "zipped" folder." (Figure 1). If the "send to" is not on the menu, then select "add to zip file" or "create archive." Any of these options will create a zip file.

There are ways to create zip files in most operating systems such as MacOS or Linux, which are very similar to the method described above. The resulting file must be a zip of the directory of trace files and can have any name.

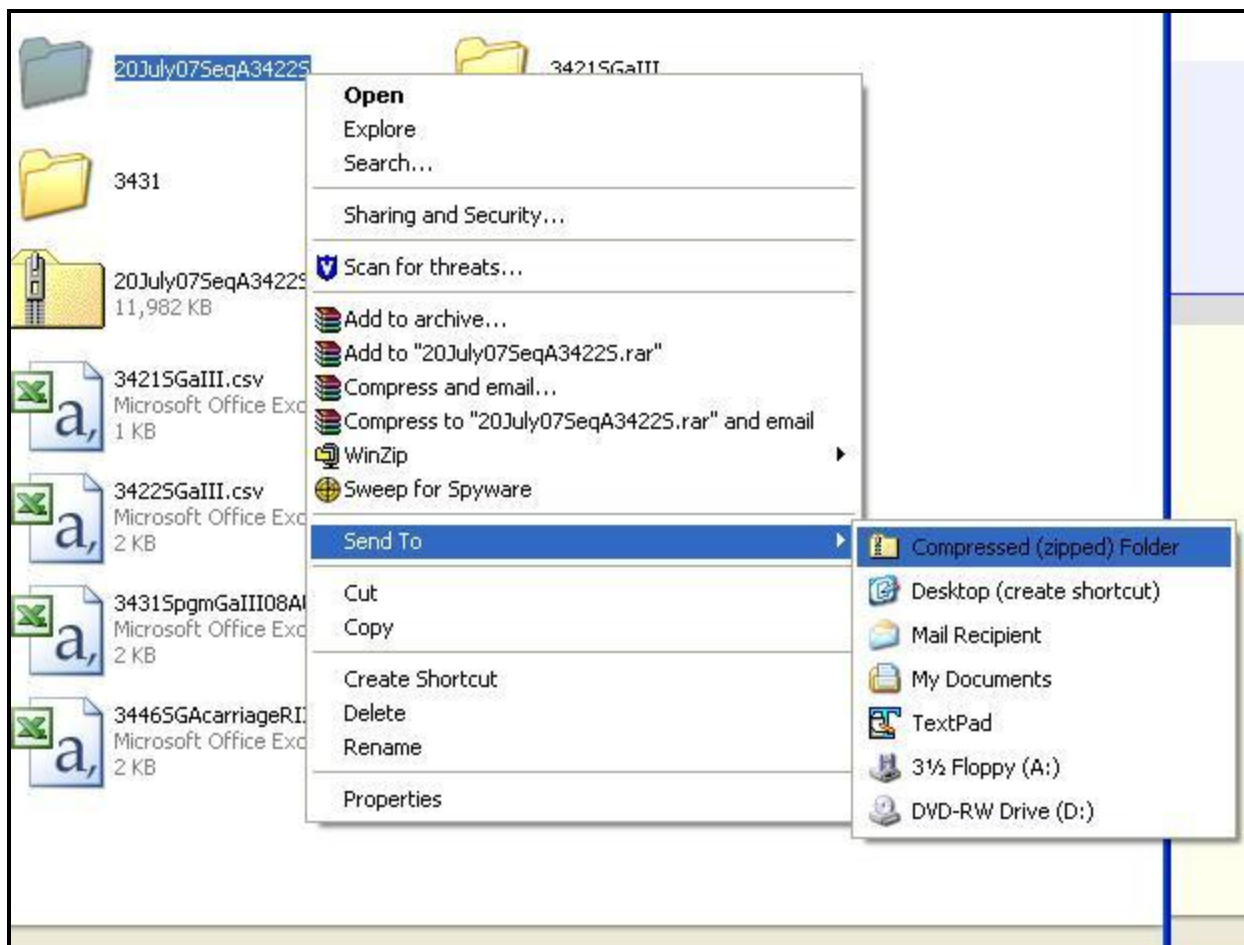


Figure 1. Creating a zip file of the sequencing data to be analyzed

2. Creating the template spreadsheet

Each cell in the spreadsheet corresponds to the appropriate well from the sequencing plate. For example, a trace file from a well might be automatically named 1433_**B01**.ab1, which corresponds to the first column, second row of your wells. Hypothetically, if the well B01 contains the locus *pgm_* from the strain M2341 and it was the forward primer, then the correct way to label the cell is M2341.MLST.pgm_.1. **The correct way to label each cell in the spreadsheet is strain.sequencingTypingMethod.locus.primers.** Any blank well should either be blank or have the word BLANK in the cell. Exact locus names and sequence typing method names can be found on the MGIP upload page at <http://mgip.biology.gatech.edu/uploadTraces.php>.

To generate an entire spreadsheet (which you may have to modify), you can use the form found on the upload page under the Automatic Spreadsheet Generation section. After viewing the resulting table from the Automatic Spreadsheet Generator, you can click the download button to save the spreadsheet. The spreadsheet must be in CSV format, which is available as a "save as..." option in Excel and in other spreadsheet programs. Therefore you

can convert any spreadsheet you have to a CSV format by opening it in a spreadsheet program and saving it as a CSV.

3. Uploading the files

Upload the zipped sequence files and template spreadsheet at: <http://mgip.biology.gatech.edu/uploadTraces.php>. After uploading, click “Analyze Trace Files”.

C. Viewing results

1. By set

Once the files are analyzed, the alignments and results can be viewed by clicking on the name of the set of sequences. Or starting from the main page, click “view results” and click on the name of the set of sequences.

From the first box, choose the data set. The sets are named after your original zip file (Figure 2).

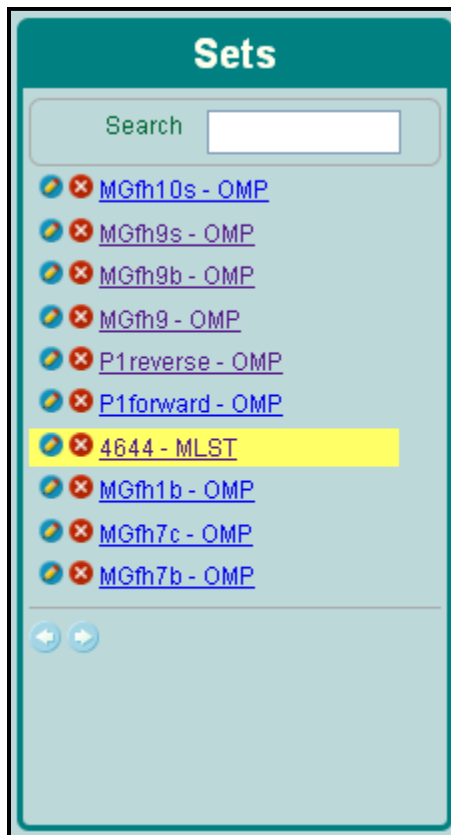


Figure 2. Selecting a set of sequence data to view

An alignment's blast results, fasta format sequence, or the actual trace files that were uploaded can be viewed (Figure 3). Find the alignment's strain/locus and click the options link to view the aforementioned choices. An editing function is available to adjust incorrect base calls, if necessary. In addition, files that were unable to be fully analyzed and need the attention of the user are marked with a red flag.

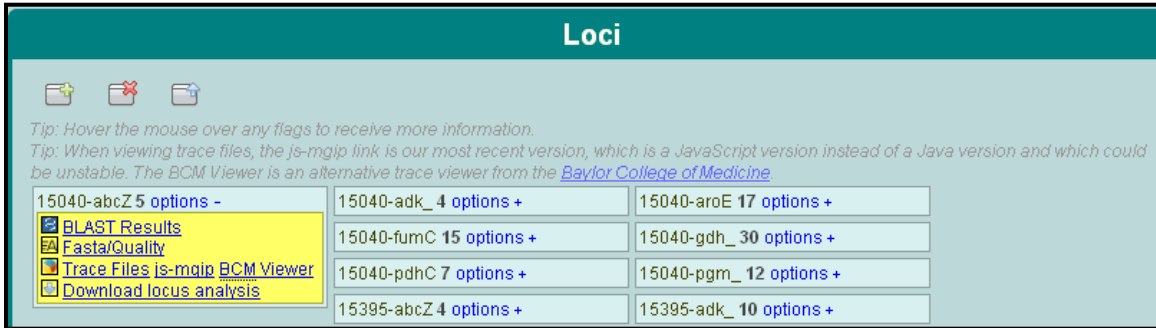


Figure 3. Allele calls and options for viewing analyzed data

2. By strain

The user can view their strains in a table alongside their alleles. Click the "strain table" link from the main menu. Strains are listed on the left, alleles in the middle, and lastly, the derived Sequence Type is on the right. If enough information is present, then the database will indicate the ST of the isolate. If insufficient data is present to designate a ST, then a list of all possible STs is given (Figure 4). In the case of a combination of alleles that are not present in the database, a designation of "novel strain" will be displayed.

The screenshot shows a web interface titled "Strain Table". At the top, there are three icons: a folder, a magnifying glass, and a download arrow. Below the icons, the text "Your strains" is displayed. The main content is a table with columns for strain, abcZ, adk, aroE, fumC, gdh, pdhC, pgm, ST, and CC. The first row shows strain 15534 with specific allele counts and a dropdown menu for ST (ST-437 ST-41/44 complex/Lineage 3) and CC (ST-41/44 complex/Lineage 3). The following rows show other strains with "View ST Possibilities" and "View CC Possibilities" links.

strain	abcZ	adk	aroE	fumC	gdh	pdhC	pgm	ST	CC
15534	9	6	9	17	9	6	9	ST-437 ST-41/44 complex/Lineage 3	ST-41/44 complex/Lineage 3
15508	9	6	9	129	9	6	9	View ST Possibilities	View CC Possibilities
15563	1	5	13	53	26	41	3	View ST Possibilities	View CC Possibilities
15040	5	4	17	15	30	7	12	View ST Possibilities	View CC Possibilities
15395	4	10	5	4	6	3	8	View ST Possibilities	View CC Possibilities
15564	4	10	5	4	6	3	8	View ST Possibilities	View CC Possibilities

Figure 4. Viewing STs by strain

D. OMP sequence analysis

See above for assembling, editing, and aligning sequences to produce a consensus sequence. Once the consensus sequences are created, they can be directly uploaded to the *Neisseria.org* website for typing:

1. PorA

Neisseria meningitidis PorA variable region database: <http://pubmlst.org/neisseria/PorA/>, click on “Single sequence query” or “Batch sequence query” under the heading “Identify PorA variable regions to determine the VR1 and VR2 types.”

2. PorB

Neisseria meningitidis PorB typing database: <http://pubmlst.org/neisseria/porB/> click on “*porB* (NEIS2020) [whole coding region]” or “*porB* [partial coding sequence]” under the heading “Identify *porB* alleles” to type the class and loop regions.

3. FetA

Neisseria meningitidis FetA variable region database: <http://pubmlst.org/neisseria/FetA/>, click on “Single sequence query” or “Batch sequence query” under the heading “Identify FetA variable regions” to determine the FetA type.

4. fHbp

Factor H-binding protein database: <http://pubmlst.org/neisseria/fHbp/>, click on “Single sequence query” or “Batch sequence query” under the heading “Identify fHbp alleles and peptides” to determine the fHpB type.

Consensus sequences not represented in the database can be submitted as a new allele. The database curator evaluates the traces of the sequence before assigning a number to the new allele and including it in the database. MGIP (see above MLST Analysis section) can also be used to obtain allele types for each of these OMPs.

IX. Pulsed-field gel electrophoresis

A. Overview

Pulsed-field gel electrophoresis (PFGE) is an agarose gel-based typing method that assesses strain inter-relatedness by comparison of complete genomes and has been applied to at least 40 pathogens (25). PFGE involves digesting genomic DNA with a restriction enzyme that cleaves chromosomal DNA infrequently to produce a small number (11-41) of fragments of different sizes. The resulting fragments are usually large and unable to be separated efficiently by conventional uni-directional electrical field gel electrophoresis. Two of the most commonly used methods to size-fractionate these large digestion products for PFGE are the contour-clamped homogenous electric field (CHEF) and field inversion gel electrophoresis (FIGE). CHEF uses a hexagonal array of 24 electrodes that produce an alternating 120° angle uniform electrical field. FIGE is based on a conventional electrophoresis in which the electric field is periodically inverted by 180° (25). In both CHEF and FIGE, the electric field used for PFGE is periodically alternated or pulsed to facilitate the migration of the DNA fragments through the gel. Larger fragments migrate through the gel slower than smaller fragments creating a size-based banding pattern that has larger fragments near the top of the gel and smaller fragments near the bottom of the gel (4, 14, 25). The resulting banding pattern can be analyzed by visual inspection or by using a computer program to determine differences in the banding patterns between isolates.

PFGE is a highly discriminatory subtyping tool and is particularly suitable for outbreak or cluster investigations. PFGE is used in combination with epidemiologic information to help identify outbreak isolates and to determine the relationships among isolates associated with the outbreak or cluster. The etiological agents in outbreaks are often clonal and produce indistinguishable PFGE patterns, but point mutations and insertions or deletions can occur during an outbreak that lead to a PFGE pattern difference of two or three fragments in isolates linked to the outbreak. Guidelines have been developed to interpret the minor variations in PFGE patterns from closely related strains, but these guidelines do not take into account the total genetic variation observed within the circulating population (27). Isolates that differ by two or three fragments are still considered epidemiologically linked subtypes of the same strain.

B. Preparation for PFGE

Time required for procedure

PFGE requires approximately 28-30 hours once overnight cultures are available, thus time management is important in planning the procedure.

Equipment

Electrophoresis equipment

CHEF system with pump and cooling unit module

Documentation system equipped with a camera that can provide computer compatible images

37°C incubator

37°C water bath

56°C water bath

50°C water bath

Orbital/shaker water bath

Turbidity meter, spectrophotometer, or McFarland standards

Scales/balances to measure solid reagents

Microwave to melt agarose

Select reagents

Appropriate agar plates for growing up cultures (see below)

Rapid resolution agarose (rapid resolution of DNA and PCR products between 1 kb and 50 kb by electrophoresis) for making plugs

A serine protease/endopeptidase such as Proteinase K (liquid or powder)

10X 1 M Tris/borate/EDTA, pH 8.0 (TBE)

1 M Tris-HCl, pH 8.0

0.5 M EDTA, pH 8.0

N-Lauroyl sarcosine sodium salt

Ethidium bromide, 10 mg/ml

Restriction enzyme and enzyme-specific buffer

Sterile distilled deionized H₂O (dd H₂O)

Supplies

PFGE plug molds (reusable or disposable)

Gel comb and holder
Casting frame, platform, and leveling table
Sterile clear polystyrene 12 X 75 mm tubes with caps
Sterile 1.5 ml microcentrifuge tubes
Sterile 2 ml round bottom tubes
Scalpels or single edge razor blade
Glass slides
Sterile disposable Petri dishes or large glass slides
Flat spatula
Container to stain gel
Sterile screw cap flasks or bottles
Sterile graduated cylinders
Sterile 50 ml screw cap centrifuge tubes
Sterile pipettes
PPE (gloves, eye, and respiratory protection)
Heat-resistant gloves

C. Reagent and solutions

Tris-HCl, 1.0 M, pH 8.0 (1 L)

1. Dissolve 121 g Tris base in 800 ml ddH₂O.
2. Adjust to pH 8.0 with concentrated HCl.
3. Mix and add sterile distilled H₂O to 1 L.
4. Autoclave or filter sterilize.

