Laboratory Manual
ANTIMICROBIAL SUSCEPTIBILITY TEST

Safety certification

Haemophilus influenzae

MEDICAL LABORATORY SCIENCE

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Any questions?

If you have any questions or need clarification at any stage of the experiment, don't hesitate to ask! Clear understanding is key to performing the experiment correctly and safely.
Here are a few points to consider when asking questions:

- Do you fully understand the purpose of the experiment?
- Are the procedures and steps clear to you?
- Do you need clarification on any of the equipment or materials being used?
- Are there any safety concerns or precautions that are not fully understood?

Remember, it's always better to ask and be sure than to proceed with uncertainty!

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Learning Objectives

- Understand the principles and importance of Antibiotic Susceptibility Testing in clinical microbiology.
- Perform AST using methods such as the disk diffusion (Kirby-Bauer) method and interpret the results based on standardized guidelines.
- Differentiate between resistant, intermediate, and susceptible bacterial strains based on zone of inhibition measurements.
- Apply the knowledge gained to make informed decisions regarding the selection of appropriate antibiotics for treatment.
- Recognize the significance of AST in preventing the spread of antibiotic resistance and promoting effective antimicrobial stewardship.

Brief Description

Antibiotic Susceptibility Testing (AST) is a laboratory procedure used to determine the effectiveness of antibiotics against specific bacterial pathogens. It helps identify which antibiotics can inhibit or kill the bacteria causing an infection.

In this process, bacterial samples are exposed to various antibiotics, and their growth is monitored. The results guide healthcare professionals in selecting the most effective antibiotic for treatment. Common methods include the disk diffusion method (Kirby-Bauer test), broth dilution, and E-test. AST is crucial for combating antibiotic resistance and ensuring proper use of antibiotics.

Principle of Test

The principle of the Antibiotic Susceptibility Test (AST) is to evaluate the effectiveness of antibiotics against bacteria. The test determines whether a specific bacterium is sensitive (susceptible) or resistant to various antibiotics.

Requirements of a Reference Laboratory

A reference laboratory differs from a clinical laboratory in that it focuses on confirming and investigating isolates sent from other labs or hospitals, and performs standardized antimicrobial susceptibility testing. This manual is designed for use in a reference or national central laboratory, where consistent quality control and ample resources are available for regular testing. Reference labs must participate in quality assurance programs at least once a year and administer such programs for other labs in their jurisdiction. The WHO encourages labs in resource-limited countries to establish national quality assessment schemes and participate in at least three surveys annually.

Due to the costs involved in maintaining time, supplies, and trained personnel, not all countries may be able to support a fully functional reference laboratory. In such cases, countries should consult regional or sub-regional reference laboratories for guidance on handling isolates that need further investigation. To meet the standards set forth in this manual and other similar protocols, reference labs must continuously invest in equipment, supplies, and human resources, which should be ensured by the Ministry of Health or an equivalent agency.

• Laboratory space

• Trained laboratory technologists

• Water (purified either by filter system or distillation apparatus)

• Stable source of electricity

• Equipment

– Water bath

– Incubator

– Refrigerator

– Freezer

– Autoclave

– Vortex mixer

– Labels and/or permanent marking pens

– Materials for record-keeping (e.g., laboratory log-books, and a computer

with printer and Internet / e-mail access)

– Antimicrobial disks and / or antimicrobial gradient agar diffusion tests

(Etests®) (depending on the organisms to be tested)

• Standard laboratory supplies (e.g., plates, tubes, pipettes, flasks, inoculating loops, other glassware or plasticware, rulers, bunsen burners or alcohol burners,pH meter, bleach, alcohol), media and reagents.

It is also of considerable importance that the reference laboratory have an open line of communication with public health authorities, including ministries of health, professionals in the medical field, and policymakers. If the laboratory is responding to an epidemic that extends across borders, an outside public health agency (e.g., the WHO) may become involved; in such situations, it is significant that data from the laboratory will enable better decision-making for clinical treatment and public health policy in more than one country.

