

Manual for the Laboratory Identification and Antimicrobial Susceptibility Testing of Bacterial Pathogens of Public Health Importance in the Developing World



Haemophilus influenzae,
Neisseria meningitidis,
Streptococcus pneumoniae,
Neisseria gonorrhoeae,
Salmonella serotype Typhi,
Shigella, and
Vibrio cholerae



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*Haemophilus influenzae, Neisseria meningitidis,
Streptococcus pneumoniae,
Neisseria gonorrhoeae, Salmonella* serotype Typhi,
Shigella, and Vibrio cholerae

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Contents

I. Introduction	1
II. Requirements of a Reference Laboratory	3

Bacterial Agents of Pneumonia and Meningitis

III. <i>Haemophilus influenzae</i>	5
• Confirmatory identification	5
• Antimicrobial susceptibility testing	13
• Data for decision-making	27
IV. <i>Neisseria meningitidis</i>	29
• Confirmatory identification	30
• Antimicrobial susceptibility testing	38
• Data for decision-making	43
V. <i>Streptococcus pneumoniae</i>	45
• Confirmatory identification	46
• Antimicrobial susceptibility testing	53
• Data for decision-making	62

Sexually Transmitted Pathogen for which there are Increasing Antimicrobial Resistance Concerns

VI. <i>Neisseria gonorrhoeae</i>	63
• Presumptive identification	64
• Confirmatory identification	67
• Antimicrobial susceptibility testing	82
• Data for decision-making	101

Bacterial Agents of Enteric Diseases of Public Health Concern

VII. <i>Salmonella</i> serotype Typhi	103
• Identification	104
• Antimicrobial susceptibility testing	111
• Data for decision-making	118
VIII. <i>Shigella</i>	121
• Identification	121
• Antimicrobial susceptibility testing	130
• Data for decision-making: Informed epidemic response	139
IX. <i>Vibrio cholerae</i>	141
• Identification	142
• Antimicrobial susceptibility testing	151
• Data for decision-making: Informed epidemic response	159
X. Conclusion	161
XI. Appendices	
1. Standard Safety Practices in the Microbiology Laboratory	163
2. Media, Reagents, and Quality Control	169
• Quality control of media	169
• Quality control of reagents	172
• Advantages of centralized acquisition of reagents	173
• Preparation of media and reagents	173
• Media for enrichment, identification, and antimicrobial susceptibility testing	174
• Transport and storage media	199
• Miscellaneous reagents	202
• Preparation of turbidity standards	209
• Sources of prepared media and reagents	214
3. Collection and Transport of Sterile Site Specimens	219
4. Isolation and Presumptive Identification of Agents from Normally Sterile Sites	227
5. Nasopharyngeal Swab Specimen Collection & Culture Methodology	251
6. Serotyping and Quellung Typing of <i>Streptococcus pneumoniae</i>	255
7. Antimicrobial Susceptibility Testing by Broth Microdilution	259

8. Specimen Collection and Primary Isolation of <i>Neisseria gonorrhoeae</i>	263
9. Fecal Specimens: Collection, Transport, and Field Supplies	275
10. Laboratory Processing of Fecal Specimens	287
11. Preservation and Storage of Isolates	299
12. Packing and Shipping of Diagnostic Specimens and Infectious Substances	309
13. Manufacturer, Supplier, and Distributor Contact Information	325
14. International Reference Laboratories	333
15. Selected References	337

List of Tables

Table	Title	Page
1.	Identification of <i>Haemophilus</i> species by their growth requirements	10
2.	Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for <i>Haemophilus influenzae</i>	19
3.	Carbohydrate utilization by some species of <i>Neisseria</i> and <i>Moraxella</i>	36
4.	Minimal inhibitory concentration (MIC) ranges for quality control of <i>Neisseria meningitidis</i> antimicrobial susceptibility testing	42
5.	Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for <i>Streptococcus pneumoniae</i>	58
6.	Results of biochemical and enzymatic tests for <i>Neisseria gonorrhoeae</i> and related species with similar colonial morphology	70
7.	Examples of quality control (QC) strains for supplemental tests used to identify <i>Neisseria gonorrhoeae</i>	71
8.	Phenotypic designations of <i>Neisseria gonorrhoeae</i>	88
9.	Acceptable limits for minimal inhibitory concentrations (MICs) and inhibition zone diameters for quality control strains of <i>Neisseria gonorrhoeae</i>	97
10.	Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for <i>Neisseria gonorrhoeae</i>	98
11.	Minimal inhibitory concentration (MIC) critical values for <i>Neisseria gonorrhoeae</i> and appropriate laboratory response	100
12.	Typical reactions of <i>Salmonella</i> spp. in screening biochemicals	110
13.	Antimicrobial agents suggested for use in antimicrobial susceptibility testing of <i>Salmonella</i> ser. Typhi	113

Table	Title	Page
14.	Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for <i>Enterobacteriaceae</i> (for selected antimicrobial disks appropriate for <i>Salmonella</i> ser. Typhi)	117
15.	Reactions of <i>Shigella</i> in screening biochemicals	125
16.	Subgroup and serotype designations of <i>Shigella</i>	130
17.	Antimicrobial agents suggested for use in antimicrobial susceptibility testing of <i>Shigella</i>	133
18.	Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for <i>Enterobacteriaceae</i> (for selected antimicrobial disks appropriate for <i>Shigella</i>)	136
19.	Reactions of <i>Vibrio cholerae</i> in screening tests	146
20.	Agglutination reactions in absorbed antiserum of serotypes of <i>Vibrio cholerae</i> serogroup O1	150
21.	Antimicrobial agents suggested for use in antimicrobial susceptibility testing of <i>Vibrio cholerae</i> O1 and O139	153
22.	Interpretive standards for antimicrobial susceptibility testing of <i>Vibrio cholerae</i> with selected antimicrobial disks	158
23.	Composition of McFarland turbidity standards	211
24.	Partial listing of materials and suppliers / manufacturers	215
25.	Submission of tubes of cerebrospinal fluid (CSF) to laboratories based on the number of tubes collected from the patient	223
26.	A checkerboard typing system for <i>Streptococcus pneumoniae</i>	258
27.	Concentrations of antimicrobial agents used in minimal inhibitory concentration (MIC) testing	260
28.	Conditions affecting the growth of <i>Neisseria gonorrhoeae</i>	264
29.	Specimen collection procedures for the laboratory diagnosis of <i>Neisseria gonorrhoeae</i>	266
30.	Colonial morphology of <i>Neisseria gonorrhoeae</i> and related species on gonococcal selective media	270
31.	Collection and transport of fecal specimens for laboratory diagnosis	276
32.	Materials needed to collect, transport, and test specimens from dysentery outbreaks for laboratories at the district level, the regional level, and the national (central) reference level	282
33.	Materials needed to collect, transport, and test specimens from cholera outbreaks for laboratories at the district level, the regional level, and the national (central) reference level	284

Table	Title	Page
34.	Appearance of <i>Salmonella</i> ser. Typhi colonies on selective plating media	290
35.	Appearance of <i>Shigella</i> colonies on selective plating media	293
36a.	Summary of labels and markings required for safe and proper shipping of different types of packages	312
36b.	Description of individual labels and markings required for safe and proper shipping of different types of packages	313