EDTA, 0.5 M, pH 8.0 (1 L)

1. Dissolve 186 g EDTA in 700 ml ddH₂O.
2. Adjust pH to 8.0 with 10 M NaOH (~50 ml).
3. Add sterile distilled H₂O to 1 L.
4. Autoclave or filter sterilize.

10% Sodium lauroyl sarcosine (Sarcosine)

1. Add 10 g of N-Lauroyl sarcosine salt to 100 ml ddH₂O.*
2. Filter through a .22 micron membrane.

*Eye and respiratory protection should be worn when weighing powdered Sarcosine.

Proteinase K (20 mg/ml)

1. Add 100 mg of Proteinase K powder to 5 ml ddH₂O.
2. Filter sterilize, aliquot, and store at -20°C.
 - Alternatively, 5 ml of a 20 mg/ml solution is available commercially.

Ethidium bromide (EtBr), 10 mg/ml

1. Dissolve 0.2 g ethidium bromide in 20 ml ddH₂O.
2. Mix well and store at 4°C in the dark in 1 ml aliquots.
 - EtBr is a powerful mutagen and should be handled with care.

Cell suspension buffer (100 mM Tris and 100 mM EDTA, pH 8.0)

1. 10 ml of 1 M Tris, pH 8.0 (sterile solution, available commercially).
2. 20 ml of 0.5 M EDTA, pH 8.0 (sterile solution, available commercially).
3. Dilute to 100 ml with ddH₂O, not tap water.
4. Can be stored at room temperature (20-25°C) for several months.

Plug wash TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0)

1. 10 ml of 1 M Tris, pH 8.0.
2. 2 ml of 0.5M EDTA, pH 8.0.
3. Dilute to 1000 ml with ddH₂O, not tap water.
4. Can be stored at room temperature (20-25°C) for several months.

TBE (Tris/borate/EDTA) electrophoresis buffer, 10X stock solution*

1. To 800 ml of ddH₂O add:
 - 108 g Tris base (890 mM).
 - 55 g boric acid (890 mM).
 - 40 ml 0.5 M EDTA, pH 8.0 (20mM).
2. Add ddH₂O to 1 L.
3. Autoclave or filter sterilize.

*To make 0.5X TBE working solution, add 100 ml 10X TBE to 1.9 L distilled H₂O.

Agarose (1.0% rapid resolution agarose)

1. Add 250 mg of rapid resolution agarose to 23.5 ml of 0.5X TBE in a 250 ml flask and microwave for 1 min, swirl, and then microwave in 15 sec increments with swirling until the agarose is fully melted and the solution is clear.
2. Place in 56°C water bath to keep agarose from hardening.
3. Agar can be stored at room temperature for several months and can be re-melted and used again.

Cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0 and 1% Sarcosine)

1. Add 25 ml of 1 M Tris, pH 8.0.
2. Add 50 ml of 0.5 M EDTA, pH 8.0.
3. Add 50 ml of 10% sodium lauroyl sarcosine (Sarcosine), membrane-filtered.
4. Dilute to 500 ml with ddH₂O, not tap water.

D. Performing PFGE**Growth of bacteria**

A pure culture of each isolate is grown on trypticase soy agar plates supplemented with 5% sheep blood for *N. meningitidis* and *S. pneumoniae*, or chocolate agar plates supplemented with hemin and NAD for *H. influenzae* in a humidified incubator for 18-24 hours at 37°C with 5% CO₂. If the isolate does not look pure or if the growth is not sufficient, subculture in a humidified incubator for 18-24 hours at 37°C with 5% CO₂.

Preparation of gel plugs

Fill water baths to the correct water level with deionized water. Turn on shaking bath at 54°C and non-shaking baths at 56°C and 37°C. Prepare or re-melt plug agarose (see above) and hold at 56°C in the water bath until used.

1. Label and set up a 12 x 75 mm plastic capped tube containing 2 ml of cell suspension buffer (CSB) for each isolate and for a control strain and a size marker.
2. Using a 1 µl disposable loop, gently harvest enough growth to make a suspension of cells reading 0.48-0.52 using a turbidity meter or spectrophotometer at O.D.₂₈₀. If a turbidity meter or spectrophotometer is not available, this amount of growth is approximately a 0.5 McFarland standard.
 - To make a uniform suspension, rub the growth onto the side-wall of the tube just above the level of the CSB until it washes into the liquid. Before making the turbidity reading, gently mix the tube by finger-tapping until the suspension appears homogeneous within the tube.
 - The bacterial concentration needs to be adjusted precisely. Achieving uniform concentrations is critical for reproducibility, resolution of the bands of similar size, and for comparison between different strains.
3. Add 400 µl of each cell suspension to a labeled 1.5 ml microcentrifuge tube. Then add 20 µl of Proteinase K (20 mg/ml) to the side-wall of each tube above the suspension to avoid premature lysis of the cells.
4. Add 400 µl of melted plug agarose and mix gently by pipetting the mixture up and down several times. Add 400 µl of the mix to fill a well of a 10-well reusable or disposable plug mold. Repeat for each suspension, filling all of the designated wells. Let the plugs harden for 5 min at 4°C or 15 min at room temperature (25°C). Extra plugs can be made from the left-over cell suspensions, if desired.
 - Avoid making bubbles and do not vortex. To prevent hardening of the agarose, keep the flask in a beaker of water at 56°C until all of the plugs are made.
5. Add 5 ml of cell lysis buffer (CLB) and 133 µl Proteinase K (20 mg/ml) to labeled 50 ml centrifuge tubes.
6. Open the plug molds and push a plug into each of the designated tubes of CLB and close the caps tightly.
7. Incubate for 1.5 to 2 hours in a 54°C shaker water bath set at 75 strokes per min.

Washing the plugs

1. Pre-warm 500 ml of sterile reagent grade water and a liter of plug wash TE buffer in a 50°C water bath.
2. Pour off CLB and add 15 ml sterile distilled water to each plug.
3. Incubate for 15 min in a 50°C shaker water bath set at 150 strokes per min.
4. Replace water with 15 ml of plug wash TE buffer and incubate for 20 min in a 50°C shaker water bath set at 150 strokes per min. Decant buffer and repeat 4X.
5. Store plugs in tubes with 2-5 ml of plug wash TE buffer at 4°C until ready for restriction digestion. Plugs are usable for up to 4 months but it is preferable to use them as soon as possible after the washing step.

Restriction digestion

The restriction enzymes to use are dependent on the type of the bacteria being tested. For *N. meningitidis* use *NheI*, and for *H. influenzae* and *S. pneumoniae* use *SmaI*. Note that for further resolution, isolates can be cut with an additional enzyme, but do not cut with two enzymes in the same reaction. This is useful if other molecular characterization such as MLST will not be performed. *SpeI* can also be used for *N. meningitidis* and *XmaI* for *H. influenzae* and *S. pneumoniae*.

1. Sterilize a single-edge razor blade and a clean 3 x 2 inch glass slide with 70% alcohol.
2. Add 180 µl of sterile reagent grade water and 20 µl of the 10X restriction buffer for the enzyme to be used to a 1.5 ml microtube for each isolate.
3. Using a narrow spatula, remove a plug from the Plug Wash TE buffer and place it on the glass slide.
4. Using the razor blade cut off and discard any uneven edges that may prevent the plug from fitting in the gel well.
5. Cut two 1-2 mm thick slices from the plugs, including the standard plug and place them into one of the 1.5 ml microcentrifuge tubes and incubate at 37°C for 15 minutes (30°C for *SmaI*).
 - The unused portions of the plugs can be returned to their storage tubes and refrigerated.
 - The overall goal is to cut plug slices that are sufficient in size to easily manipulate, fit in the well, and that contain enough DNA to create a clear, easily readable banding pattern. The size of the band may need to be optimized for the bacteria being tested.
6. Carefully aspirate the liquid from the tubes with a pipette, taking care not to damage or remove the plug slice.

7. Replace the liquid with 170 μ l of sterile distilled water, 20 μ l of 10X restriction buffer, and 50 units of the appropriate enzyme.
8. This can be prepared as a “master-mix” or each reagent can be added separately with gentle mixing.
9. Incubate plug slices in a 37°C (30°C for *Sma*I) water bath for 1.5-3 hours.
 - Note that *Sma*I loses 50% activity after one hour at 37°C.
10. Carefully aspirate the liquid from the tubes and add 150 μ l of 0.5X TBE buffer.

Gel preparation and loading

1. Make agarose (1.0% rapid resolution agarose) and place in a 56°C water bath until use.
 - Remove 2 ml to a sterile tube and hold at 56°C to use later in the procedure.
2. Assemble the gel-casting mold and make sure it is level on the leveling stand. Adjust the height of the comb teeth so that, when upright, the teeth touch the gel platform.
3. Once the comb is in place, add molten agarose to the apparatus until the agarose is nearly to the top of the teeth of the comb. Take care that the agarose is well-mixed to ensure a uniform gel.
4. Once the gel has hardened, carefully remove the comb.
5. Lay the comb flat and using a narrow spatula remove the plug slices from each tube and place each one at the bottom of its designated comb tooth and allow plug slices to air-dry at ambient temperature for 15 minutes.
 - Run one plug slice per isolate and save extra plug slices.
6. Position the comb in the upper pair of slots of the gel casting stand and slowly pour the molten agarose from the flask into the mold and let the gel solidify for 25-30 min.
7. Remove the comb and seal the wells with the 2 ml of molten agarose set aside.
8. Transfer the gel on its platform into the chamber, be sure it is positioned properly in its frame and is immersed in the 0.5X TBE buffer. Typically 2 L of buffer is required for the gel to be submersed with 1 cm of buffer over the gel. Close the cover of the chamber and begin the run after setting the following parameters on the power supply:

Table 45. Electrophoresis parameters for PFGE

Initial switch time	2.2 seconds
Final switch time	35 seconds
Run time	18 hours
Angle	120 degrees
Gradient	6.0 volts/cm
Temperature	14°C
Ramping factor	Linear

Staining the gel and documenting the image

1. Turn off the equipment at the end of the run.
2. Set up a tray containing EtBr staining solution (400 ml of deionized water with 40 µl of [10 mg/ml] EtBr). Note that EtBr is a carcinogen and gloves and eye protection should be used.
 - It is important that the gel box and cooling unit be cleaned after each run. To do this drain the buffer and pour 1 L of distilled H₂O into the gel box and circulate for 2-3 min. Drain the distilled H₂O and dry the apparatus.
3. Remove the gel from the chamber and platform and immerse it in staining solution.
4. Cover the tray to shield it from light and place it on a rotator or oscillating platform shaker and rotate slowly for 30 min.
5. Pour off the EtBr staining solution according to safety regulations and destain the gel with 500 ml cold deionized water for 30 min on the rotator. Remove water and repeat 2X.
6. Transfer the gel to an imaging system to photograph the image under UV light and save it on the computer as a tagged image file format (TIFF) file for further analysis. TIFF is the preferred format because of its greater resolution but the files can be saved as .jpeg if file size is a problem. If an imaging system is unavailable, a photo of the agarose gel can be taken.

Analysis of image and interpretation

Visual analysis can be used to directly compare the band patterns of a limited number of isolates on a gel. However, several computer software programs have been developed to analyze gel images, compare multiple gel images, match banding patterns, construct dendrograms, and store gel data to allow for more accurate and sophisticated data analyses. The banding patterns are analyzed with the Dice coefficient, an optimization of 1.0%, and a position tolerance of 1.5% for the band migration distance. A PFGE-based clonal group is defined as a group of isolates with genetically related PFGE patterns. In general, the PFGE patterns of strains categorized within a clonal group have six or fewer differences from each other and $\geq 80\%$ genetic relatedness on the

dendrogram. When comparing isolates associated with an outbreak, it is helpful to include isolates that do not have an epidemiological link to the outbreak to determine if the outbreak clone is currently circulating within the population or if it has been recently introduced. After results are obtained using the software, it is recommended that the results be visually compared to the bands on the gel to be sure the results make sense.

For *S. pneumoniae*, comparisons of PFGE profiles to those of major global clones should be made by using the profiles described by the Pneumococcal Molecular Epidemiology Network (PMEN) or reference isolates can be requested from this group to include in PFGE runs. (http://www.sph.emory.edu/PMEN/pmen_clone_collection.html).

E. Technical considerations

Use of a standard

A standard or molecular weight size marker is used to provide an accurate fragment size estimate and assists with normalization and correction of gel patterns due to variations in electrophoresis. It should be included in each gel run to allow for inter- and intra-gel comparison of isolates. A common commercial marker is the lambda bacteriophage, which consists of concatamers of the bacteriophage lambda DNA. This marker is available in both high molecular weight and low molecular weight varieties and some laboratories use both on a gel. However, inconsistency in the DNA concentration and quality of the commercially available lambda ladders has been observed between vendors and in lot-to-lot variation of the ladder. Alternatively, a bacterial strain standard can be used that produces bands of known molecular weight. The bacterial standard does not necessarily have to be of the same strain being tested. The caveat with using a bacterial strain is that it must be prepared in the lab and mutations or genomic rearrangements can occur to change a cleavage site, thus changing the size of two or more of the bands. Regardless of the type of marker used, 3 lanes of the gel spaced evenly apart, but not on the edge of the gel, should be used for markers.

Care needs to be taken in the interpretation of results, especially in inter-laboratory studies, as small differences in electrophoresis conditions can alter the distance migrated by each band, complicating the comparison between isolates in different gels.

Quality control

Quality control (QC) is a measure of precision and a way to ensure that test results are correct, consistent and reproducible. Thus, QC for reagents used in a test is critical to the overall result and interpretation of the test. QC measures are listed below:

1. Use clean glassware that is free of detergents to prepare reagents.
2. Use sterile distilled water, not tap water, to prepare reagents.
3. Perform regular maintenance on equipment; keep equipment clean; pipettors calibrated; and keep a maintenance/calibration log.

4. New plugs to be used as standards should be run as an unknown to verify that the new standard produces the same banding pattern and intensity as the old standard.
5. Test new lots of restriction enzyme to verify that the new enzyme is working properly and produces a consistent pattern.
6. Sterilize the following reagents by filtration or by autoclaving before use: 10% Sarcosine, 1 M Tris-HCl, pH 8.0; 0.5 M EDTA, pH 8.0; and 10X Tris/Borate/EDTA buffer (TBE).

Troubleshooting

Unexpected results can be attributed to equipment failure, incorrect calculations, and improperly made reagents. When errors arise in an assay, it is helpful to review the equipment, reagents, and steps used in the procedure to look for errors. Listed below are some common problems encountered when performing PFGE with suggestions on how to correct them.

1. No power to equipment:

- Check that equipment is plugged in and that the fuse in the back of the power supply is functional.

2. Gel lanes are curved or slanted:

- Examine and replace any damaged electrodes.
- Check chamber for foreign objects and remove them if present.
- Be sure agarose plugs are firm enough for loading as fragmented plugs will not run correctly.
- Verify that the electrophoresis chamber is level. The gel must be poured level.
- Verify that the gel is securely loaded into the gel chamber.

3. Bands have poor resolution:

- Fresh buffer should always be used and the buffer tank and circulation lines should be cleaned and flushed after each use.
- The level of the buffer used to run the gel could be incorrect or have changed during the run creating an electrical field that is not appropriate; therefore check buffer levels carefully and add or drain buffer as needed.
- High buffer concentration elevates the temperature of the run buffer. If this is suspected, remake the running buffer
- Check chiller to make sure it is working properly to cool the run buffer.
- Remove any kinks in the tubing. This may reduce the flow rate of the pump which would increase the temperature of the buffer.
- The percentage of agarose used may have been too low.