Bacterial Agents of Pneumonia and Meningitis

Haemophilus influenzae

Neisseria meningitidis

Streptococcus pneumoniae

Haemophilus influenzae

CONFIRMATORY IDENTIFICATION AND

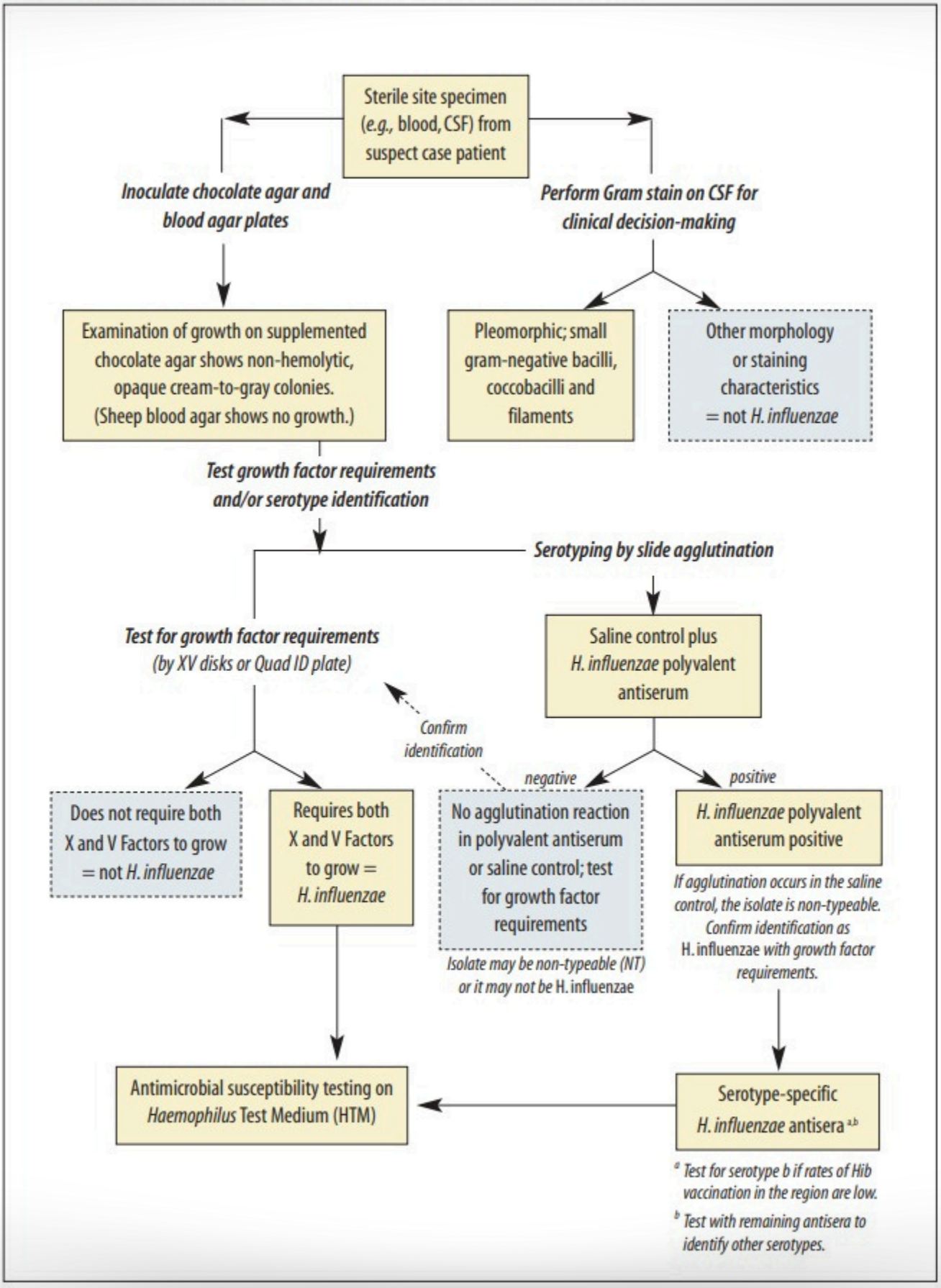
ANTIMICROBIAL SUSCEPTIBILITY TESTING

Haemophilus *influenzae* is a common etiologic agent of diseases such as pneumonia, meningitis, otitis media, and conjunctivitis. Meningitis caused by H. influenzae occurs almost exclusively in children less than five years of age, and most invasive H. influenzae disease is caused by organisms with the type b polysaccharide capsule (H. influenzae type b, commonly abbreviated as Hib). There are conjugate vaccines to prevent H. influenzae infections caused by serotype b, though they are not widely available in some parts of the world. No vaccines for the other serotypes or for unencapsulated strains have been developed. Although meningitis is the most severe presentation of disease, H. influenzae pneumonia causes more morbidity than H. influenzae meningitis.