List of Figures

Figure	Title	Page
1.	Flowchart for laboratory identification of <i>Haemophilus influenzae</i>	6
2.	Techniques to properly mix antiserum and suspension for slide agglutination	9
3.	Growth factor requirements: X and V factors on paper disks	11
4.	Growth factor requirements: <i>Haemophilus</i> Quad ID plate	12
5.	Sample form for recording antimicrobial susceptibility test results for <i>Haemophilus influenzae</i>	15
6.	The antimicrobial susceptibility disk diffusion test: disk placement and measurement of inhibition zone diameters	18
7.	Proper placement of Etest® strips on dry, inoculated plates	22
8.	Guidance for reading Etest® results	23
9.	Flowchart for laboratory identification of <i>Neisseria meningitidis</i>	31
10.	Kovac's oxidase test: a positive reaction on filter paper	33
11.	Positive and negative agglutination reactions on a slide: grouping antisera and saline control with <i>Neisseria meningitidis</i>	35
12.	Cystine trypticase agar sugar reactions for acid production from carbohydrates by <i>Neisseria meningitidis</i>	37
13.	Sample form for recording antimicrobial susceptibility test results for <i>Neisseria meningitidis</i>	39
14.	A properly streaked blood agar plate with pneumococci and viridans streptococci	47
15.	Flowchart for laboratory identification of <i>Streptococcus pneumoniae</i>	48
16.	Optochin susceptibility test for identification of <i>Streptococcus pneumoniae</i>	49

Figure	Title	Page
17.	Positive and negative results of the bile solubility test	51
18.	Sample form for recording antimicrobial susceptibility test results for <i>Streptococcus pneumoniae</i>	54
19.	Flowchart for isolation and presumptive identification of <i>Neisseria gonorrhoeae</i>	66
20.	Kovac's oxidase test: a positive reaction on a swab	67
21.	Flowchart exhibiting one algorithm for confirmatory identification of <i>Neisseria gonorrhoeae</i>	69
22.	Positive and negative reactions in superoxol (30% H ₂ O ₂) and catalase (3% H ₂ O ₂) reagents	73
23.	Positive and negative results of the polysaccharide-production test on sucrose medium	76
24.	Acid production commercial test kit results for <i>Neisseria gonorrhoeae</i> and related organisms	78
25.	Reactions of <i>Neisseria gonorrhoeae</i> and related organisms in a commercial enzyme substrate test	80
26.	Schematic representation of the nitrate reduction test	83
27.	Sample form for recording antimicrobial susceptibility test results for <i>Neisseria gonorrhoeae</i>	92
28.	Disk diffusion testing: disk placement for <i>Neisseria gonorrhoeae</i> and measurements of inhibition zone diameters	96
29.	Flowchart for the isolation and identification of <i>Salmonella</i> serotype Typhi	106
30.	Sample worksheet for <i>Salmonella</i> serotype Typhi test results	107
31.	<i>Salmonella</i> ser. Typhi colonies on triple sugar iron (TSI) agar	109
32.	Use of a bent loop to dispense small amounts of antiserum for slide agglutination tests	112
33.	Flowchart of the general procedure for antimicrobial susceptibility testing by disk diffusion	114
34.	Inoculation of Mueller-Hinton medium for antimicrobial susceptibility tests	116
35.	Sample form for recording antimicrobial susceptibility test results for <i>Salmonella</i> ser. Typhi	119
36.	Flowchart for the isolation and identification of <i>Shigella</i>	123
37.	Sample worksheet for <i>Shigella</i> test results	124

Figure	Title	Page
38.	Reaction typical of <i>Shigella</i> in Kligler iron agar (KIA): alkaline slant and acid butt	126
39.	Motility medium showing a non-motile organism in the left tube and a motile organism (seen as clouding) in the right tube	127
40.	Reactions in urea medium	127
41.	Reactions in lysine iron agar (LIA) medium	129
42.	Serologic identification: agglutination reactions of <i>Shigella</i>	131
43.	Sample results of the disk diffusion assay	137
44.	Sample form for recording antimicrobial susceptibility test results for <i>Shigella</i> isolates	138
45.	Flowchart for the isolation and identification of <i>Vibrio cholerae</i>	143
46.	Sample worksheet for <i>Vibrio cholerae</i> test results	144
47.	A positive string test with <i>Vibrio cholerae</i>	147
48.	Reactions of <i>Vibrio cholerae</i> in Kligler iron agar (KIA) and triple sugar iron agar (TSI)	148
49.	Sample form for recording antimicrobial susceptibility test results for <i>Vibrio cholerae</i>	157
50.	Flowchart procedure for preparation and quality control of the 0.5 McFarland turbidity standard	210
51.	Comparison of the 0.5 McFarland turbidity standard with inoculum suspension	211
52.	Background lines for viewing turbidity of a suspension in comparison to a turbidity standard	212
53.	Collection of blood from an arm	222
54.	Kit for collection of cerebrospinal fluid (CSF)	224
55.	Collection of cerebrospinal fluid (CSF) by lumbar puncture	225
56.	Proper streaking and growth of <i>Neisseria meningitidis</i> on blood agar	230
57.	Proper streaking and growth of <i>Streptococcus pneumoniae</i> on blood agar	230
58.	Proper streaking and growth of <i>Haemophilus influenzae</i> on chocolate agar	231
59a.	Growth of <i>Salmonella</i> ser. Typhi on MacConkey agar	231
59b.	Growth of <i>Salmonella</i> ser. Typhi on blood agar	231
60.	Presumptive identification of <i>Haemophilus influenzae</i> , <i>Neisseria meningitidis</i> , and <i>Streptococcus pneumoniae</i>	233

Figure	Title	Page
61.	Growth of <i>Haemophilus influenzae</i> , <i>Neisseria meningitidis</i> , and <i>Streptococcus pneumoniae</i> on sectioned blood agar and chocolate agar plates	233
62.	<i>Haemophilus influenzae</i> and <i>Streptococcus pneumoniae</i> colonies growing on the same chocolate agar plate	234
63.	Sample worksheet for the presumptive laboratory identification of bacterial agents of pneumonia and meningitidis	235
64.	Alpha, alpha-prime, and beta hemolysis on sheep blood agar	236
65.	<i>Haemophilus influenzae</i> colonies on chocolate agar	237
66.	Overnight growth of <i>Neisseria meningitidis</i> on blood agar and chocolate agar	238
67.	<i>Streptococcus pneumoniae</i> colonies on blood agar	239
68.	<i>Streptococcus pneumoniae</i> and <i>Staphylococcus aureus</i> growing together on the same blood agar plate	239
69.	Gram stain of <i>Salmonella</i> ser. Typhi	240
70.	Nomograph for calculation of relative centrifugal force (RCF)	241
71.	Processing of cerebrospinal fluid (CSF)	243
72.	Gram stain of cerebrospinal fluid (CSF) with <i>Neisseria meningitidis</i>	245
73.	Gram stain of cerebrospinal fluid (CSF) with <i>Streptococcus pneumoniae</i>	245
74.	Gram stain of cerebrospinal fluid (CSF) with <i>Haemophilus influenzae</i>	246
75.	Trans-Isolate (T-I) medium	247
76.	Collection of a nasopharyngeal (NP) swab	253
77.	A Quellung reaction	257
78.	Streaking a plate with a specimen swab for primary isolation of <i>Neisseria gonorrhoeae</i> : “Z-streak” inoculation method	271
79.	Colonial morphology typical of <i>Neisseria gonorrhoeae</i>	272
80.	Gram stain and simple single Loeffler’s methylene blue stain of <i>Neisseria gonorrhoeae</i>	273
81.	Cary-Blair semisolid transport medium	277
82.	Sample data sheet for collecting and recording patient information with stool specimens during a diarrheal outbreak	280
83.	<i>Salmonella</i> ser. Typhi colonies on bismuth-sulfite (BS) agar	290