4. Faint bands or no bands on gel:

- Low amounts of DNA or degraded/sheared DNA in the plugs will not produce good results. If this problem is suspected, remake the plugs.
 - The bacteria in the plug may not have been completely lysed. Verify that a sufficient amount of Proteinase K was used and that the cell lysis buffer was made properly and used at the correct temperature.
 - Verify that the gel was stained with EtBr made at the correct concentration.
5. The gel has unspecific signal in areas where no signal is expected (background):
 - Plugs may not have been washed thoroughly. Rewash the plug and repeat restriction digest.
 - The DNA concentration in the plug was too high. Use a thinner plug slice or remake the plug with the proper cell concentration.
 6. Faint bands that appear on the gel between normal bands:
 - The agarose plug may not have been properly digested with restriction endonuclease. Digest the plug again making sure that the correct concentration of enzyme is used for the proper length of time, that the proper enzyme buffer was used, and that the plug slice is completely submerged during digestion.
 7. Specks appear in stained gel.
 - Verify that the gel does not contain undissolved agarose. If so, remake the gel with agarose completely dissolved and thoroughly mixed.
 - Clean the surface of imager and lens to remove particles, if present.
 - Wear non-powdered gloves during the procedure as powdered gloves leave powder that gets on the gel and fluoresces when exposed to UV light.
 - The EtBr may be incompletely dissolved.

Appendix 1

Fast DNA extraction protocol for *N. meningitidis* and *H. influenzae* (gram-negative)

1. Dispense 1.0 ml of 10 mM Tris (pH 8.0) buffer into vials and label.
2. Harvest colonies from 18-24 hour pure cultures of *H. influenzae* and/or *N. meningitidis* using a sterile polyester or rayon-tipped swab and swirl the swab in the Tris buffer to make a turbid suspension (equivalent to McFarland 3.0 standard). Be careful not to pick up pieces of agar on swab.
3. Vortex briefly and boil cell suspension at 100°C for 10 minutes.
4. Proceed immediately with PCR or store at -20°C.

The procedure above is not vigorous enough to completely lyse the more robust cell wall of *S. pneumoniae*, a gram positive organism. Use the protocol below to prepare DNA from isolates of *S. pneumoniae*. Also use the protocol below if the identity of the bacterial isolate is unknown.

Fast DNA extraction protocol for *S. pneumoniae* (gram-positive)

1. Dispense 300 μ l of 0.85% NaCl into vials and label.
2. Harvest colonies from 18-24 hour pure cultures of *S. pneumoniae* using a sterile polyester or rayon-tipped swab and swirl the swab in the 0.85% NaCl to make a turbid suspension (equivalent to McFarland 3.0 standard). Be careful not to pick up pieces of agar on the swab.
3. Vortex briefly and incubate at 70°C for 15 minutes.
4. Microcentrifuge at 12,000 x g for 2 minutes and remove the supernatant.
5. Re-suspend in 50 μ l TE buffer (10 mM Tris-HCl, 100 μ M EDTA, pH 8.0) and add 10 μ l mutanolysin (3000 U/ml)* and 8 μ l of hyaluronidase (30 mg/ml)**
6. Incubate at 37°C for 30 minutes up to 18 hours (overnight).
7. Heat-inactivate the enzymes in the suspension by boiling at 100°C for 10 minutes.
8. Microcentrifuge at 12,000 x g for 4 minutes and remove supernatant for use as DNA template.
9. Proceed immediately with PCR or store at -20°C.

*Mutanolysin (10,000 U). Dilute in 3.3 ml of TE buffer to make 3000 U/ml stock solution, store at -20°C as 500 μ l aliquots.

**Hyaluronidase (100 mg). Dilute in 3.3 ml of TE buffer to make 30 mg/ml solution, store at -20°C as 500 μ l aliquots.

Methods for DNA extraction that will provide purified DNA can be found in Chapter 10: PCR Methods.

Appendix 2

Analysis of PCR products on an agarose gel

To check for a successful PCR amplification, run an aliquot of the end-products on a 1% agarose gel.

1. Briefly spin the PCR plate or tubes at 500 x g to ensure all liquid is at the bottom of the wells.

2. Mix 5 μ l of PCR reaction with 1 μ l of 6X loading dye (see Table 46 for protocol). It is important to include DNA size markers in one of the wells.
3. Make a 1% agarose gel. Add 1 g of electrophoresis grade agarose to 100 ml of 1X Tris/Borate/EDTA (TBE) buffer (see below) in a 250 ml flask and melt the agarose in a microwave. Microwave for 1 min, swirl, and then microwave in 15 sec increments with swirling until the agarose is fully melted and the solution is clear. Be cautious as the molten agarose will be extremely hot. Once the agar has cooled to approximately 55°C add 1-2 μ l of EtBr (see below) and swirl. Pour into a gel casting box, insert the comb, and allow time for hardening. Remove the comb after the gel has hardened.
 - a. EtBr is a powerful carcinogen and must be handled with care.
4. Add 1X TBE buffer to the gel box until the buffer is just over the surface of the gel and pipette the DNA/loading dye mixtures into the wells.
5. Electrophorese the gel at 50-100 volts for 15-20 minutes or until the Bromophenol Blue dye band is halfway down the gel. The dye runs at approximately the same rate as a 500 base-pair DNA fragment.
6. Visualize the gel under a UV light and print out or save the image, if possible.
7. Each reaction should give a single band. If multiple bands are consistently present, annealing temperature optimization may be required.
8. Store the remainder of the amplicon at -20°C unless proceeding directly to DNA purification.

Table 46. Protocol for making 6X loading dye

Reagents	Needed for 10mls	Notes
2.5% Ficoll 400	0.25 ml	
11 mM EDTA, pH 8.0	1.1 ml 0.1 M EDTA	See recipe below
3.3 mM Tris-HCl, pH 8.0	0.3 ml 0.1 M Tris-HCl	See recipe below
0.017% SDS	0.17 ml 1% SDS	1 g SDS* to 100 ml sterile distilled H ₂ O for 1% SDS solution
0.015% Bromophenol Blue	0.15 ml 1% Bromophenol Blue	0.1 g Bromophenol Blue in 10 ml sterile distilled H ₂ O for 1% solution
sterile distilled H ₂ O	8.03 ml sterile distilled H ₂ O	Make 0.5-1.0 ml aliquots of the batch of 6X loading dye

*Eye and respiratory protection should be worn when weighing out powdered SDS.

Stock solutions for reagents:

EDTA, 0.5 M, pH 8.0 (100 ml)

1. Dissolve 18.6 g EDTA in 70 ml ddH₂O.

2. Adjust pH to 8.0 with 10 M NaOH (~5 ml).
3. Add ddH₂O to 100 ml.
4. Autoclave or filter sterilize.

EDTA, 0.1 M, pH 8.0 (100ml)

1. Dissolve 3.7 g EDTA in 70 ml ddH₂O.
2. Adjust pH to 8.0 with 10 M NaOH.
3. Add ddH₂O to 100 ml.
4. Autoclave or filter sterilize.

Ethidium bromide (EtBr), 10 mg/ml

1. Dissolve 0.2 g ethidium bromide in 20 ml ddH₂O.
2. Mix well and store at 4°C in the dark in 1 ml aliquots.
 - EtBr is a powerful mutagen and should be handled with care.

Tris-HCl, 0.1 M, pH 8.0

1. Dissolve 1.2 g Tris base in 80 ml ddH₂O.
2. Adjust to pH 8.0 with concentrated HCl.
3. Mix and add sterile distilled H₂O to 100 ml.
4. Autoclave or filter sterilize.

TBE (Tris/borate/EDTA) electrophoresis buffer, 10X stock solution*

1. To 900 ml of ddH₂O add:
 - 108 g Tris base (890 mM).
 - 55 g boric acid (890 mM).
 - 40 ml 0.5 M EDTA, pH 8.0 (20mM).
2. Add ddH₂O to a total volume of 1000 ml.
3. Autoclave or filter sterilize.

*10X TBE will have to be diluted 1:10 to 1X in ddH₂O before use.

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CHAPTER 13

Quality Control/Quality Assurance

Quality Control/Quality Assurance (QC/QA) can be defined as the set of planned and systematic activities focused on providing confidence that quality requirements will be fulfilled. It covers a wide range of matters that influence the quality of a product or service. In a medical laboratory, the quality can be defined as accuracy, reliability, and timeliness of the reported test results (1). QC refers to those measures that must be included in each assay to verify that the test is working properly. QA is defined as the overall program that ensures that the final results reported by the laboratory are as correct and accurate as possible.

I. Negative consequences of laboratory errors

This broad concept applies to any kind of laboratory testing, including diagnostic testing for bacterial meningitis. Inaccurate meningitis diagnostic results can have significant consequences at the patient care or public health level. At the patient care level, errors can lead to:

- Failure to provide proper treatment to the patient
- Unnecessary treatment, treatment complications, or additional expenses
- Delay in correct diagnosis
- Additional and unnecessary diagnostic testing

At a public health level, laboratory errors on the species, serotype or serogroup identification, as well as antibiotic susceptibility profiles, can impact a cornucopia of public health decisions on the following matters:

- Delay in determining when the epidemic threshold has been reached and implementing public health measures
- Inadequate national control measure recommendations or treatment algorithms
- Inappropriate choice of antibiotics or vaccines

These consequences result in increased cost in time, personnel effort, other resources, and poor patient outcomes in terms of morbidity and mortality.

II. Quality management system

To achieve the highest level of accuracy and reliability, standard QC/QA testing procedures and conditions must be practiced in laboratories on an every day basis. A quality management system, which oversees the entire system, is very important for achieving optimal laboratory performance. Laboratory processes can be grouped into pre-examination, examination, and post-examination categories. Quality management measures should be applied during the entire path of workflow that begins with the patient and ends in interpreting and reporting results. The quality management system is not only concerned with monitoring QC/QA programs, but should

also include administrative considerations that may indirectly influence the quality and efficiency of the laboratory operation.

A widely used quality management system model organizes all of the laboratory activities into twelve quality system essentials, which are a set of coordinated activities that serve as building blocks for quality management (2):

1. Organization and supervision
2. Personnel
3. Equipment
4. Purchasing and inventory
5. Documents and records
6. Process control
7. Information management
8. Occurrence management
9. Assessment
10. Customer service
11. Process improvement
12. Facilities and safety

Some of these elements constitute management requirements (i.e., organization, documents and records, and purchasing and inventory) while other constitute technical requirements (personnel, equipment, and process control with the examination procedures) (3). Many of the twelve quality system essentials overlap each other (i.e., there is a close relationship between documents and records, and information management).

A. Quality systems essentials for meningitis diagnostic laboratories

Most of the quality systems essentials described above are not specific to the meningitis diagnostic laboratory and should apply to all laboratory disciplines regardless of the nature of the specimens, pathogens, or assays. However, some specific meningitis laboratory quality assurance measures can be highlighted. Many of them are detailed again in the respective chapters of this manual.

1. Organization and supervision

The structure and management of the laboratory must be organized so that quality management policies can be established and implemented. The laboratory should prepare an organizational chart that reflects the hierarchy and lines of authority with functions and responsibilities of each post. The current duties and responsibilities of staff should be specified in written job descriptions including training required and necessary experience. The director's commitment is crucial. A quality manager should be designated to ensure the implementation and monitoring of the quality policies.

2. Personnel

The most important laboratory resource is a competent, trained, and motivated staff. Continuous education opportunities should be offered to the staff and recorded, especially if new tests or methods are introduced. Regular competency assessment and proficiency testing should be conducted and documented. This can be done by direct observation of the personnel, records monitoring, and/or by analyzing the quality control or the external quality assessment results.

3. Equipment

Specimen identification and characterization requires many types of equipment, such as microscopes, incubators, autoclaves, biosafety cabinets, refrigerators, freezers, water baths, automated identification systems, or antibiotic susceptibility systems. Choosing the right equipment, installing it correctly, assuring that the staff is properly trained to use the equipment, and assuring that the new equipment works properly and receives proper maintenance are crucial. Equipment manuals should be available in the laboratory area for easy reference. An inventory of equipment including records of maintenance and repair should be maintained.

4. Purchasing and inventory

Proper management of purchasing and inventory of reagents, media, and supplies can produce cost savings in addition to assuring accurate and timely reporting of laboratory results. The procedures should be written and implemented to assure that all reagents and supplies are correctly selected, purchased, used, and stored in a manner that preserves integrity and reliability. The inventory should be kept up to date including information on reception, storage, and issuance. Package inserts and Material Safety Data Sheets (MSDS) should be archived as part of records keeping.

5. Documents and records

Documents provide written information about policies, processes, and testing procedures and should be stored in the laboratory quality manual for each laboratory. This manual should serve as a basis for writing the laboratory Standard Operating Procedures (SOPs) which need to be adapted to the laboratory's role and capacity. The SOPs, QC/QA procedures, specimen testing request forms, report forms, and other laboratory forms are all important components of the quality manual, which documents the quality management system.

An SOP should be written for all procedures in the laboratory, including specimen collection, transport, storage, waste disposal, Gram stain, microscopy, biochemistry measurements, culture, identification, antimicrobial susceptibility testing, typing methods (serological or molecular methods), reagents and media preparation, equipment use and maintenance, and SOP writing.

Examples of records include request forms, report forms, logbooks, quality control results, patient reports, critical communications, and notices from hospitals or public health authorities. See Chapter 3: Results Management and Reporting of Data for items that should be included in the request and report forms.

6. Process control

Process control refers to control of all activities involved in the operation of a laboratory, from pre-examination to post-examination steps.

One of the most familiar process control measures is the use of internal QC to monitor the performance of examination methods. Internal QC of specimen identification and characterization should include:

- Regular QC to check media and reagents, such as agar plates and serogrouping or serotyping antisera. QC should be performed quarterly as well as when a new lot is received in the laboratory.
- Well-characterized reference control strains (gram-positive and gram-negative) should be used to assess the following tests: Gram stain, culture procedures, identification, serogrouping or serotyping, PCR, and antimicrobial susceptibility testing. If access to QC strains is difficult, well-characterized clinical isolates confirmed and characterized in a reference laboratory (such as a WHO Collaborating Center) are available. Isolates received from another laboratory as part of a proficiency testing program received in the scope of External Quality Assessment Schemes can also be used.
- Internal QCs included in rapid diagnostic tests are to be used each time the kit is used.

7. Information management

Written SOPs should be developed for data management, cleaning, and reporting. See Chapter 3: Results Management and Reporting of Data.

8. Occurrence management

An “occurrence” is an error or an event that should not have happened. A system is needed for detecting and documenting these occurrences, for handling them properly, and for taking corrective action to reduce the chance of recurrence. Common errors include:

- Patient identification error
- Specimen misplacement
- Specimen transport delayed or at insufficient temperature
- Contaminated specimens
- Performing an inappropriate test
- Performing a test inconsistent with the written procedure
- Lack of QC/QA
- Transcription and clerical errors

Occurrences are detected through various means, such as supervisory review, physicians’ or patients’ complaints, QC/QA results, or findings from external audits. Immediate remedial corrective action should be undertaken before the result is reported to the clinician or public health authorities. Ultimately, corrective actions should be implemented to prevent similar errors from recurring.

9. Assessment

Assessment is a tool for examining laboratory performance and comparing it to known standards or to performance of other laboratories. Assessment may be internal, performed by the laboratory's own staff, or may be external, conducted by an external group or agency outside the laboratory.