Confirmatory identification of H. influenzae H. influenzae are characterized as small, gram-negative bacilli or coccobacilli that require X and V growth factors, grow on chocolate agar (but not on sheep blood agar), and have a pungent indol smell. Methods for the isolation and presumptive identification of H. influenzae are included in Appendix 4. Figure 1 presents a schematic flowchart of confirmatory identification of H. influenzae.

Identification of the H. influenzae serotype Laboratory identification of H. influenzae includes testing for X and V factor requirements and then performing serotyping; this sequence of testing is an efficient way to save costly antisera. However, when the laboratory results must be obtained rapidly for clinical decision-making, serotyping should be performed first the prompt presumptive identification of H. influenzae. Isolates identified as H. influenzae with typing antisera should still be confirmed by testing for X and V factor requirements

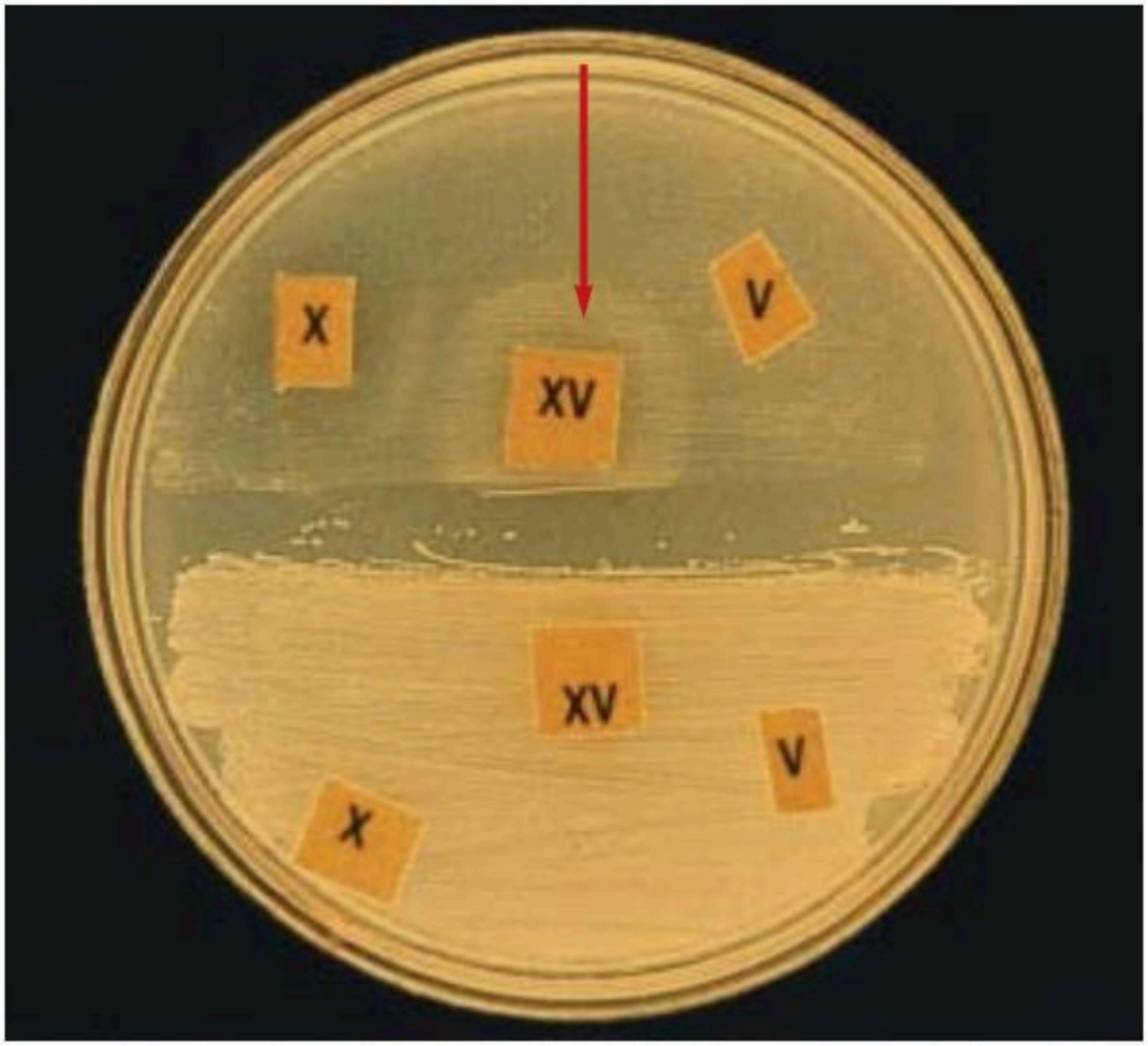
FIGURE 1: Flowchart for laboratory identification of *Haemophilus influenzae*