Figure	Title	Page
84.	Method of streaking plating medium for isolation of <i>Shigella</i> and <i>Vibrio</i> species	292
85.	<i>Shigella dysenteriae</i> 1 colonies on xylose lysine desoxycholate (XLD) agar	293
86.	<i>Shigella flexneri</i> colonies on xylose lysine desoxycholate (XLD) agar	294
87.	<i>Shigella flexneri</i> and <i>Escherichia coli</i> colonies on xylose lysine desoxycholate (XLD) agar	294
88.	<i>Shigella flexneri</i> and <i>Escherichia coli</i> colonies on MacConkey (MAC) agar	295
89.	Growth of <i>Vibrio cholerae</i> on thiosulfate citrate bile salts sucrose (TCBS) agar	296
90.	Silica gel packets for transport (and short-term storage)	301
91.	Proper packing and labeling of the secondary container for shipping of infectious substances	319
92.	Proper packing and labeling of the secondary container for shipping of diagnostic specimens	320
93.	Information required for proper completion of the “Shipper’s Declaration for Dangerous Goods” form	323



List of Abbreviations Used in This Document

APW	Alkaline peptone water
ASM	American Society for Microbiology
ATCC	American Type Culture Collection
BS	Bismuth sulfite agar
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CSF	Cerebrospinal fluid
CTA	Cystine trypticase agar
DCA	Desoxycholate citrate agar
DE	Dorset egg medium
DGR	Dangerous Goods Regulations (publication)
GC	<i>Neisseria gonorrhoeae</i> (or, gonococcus)
GN	Gram-negative broth
HE	Hektoen enteric agar
HIA	Heart infusion agar
Hib	<i>Haemophilus influenzae</i> serotype b
HTM	<i>Haemophilus</i> test medium
IATA	International Air Transport Association
ICAO	International Civil Aviation Organization
ICG	International Collaboration on Gonococci
KIA	Kligler iron agar
LIA	Lysine iron agar
MAC	MacConkey agar
MIC	Minimal inhibitory concentration
ML	Martin-Lewis medium

MTM	Modified Thayer-Martin medium
NAD	Nicotinamide adenine dinucleotide (V factor)
NCCLS	Formerly known as the “National Committee on Clinical Laboratory Standards,” NCCLS is an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.
NP	Nasopharyngeal
PBS	Phosphate buffered saline
QC	Quality control
RCF	Relative centrifugal force (measured in xg)
SEL	Selenite broth
SIM	Sulfide-indole-motility medium
SPS	Sodium polyanetholesulfonate
SS	<i>Salmonella-Shigella</i> agar
STGG	Skim-milk tryptone glucose glycerol medium
STI	Sexually transmitted infection
TCBS	Thiosulfate citrate bile salts sucrose agar
T-I	Trans-isolate medium
TSA	Tryptone-based soy agar
TSB	Tryptone-based soy broth
TSI	Triple sugar iron agar
UN	United Nations
WHO	World Health Organization
XLD	Xylose lysine desoxycholate agar

Introduction

Respiratory and enteric diseases comprise a substantial proportion of the burden of morbidity and mortality in the developing world; acute respiratory infection and diarrheal illness are the top two killers of children less than five years of age worldwide. Reproductive tract pathogens cause uncomplicated infections of the mucosal membranes; however, if left untreated, infections with these pathogens can also lead to pelvic inflammatory disease, ectopic pregnancies and infertility, and may facilitate the transmission of HIV. Public health interventions such as access to safe water, improved sanitation, hygiene, immunizations, education, health communication, and access to acute medical care with appropriate case management have contributed to on-going improvements in health, and in social and economic development. One outcome of the increased availability of antimicrobial agents for symptomatic treatment of illness in hospitals and community environments, however, has been the emergence of antimicrobial resistance in pathogens of public health concern.

Antimicrobial resistance is an issue of great significance for public health at the global level. However, it is of particular concern in the developing world because fewer affordable and appropriate treatment options are readily available. It has become increasingly important to monitor patterns of resistance as the antimicrobial susceptibility of bacterial pathogens which contribute significantly to the burden of respiratory, febrile, reproductive tract, and diarrheal illness has declined. Because antimicrobial susceptibility testing is resource-intensive, the World Health Organization (WHO) recommends that only one or two reference laboratories in a country perform these tests. Until now, however, there has not been a technically appropriate source of standardized information for laboratory detection of antimicrobial resistance that is practical for use in regions with limited resources.

This laboratory manual focuses on seven bacterial pathogens of public health importance in the developing world: *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Salmonella* serotype Typhi, *Shigella*, and *Vibrio cholerae*. Methods for the isolation and identification of each of these bacterial agents from clinical specimens are presented, and

standardized antimicrobial susceptibility testing techniques and criteria for interpretation are described. To benefit from the information presented in this manual, laboratorians must have received training in proper basic microbiological techniques and be comfortable with such tasks as sterilization of instruments and media preparation. Flow charts of procedures and color figures of bacterial colonies and typical reactions have been provided as supplements to the text for ease of comparative identification. Procedural accuracy and methodological standardization are critical to the performance of antimicrobial susceptibility testing, and adherence to protocols of quality control is also vital to ensure that test results are valid and meaningful.

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. Antimicrobial susceptibility test methods must be performed as described according to internationally recognized clinical guidelines such as those provided by NCCLS (an international, interdisciplinary, nonprofit, educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis) in order to provide meaningful results for clinical and epidemiological interpretation. Laboratory staff must be afforded the appropriate time and resources to carry out the procedures described in this manual if the results are to be meaningful and applicable to clinical and policy decisions.

As resistance to antimicrobial agents in the pathogens causing these diseases grows and changes, strategies of response also must evolve. Resistant pathogens can translate to fewer treatable infections and thus higher morbidity and mortality, a drain on resources, and an obstacle to social, economic, and health development overall. Timely communication between the laboratory and public health officials is essential to the shaping of locally treatment appropriate policies; the data collected in the laboratory are crucial components of the decision-making process for clinical and public health policies.

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Requirements of a Reference Laboratory

FOR THE PERFORMANCE OF STANDARDIZED ANTIMICROBIAL SUSCEPTIBILITY TESTING

A reference laboratory differs from a clinical laboratory in that microbiologists are able to dedicate their time to confirmation and investigation of isolates sent in from other laboratories or hospitals and (for the purposes of this manual) then perform standardized antimicrobial susceptibility testing. This manual is written and intended for use in a reference laboratory or national central laboratory setting, where material resources are consistently quality controlled and available in sufficient quantities for regular testing of isolates. Reference laboratories must participate in a quality assurance program at least once per year and should also administer quality assurance programs for laboratories in their jurisdiction; the World Health Organization (WHO) encourages central public health laboratories in countries with limited resources to establish national quality assessment schemes and to participate in at least three surveys per year. Time, supplies, and personnel can be costly; as a result, it is anticipated that not every country will be able to support a reference laboratory meeting these requirements. A country that can not establish a reference laboratory should consult a regional or sub-regional reference laboratory for further guidance and for advice on where to send isolates requiring further investigation.