- External Quality Assessment (EQA) is a system for objectively checking the laboratory's performance using an external agency or facility. There are three commonly used EQA methods or processes:
 - Proficiency testing (PT) through a panel of unknown specimens sent regularly to the laboratory by an organizer. The laboratory reports the results back to the organizer who will compare the test results with known results and record a pass (all results concordant) or fail (any discrepant results) for the PT.
 - Confirmation by sending a subset of isolates to a reference laboratory for re-identification and characterization.
 - Site visits conducted by inspection, certification, or accreditation bodies.
- Internal audits can be conducted by the staff of the laboratory to identify weaknesses and undertake corrective actions.
- Quality indicators can be defined by the laboratory management and staff to complement the use of internal QC. While internal QC primarily assesses the examination steps, other quality indicators can be designed to monitor the pre- and post-examination steps:
 - Percentage of cerebrospinal fluid (CSF) specimens received from remote areas and not transported in Trans-Isolate (T-I) medium (if T-I was available but not used). This indicator provides information on the pre-examination performance.
 - Percentage of CSF specimens received without appropriate identification, which provides information on the pre-examination performance.
 - Discrepancies between the CSF macroscopic examination and the cell count, which provides information on the examination performance.
 - Inappropriate antibiotic treatment after the antimicrobial susceptibility testing report is given to the physician, which provides information on the reporting system performance, physician error, or if the proper antibiotic is not available.
 - Percentage of results reported on time, which provides information on examination and reporting performance.

10. Customer service

The laboratory should understand who their customers are (the patients, the physicians, or the public health authorities), assess their needs, and use customer feedback for making improvements. Customers' satisfaction can be assessed by means of questionnaire, interviews, or meetings.

11. Process improvement

The primary goal of a quality system is continuous improvement of the laboratory processes in a systematic manner. A number of tools have been described above to identify errors, such as customer service surveys, internal QC, EQA, auditing, and quality indicators. A rigorous analysis of all of these indicators should lead to improvements in procedures and practices. These changes should be recorded and reflected in the SOPs and implemented in the laboratory. Open communication among staff members is also important to encourage suggestions that may improve the quality and efficiency of the laboratory.

12. Facilities and safety

The laboratory should develop SOPs for biosafety, basic safe operating procedures, and waste management that are adapted to their specific role in the laboratory and in conjunction with institutional policies. See Chapter 4: Biosafety.

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CHAPTER 14

Storage and Shipping of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*

I. Preservation and storage of isolates

It is often necessary for an isolate to be re-examined or further characterized at a time after the culture was initially obtained and tested. If isolates need to be sent to a national reference laboratory for confirmation of identity and/or further testing, they must be stored properly prior to packing and shipping. Selection of a storage method depends on the length of time the organisms are to be stored and the laboratory equipment and facilities available. Isolates to be prepared for either short-term or long-term storage should be confirmed as pure cultures before proceeding with any of these methodologies. Fresh cultures (i.e., 18-24 hour growth) should always be used for the preparation of isolates to be stored.

N. meningitidis, *S. pneumoniae*, and *H. influenzae* are fragile bacteria and care must be employed to preserve and transport them under the most ideal conditions possible. Aseptic techniques should be used at all times during the preparation of isolates for storage and/or transport to avoid contamination.

A. Short-term storage

N. meningitidis, *S. pneumoniae*, and *H. influenzae* can only survive for 3-4 days on blood agar plates (BAP) and/or chocolate agar plates (CAP) and do not survive for long periods of time in broth; hence the need for effective and practical short-term and long-term storage methods. Short-term storage methods are appropriate for bacterial isolates that only need to be stored for several days to a few weeks at a time. These methods include Dorset Transport medium, chocolate agar slants, and silica gel packages.

Dorset Transport medium

Dorset Transport medium can be used for room temperature (25°C) storage of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* (1). On Dorset Transport medium, *N. meningitidis* and *H. influenzae* can be stored for approximately 3 weeks, whereas *S. pneumoniae* can be stored for approximately 6 weeks. Instructions for preparation of Dorset Transport medium are included in the Annex. Dorset Transport medium is typically produced as a 4 ml slant in a 7 ml screw-cap tube. It should be stored at 4°C when not in use and warmed to room temperature (25°C) before use.

1. Grow the pure isolate(s) to be stored for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Label the Dorset Transport slant with the Lab ID and the date.

3. Use a disposable plastic loop to collect a few colonies of the overnight growth on the BAP or CAP and inoculate the Dorset Transport slant.
4. Incubate overnight at 37°C in 5% CO₂ then store the Dorset Transport slant at room temperature (25°C).

Chocolate agar slants

If Dorset Transport medium is not readily prepared or used by the laboratory, short-term storage of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* can be carried out on chocolate agar slants for up to 1 week. Instructions for preparation of chocolate agar slants are included in the Annex. Chocolate agar slants are typically produced as a 4 ml slant in a 7 ml screw-cap tube. They should be stored at 4°C when not in use and warmed to room temperature (25°C) before use.

1. Grow the pure isolate(s) to be stored for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Label the chocolate agar slant with the Lab ID and the date.
3. Use a disposable plastic loop to collect a few colonies of the overnight growth on the BAP or CAP and inoculate the chocolate agar slant.
4. Incubate the slant for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
5. Store the slant at room temperature (25°C).
 - For *N. meningitidis*, solid screw-caps should be loosened during storage but permeable membrane screw caps (which allow for an exchange of gases and are commercially available) should be used when possible. An overlay of trypticase soy broth (TSB) may also be helpful and might increase viability. *N. meningitidis* slants should not be refrigerated.
 - Viability is best for *S. pneumoniae* and *H. influenzae* if the slants are maintained at 4°C with the cap tightened to avoid drying after incubation.

Silica gel packages

N. meningitidis, *S. pneumoniae*, and *H. influenzae* can also be stored short-term on swabs stored in silica gel packets, which are typically 1.5 g foil bags, with 75% white gel and 25% blue gel (the blue gel is added to detect moisture). Isolates can survive approximately 2 weeks at 4°C and perhaps slightly shorter at room temperature (25°C). The packets are inexpensive and easy to use (Figure 1), but are not often available from commercial manufacturers. Silica gel packages can be stored at room temperature (25°C) when not in use.

1. Grow the pure isolate(s) to be stored for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).

2. Label the silica gel package with the Lab ID.
3. Cut open the silica gel package near the adhesive cover with sterile scissors.
 - Inspect the contents of the package. Blue and white gels should be visible. If only white gels are visible, that indicates that moisture was introduced into the silica gel package and the package should not be used.
4. Collect the overnight pure culture growth from the BAP or CAP with a single sterile polyester swab.
 - Do not use cotton swabs as cotton has a bacteriostatic effect which will inhibit bacteria growth.
5. Place the swab in the silica gel package with the tip inserted into the silica and the shaft sticking out the top of the package.
6. Remove the cover of adhesive tape on the silica gel package and fold down the corners to seal the package.
7. Place additional tape around the shaft of the swab and folded corners to secure the swab and seal the package.

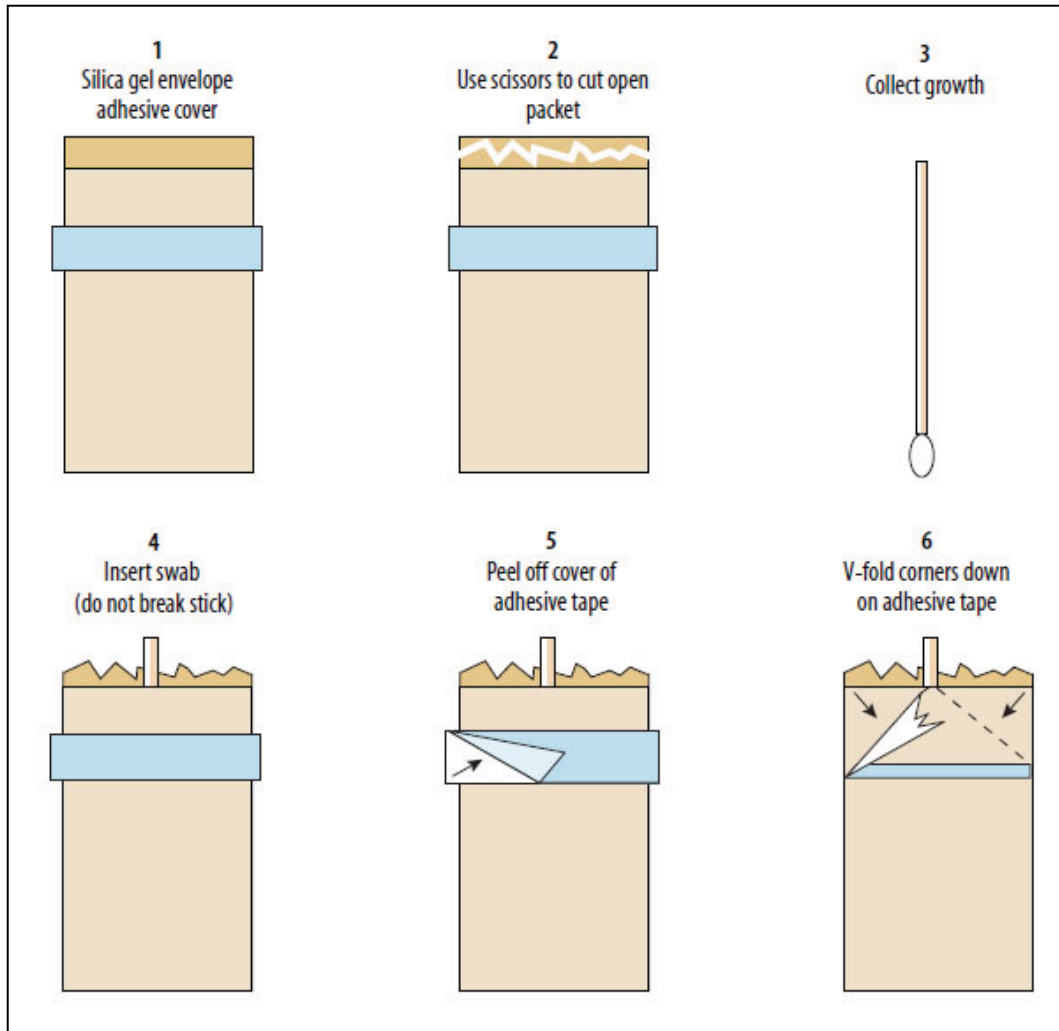


Figure 1. Procedure for inoculating silica gel packages for short-term storage

B. Long-term storage

Long-term storage of bacterial isolates is best accomplished by either freezing or lyophilization (freeze-drying). Freezing is the most convenient storage method for frequently recovered isolates as the cultures do not need to thaw completely each time they are removed from frozen storage. Lyophilized bacteria can be stored for long periods at 4°C or -20°C and can be transported without refrigeration. However, the equipment required for this procedure is expensive and not all laboratories have the ability to lyophilize isolates. Reference laboratories choosing to lyophilize bacteria should always maintain a frozen preparation in addition to larger quantities of lyophilized strains as some lyophilized preparations may be nonviable upon reconstitution. Bacterial cultures may be stored frozen or lyophilized in a variety of storage media formulated for that purpose.

Frozen storage

Skim milk with glycerol, defibrinated sheep, horse, or rabbit blood, or Greaves medium is used for freezing. Instructions for preparing these media are listed in the Annex. Human blood should not be used due to safety issues (e.g., HIV and hepatitis transmission) and the possible inhibition of growth of isolates resulting from antibodies or residual antibiotics. Glass ampoules or vials for freezing in liquid nitrogen should not be used because they can explode upon removal from the freezer.

1. Grow the pure isolate(s) to be stored for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Label the storage tube with the Lab ID and the date.
3. Use a sterile Pasteur pipette to add 1 ml of well-mixed, sterile storage media to a 2 ml, externally-threaded, screw-cap cryovial.
4. Use a sterile polyester-tipped swab to harvest all of the pure overnight growth from the BAP or CAP and inoculate the storage media by swirling the swab to release the organisms.
 - Do not use cotton swabs as cotton has a bacteriostatic effect which will inhibit bacteria growth.
 - Squeeze the excess media from the swab by rotating it against the sides of the cryovial before carefully withdrawing it. Discard the swab in disinfectant.
5. If possible, rapidly freeze the suspension in a bath of 95% alcohol and dry ice pellets.
6. Place the cryovials in a -70°C freezer or a liquid nitrogen freezer (-120°C). A -20°C freezer can be used, but some loss of viability can be expected.
 - Freezers with automatic defrost cycles should never be used for the laboratory.

Lyophilization

Some laboratories may have lyophilization facilities. Serum-based media, skim milk, or polyvinylpyrrolidone (PVP) medium are generally used for lyophilization.

1. Grow the pure isolate(s) to be stored for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Label the storage tube and/or lyophilization vial with the Lab ID and the date.
3. Use a sterile Pasteur pipette to add 1-2 ml of well-mixed, sterile lyophilization medium to a 2 ml, externally-threaded, screw-cap cryovial.

4. Use a sterile polyester-tipped swab to harvest all of the pure overnight growth from the BAP or CAP and suspend it into 1-2 ml of sterile lyophilization medium by swirling the swab to release the organisms.
 - Do not use cotton swabs as cotton has a bacteriostatic effect which will inhibit bacteria growth.
 - Squeeze the excess media from the swab by rotating it against the sides of the cryovial before carefully withdrawing it. Discard the swab in disinfectant.
5. Place approximately 0.5 ml of the cell suspension into a sterile ampoule or lyophilization vial.
 - Several vials can be prepared from a single plate, if desired.
 - Sterility should be maintained at all times during preparation of the vial.
6. Shell-freeze the cell suspension on the walls of the lyophilization vial. This is accomplished using one of the following two methods:
 - Keep the lyophilization vial at -70°C until just before the cell suspension is added. Add the cell suspension and rapidly rotate the vial to freeze the suspension to the wall. Return the vial to the -70°C freezer until it is ready to be attached to the lyophilizer.
 - Alternatively, if a -70°C freezer is not available, prepare a mixture of alcohol (95% ethanol) and dry ice. Add the cell suspension and rapidly rotate the lyophilization vial at a $45\text{-}60^{\circ}$ angle in the alcohol and dry-ice mixture.
7. Attach the vial to the lyophilizer and follow the manufacturer's instructions for lyophilization as the type of apparatus may vary slightly with each instrument.
 - The time of lyophilization will depend on the number of vials being lyophilized and the capacity of the instrument. On an average machine, 4-5 hours are required to completely dry 10-20 small vials.
8. Once the run is completed, seal the vials using a heat source or a capping mechanism while they are still attached to the lyophilizer and under a vacuum. The vials can be stored at 4°C or -20°C after being sealed.

C. Recovery of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* isolates

Whether recovering an isolate from short-term or long-term storage, be sure to label all agar plates with the appropriate Lab ID and date using a permanent marker. Use the appropriate agar medium for each organism as specified in Chapter 6: Primary Culture and Presumptive Identification.

Dorset Transport medium and chocolate agar slants

1. Use a sterile 10 μ l loop to remove a loopful of growth from the Dorset Transport or chocolate agar slant and streak for isolation on a BAP or a CAP.
2. Incubate the plate for 18–24 hours at 35–37°C with ~5% CO₂ (or in a candle jar) and observe for growth
3. If no growth is observed on the plate, repeat the above steps with another loopful of growth from the Dorset Transport or chocolate agar slant and streak for isolation on a BAP or a CAP.

Silica gel packages

1. Remove the swab from the silica gel package and streak a vertical line down 1/3 of the center of a BAP or a CAP and then cross-streak 1/3 of the plate using the swab.
 - Be sure that the entire surface area that contained the culture comes in contact with the plate.
2. After streaking 1/3 of the plate, place the swab in approximately 1 ml of brain heart infusion (BHI) broth and incubate the broth for 18–24 hours at 35–37°C with ~5% CO₂ (or in a candle-jar).
3. Streak the remaining 2/3 of the plate for isolation with a 10 μ l loop.
4. Incubate the plate for 18–24 hours at 35–37°C with ~5% CO₂ (or in a candle-jar) and observe for growth.
5. If the organism is nonviable after overnight incubation, pipet 10 μ l of the broth onto a new plate and streak for isolation. Incubate the plate for 18–24 hours at 35–37°C with ~5% CO₂ and check for growth the next day.