Growth factor test using Haemophilus Quad ID Plates

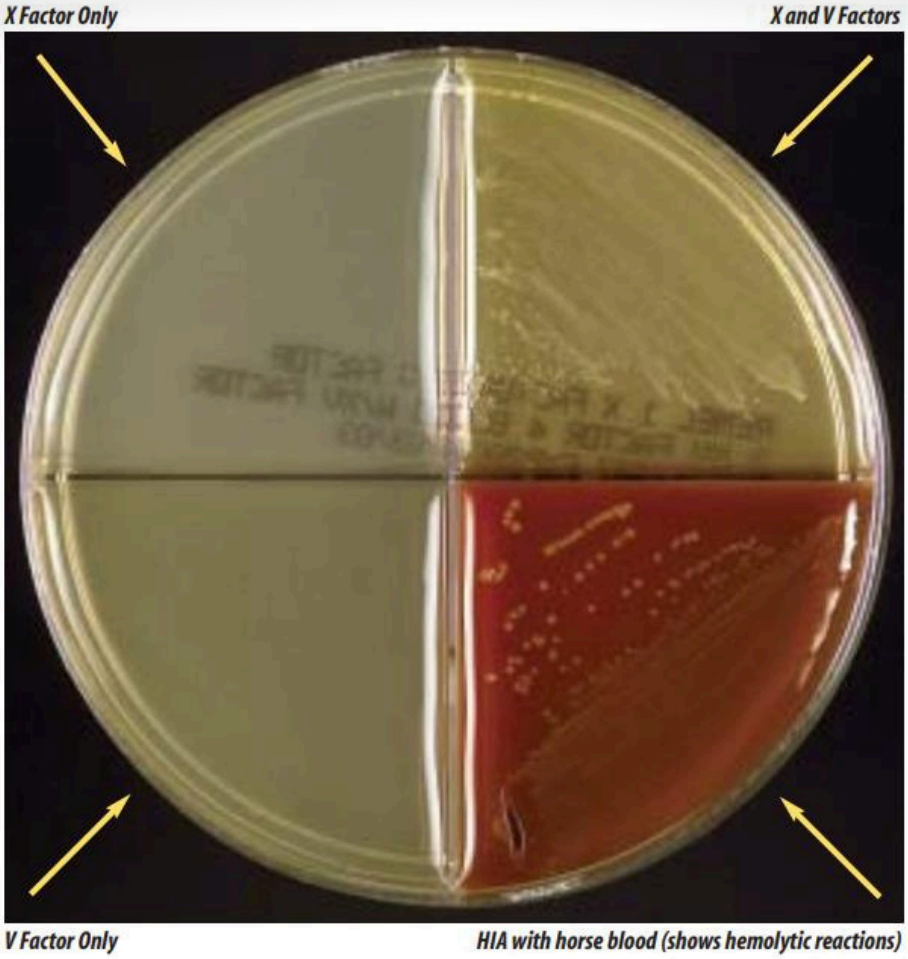
Quad ID plates are another, although more expensive, method for determining growth factor requirements of Haemophilus isolates (Figure 4). Available commercially, the Quad ID plate is divided into four agar quadrants: one quadrant includes medium containing haemin (X factor); one quadrant includes medium containing NAD (V factor); another quadrant contains medium that includes both X and V factors; and, the final quadrant contains heart infusion agar or blood agar base with 5% horse blood for differentiating H. haemolyticus, an oral species requiring X and V factors, from H. influenzae. Quadrant location of the growth factors may vary with commercial brand of the Quad ID plate.