In order to carry out the standardized procedures referred to in this laboratory manual (and many others), the laboratory must be able to make ongoing investments in equipment, supplies, and human resources (*i.e.*, trained laboratorians). The Ministry of Health (or similar appropriate agency) should therefore ensure that its central public health laboratory has the following items of great importance:

- 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

H*aemophilus 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.

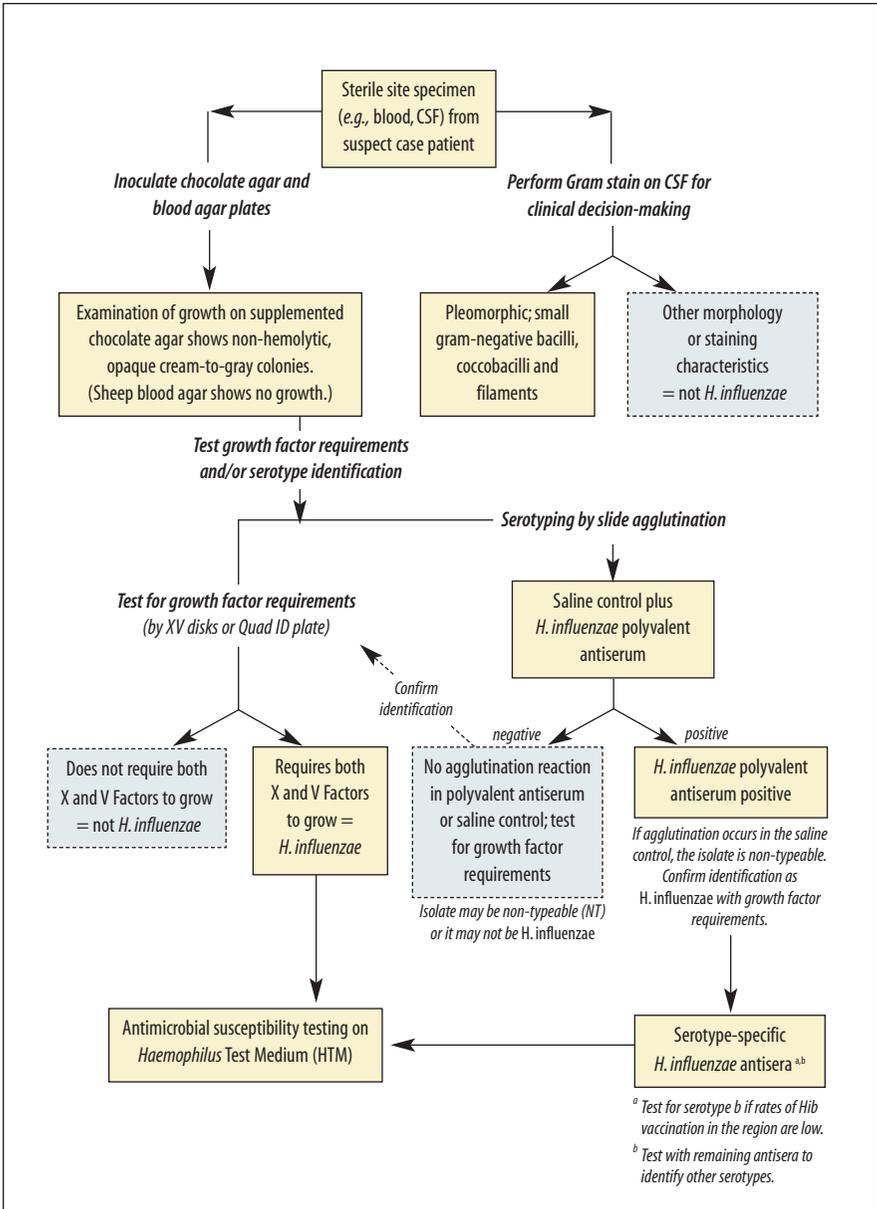
Confirmatory identification of *H. influenzae*

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*



H. influenzae is currently recognized to have six serotypes: a, b, c, d, e, and f. *H. influenzae* type b (Hib) is the major cause of both *H. influenzae* meningitis and of meningitis overall in unvaccinated children in many parts of the world. Suspected Hib isolates should be tested with Hib antiserum, an antiserum to one of the other groups, and saline. **A strongly positive (3+ or 4+) agglutination reaction with type b antiserum and no agglutination with antiserum to the other serotypes and saline is rapid evidence of Hib.**¹

Antisera should be stored in the refrigerator at 4°C when not in immediate use. Screening an isolate first with polyvalent antiserum (which contains antisera to all six recognized serotypes) and a saline control is convenient and saves resources (*i.e.*, type-specific antisera).

- **If an isolate is positive in polyvalent antiserum** and negative in the saline control, proceed by testing the isolate with type b antiserum if Hib vaccination is uncommon in the patient's geographic region. If the serotype b reaction is negative, test with the remaining type-specific antisera (*i.e.*, a, c, d, e, and f).
 - If Hib disease is unlikely because of widespread vaccination, the culture should be tested with all the type-specific antisera (*i.e.*, a through f).
- **If an isolate is non-agglutinating in the polyvalent antiserum**, it is either non-typeable or is not *H. influenzae*. Therefore, growth factor requirements must be determined to confirm the identity of the isolate as *H. influenzae* or another species of *Haemophilus*.

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

- a) Clean a glass slide with alcohol (optional if slides are pre-cleaned). Divide the slide into equal sections (*e.g.*, three 25-mm [1-inch] sections for a 25-mm x 75-mm [1-inch x 3-inch] slide) with a wax pencil or other marker.
- b) Collect a small portion of growth from the surface of an overnight culture on chocolate agar (**without bacitracin**), a *Haemophilus* ID plate, or *Haemophilus* test medium (HTM) plate with a sterile inoculating loop. Make a moderately milky suspension of the test culture in a small vial with 250 µl (0.25 ml) of formalinized physiological saline. Vortex the suspension, if possible.
 - If only working with several isolates, another option is to make the suspension directly on the slide in 10 µl of formalinized physiological saline per droplet.

¹ Laboratorians are often tempted to test suspect *H. influenzae* isolates only with type b antiserum since because serotype b (Hib) is vaccine preventable; however, **it is of great importance to screen the isolate with a saline control and at least one other antiserum in addition to type b.** Observing agglutination reactions with several antisera in different portions of the same slide permits comparisons and provides evidence that any agglutination in type b antiserum is not just a mild cross-reaction with a different serotype, providing the laboratorian and clinician with a more informed definition of a 'positive' reaction.