Frozen isolates

1. Allow frozen isolate to thaw at room temperature, just enough so that 10 μ l of freezing medium can be removed from the top.
 - Return frozen stock to the freezer immediately after collection. Once completely thawed, the frozen culture will begin to lose viability.
2. Place 10 μ l of freezing medium onto a BAP or a CAP and streak for isolation.
3. Incubate the plate for 18–24 hours at 35–37°C with ~5% CO₂ (or in a candle-jar) and observe for growth.

4. If no growth is observed on the plate, repeat above steps with another 10 μ l of the frozen culture.

- Isolates grown from frozen cultures should be subcultured at least once prior to being used in tests.

Lyophilized cultures

1. Suspend lyophilized isolates in 0.25-0.5 ml of broth (e.g., TSB or Mueller-Hinton).
2. Add 10 μ l of the cell suspension to a BAP or a CAP and streak for isolation.
3. Add approximately 50 μ l of the suspension to a liquid broth containing 50 μ l of blood (sheep, rabbit, goat, or horse blood, but not human blood).
4. Incubate the plate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) and observe for growth.
5. If growth of the appropriate bacteria is visible, the broth tube can be discarded.
6. If no growth is observed on the plate, place 10 μ l of the broth tube contents onto a new agar plate, streak for isolation, and incubate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
 - If the broth tube is not turbid, it is likely that the lyophilized sample is nonviable. This is why it is strongly suggested that a specimen be prepared for long-term frozen storage in addition to lyophilization.
 - Isolates grown from lyophilized cultures should be subcultured at least once prior to being used in tests

II. Preparation of infectious substances and diagnostic/patient specimens for packaging and shipping

Transport of diagnostic specimens and etiologic agents (infectious substances) should be done with care not only to minimize the hazard to humans and the environment, but also to protect the viability of the suspected pathogens. Transport of infectious material by public or commercial delivery systems may be subject to local, national, and international regulations.

If possible, specimens should be shipped so that they arrive in the receiving laboratory during working hours to ensure proper handling, prompt plating, and storage of the specimens. The receiving laboratory should be informed that the specimens are being shipped, preferably before the specimens are sent, so that appropriate arrangements can be made.

Depending on local conditions, within-country transport may be by ground or by air. If specimens are sent by a messenger, the messenger must know the location of the laboratory and

the appropriate person to contact. The sender should identify a quick, inexpensive, and reliable mode of transport in advance and coordinate the shipping schedule and funding mechanism. For longer distances and international shipments, the quickest transport service may be air-freight or expedited delivery service. Because ice packs or dry ice for cold shipments will last only 24-48 hours, arrangements should be made for immediate collection at the receiving airport. In addition, the sender should coordinate with a contact at the destination to identify any special arrangements or criteria that need to be met for customs in that country. When specimens are shipped by air, the following information should be communicated immediately to the receiving laboratory: air freight company, air waybill number, flight number, times and dates of departure and arrival of the flight, and contents of the package.

A. Preparation for transport of infectious specimens and cultures

N. meningitidis, *S. pneumoniae*, and *H. influenzae* specimens and isolates can be shipped frozen or on chocolate agar slants, in silica gel packages, or as lyophilized cultures. They should be packaged for transport as indicated below.

Frozen specimens and isolates

Frozen specimens and isolates should be shipped using a sufficient amount of ice packs or dry ice to maintain the proper cold temperature throughout the duration of the transportation, especially when shipping long distances or internationally. Ice packs will remain frozen for a day or two after the dry ice has dissipated. Glass vials should not be used when shipping frozen isolates. Guidelines for packaging infectious substances and clinical specimens are listed in Sections IV and V, respectively.

Chocolate agar slants

Chocolate agar slants in screw-cap tubes should be shipped at room temperature (25°C). Isolates can survive for at least one week under these conditions.

Silica gel packages

Prepared silica gel packages should be shipped at 4°C (preferable) or at room temperature (25°C). Isolates can survive for up to 2 weeks in silica gel packages. Silica gel packages should be enclosed in 2 sealable bags within a plastic shipping container.

Lyophilized cultures

Lyophilized culture vials should be packaged according to the regulations specified in the WHO Laboratory Safety Manual, which is available at: http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/. Each vial or tube should be individually wrapped before being enclosed in a metal container along with enough absorbent material to absorb all liquid in the event of a spill. This container should then be enclosed in a cardboard shipping container and can be transported at room temperature (25°C). An address label and an etiological agent-warning label (EA label) should

be attached to the shipping container. No more than 50 ml of culture can be shipped in one package.

III. Regulations regarding transport and shipment of infectious substances and diagnostic/patient specimens

A. Regulatory organizations

The United Nations Committee of Experts on the Transport of Dangerous Goods is continually developing recommendations for the safe transport of dangerous goods. The International Civil Aviation Organization (ICAO) has used these recommendations as the basis for developing regulations for the safe transportation of dangerous goods by air. The regulations of the International Air Transport Association (IATA) contain all the requirements of the ICAO Technical Instructions for the Safe Transport of Dangerous Goods. However, IATA has included additional requirements that are more restrictive than those of ICAO. Member airlines of the IATA have adopted the use of the IATA regulations governing dangerous goods and shippers must comply with these regulations in addition to any applicable regulations of the state of origin, transit, or destination.

The shipment of infectious substances or diagnostic specimens by air must comply with local, national, and international regulations. International air transport regulations may be found in the IATA publication titled *Dangerous Goods Regulations*. This reference is published annually in January and the regulations are updated each year. A copy of the IATA regulations in English, Spanish, French, or German may be obtained from one of the following regional offices.

Orders for IATA regulations from the Americas, Europe, Africa, and the Middle East:

Customer Service Representative
International Air Transport Association
800 Place Victoria, P.O. Box 113
Montreal, Quebec
CANADA H4Z 1M1
Telephone: +1 514 390 6726
Fax: +1 514 874 9659
Teletype: YMQTPXB

Orders for IATA Regulations from Asia and the Pacific:

Customer Service Representative
International Air Transport Association
77 Robinson Rd.
No. 05-00 SIA Building
SINGAPORE 068896
Telephone: +65 438 4555
Fax: +65 438 4666
Telex: RS 24200 TMS Ref: TM 2883

Cable: IATAIATA
Teletype: SINPSXB






Internet information:
www.iata.org

For internet orders, send e-mail to:
sales@iata.org

B. Shipping regulations for infectious substances and diagnostic/patient specimens

Packages that are shipped by air via commercial and cargo carriers (such as Federal Express, DHL, and passenger aircraft) are affected by IATA regulations. These regulations are outlined in this section of the laboratory manual to provide examples of acceptable packaging procedures for infectious materials. However, because they may not reflect current national or IATA requirements for packaging and labeling for infectious substances, anyone packaging isolates or infectious specimens should consult the appropriate national regulations and the current edition of the IATA *Dangerous Goods Regulations* before packing and shipping infectious substances by any means of transport (2). Table 1 includes images of labels and packages appropriate for shipping and different classifications of packages under IATA regulations. Note that a completed Shipper's Declaration for Dangerous Goods form is required for shipping hazardous materials including infectious substances. Instructions for completing this form are provided at the end of this section.

Table 1. Description of individual labels and markings required for safe and proper shipping of different types of packages

	<p>This orientation label should clearly mark which side is “Up”. Two labels are required on all boxes with each one on opposite sides of the package.</p>
<p>Inner Packages Comply With Prescribed Specifications</p>	<p>This marking must appear on an overpack when the regulations require the use of packages bearing UN Specification Markings.</p>
	<p>This marking is required when shipping patient/diagnostic specimens.</p>
	<p>This label is required when shipping a substance or specimen on dry ice.</p>
	<p>This label is required when shipping infectious substances. Please note when shipping infectious substances you must use UN certified 6.2 Infectious Substance Packaging.</p>
	<p>This label is required when shipping ≥ 50 ml of an infectious substance.</p>

C. Definition of infectious substances

According to IATA, infectious substances are defined as substances known or reasonably expected to contain pathogens. Pathogens are microorganisms (including bacteria, viruses, rickettsia, parasites, fungi) and other agents such as prions, which cause infectious disease in humans or animals.

D. Definition of diagnostic/patient specimens

According to IATA, diagnostic/patient specimens are those collected directly from humans or animals, including, but not limited to, excreta, secretions, blood and its components, tissue and tissue fluid swabs, and body parts being transported for purposes such as research, diagnosis, investigational activities, disease treatment and prevention.

IV. Guidelines for packaging and labeling infectious substances

Persons who ship infectious agents must comply with all local, national, and international regulations pertaining to the packaging and handling of these materials. They must ensure that specimens arrive at their destination in good condition and that they present no hazard to humans or other organisms during transport.

A. The inner packaging of infectious substance shipments must include the following:

1. An inner watertight primary container that is glass, metal, or plastic and has a leak-proof seal.
2. Packaged infectious agents or diagnostic specimens which are placed in the primary container.
 - Agar slants with screw-cap tops should be reinforced with adhesive tape. Petri plates should not be shipped.
3. A watertight, impact-resistant secondary container.
 - United Nations (UN) Specification Packaging that has been rigorously tested and certified for infectious substances.
4. The primary receptacle or the secondary packaging must be capable of withstanding, without leakage, an internal pressure producing a pressure differential of not less than 95 kPa and a temperature range of -40°C to 55°C (-40°F to 130°F).
5. Absorbent material (such as cotton wool) between the primary container and the secondary container.
 - If multiple primary containers are placed in a single secondary packaging, they must be wrapped individually, separated, and supported to ensure that contact between them is

prevented. The absorbing material must be sufficient to absorb the entire contents of all primary containers.

6. An itemized list of contents, placed between the secondary packaging and the outer packaging.

B. The outer packaging of infectious substance shipments must meet the following requirements:

1. Container should be of sufficient strength to adequately protect the contents.
2. Size must be at least 100 mm (4 inches) in its smallest overall external dimension and of sufficient size to accommodate all labels to be placed on a single surface without overlapping.
3. Package must be durably and legibly marked on the outside with the address and telephone number of the shipper and the consignee (the intended recipient).
4. Infectious substance label must be affixed to the outside of the outer container and must bear the inscription, "Infectious substance. In case of damage or leakage, immediately notify public health authority."
5. Marked with UN Specification Markings denoting that the packaging has been tested and certified for shipping infectious substances.
6. Marked with the infectious substance marking (UN 2814): "Infectious substance, affecting humans (Genus species or technical name) x total number of milliliters or grams." The species can be specified or otherwise indicated as "spp." Note that this marking can be written by hand and does not require a special adhesive label. For example: "Infectious substance, affecting humans (*N. meningitidis*) x 5.0 ml" or "Infectious substance, affecting humans (*Streptococcus* spp.) x 5.0 ml" or "Infectious substance, affecting humans (HIV) x 0.5 ml".
7. Labeled with a set of two up-arrows on at least two opposite sides of the outer box to indicate the proper package orientation for the closures to be in the upright position.
8. Labeled on the top of the box with the statement "This End Up" or "This Side Up."
9. Labeled with a "Cargo Aircraft Only" label if the total volume of the infectious substance per outer shipping container is ≥ 50 ml.
10. Marked with the name and telephone number of the person responsible for the shipment.

The packaging requirements for transport of infectious substances are illustrated in Figure 2.

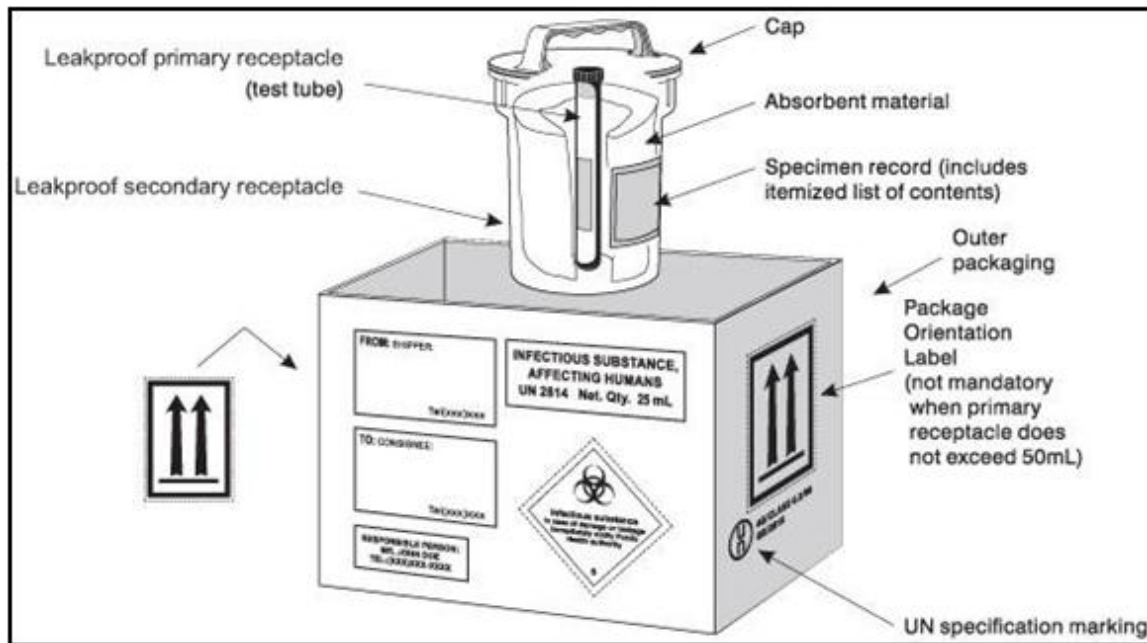


Image source: IATA Guidance Document, Infectious Substances, Annex 3, Montreal, Canada

Figure 2. Proper packing and labeling of the secondary container for shipping of infectious substances

V. Guidelines for packaging and labeling diagnostic/patient specimens

Diagnostic/patient (clinical) specimens with a low probability of containing an infectious agent must be packaged as follows, so that will not leak after a 1.2 meter drop test procedure:

1. Contents should be triple packed with a watertight primary container, a leak-proof secondary container, and sufficient absorbent material to absorb all liquid in case of a spill packed in between the primary and secondary containers.
2. The primary receptacle or the secondary packaging must be capable of withstanding, without leakage, an internal pressure differential of 95 KiloPascals between -40°C and 55°C. Infectious substance containers exceed these criteria and are therefore acceptable for use for packing and shipping of diagnostic specimens.
3. Include an itemized list of contents between the secondary packaging and the outer packaging.
4. Marked with the “diagnostic specimens” statement on the outside of the outer container: “Diagnostic specimen. UN 3373. Packed in compliance with IATA Packing Instruction 650.” This marking can be written by hand and does not require a special adhesive label.
5. If being shipped by air, the diagnostic specimens statement “Diagnostic specimen. UN 3373. Packed in compliance with IATA Packing Instruction 650” must be present on the air waybill as well as on the outer container. The packaging requirements for transport of diagnostic specimens are illustrated in Figure 3.