FIGURE 3: Growth factor requirements: X and V factors on paper disks



The top strain is only growing around the disk containing both X and V factors and can therefore be considered presumptive *H. influenzae*.

FIGURE 4: Growth factor requirements: *Haemophilus* Quad ID plate



The Quad ID plate is divided into four compartments. One quadrant (*upper left*) includes medium containing haemin (X factor). One quadrant (*lower left*) includes medium containing nicotinamide dinucleotide (NAD, or V factor). Another quadrant (*upper right*) contains medium that includes both X and V factors. The fourth quadrant (*lower right*) contains heart infusion agar (HIA) with 5% horse blood to detect hemolytic reactions of *Haemophilus* species.

Methods

a) Inoculate the Quad ID plate by suspending the growth from a young, pure culture of suspected Haemophilus in tryptone soy broth (TSB) or distilled water to a light, milky suspension (equivalent to a 0.5 McFarland turbidity standard). Using a bacteriological loop, streak one loopful of this suspension on each quadrant of the plate, beginning with the V quadrant and ending with the blood quadrant. Streak the entire quadrant, starting at the periphery and streaking toward the center of the plate. Stab into the blood agar for detection of weak hemolysis

b) Invert the plate and incubate under a CO2-enhanced atmosphere (in a candle-jar or CO2-incubator) for 18–24 hours at 35˚C.

c) After incubation, examine the blood section for hemolysis and the other sections for growth. H. influenzae typically shows growth in the XV quadrant and in the (horse-)

blood quadrant with no hemolysis. If strong growth occurs in either one of the X or V quadrants besides XV, the organism is probably another species of Haemophilus. If growth occurs in every quadrant, the culture is probably not a species of Haemophilus. (Note: Occasionally, H. influenzae may show slight growth in the V-factor quadrant.) Read and record results.

Hemolytic reactions of Haemophilus species

Although most laboratories will not need to determine the hemolytic reaction of each Haemophilus spp. (because too few Haemophilus strains will be isolated), some laboratories may want to determine the hemolytic reaction to definitively identify both H. influenzae and H. haemolyticus.

• If X, V, and XV factor disks or strips were used to test growth factor requirements, a separate test to detect hemolytic reactions must be performed by inoculating a broth suspension of the strain on HIA + 5% rabbit blood (or agar infusion base containing horse blood); the hemolytic reaction permits determination the species.

• If a Quad ID plate was used to test for growth factor requirements, the hemolytic reaction of the organism is tested in the (horse-) blood agar quadrant of the plate; thus no separate test is required. H. influenzae should be α-hemolytic (i.e., causing a greening in the agar around the colony) or γ-hemolytic (non-hemolytic) on the HIA plate containing 5% rabbit blood, while H. haemolyticus will exhibit ß-hemolysis (i.e., a clearing of the blood cells in the agar surrounding the colonies on the plate). A summary of test results used in the identification of H. influenzae and most closely related Haemophilus species is shown in Table 1. Proper determination of the hemolytic reaction is the only way to differentiate H. influenzae from H. haemolyticus.