- It is not necessary to make a standard suspension for slide serology; however, it should be noted that a “moderately milky suspension” is roughly comparable to a 6 McFarland turbidity standard.
- c) For the agglutination reaction, use a micropipettor or a bacteriologic loop to transfer a drop (5–10 μl) of the cell suspension to the lower portion of two sections of the slide prepared in step a, above. Use enough suspension in the droplet so that it does not dry on the slide before testing with the antisera.
 - d) Add 5–10 μl of polyvalent antiserum above the drop of suspension in one of the test sections on the slide.² In an adjacent section of the slide, use the same method to add a (5–10 μl) drop of saline above the final drop of suspension.
 - **The loop used in the antiserum must not touch either the cell suspension or the other antisera being tested; if it does, it must not be placed back into the source bottle of antiserum.** If the source antiserum is contaminated, a new bottle must be used.
 - e) **Using a separate toothpick (or sterile loop) for each section**, mix the antiserum (and control saline) with the corresponding drop of cell suspension. Avoid contamination across the sections of the slide.
 - f) **Gently rock the slide with a back and forth motion** for up to 1 minute. Do not use a circular motion while rocking, because it can cause the mixtures to run together and contaminate each other. After one minute of rocking, observe the mixed drops and read the slide agglutination reactions under bright light and over a black background, as shown in Figure 2.
 - g) **Only strong agglutination reactions (3+ or 4+) are read as positive.** In a strong reaction, all the bacterial cells will clump and the suspension fluid will appear clear (see Figures 11 and 42). When a strain reacts with more than one antiserum, or agglutinates in saline, the result is recorded as non-typeable.
 - *If strong agglutination occurs in the polyvalent antiserum:* Using the methods described in steps a through f (above), continue testing the isolate with type b antiserum and other type-specific antisera to identify the serotype.
 - *If agglutination does not occur in the polyvalent antiserum:* The isolate is either non-typeable or not *H. influenzae*. Continue by testing the isolate for X and V growth factor requirements to confirm identification as *H. influenzae*.

² This laboratory manual suggests using a micropipettor or a loop to transfer antiserum from the bottle to the slide (rather than the dropper provided with the bottle of antiserum) because they conserve costly antiserum resources. (Micropipettors permit the precise measurement of antiserum, and the loop method collects only approximately 5–10 μl of antiserum on average; in contrast, the dropper transfers several times this amount in each drop.) Because only 5–10 μl of antisera are required for agglutination reactions to occur using the methods presented here, **using a micropipettor or a loop to transfer antiserum from the bottle to the slide is more cost-effective.**

- *If agglutination occurs in the saline control:* The isolate is recorded as non-typeable. Continue by testing the isolate for X and V growth factor requirements to confirm identification as *H. influenzae*.

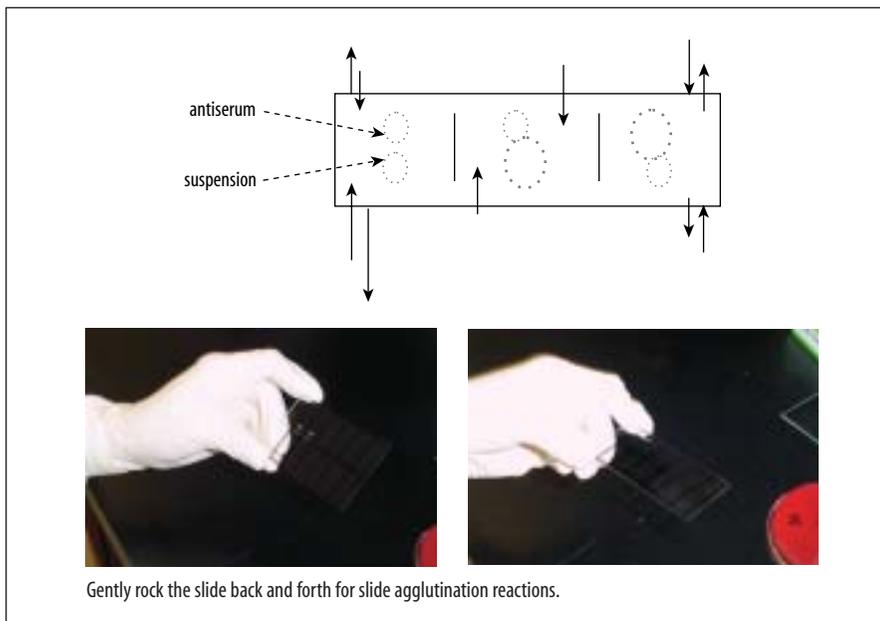
Record results and report to attending clinicians, as appropriate.

Growth factor (X and V) requirements

H. influenzae is a fastidious organism requiring media containing haemin (X factor) and nicotinamide adenine dinucleotide (NAD, V factor) for growth. The standard medium is chocolate agar, which is often prepared with horse blood, a good source of both X and V factors (Appendix 2). Heating the blood is necessary to make both factors available to the organism. Chocolate agar with added supplements (e.g., IsoVitaleX, Supplement B, or Vitox) is available commercially or can be prepared in the laboratory. Supplemented chocolate agar is superior to unsupplemented medium for growth of *H. influenzae* and is the medium of choice. Although some strains of *H. influenzae* may grow on unsupplemented chocolate agar, **supplements must be added to reliably support the growth of most strains.**

H. influenzae is identified on the basis of its growth requirements for X and V factors (Table 1). *H. influenzae* can be differentiated from most other species of *Haemophilus* by its requirement for both X and V factors for growth.

FIGURE 2: Techniques to properly mix antiserum and suspension for slide agglutination



Gently rock the slide back and forth for slide agglutination reactions.

H. haemolyticus is the only other species requiring X and V factors but this species differs from *H. influenzae* by producing hemolysis on horse- or rabbit blood agar.

Tests to identify X and V growth factor requirements: paper disks and strips or Quad ID plates

Growth factor requirements can be identified with paper disks or strips (using the principles of agar diffusion) or by using Quad ID plates (which contain four types of media with and without X and V factors).

- **Growth factor test using X, V, and XV factor paper disks or strips**

A medium completely without X and V factors, such as tryptone-based soy agar (TSA) or heart infusion agar (HIA), must be used for this test.

Methods

- Prepare a heavy suspension of cells (1 McFarland turbidity standard, see Appendix 2) from a primary isolation plate in a suitable broth (e.g., tryptone-based soy broth (TSB) or heart infusion broth). If the primary isolation plate contains insufficient growth or is contaminated, make a subculture on a chocolate agar plate. When preparing the broth **avoid transfer of agar medium to the broth**; even the smallest sample of agar will affect the test and **may lead to misidentification** of the bacteria because the agar contains X and V factors.
- Inoculate a HIA or TSA plate. A sterile swab or sterile loop of the suspension should be streaked over one-half of the plate (with streaking in at least two directions to ensure confluent growth). Two strains can be tested on one 100-mm plate, but care must be taken to ensure the isolates do not overlap. Paper strips or disks containing X, V, and XV factors are placed on the inoculated plate after the inoculum has dried. **When two bacterial strains are tested on the same plate, as shown in Figure 3, the disks should be placed in the exact manner shown.**
- Carefully invert the plate and place it in a CO₂-incubator or candle-extinction jar. Incubate it for 18–24 hours at 35°C. *H. influenzae* will grow

TABLE 1: Identification of *Haemophilus* species by their growth requirements

Species	X- and V-Factor Requirements		β-hemolysis on rabbit blood agar
	X	V	
<i>H. influenzae</i>	+	+	–
<i>H. parainfluenzae</i> *	–	+	–
<i>H. haemolyticus</i>	+	+	+
<i>H. parahaemolyticus</i>	–	+	+
<i>H. aphrophilus</i>	+	–	–
<i>H. paraphrophilus</i> *	–	+	–