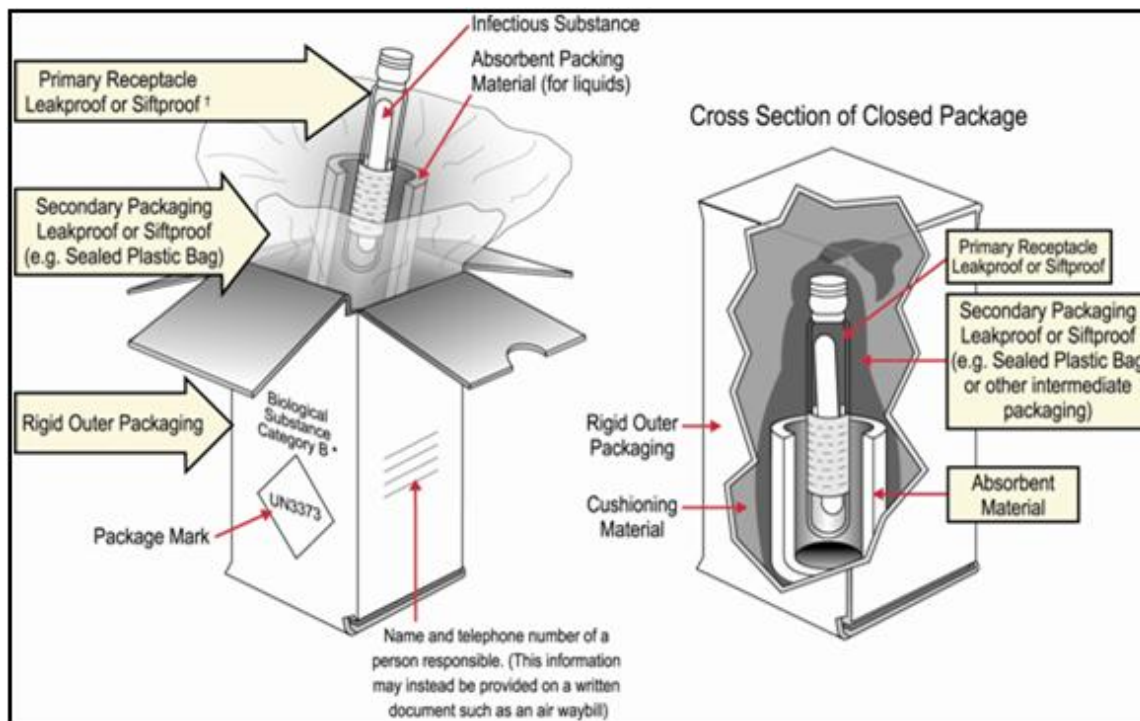


Image source: US Department of Transportation

Figure 3. Proper packing and labeling of the secondary container for shipping of diagnostic/patient specimens

VI. Guidelines for packaging and labeling of specimens shipped on dry ice (CO₂)

1. Dry ice must be placed outside the secondary packaging in an overpack and interior supports must be provided to secure the secondary packaging in the original position after the ice has dissipated.
2. Dry ice must be packed according to IATA Packing Instruction 954: the outer packaging must permit the release of carbon dioxide (CO₂) gas. Cardboard and polystyrene foam are two examples of materials suitable for the packaging of dry ice. In a temperate climate, approximately 6 pounds of dry ice will dissipate in a 24 hour period and are therefore suitable for a 24 hour shipment. This amount should be adjusted accordingly for warmer climates and size of the box to ensure that the contents remain frozen. For air transport, the maximum dry ice allowed in a single outer container is 200 kg (approximately 440 pounds).
3. Packages containing dry ice must be properly marked with the statement “Carbon dioxide, solid (dry ice); UN1845; (and net weight of the dry ice in kg),” and a preprinted Class 9 “Miscellaneous Dangerous Goods” label, as shown in Table 1.
4. When an overpack is used, it must be marked with the statement “Inner packages comply with prescribed specifications” because the UN Specification Markings will not be visible on the outer-most packaging.

VII. Guidelines for completion of the “Shipper’s Declaration for Dangerous Goods” form

All shipments of hazardous materials, including infectious substances, must be accompanied by two original completed copies of the “Shipper’s Declaration for Dangerous Goods” form, along with the other shipping documents. It is important to remember the following in order to reduce the risk of a shipment being refused and returned to the laboratory of origin:

1. International regulations require the diagonal hatch marks in the left and right margins to be printed in red. Therefore, black and white photocopies of this form may not be used.
2. The form must be completed in English, although translations may accompany it on the same form.
3. Specific terms, spellings, and nomenclature must be used. For example, a cardboard box must be referred to as “fibreboard box” (spelled with R before E), and there must be a comma after the term “infectious substance” within the statement “infectious substance, affecting humans”.
4. The person responsible for the shipment must be listed in one of the address boxes. If the person responsible for the shipment is different than the shipper or recipient, the responsible person’s telephone number should be included alongside the name.
5. Under the “Transport Details” portion of the form, cross out the option that does not apply. If the shipment is less than 50 ml, cross out “cargo aircraft only.” If the shipment is greater than 50 ml, cross out “passenger and cargo aircraft.”
6. Under the “Nature and Quantity of Dangerous Goods” portion of the form, the proper shipping name for infectious substances is “Infectious substance, affecting humans (technical name).” The technical name of the infectious substance(s) must be included in parentheses after the proper shipping name; however, the specific species is not required and “spp.” may follow the genus. The technical name of the infectious substance *Neisseria meningitidis* should be listed as either “(*Neisseria meningitidis*)” or “(*Neisseria* spp.).”
7. For “Infectious substances, affecting humans (technical name)”: the proper class is Division 6.2, the UN number is UN2814, and the packing instruction is 620.
8. For “Carbon dioxide, solid (dry ice)”: the proper class is Class 9 Miscellaneous Dangerous Good, the UN number is UN1845, the packing group is III, and the packing instruction is 954.
9. For infectious substances, the quantity must be noted in ml under the “Quantity and Type of Packing” portion of the form.
10. For dry ice, the quantity must be noted in kg (measured in whole numbers) under the “Quantity and Type of Packing” portion of the form.

11. If the UN specification marking is not visible on the outer package, the declaration must contain the statement “OVERPACK USED” under the “Quantity and Type of Packing” portion of the form.
12. Under the “Additional Handling Information” portion of the form, the 24 hour emergency contact telephone number must be answered by a person knowledgeable about emergency response procedures for damaged and leaking boxes.
13. The “Shipper’s Declaration for Dangerous Goods” form is a legal document and must be signed.

It is important to communicate shipping details to the intended recipient prior to shipment of the package. In addition, arrangements should be made for proper handling during shipping and legal importation of the infectious substance to ensure delivery without delay. These guidelines are in accordance with IATA regulation 1.3.3.1.

References

1. **Wasas, A.D., Huebner, R.E., and Klugman, K.** 1999. Use of Dorset Egg Medium for Maintenance and Transport of *Neisseria meningitidis* and *Haemophilus influenzae* Type b. *Journal of Clinical Microbiology* **37**:2045–6.
2. International Air Transport Association *Dangerous Goods Regulations* 52nd Edition, Effective January 1, 2011. Montreal.

ANNEX

Preparation of Media and Reagents

Quality control (QC) of media

Each batch of media prepared in the laboratory and each new manufacturer's lot number of media should be tested using appropriate QC reference strains for sterility, the ability to support growth of the target organism(s), and/or the ability to produce proper biochemical reactions. A QC record should be maintained for all media prepared in the laboratory and purchased commercially; including preparation or purchase date and QC test results. Any unusual characteristic of the medium, such as color or texture, or slow growth of reference strains should be noted.

I. Routine agar and broth media

All agar media should be aseptically prepared and dispensed into 15x100 mm Petri dishes (15-20 ml per dish). After pouring, the plates should be kept at room temperature (25°C) for several hours to prevent excess condensation from forming on the covers of the dishes. For optimal growth, the plates should be placed in a sterile plastic bag and stored in an inverted position at 4°C until use. All broth media should be stored in an appropriate container at 4°C until use.

A. Blood agar plate (BAP): trypticase soy agar (TSA) + 5% sheep blood

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

Media preparation

1. Prepare the volume of TSA needed in a flask according to the instructions given on the label of the dehydrated powder.
 - It is convenient to prepare 500 ml of molten agar in a 1-2 liter flask. If TSA broth powder is used, add 20 g agar into 500 ml of distilled water.
 - The media should be heated and fully dissolved with no powder on the walls of the vessel before autoclaving.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 60°C in a water bath.
4. Add 5% sterile, defibrinated sheep blood (5 ml sheep blood can be added to 100 ml of agar).

- If a different volume of basal medium is prepared, the amount of blood added must be adjusted accordingly to 5% (e.g., 50 ml of blood per liter of medium). Do NOT use human blood.
5. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
 6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

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

Passing result:

- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis).
- *N. meningitidis* should appear as large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- After 48 hours, the sterility test plate should remain clear.

B. Blood culture broth

Blood culture medium is used to grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*.

Media preparation

1. Follow the manufacturer's instructions on the label of each bottle of dehydrated trypticase soy broth (TSB).
2. Add 0.25 g sodium polyanetholsulfonate (SPS) per liter of medium.
 - SPS is especially important for recovery of *H. influenzae*.
3. Dispense in 20 ml (for a pediatric blood culture bottle) and 50 ml (for an adult blood culture bottle) amounts into suitable containers (tubes or bottles) with screw-caps with rubber diaphragms.
 - The amount of liquid in the containers should make up at least two-thirds of the total volume of the container.

4. Autoclave at 121°C for 15 minutes.
5. Allow to cool and store medium at room temperature (25°C).

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a BAP or CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Add 1-3 ml of sterile rabbit, horse, or human blood to 3 bottles of freshly prepared blood culture media.
3. Collect a loopful of overnight growth from each of the plates of bacteria and suspend it in 1-2 ml of blood culture broth (a different organism for each bottle).
4. Inoculate the bacterial suspensions into the 3 blood culture bottles.
5. Incubate the blood culture bottles at 35-37°C with ~5% CO₂ (or in a candle-jar) for up to 7 days and observe for growth.
6. Subculture bacteria onto appropriate media at 14 hours and 48 hours.
7. As a sterility test, incubate an uninoculated blood culture bottle for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- All three bacteria should be recovered on appropriate media after 24 and 48 hours.
- After 48 hours, the sterility test plate should remain clear.

C. Chocolate agar plate (CAP)

CAP is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO₂ atmosphere. CAP has a reduced concentration of agar, which increases the moisture content of the medium. It can be prepared with heat-lysed horse blood, which is a good source of both hemin and NAD, although sheep blood can also be used. Growth occurs on a CAP because NAD is released from the blood during the heating process of chocolate agar preparation (the heating process also inactivates growth inhibitors) and hemin is available from non-hemolyzed as well as hemolyzed blood cells.

Media preparation

1. Heat-lyse a volume of horse or sheep blood that is 5% of the total volume of media being prepared very slowly to 56°C in a water bath.

2. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
3. Place the plates in sterile plastic bags and store at 4°C until use.
4. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the CAP for specific colony morphology and hemolysis.

Passing result:

- *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.
- *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.
- After 48 hours, the sterility test plate should remain clear.

D. CAP with bacitracin

CAP with bacitracin is a selective medium used to improve the primary isolation of *H. influenzae* from specimens containing a mixed flora of bacteria and/or fungi.

Media preparation

1. Prepare double strength TSA (20 g into 250 ml distilled water) as the basal medium.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 50°C in a water bath.
 - Use a thermometer to verify the temperature in the water bath.
4. Prepare a solution of 2% hemoglobin (5 g in 250 ml distilled water). Mix the hemoglobin in 5-6 ml of distilled water to form a smooth paste. Continue mixing as the rest of the water is added.
5. Autoclave at 121°C for 20 minutes.
6. Cool to 50°C in a water bath.
7. Add the hemoglobin solution to the double strength TSA and continue to hold at 50°C.

If a hemoglobin solution is unavailable, use the alternative method with defibrinated sheep blood described below:

1. Add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood to 100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water).
 - Do NOT use human blood.
2. Heat to 56°C in a water bath then cool to 50°C.
8. After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50°C, add the growth supplement containing hemin and NAD to a final concentration of 1%. Mix the ingredients by gently swirling the flask in a figure 8 motion on the counter.
 - Avoid forming bubbles.
9. Prepare a stock solution of bacitracin by suspending 3 g bacitracin in 20 ml distilled water. Filter-sterilize, dispense into 1 ml amounts, and store at -20°C or -70°C.
10. While the medium is still at 50°C, add 1 ml stock solution of bacitracin (prepared in step 9) per 500 ml chocolate agar.
11. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
12. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

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

Passing result:

- *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with bacitracin with no discoloration of the medium.
- After 48 hours, the sterility test plate should remain clear.

E. Chocolate agar with TSA and growth supplement

Chocolate agar with TSA and growth supplements is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO₂ atmosphere.

Media preparation

1. Prepare double strength TSA (20 g into 250 ml distilled water) as the basal medium.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 50°C in a water bath.
 - Use a thermometer to verify the temperature in the water bath.
4. Prepare a solution of 2% hemoglobin (5 g in 250 ml distilled water). Mix the hemoglobin in 5-6 ml of distilled water to form a smooth paste. Continue mixing as the rest of the water is added.
5. Autoclave at 121°C for 20 minutes.
6. Cool to 50°C in a water bath.
7. Add the hemoglobin solution to the double strength TSA and continue to hold at 50°C.

If a hemoglobin solution is unavailable, use the alternative method with defibrinated sheep blood described below:

 1. Add 5% sterile defibrinated sheep, rabbit, guinea pig, or horse blood (5 ml blood to 100 ml agar) to full-strength TSA agar base (20 g in 500 ml distilled water).
 - Do NOT use human blood.
 2. Heat to 56°C in a water bath then cool to 50°C.
8. After the hemoglobin solution or the defibrinated blood has been added to the base medium and the medium has cooled to 50°C, add the growth supplement containing hemin and NAD to a final concentration of 1%. Mix the ingredients by gently swirling the flask in a figure 8 motion on the counter.
 - Avoid forming bubbles.
9. Dispense 20 ml in each 15x100 mm Petri dish. Allow the media to solidify and condensation to dry.
10. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the chocolate agar with TSA and growth supplements for specific colony morphology and hemolysis.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.
- *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.
- After 48 hours, the sterility test plate should remain clear.

F. Chocolate agar with gonococcus medium (GC) base and growth supplement

Chocolate agar with GC base and growth supplement is a medium that supports the special growth requirements (hemin and NAD) needed for the isolation of fastidious organisms, such as *H. influenzae*, when incubated at 35-37°C in a 5% CO₂ atmosphere.

Media preparation

1. Suspend 7.2 g of GC agar base in 100 ml distilled water in a flask. 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.
3. Cool to 50°C in a water bath.
4. Add 100 ml of warm distilled water to 2 g of soluble hemoglobin powder. Mix the powder with 5-10 ml of distilled water until a smooth paste is achieved. Gradually add the balance of water until the solution is homogenous. Continually stir the solution during the addition of water.
 - Alternatively, 100 ml ready-made 2% sterile hemoglobin solution, warmed to 50°C can be used.
5. Autoclave the solution at 121°C for 15 minutes. Cool to 50°C in a water bath.
6. Reconstitute lyophilized growth supplement containing NAD and hemin by aseptically transferring 10 ml of the accompanying diluent with a sterile needle and syringe. Shake to

assure complete solution. After reconstitution, use immediately, or store at 4°C and use within 2 weeks.

7. Aseptically add 100 ml sterile hemoglobin solution and growth supplement to 100 ml of the GC agar base solution. Mix gently, but thoroughly, to avoid air bubbles in the agar.
8. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
9. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Observe the chocolate agar with TSA and growth supplements for specific colony morphology and hemolysis.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *N. meningitidis* and *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.
- *S. pneumoniae* should appear as small grey to green colonies with a zone of alpha-hemolysis (only slightly green) on the CAP.
- After 48 hours, the sterility test plate should remain clear.

G. Chocolate agar slants

Chocolate agar slants for transport and short-term storage can be prepared in the same manner as described for agar plates with one difference: 4 ml of the medium should be dispensed into 16x125 mm screw-cap tubes and slanted before solidifying. Chocolate agar slants should look brown to brownish-red in color. Chocolate agar slants should be tested for QC using the same methods used for QC testing of CAP.

H. Cystine trypticase agar (CTA) with 1% carbohydrate (a semi-solid medium)

Media preparation

1. Follow the manufacturer's instructions for the amount of CTA medium to suspend 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.