Antimicrobial susceptibility testing of H. influenzae

The results of antimicrobial susceptibility tests will be used to select the most effective antimicrobial agent to use for treating patients. This laboratory manual describes susceptibility testing of Haemophilus 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 Etest® provides more detailed information about the minimal inhibitory concentration (MIC) of an antimicrobial agent. The accuracy and reproducibility of these tests are dependent on following a standard set of procedures and conditions in laboratories on an on-going basis. A sample worksheet for recording antimicrobial susceptibility test results for H. influenzae is included in Figure 5.

Media and disks for antimicrobial susceptibility testing

Antimicrobial susceptibility can be determined using the disk diffusion method. The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS;3 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 are dependent on the consistent use of a standard set of procedures in laboratories. This section describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results.

The recommended medium for antimicrobial susceptibility testing for H. influenzae is Haemophilus test medium (HTM) (Appendix 2). The MuellerHinton agar used for this test should be thymidine-free to obtain consistent results with trimethoprim-sulfamethoxazole (also referred to as cotrimoxazole). All media used for antimicrobial susceptibility testing should be freshly prepared. Recommended antimicrobial agents for testing are ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole.

The 10-µg ampicillin disk predicts both intrinsic (i.e., penicillin-binding protein mediated, or "PBP") and ß-lactamase (beta-lactamase) mediated penicillin and ampicillin resistance and should be used when testing H. influenzae. (Methods for ß-lactamase testing of H. influenzae are listed after the direct antimicrobial susceptibility testing methods in this section.) For H. influenzae, a 30-µg chloramphenicol disk is used for predicting resistance to chloramphenicol, and a 1.25/23.75-µg trimethoprim-sulfamethoxazole disk is used for predicting trimethoprim-sulfamethoxazole resistance. The zone diameter sizes can only be properly interpreted when HTM is used, as per NCCLS standards.

Quality control of antimicrobial susceptibility testing of H. influenzae

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. H. influenzae ATCC 49247 is the control strain used when testing H. influenzae for most antimicrobial agents (e.g., ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole), although ATCC 49766 is appropriate for some others. (Consult NCCLS document M100- S12 [2002] for more complete information.) Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits, which are included in Table 2. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

FIGURE 5: Sample form for recording antimicrobial susceptibility test results for *Haemophilus influenzae*

Date of Testing: _____/_____/______
*Test performed by:*____________________

Interpretation of susceptibility: *S* = susceptible *I* = intermediate *R* = resistant

Specimen number	Meningitis isolate?	Organism	Chloramphenicol	Trimethoprim-sulfamethoxazole	Ampicillin	*(other drug)* ______
			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*
ATCC 49247	N/A	*H. influenzae* NCCLS QC strain *QC in range?* →	mm µg/ml Yes No	mm µg/ml Yes No	mm µg/ml Yes No	mm µg/ml Yes No

Reveiwed by: ________ ***Date:*** ___/___/____

Note: After 16 – 18 hours of incubation, check the results for the quality control (QC) strain 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 Table 2.)*

- Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other environmental factors. The medium used may be a source of error if it fails to conform to NCCLS 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.
- 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.
- 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 the inoculum is too light. 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 trimethoprimsulfamethoxazole disks.

Quality control tests should be performed once per week if antimicrobial susceptibility tests are performed daily (after 30 days of in-control results), or with every group of tests when testing is done less frequently. They should also be done with each new batch of test medium and every time a new lot of disks is used.

Antimicrobial susceptibility testing of H. influenzae by the disk diffusion method

Prepare the inoculum for seeding the antimicrobial susceptibility media with H. influenzae from fresh, pure cultures of H. influenzae (i.e., from isolates grown overnight on supplemented chocolate agar). Prepare cell suspensions of the bacteria to be tested in broth or sterile physiological saline; use a suspension equal to a density of a 0.5 McFarland turbidity standard for the inoculum. (Preparation of a McFarland turbidity standard is described in Appendix 2.

a) Suspend viable colonies from an overnight chocolate agar plate in a tube of broth 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. This suspension should be used within 15 minutes.