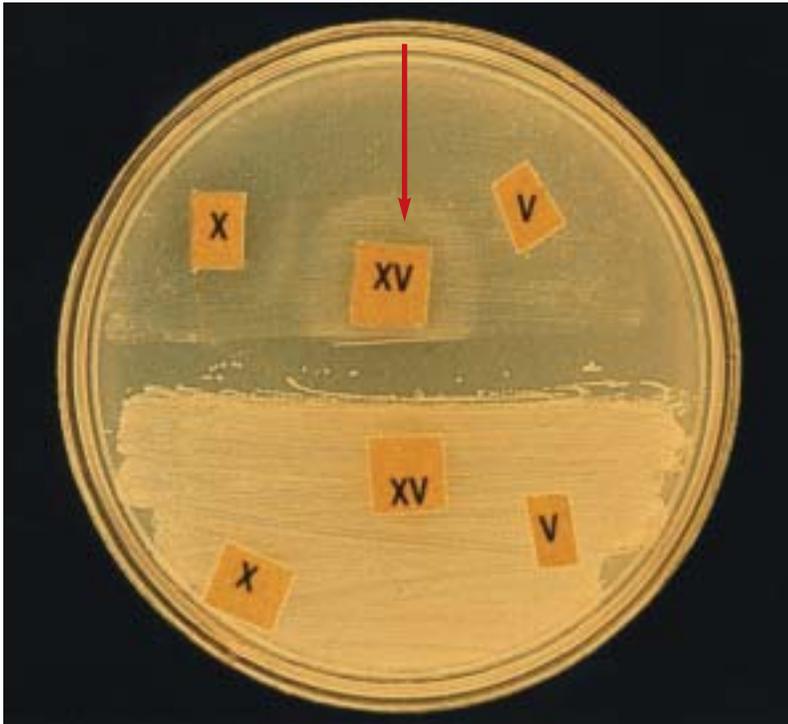
* *H. paraphrophilus* is ornithine negative, whereas *H. parainfluenzae* is ornithine positive.

only around the XV disk (*i.e.*, the disk containing both X and V factors), as shown on the upper half of the plate in Figure 3.

- **Growth factor test using *Haemophilus* Quad ID Plates**

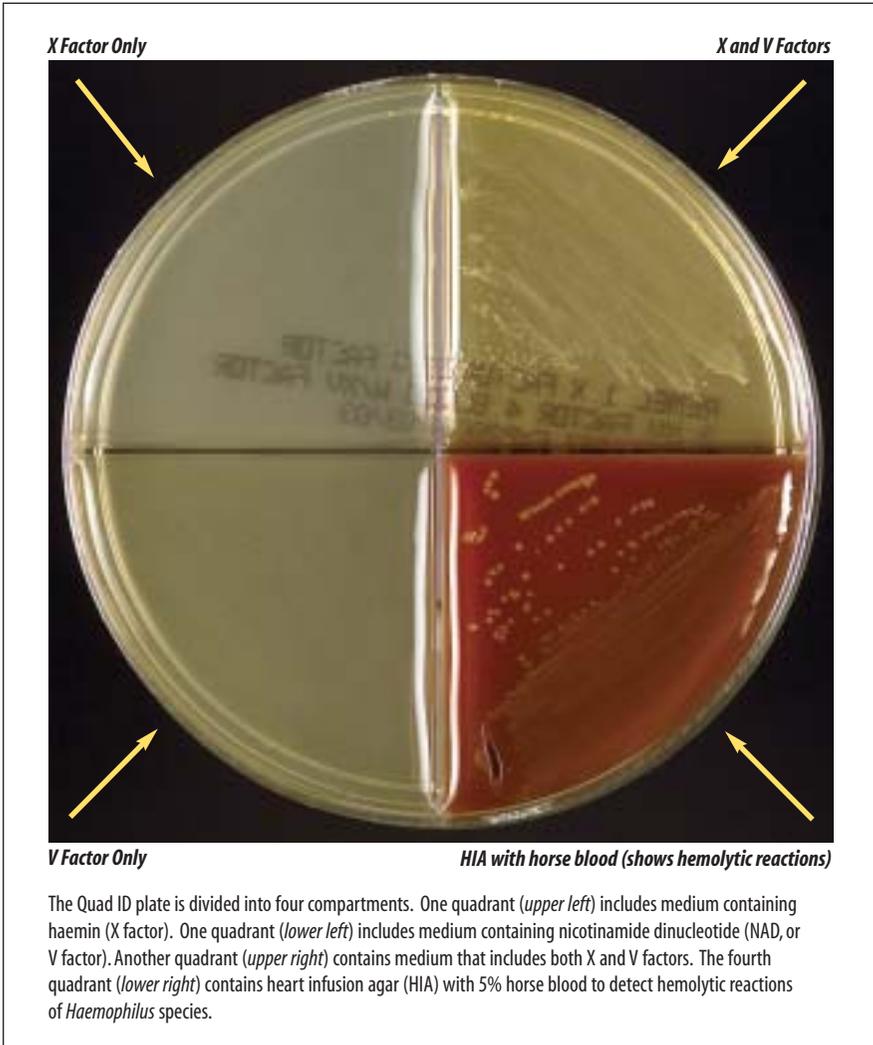
Quad ID plates are another, although more expensive, method for determining growth factor requirements of *Haemophilus* isolates (Figure 4). Available commercially, the Quad ID plate is divided into four agar quadrants: one quadrant includes medium containing haemin (X factor); one quadrant includes 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



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 β -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 (Etest®) 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 Mueller-Hinton 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- μ g ampicillin disk predicts both intrinsic (*i.e.*, penicillin-binding protein-mediated, or “PBP”) and β -lactamase (beta-lactamase) mediated penicillin and ampicillin resistance and should be used when testing *H. influenzae*. (Methods for β -lactamase testing of *H. influenzae* are listed after the direct antimicrobial susceptibility testing methods in this section.) For *H. influenzae*, a 30- μ g chloramphenicol disk is used for predicting resistance to chloramphenicol, and a 1.25/23.75- μ g 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

³ Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

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
ATCC 49247	N/A	<i>H. influenzae</i> NCCLS QC strain QC in range? <input type="checkbox"/>	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R	mm µg/ml S I R

Revised 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)

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.

- **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 trimethoprim-sulfamethoxazole 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

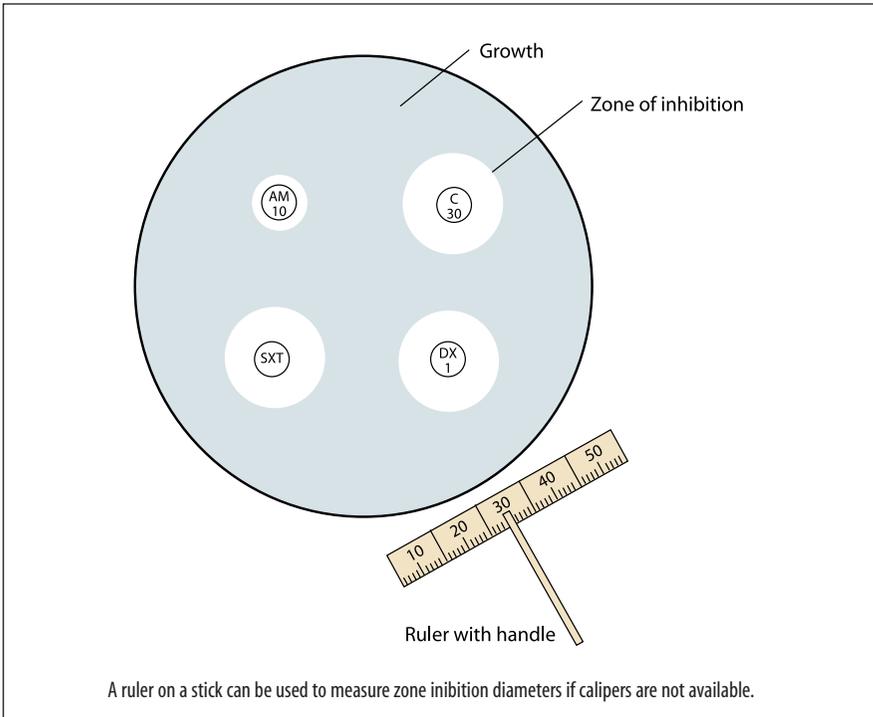


TABLE 2: Antimicrobial susceptibility test breakpoints and quality control (QC) ranges for *Haemophilus influenzae*

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

^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.

susceptibility test results to trimethoprim-sulfamethoxazole detected by disk diffusion testing with MIC testing.