3. Cool to 50°C in a water bath.
4. Prepare a 10% glucose (also called dextrose) solution by adding 10 g glucose to 100 ml distilled water. Filter-sterilize using a 0.22 micron filter.
5. Aseptically add 100 ml of the 10% glucose solution from step 4 to 900 ml of CTA medium to obtain a final concentration of 1% glucose.
6. Aseptically dispense 7 ml of the medium into 16x125 mm screw-cap glass tubes.
7. Repeat this procedure for the remaining 3 carbohydrates: maltose, lactose, and sucrose.
8. Store at 4°C and warm to room temperature (25°C) before use.

Quality control

1. Grow *N. meningitidis*, *N. lactamica*, and *N. sicca* QC strains to be tested for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Allow the 4 CTA sugars, glucose, maltose, lactose, and sucrose, to warm to room temperature (25°C) and label the tubes with the name of the QC strain.
3. Remove 3-5 colonies from overnight growth on the BAP using a 1 µl disposable loop.
4. Stab the CTA sugar several times into the upper 10 mm of medium. Approximately 8 stabs with the same loopful are sufficient.
 - Use a separate disposable loop for inoculating each carbohydrate to be tested.
5. Fasten the screw-cap of each tube loosely and place the tubes in a 35-37°C incubator without CO₂. Incubate the CTA sugars for at least 72 hours (and up to 5 days) before discarding as negative.
6. Observe the CTA sugars for development of visible turbidity and color change to yellow.

Passing result:

- Development of visible turbidity and a yellow color in the upper portion of the medium indicates growth of bacteria and production of acid and is interpreted as a positive test.
- *N. meningitidis* should utilize glucose and maltose, but not lactose or sucrose.
- *N. lactamica* should utilize glucose, maltose, and lactose, but not sucrose.
- *N. sicca* should utilize glucose, maltose, and sucrose, but not lactose.

I. *Haemophilus* test medium (HTM) plates

HTM is used for antimicrobial susceptibility testing for *H. influenzae*.

Media preparation

The Mueller-Hinton agar used to make HTM should be thymidine free to obtain consistent results if susceptibility to cotrimoxazole is to be tested.

1. Prepare a fresh hemin stock solution by dissolving 50 mg of hemin powder in 100 ml of 0.01 mol/L NaOH with heat and stirring until the powder is thoroughly dissolved.
2. Prepare a nicotinamide adenine dinucleotide (NAD) stock solution by dissolving 50 mg of NAD in 10 ml of distilled water; filter-sterilize.
3. Prepare Mueller-Hinton agar (MHA) from a commercially available dehydrated base according to the manufacturer's directions (or see protocol in section I.M.), adding 5 g of yeast extract and 30 ml of hemin stock solution to 1 L of MHA.
4. After autoclaving, cool the medium to 45°C to 50°C in a water bath.
5. Aseptically add 3 ml of the NAD stock solution.
6. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.
 - Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.
 - Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
 - Using more or less agar will affect the susceptibility results.
7. Allow the media to solidify and condensation to dry.
8. The pH should be 7.2-7.4.
 - Do not attempt to adjust the pH of the MHA test medium if it is outside the range.
9. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a *H. influenzae* QC strain for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Prepare a moderately heavy suspension of cells (comparable to a 1.0 McFarland standard) from overnight growth on the CAP in a suitable broth (trypticase soy, heart infusion, or peptone water) and mix well using a vortex.

3. Inoculate a HTM plate with 10 μ l of the cell suspension using a sterile loop and streak for isolation.
4. Observe HTM plate for specific colony morphology.
5. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the HTM plate with no discoloration of the medium.
- After 48 hours, the sterility test plate should remain clear.

J. Heart infusion agar (HIA) and trypticase soy agar (TSA)

HIA and TSA are general purpose media used with or without blood for isolating and cultivating a number of microorganisms. The media should appear straw colored (yellowish to gold coloring). HIA and TSA are also used for determining the hemin (X factor) and NAD (V factor) growth requirements of *H. influenzae*.

Media preparation

1. Prepare the volume of HIA or TSA needed in a flask, according to the instructions on the label of the dehydrated medium.
 - These 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 in a water bath and dispense 20 ml into each 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
4. Place the plates in plastic bags and store at 4°C until use.

Quality control

1. Grow a *H. influenzae* QC strain for 18-24 hours on a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. Prepare a moderately heavy suspension of cells (comparable to a 1.0 McFarland standard) from overnight growth on the CAP in a suitable broth (trypticase soy, heart infusion, or peptone water) and mix well using a vortex.

- Do not transfer any of the chocolate agar media from the plate to the cell suspension as even the smallest amount of agar will affect the test and may lead to misidentification of the bacteria.
3. Inoculate one half of the HIA or TSA plate with 10 μ l of the cell suspension using a sterile loop or swab and allow the suspension to dry.
 4. Place paper disks or strips containing hemin, NAD, and hemin/NAD on the inoculated plate after the inoculum has dried.
 5. Carefully invert the plates and incubate for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
 6. Observe growth on the HIA or TSA plate around the paper disks or strips.
 7. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing Result:

- *H. influenzae* should grow only around the hemin/NAD disk.
- After 48 hours, the sterility test plate should remain clear.

K. 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, it should be discarded and a new batch should be prepared. Horse blood may be substituted for rabbit blood in this medium.

Media preparation

1. Prepare the volume of HIA needed in a flask according to the instructions on the label of the dehydrated medium.
2. Autoclave at 121°C for 20 minutes.
3. Cool to 50°C in a water bath.
4. Add 5% sterile, defibrinated rabbit blood (alternatively, 5 ml sheep blood can be added to 100 ml of agar).
5. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a hemolytic *H. haemolyticus* QC strain for 18-24 hours on a HIA-rabbit blood at 35-37°C with ~5% CO₂ (or in a candle-jar).
 - After streaking the plate with the QC stain, stab the media with the inoculating loop.
2. Observe the HIA-rabbit blood for specific colony morphology and beta-hemolysis (clear).
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *H. haemolyticus* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies and be surrounded by a distinct zone of complete hemolysis which appears as a clear halo surrounding the colonies.
- After 48 hours, the sterility test plate should remain clear.

L. Horse blood agar (blood agar base)

This highly nutritive medium may be used for the primary isolation of *H. influenzae* and for the determination of the hemolysis with *H. haemolyticus* or other bacteria.

Media preparation

1. Prepare the volume of blood agar base needed in a flask according to the manufacturer's instructions on the label of the dehydrated medium.
2. Autoclave at 121°C for 15 minutes.
3. Cool to 50°C in a water bath.
4. Add 5 ml horse blood per 100 ml of the medium.
5. Mix well and dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a hemolytic *H. haemolyticus* QC strain for 18-24 hours on a horse blood agar plate at 35-37°C with ~5% CO₂ (or in a candle-jar).
 - After streaking the plate with the QC stain, stab the media with the inoculating loop.

2. Observe the horse blood agar for specific colony morphology and beta-hemolysis (clear).
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *H. haemolyticus* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies and be surrounded by a distinct zone of complete hemolysis which appears as a clear halo surrounding the colonies.
- After 48 hours, the sterility test plate should remain clear.

M. MacConkey (MAC) agar

MacConkey Agar is used for the isolation and differentiation of lactose-nonfermenting, gram-negative enteric bacteria from lactose-fermenting organisms. It is recommended that MAC medium be purchased commercially because preparing it with individual ingredients produces variability among lots.

Media preparation

1. Prepare MAC according to manufacturer's instructions.
2. Sterilize the medium by autoclaving at 121°C for 15 minutes.
3. Cool to 50°C in a water bath.
4. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
5. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

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

Passing result:

- *E. coli* should appear as pink to red colonies.
- After 48 hours, the sterility test plate should remain clear.

N. Modified Thayer-Martin (MTM) agar medium

MTM is a selective medium used to improve the primary isolation of *N. meningitidis* from specimens containing a mixed flora of bacteria and/or fungi. MTM is a chocolate agar base containing vancomycin, colistin, nystatin, and trimethoprim lactate.

Media preparation

1. Suspend 7.2 g of GC agar base in 100 ml distilled water in a flask. 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.
3. Cool to 50°C in a water bath.
4. Add 100 ml of warm distilled water to 2 g of soluble hemoglobin powder. Mix the powder with 5-10 ml of distilled water until a smooth paste is achieved. Gradually add the balance of water until the solution is homogenous. Continually stir the solution during the addition of water.
 - Alternatively, 100 ml ready-made 2% sterile hemoglobin solution, warmed to 50°C can be used.
5. Autoclave the solution at 121°C for 15 minutes. Cool to 50°C in a water bath.
6. Reconstitute lyophilized growth supplement containing NAD and hemin by aseptically transferring 10 ml of the accompanying diluent with a sterile needle and syringe. Shake to assure complete solution. After reconstitution, use immediately or store at 4°C and use within 2 weeks.
7. Aseptically add 100 ml sterile hemoglobin solution and growth supplement to 100 ml of the GC agar base solution. Mix gently, but thoroughly, to avoid air bubbles in the agar.
8. To the agar base solution, add the following ingredients:
 - 3.0 µg/ml vancomycin
 - 7.5 µg/ml colistin
 - 12.5 units/ml nystatin
 - 5.0 µg/ml trimethoprim lactate
9. Dispense 20 ml into 15x100 mm Petri dishes. Allow the media to solidify and condensation to dry.
10. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a *N. meningitidis* QC strain for 18-24 hours on a MTM at 35-37°C with ~5% CO₂ (or in a candle-jar).

2. Observe the MTM for specific colony morphology.
3. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *N. meningitidis* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the MTM with no discoloration of the medium.
- After 48 hours, the sterility test plate should remain clear.

O. Mueller-Hinton agar

Mueller-Hinton agar (MHA) is used for making the media required for susceptibility testing for *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. It is recommended that dehydrated Mueller-Hinton agar medium be purchased commercially because preparing it with individual ingredients can diminish the quality.

Media Preparation

1. Follow manufacturer's instructions to prepare MHA from a commercially available dehydrated base.
2. After autoclaving, cool the agar in a 45°C to 50°C water bath.
3. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.
 - Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.
 - Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
 - Using more or less agar will affect the susceptibility results.
4. Allow the media to solidify and condensation to dry.
5. The pH of MHA should be 7.2-7.4.
 - Do not attempt to adjust the pH of the MHA test medium if it is outside the range.
6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow an *E. coli* QC strain for 18-24 hours on a MHA plate at 35-37°C with ~5% CO₂ (or in a candle-jar).

2. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- Observe for *E. coli* growth.
- After 48 hours, the sterility test plate should remain clear.

P. Mueller-Hinton agar with 5% sheep or horse blood

Mueller-Hinton agar with 5% sheep or horse blood is used for susceptibility testing for *N. meningitidis* and *S. pneumoniae*. It is recommended that dehydrated MHA medium be purchased commercially because preparing it with individual ingredients can diminish the quality.

Media preparation

1. Prepare MHA as described above in Section I.M. through step 2.
2. Add 5% sterile defibrinated sheep or horse blood to the medium at 5% (i.e., 50 ml blood per liter of medium or 25 ml blood to 500 ml medium).
3. The pH of MHA after the addition of blood should be 7.2-7.4.
 - Do not attempt to adjust the pH if it is outside the range.
4. Pour agar into flat-bottom glass or plastic Petri dishes on a level pouring surface.
 - Measure 60-70 ml medium per plate into 15x150 mm plates or measure 25-30 ml per plate into 15x100 mm plates to give a uniform depth of approximately 4 mm.
 - Plates should be uniformly 3-4 mm thick as the rate of diffusion of the antimicrobial agents or the activity of the drugs may be affected.
 - Using more or less agar will affect the susceptibility results.
5. Allow the media to solidify and condensation to dry.
6. Place the plates in sterile plastic bags and store at 4°C until use.

Quality control

1. Grow a *S. pneumoniae* or a *N. meningitidis* QC strain for 18-24 hours on Mueller-Hinton agar with 5% sheep or horse blood at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. As a sterility test, incubate an uninoculated plate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).

Passing result:

- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis).
- *N. meningitidis* should appear large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- After 48 hours, the sterility test plate should remain clear.

Q. Mueller-Hinton Broth

Mueller-Hinton broth is used to prepare the Mueller-Hinton broth (cation-adjusted).

Media Preparation

1. To 750 ml deionized H₂O add:
 - 3.0 g beef extract
 - 17.5 g acid hydrolysate of casein (casamino acids)
 - 1.5 g soluble starch
2. Adjust final volume to 1 liter.
3. Adjust pH to 7.3.
4. Autoclave the broth at 121°C for 20 minutes to sterilize.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

R. Mueller-Hinton broth (cation-adjusted)

Mueller-Hinton broth (cation-adjusted) is used to prepare dilutions equivalent McFarland standards with minimal loss of viability.

Media Preparation

1. Prepare a magnesium stock solution by dissolving 8.36 g of MgCl₂·6H₂O in 100 ml deionized H₂O to a final concentration of 10 mg/ml Mg²⁺.
2. Prepare a calcium stock solution by dissolving 3.68 g of CaCl₂·2H₂O in 100 ml deionized H₂O to a final concentration of 10 mg/ml Ca²⁺.
3. Filter-sterilize both stock solutions.

4. To Mueller-Hinton broth, add the magnesium stock solution to a final concentration of 10-12.5 µg/ml Mg²⁺.
5. Add the calcium stock solution to a final concentration of 20-25 µg/ml Ca²⁺.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

S. Trypticase soy broth (TSB)

TSB is used for making suspensions of *H. influenzae* prior to testing for hemin and NAD requirements. Heart infusion broth, sterile saline, or PBS may be substituted for TSB.

Media preparation

1. Prepare the volume of TSB needed in a flask according to the instructions on the label of the dehydrated medium.
2. Dispense 5 ml into 15x125 mm tubes.
3. Autoclave at 121°C for 20 minutes.
4. Cool and store at 4°C.

Quality control

1. Grow a *S. pneumoniae* QC strain for 18-24 hours on a BAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
 - *H. influenzae* does not grow in TSB, but the medium should not be toxic to other bacteria. Therefore, *S. pneumoniae* should be used to QC for toxicity.
2. Inoculate a tube of TSB with a loop of overnight growth from the BAP and incubate overnight at 35°C.
3. The broth should be turbid the next day. Subculture the broth onto a BAP to test for proper growth characteristics of *S. pneumoniae*.
4. Observe the BAP for specific colony morphology and hemolysis.

Passing result: *S. pneumoniae* colonies are small and appear grey to grey-green surrounded by a distinct green halo (alpha-hemolysis).

II. Storage and transport media

A. Defibrinated sheep blood

Defibrinated sheep blood is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. Mechanically shake 30 ml sheep blood with sterile glass beads or a wooden stick device in a 125-250 ml Erlenmeyer flask at approximately 90 rpm for 7-9 minutes.
 - Clotting factors will be visible in the flask as a translucent, fibrous web.
2. Remove the clotting factors using sterile forceps.
3. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

B. Dorset Transport medium

Dorset Transport medium is used for short term storage of *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* isolates.

Media preparation

1. Combine sterile 0.85 % saline solution with beaten whole hen's eggs at a 1:3 ratio.
2. Inspissate (i.e., thicken) the mixture in an electric inspissator at 80°C for 60 minutes.
3. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

C. Greaves solution

Greaves solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. To 200.0 ml distilled water, add:
 - 10.0 g bovine albumin, fraction V
 - 10.0 g L-glutamic acid, sodium salt
 - 20.0 ml glycerol
2. Mix all ingredients listed below until they are completely dissolved.
3. Filter-sterilize the solution and transfer to a sterile flask.
4. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

D. Modified Trans-Isolate (MT-I) medium

Modified Trans-Isolate medium (MT-I) was designed and developed to be a simple and inexpensive medium for transport of CSF and growth of meningococci in large scale epidemics of meningococcal disease. It is less expensive and easier to produce than the standard T-I, and can be produced in most microbiology laboratories at approximately USD \$0.50/bottle, providing a relatively inexpensive, rapidly available (reducing the production and shipping time and cost) transport medium and diagnostic tool for use during a meningitis epidemic. The ingredients of the MT-I formulation are similar to those in T-I, with modifications to eliminate costly ingredients and save time.