b) Compare the suspension to the 0.5 McFarland turbidity standard by holding the suspension and the McFarland turbidity standard in front of a light against a white background with contrasting black lines and compare the density (see Figures 51 and 52). If the density of the suspension is too heavy, the suspension should be diluted with additional broth. If the density of the suspension is too light, additional bacteria should be added to the suspension.

c) When the proper density is achieved, dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid.

d) Use the swab to inoculate the entire surface of the HTM plate three times, rotating the plate 60 degrees between each inoculation (see Figure 34). Use the same swab with each rotated streak, but do not re-dip the swab in the inoculum (i.e., the bacterial cell suspension).

e) Allow the inoculum to dry before the disks are placed on the HTM 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.)

f) After the plate is dry, antimicrobial disks should be placed on the HTM plate as shown in Figure 6. The disks should be placed on the agar with sterile forceps and tapped gently to insure adherence to the agar. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

g) Invert the plate and incubate it in a CO2-enriched atmosphere (5% CO2-incubator or candle-extinction jar) for 16–18 hours at 35˚C.

Note: If this is a new batch of HTM, the antimicrobial disks are new, or it is an otherwise appropriate time to perform quality control, follow steps a through g- above and run parallel tests on the reference strain(s). Appropriate disk diffusion zone sizes for the reference quality control strain (for the antimicrobial agents included in this chapter) are presented in Table 2.

h) After overnight incubation, measure the diameter of each zone of inhibition. 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 (Figure 6).

• Care should be taken not to touch the disk or surface of the agar. Sterilize 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.

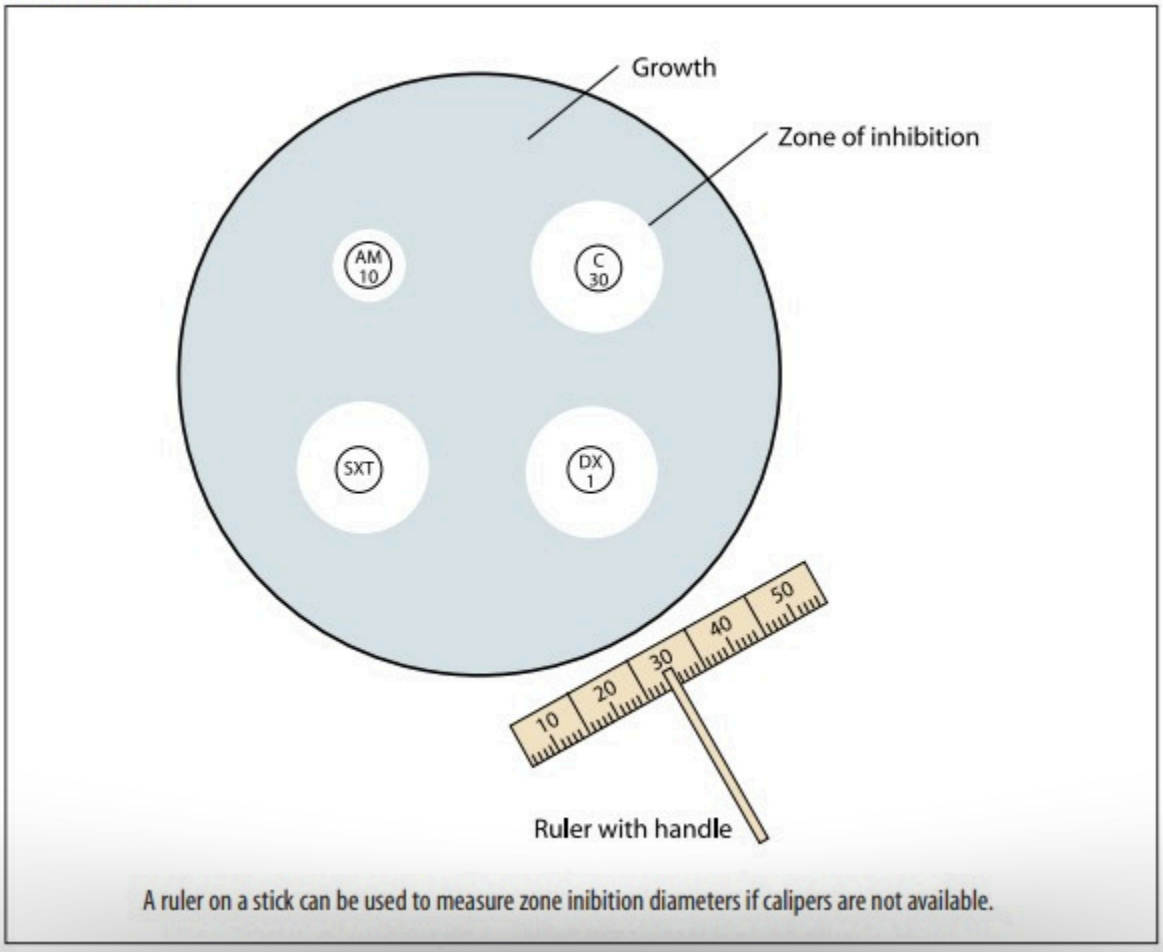
Record the results in millimeters (mm). Figure 5 provides a sample form for recording results.