MIC testing by dilution can be expensive and challenging; because of the technical complexity required for these tests, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. In countries where MIC testing is done at more than one laboratory, standardization and quality control should be conducted as described earlier in this chapter.

With increasing antimicrobial resistance testing being performed outside of international reference laboratories, the Etest[®] serves as a test method that is both convenient and reliable.⁴ The Etest[®] requires less technical expertise than MIC testing by dilution methods, but it gives comparable results. **Etest[®] strips must be consistently stored in a freezer at -20°C.**

The Etest[®] is 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 ($\mu\text{g/ml}$). 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.⁵

The continuous concentration gradient of stabilized, dried antibiotic is equivalent to 15 log₂ dilutions by a conventional reference MIC procedure as suggested by the

⁴ The Etest[®] can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

NCCLS. The Etest® has been compared with and evaluated beside both the agar and broth dilution susceptibility testing methods recommended by the NCCLS. Authoritative reports indicate that an (approximately) 85% – 100% correlation exists between the accepted conventional MIC determinations and the MIC determined by the Etest® procedure for a variety of organism-drug combinations (see, e.g. Jorgensen *et al.* [1994] and Barry *et al.* [1996] in Appendix 15). Some studies have cited Etest® MICs as approximately one dilution higher than MICs determined by standard dilution methods.

Although this manual serves as a general guide to use of the Etest® antimicrobial gradient strip, **always follow the manufacturer’s directions for use of the Etest®**, as certain antibiotic-bacteria (“drug-bug”) combinations have special testing requirements.

Methods for antimicrobial susceptibility testing with the Etest®

For *H. influenzae*, HTM is used when performing antimicrobial susceptibility testing. Follow the directions on the package insert included with the Etest® strips. Either 150-mm or 100-mm plates can be used, depending on the number of antimicrobial agents to be tested per isolate. Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and although the manufacturer states that up to six Etest® 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 Etest® strips be used on a 150-mm plate (see Figure 7).

- a) Suspend viable colonies from an overnight chocolate agar plate into a broth tube 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. **This suspension should be used within 15 minutes.**
- b) Dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid. Inoculate the entire surface of the agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure confluent growth of the bacteria (see Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.
- c) 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 Etest® strips from the -20°C freezer and allow the strips that will be used in the batch of testing to warm to room temperature. Return the strips that will not be used in this batch of testing to the -20°C freezer.

⁵ Antimicrobial susceptibility testing with an antimicrobial gradient strip such as the Etest® can be considered to be a semi-quantitative method (because although the suspension used to inoculate a plate for Etest® is standardized, the inoculum itself is not standardized). However, results are generally comparable to quantitative results of standard broth microdilution or agar dilution MIC tests.

- d) Place the Etest® strips onto the dried, inoculated agar plate with an Etest® applicator or sterile forceps, oriented as shown in Figure 7. (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].) **Once applied, do not move the antimicrobial gradient strips.**
- e) Incubate the plates in an inverted position in a CO₂-enriched atmosphere (2% – 5% CO₂) for 16–18 hours at 35°C. A candle-extinction jar may be used if a CO₂-incubator is not available.
- f) After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **Quality control results must be reviewed before reading and interpreting the Etest® MIC.**

MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® strip. Use oblique light to carefully examine the end point. A magnifying glass may be used if needed. Read the MIC at the point of complete inhibition of all growth including hazes and isolated colonies. Figure 8 presents a reading guide for the Etest®,⁶ and shows drug-related effects, technical and handling effects, organism-related effects and resistance-mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) 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 a *H. influenzae* isolate to ampicillin, an MIC recorded from the graduations on the Etest® strip might be 0.75 mg/ml; however, the reported MIC would be 1.0 µg/ml.

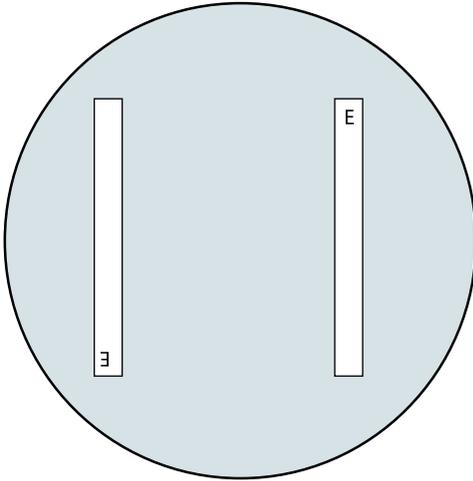
Breakpoints for interpretation of MICs follow the NCCLS guidelines, **unless exceptions made by the manufacturer are provided in the package insert.** NCCLS breakpoints for antimicrobial agents used for *H. influenzae* are included in Table 2.

Surveillance for emerging antimicrobial resistance in *H. influenzae*

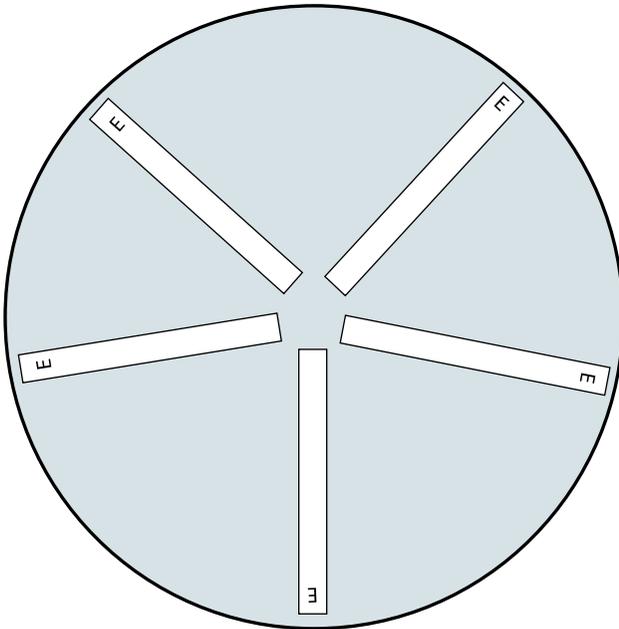
Laboratories may wish to help detect the emergence of new strains of *Haemophilus* by testing isolates against a panel of drugs in which reduced susceptibility is not expected to be found. A laboratory might look at specific drugs or characteristic groupings (such as, for example, β-lactamase negative, ampicillin resistant

⁶ AB Biodisk also maintains a website with an Etest® reading guide: <http://www.abiodisk.com>.

FIGURE 7: Proper placement of Etest® strips on dry, inoculated plates



Up to two Etest® strips can be placed on a 100 mm plate, as shown.