Laboratory evaluation has demonstrated that growth/survival of isolates of meningococci was equal in T-I and MT-I under several environmental conditions. It has not been evaluated under field conditions with clinical specimens yet. It does not support the growth of *H. influenzae*; therefore, it is not used for routine surveillance of agents of bacterial meningitis.

0.5 oz, sterile round, clear glass screw-cap (with rubber liner) bottles should be used. MT-I bottles should be stored upright at 4°C when not in immediate use and warmed to room temperature (25-30°C) before use. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature. MT-I media has a shelf life of 1 year with proper storage.

Media preparation

1. Weigh all ingredients listed below and place them into a 500mL Erlenmeyer flask.

8 g 2.0% gelatin
2 g 0.5% agar
2.5 g Tris-HCl
0.5 g Tris base
12 g 3.0% Tryptic Soy Broth

2. Add 350 ml distilled, deionized water to the flask (pH should be 7.5 at 25°C).
3. Place a magnetic stirring bar in the flask and place the flask on hot plate stirrer.
4. Bring the solution to boiling (90-100°C) to melt and dissolve the gelatin until the medium is completely clear (about 30-45 minutes).
5. Remove the flask from the hot plate stirrer.
6. Mix 2.6 g 0.5% soluble starch with a small amount of cold water and dissolve completely.
7. Once the starch is evenly distributed throughout medium, add 1.6 g 0.4% activated charcoal and adjust water to 400ml.
8. Return the flask to the hot plate stirrer until all ingredients are thoroughly mixed (about 10 minutes).
 - The solution should be liquid, appear black in color, and should not have any clumps.
9. Turn down the heat to low so the flask can be handled comfortably while dispensing the medium.
 - Optional: At this step, the procedure can be stopped overnight if there is not enough time to dispense. Split the media into two flasks and place both flasks at 4°C overnight. The next day, apply heat until fully melted and aliquot (see step 10).
10. While the media is being stirred, use a sterile serological pipette to remove 7 ml of medium and dispense into each bottle.
11. Cap each bottle loosely with a screw cap.
12. Autoclave the bottles for 15 minutes at 121°C.
13. Tighten the caps as soon as possible after autoclaving.
14. Swirl the bottles to avoid the charcoal settling.
15. Slant the bottles overnight (or at least 4 hours) on wooden slanting stick (35 mm, 35 mm, 500 mm).
 - When slanted, the liquid should reach shoulder of the bottle.

16. Once the medium is firm, stand the bottles upright.
17. In approximately 1 hour, the weak slant should release some of the broth and the medium should appear biphasic.
18. Store at 4°C when not in use.

Quality control

1. Grow *N. meningitidis* and *S. pneumoniae* QC strains for 18-24 hours on a BAP or a CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. For each organism, make a cell suspension equivalent to a 0.5 McFarland standard (~equivalent to 1.5x10⁸ CFU/ml) and serially dilute it to achieve an inoculum size of 10³ CFU/ml in brain heart infusion (BHI) broth.
3. Remove the screw cap from 3 MT-I bottles.
4. Inoculate the MT-I bottles with 100 µl of the 10³ CFU/ml suspensions within 15 minutes of preparation (inoculum size is 100 CFU) and replace the screw caps tightly.
 - After inoculation and replacement of the screw-caps, invert each MT-I bottle several times to mix.
5. Slightly loosen the screw-caps of the MT-I bottles to allow some air exchange.
6. Incubate the MT-I bottles for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
7. Close the screw-caps tightly and invert each MT-I bottle several times to mix.
8. Remove 10 µl of broth from each MT-I bottle and inoculate a BAP for the MT-Is containing *N. meningitidis* and *S. pneumoniae* and a CAP for the MT-I containing *H. influenzae*.
9. Streak for isolation with a sterile loop and incubate plates for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to detect growth of the QC strains.

Passing results:

- *N. meningitidis* should appear large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis) on the BAP.

E. 10% skim milk and 15% glycerol solution

10% skim milk and 15% glycerol solution is used for long term preservation of isolates by freezing at -70°C.

Media preparation

1. Place 10 g dehydrated skim milk and 85 ml distilled water into flask A. Swirl to mix.
2. Place 15 ml of glycerol into flask B.
3. Autoclave both flasks at 115°C for 10 min, and exhaust the pressure carefully.
4. While still hot, pour the contents of flask A into flask B in a safety cabinet.
5. Store at 4°C when not in use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

F. Skim milk tryptone glucose glycerol (STGG) medium

STGG medium is used for transport and short term storage of nasopharyngeal swabs.

Media preparation

1. Add the following ingredients to 100 ml distilled water:
 - 2 g skim milk powder
 - 3 g TSB
 - 0.5 g glucose
 - 10 ml glycerol
2. Mix to completely dissolve all ingredients.
3. Dispense 1.0 ml amounts into 1.5 ml screw-cap vials.
4. Loosen the screw-caps and autoclave at 121°C for 10 minutes.
5. Tighten the caps after autoclaving and store at -20°C until use.

Quality control

1. Streak 10 µl onto a BAP or a CAP and incubate for 48 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to verify the sterility of the solution.

Passing result: no growth should be observed.

G. Trans-Isolate (T-I) medium

T-I is used to transport CSF and grow *N. meningitidis*, *H. influenzae*, and *S. pneumoniae* from CSF. 10 cc tubing vials with rubber septum plugs and aluminum crimp seal caps should be used. T-I bottles should be stored upright at 4°C when not in immediate use. T-I media should be warmed to room temperature (25°C) before use. In the refrigerator, the liquid phase becomes gelatinous but re-liquefies at room temperature. T-I media has a shelf life of 1 year with proper storage.

Media preparation

Diluent for solid and liquid phases: 3- (N-morpholino) propanesulfonic acid (MOPS) buffer; 0.1 M, pH 7.2

1. Dissolve 20.93g MOPS in 900ml distilled water.
2. Adjust to pH 7.2 with 1N NaOH.
3. Adjust volume to 1000 ml with distilled water.

Solid phase

Activated charcoal	2.0 g
Soluble starch	2.5 g
Agar	10.0 g

1. Suspend activated charcoal, soluble starch, and agar into 500 ml of MOPS buffer in a flask and add a magnetic bar to the flask.
2. Heat on a magnetic stirrer hot plate to dissolve the charcoal and starch and melt the agar.
3. While stirring to keep the charcoal in suspension, use a sterile serological pipette to remove 5.0 ml and dispense into each serum bottle.
4. Cap each bottle with a piece of aluminum foil and autoclave at 121°C for 20 minutes.
5. Remove from the autoclave and slant the bottles until they reach room temperature (25°C), so that the apex of the agar reaches the shoulder of each bottle.

Liquid phase

Tryptic soy broth (TSB)	30.0 g
Gelatin	10.0 g
MOPS buffer	500.0 ml

1. Heat the TSB, gelatin, and MOPS buffer to completely dissolve the gelatin and avoid coagulation.

2. Autoclave the media at 121°C for 15 minutes.

Optional additive for growth of *H. influenzae*

1. Once the bulk liquid phase medium has cooled to room temperature (25°C) after autoclaving, add 10 ml of a sterile liquid growth supplement containing NAD and hemin aseptically to help support growth of *H. influenzae*.
 - Alternatively, aseptically add 0.1 ml of the supplement to an individual T-I bottle (1% of the volume of both phases) or to a limited number of bottles, as needed.
 - If a commercial growth supplement is used (preferred method), it should be a sterile product and can be added directly to the T-I medium.
 - If the growth supplement is prepared in the laboratory, it should be filter-sterilized prior to being added to the T-I medium. To prepare the supplement in the laboratory:
 1. Dissolve the lyophilized supplement into an appropriate diluent (usually water).
 2. Filter-sterilize using a 0.45 micron pore size membrane.
 3. Use immediately.

T-I medium

1. Dispense 5 ml of the liquid phase aseptically into each of the bottles containing the solid phase slants.
2. Seal the bottles with sterile rubber septum plugs and aluminum caps. Use a hand crimping tool to fasten the aluminum caps if an automated system is not available.
3. Store at 4°C when not in use.

Quality control

1. Grow *N. meningitidis*, *S. pneumoniae*, and *H. influenzae* QC strains for 18-24 hours on a BAP or CAP at 35-37°C with ~5% CO₂ (or in a candle-jar).
2. For each organism, make a cell suspension equivalent to a 0.5 McFarland standard (~equivalent to 1.5X10⁸ CFU/ml) and serially dilute to achieve an inoculum size of 10³ CFU/ml in brain heart infusion (BHI) broth.
3. Using sterile forceps pull the aluminum cover of 3 T-I bottles away from the rubber stopper and wipe the stopper with 70% isopropanol or ethanol.
 - Do not use povidone-iodine.

4. Use a sterile syringe and needle to inoculate the T-I bottles with 100 μ l of the 10^3 CFU/ml suspensions within 15 minutes of preparation (inoculum size is 100 CFU).
 - Use a new needle and syringe for each T-I. After inoculation and removal of the syringe and needle from the rubber stopper, invert each T-I bottle several times to mix.
5. Insert a sterilized T-I venting needle through the rubber stopper of each of the inoculated T-I bottles.
 - Be sure that the venting needles do not touch the broth.
6. Incubate vented T-I bottles for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar).
7. Remove and discard venting needles. Invert each T-I bottle several times to mix.
8. Use a sterile syringe and needle to remove 100-200 μ l of broth from each T-I bottle and place the broth into a sterile, labeled 1.5 ml microcentrifuge tube. Inoculate a BAP for the T-Is containing *N. meningitidis* and *S. pneumoniae* and a CAP for the T-I containing *H. influenzae* with 10 μ l of this broth.
9. Streak for isolated colonies with a sterile loop and incubate plates for 18-24 hours at 35-37°C with ~5% CO₂ (or in a candle-jar) to detect growth of the QC strains.

Passing results:

- *N. meningitidis* should appear as large, round, smooth, moist, glistening, and convex, grey colonies with a clearly defined edge on the BAP.
- *S. pneumoniae* should appear as small, grey to grey-green colonies surrounded by a distinct green halo (alpha-hemolysis) on the BAP.
- *H. influenzae* should appear as large, round, smooth, convex, colorless-to-grey, opaque colonies on the CAP with no discoloration of the medium.

III. Miscellaneous reagents

A. Gram stain reagents

Ammonium oxalate-crystal violet

1. Dissolve 2.0 g certified crystal violet into 20.0 ml of 95% ethyl alcohol.
2. Dissolve 0.8 g ammonium oxalate into 80.0 ml distilled water.
3. Mix the two solutions together and allow them to stand overnight at room temperature (25°C).
4. Filter through coarse filter paper before use.

5. Store at room temperature (25°C).

Gram's iodine (protect solution from light)

1. Grind 1.0 g iodine (crystalline) and 2.0 g potassium iodide in a mortar. Small additions of distilled water may be helpful in preparing the solution.
2. Add to 300.0 ml distilled water.
3. Store at room temperature (25°C) in a foil-covered bottle.

Decolorizer is 95% ethyl alcohol

Counterstain (there are 2 options: safranin or carbol-fuchin)

Safranin

1. Add 2.5 g certified safranin-O to 100.0 ml 95% ethyl alcohol.
2. Add 10.0 ml safranin and ethyl alcohol solution made in step 1 to 90.0 ml distilled water.
3. Store at room temperature (25°C).

Ziehl-Nielsen carbol-fuchsin (may be a more effective counterstain than safranin)

1. Dissolve 0.3 g basic fuchsin in 10.0 ml 95% ethyl alcohol.
2. Add 5.0 ml melted phenol crystals to 95.0 ml distilled water.
3. Add the 5% phenol solution to the fuchsin solution and let stand overnight.
4. Filter through coarse filter paper.
5. Store at room temperature (25°C) in a foil-covered bottle for up to 1 year.

B. Iodine tincture

Iodine tincture is used as a skin antiseptic and should not be used to disinfect the rubber stoppers of T-I and blood culture bottles.

1. Add 1 g of iodine to 100 ml of 70% isopropyl alcohol.
2. Store at room temperature (25°C) in a foil-covered bottle for up to 1 year.

C. McFarland turbidity standards

1. Prepare a 1% solution of anhydrous BaCl₂ (barium chloride).
2. Prepare a 1% solution of H₂SO₄ (sulfuric acid).
3. Combine and completely mix the barium chloride and sulfuric acid solutions to form a turbid suspension of BaSO₄ in a specific proportion for each McFarland turbidity standard as shown in Table 1.
4. Place the resulting mixture in a foil-covered screw-cap tube.
5. Store the McFarland standard at room temperature (25°C) when not in use. Prepare a fresh standard solution every 6 months. McFarland standard density solution will precipitate and clump over time, and it needs vigorous vortexing before each use. Mark the tube to indicate the level of liquid, and check before use to be sure that evaporation has not occurred.

Table 1. McFarland turbidity standards

McFarland turbidity standard no.	0.5	1	2	3	4
1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1x10 ⁸ CFU/ml)	1.5	3.0	6.0	9.0	12.0

D. Phosphate buffered saline (PBS)

1. To 1000 ml distilled water, add the following ingredients for 0.1 M PBS:
 7 g sodium dihydrogen phosphate
 7 g disodium hydrogen phosphate
2. Mix to completely dissolve ingredients.
3. Adjust pH to 7.2 with 1 N acid or base.
4. Dispense buffer into a flask and autoclave at 121°C for 15 minutes.
5. Store at room temperature (25°C) for up to one year.

E. Physiological saline

1. Dissolve 8.5 g NaCl into 1 L distilled water.
2. Autoclave at 121°C for 15 minutes or sterilize using membrane filtration.
3. Store at room temperature (25°C) for up to 6 months.

Summary

This manual is the 2nd edition of the World Health Organization Manual “Laboratory Methods for the Diagnosis of Meningitis Caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*.” The first edition was published in 1999 and summarized the laboratory techniques used in isolation and identification of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* from the blood or cerebrospinal fluid of patients with bacterial meningitis.

In the twelve years since the first edition of this manual, important changes have occurred both in the epidemiology of bacterial meningitis and in the available laboratory techniques for isolating, identifying, and characterizing the causative organism. Great progress has been made in increasing worldwide access to vaccines to prevent pneumococcal, Hib, and meningococcal disease. These developments have prompted a revision of the manual to produce this second edition. The revision follows the format of the first edition, but has been expanded to include specific chapters on Results Management and Reporting of Data, Biosafety, PCR for Detection and Characterization of Bacterial Meningitis Pathogens, Antimicrobial Susceptibility Testing, Characterization by Molecular Typing Methods and Quality Control/Quality Assurance.

Surveillance for diseases caused by infectious agents targeted by newer vaccines are likely to require a syndromic approach, that is identifying persons who are ill with a specific group of symptoms that may indicate the presence of a specific disease that must be confirmed by laboratory testing. The laboratory plays a crucial role in the diagnosis of bacterial meningitis. Patients diagnosed with meningitis syndrome may all exhibit similar symptoms (i.e., fever, headache, stiff neck) but each individual’s disease could be caused by a variety of organisms including the bacterial meningitis pathogens *N. meningitidis*, *S. pneumoniae*, and *H. influenzae*. Hence, a strong laboratory component of surveillance must complement the clinical syndromic surveillance to allow for diagnostic confirmation of the specific disease.

Publication date: 01 DEC 2011