i) Interpretation of the antimicrobial susceptibility is obtained by comparing the results obtained and recorded (in the manner described in this protocol) to the NCCLS standard inhibition zone diameter sizes presented in Table 2.

Minimal inhibitory concentration testing of H. influenzae isolates

Laboratorians determining the minimal inhibitory concentration (MIC) for resistant isolates must be highly skilled in performing these tests and committed to obtaining accurate and reproducible results. In addition, a national (or regional) reference laboratory must have the ability and resources to store isolates either by lyophilization or by freezing at -70 ˚C. Antimicrobial susceptibility testing by disk diffusion indicates whether an organism is susceptible or resistant to an antimicrobial agent. For surveillance purposes, a laboratory may want to quantify "intermediate" antimicrobial

FIGURE 6: The antimicrobial susceptibility disk diffusion test: disk placement and measurement of inhibition zone diameters



A ruler on a stick can be used to measure zone inibition diameters if calipers are not available.

TABLE 2: Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for *Haemophilus influenzae*

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml) [a]			NCCLS QC strain *H. influenzae* ATCC 49247 [b]
		Susceptible	Intermediate	Resistant	
Chloramphenicol	30 µg	> 29 mm (< 2 µg/ml)	26– 28 mm (4 µg/ml)	< 25 mm (>8 µg/ml)	31 – 40 mm (0.25 – 1 µg/ml)
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25/ 23.75 µg	≥ 16 mm (< 0.5/9.5 µg/ml)	11 mm – 15 mm (1/18 – 2/36 µg/ml)	≤ 10 mm (> 4/76µg/ml)	24 – 32 mm (0.03/0.59 – 0.25/4.75 µg/ml)
Ampicillin	10 µg	≥ 22 mm (< 1 µg/ml)	19 mm – 21 mm (2 µg/ml)	≤ 18 mm (> 4 µg/ml)	13 – 21 mm (2 – 8 µg/ml)

[a] *Source:* NCCLS (2002) *Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement.* NCCLS document M100-S12 [ISBN 1-56238-454-6]. NCCLS 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA.

[b] The quality control strain *H. influenzae* ATCC 49247 is appropriate for the testing of the antimicrobial agents included in this table and this laboratory manual overall; however, for testing of some other antimicrobial agents, NCCLS recommends that a different QC strain be used. Laboratories testing the susceptibility of *H. influenzae* to *antimicrobial agents other than those listed* should therefore refer to the NCCLS document M100-S12 (or subsequent updates) for appropriate methods.

Data for decision-making

Once the laboratory has assessed the serotype and antimicrobial susceptibility patterns of H. influenzae isolates, the information should be reported back to public health officials promptly. Factors to consider in the development of treatment policy include:

- Childhood immunizations should be considered if H. influenzae type b is a major local cause of invasive disease.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or able to be obtained quickly).

Consideration of such factors when making data-based decisions will help public health officials meet needs in a manner appropriate to the local situation and the specific antimicrobial susceptibility profile. National recommendations for empiric antibiotic utilization should be developed after considering antimicrobial susceptibility data, cost, availability, convenience, and other factors.