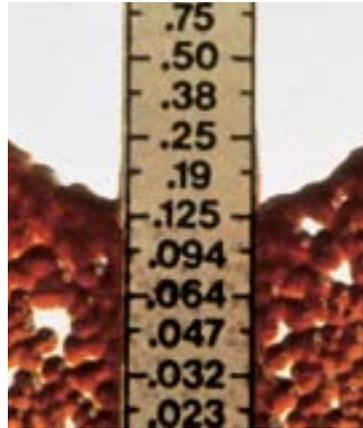


Up to five Etest® strips can be placed on a 150 mm plate, as shown.

FIGURE 8a: Guidance for reading Etest® results



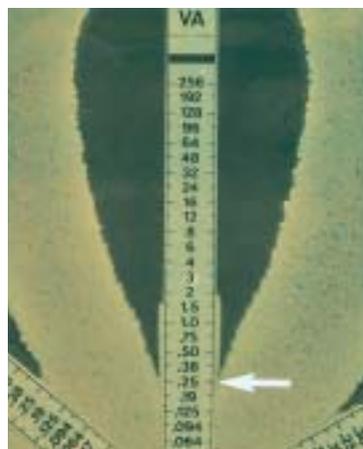
If the strip is backwards,
MIC = INVALID!
Retest and position the strip with the MIC scale
facing the opening of the plate.



Intersection in between markings.
Read the next higher value. MIC 0.19 µg/ml.



Different intersections on either side of the strip.
Read the higher value; if the difference is >1
dilution, repeat the test. MIC 0.5 µg/ml.



Ignore a thin line of growth at the edge of the strip
caused by organisms growing in a tunnel of water.
MIC 0.25 µg/ml.

The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).

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FIGURE 8b: Guidance for reading Etest® results



Bacteriostatic drugs such as trimethoprim and sulphonamides can give diffuse edges. Read at 80% inhibition. MIC 3 µg/ml.



Isolated resistant colonies due to low-level mutation. MIC >256 µg/ml.



Paradoxical effect showing partial regrowth after an initial inhibition. MIC 8 µg/ml.



Induction of β -lactamase production by clavulanic acid at the higher MIC range. MIC 96 µg/ml.

The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).

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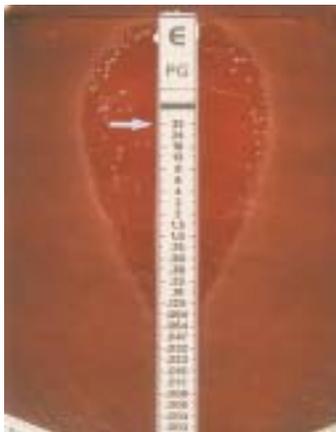
FIGURE 8c: Guidance for reading Etest® results



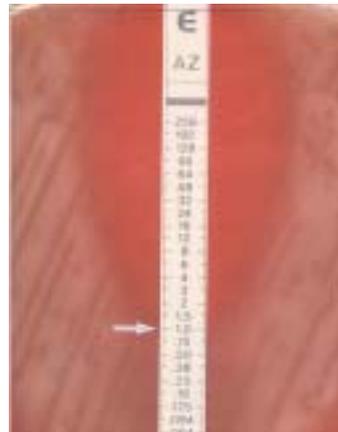
Tilt the plate to visualize pin-point colonies and hazes. This is particularly important for pneumococci. MIC 1 µg/ml.



Scrutinize pneumococcal end-points carefully to pick up all microcolonies. Tilt the plate and/or use a magnifying glass. MIC 2 µg/ml.



A highly resistant subpopulation in pneumococci. MIC >32 µg/ml.



Encapsulated strains may not give a confluent intersection. MIC 1 µg/ml.

The way in which the strip is placed on the medium can affect growth of the organisms and interpretation of the minimal inhibitory concentration (MIC).

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[BLNAR] *H. influenzae*). These strains are believed to be rare at present, but are of great interest to public health policy and clinicians because although they may exhibit *in vitro* susceptibility to certain drugs (e.g., amoxicillin + clavulanic acid, cefprozil, cefuroxime, and others), they should still be considered resistant *in vivo* [NCCLS 2002].

Testing for emerging resistance should not be done with each batch of antimicrobial susceptibility tests, nor with each new batch of media. Instead such testing could be done periodically (e.g., on an annual basis), for example on a sampling of preserved isolates in storage on an annual basis. Methods for preservation and long-term storage of isolates can be found in Appendix 11. Antimicrobials of interest could include (but are not necessarily limited to) ceftriaxone and fluoroquinolones. Appropriate zone diameter sizes can be found in NCCLS documents, which are updated regularly. **If any of these rare strains with reduced susceptibility are found in the course of this surveillance, notify an international reference laboratory and submit the isolate for further investigation.** A list of international reference laboratories is included in Appendix 14.

Testing *H. influenzae* for β -lactamase production

Testing the *H. influenzae* isolates for the presence of β -lactamase will identify most of ampicillin-resistant strains, because most (but not all) ampicillin resistance among *H. influenzae* is caused by the presence of β -lactamase. Several techniques are available for the detection of β -lactamases. All the tests are based on determination of breakdown products and use either a natural substrate (e.g., penicillin) or a chromogenic substance (e.g., nitrocefin). Two methods for detection of β -lactamase are presented in this manual: the nitrocefin test and the acidometric agar plate method.

- **Nitrocefin** can be used to screen for β -lactamase either as a reagent dropped onto colonies or in the form of a treated disk onto which colonies are rubbed. (This manual suggests using the disk method unless a laboratory is screening large numbers of isolates because the materials for the reagent tend to be available in bulk and costs can be high; methods for testing with the liquid nitrocefin reagent are included in the *N. gonorrhoeae* chapter [Chapter VI].)
 - a) Using sterile forceps or tweezers, place a nitrocefin disk on a clean slide; add a drop of distilled water.
 - b) Touch a sterile swab or loop to a characteristic colony from fresh, pure culture.
 - c) Rub the swab onto the moistened disk.
 - d) Observe the disk for five minutes; if the reaction is positive (β -lactamase producing strain), the areas of the disk containing growth will turn a characteristic red/pink color.
- A **modified acidometric agar plate method** is a differential agar method for testing *H. influenzae* isolates for the presence of β -lactamase activity [Park *et al.*

1978; Lucas 1979]. Penicillin and phenol red 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) Place a clump of isolated colonies in a discrete spot on the β -lactamase agar plate. Many strains can be tested on one plate; be certain to note their specific positions with proper labels.
- b) Apply known β -lactamase-positive and β -lactamase-negative control strains to the plate; label their positions.
- c) Incubate the plate in ambient air at 35°C for 15 minutes.
- d) Observe the plate for color change in the agar surrounding each discretely spaced colony. The agar surrounding positive-control strain should be yellow, whereas the agar surrounding the negative-control strain should not exhibit any change in color.

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.

Neisseria meningitidis

CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

N*eisseria meningitidis* is the etiologic agent of meningococcal disease, most commonly meningococcal bacteremia and meningitis. These two clinically overlapping syndromes may occur simultaneously, but meningitis alone occurs most frequently. *N. meningitidis* is an encapsulated bacterium and is classified into serogroups based on the immunological reactivity of the capsule's polysaccharide. The most common serogroups causing disease are A, B, C, Y, and W135. During the past 20 years, serogroups B and C have been responsible for most meningococcal disease in the Americas and Europe; serogroup A accounts for most meningococcal disease cases in Africa and some parts of Asia.

Meningococcal disease differs from the other leading causes of bacterial meningitis because of its potential to cause large-scale epidemics. Historically, these epidemics have been typically caused by serogroup A and, to a lesser extent, serogroup C. In Africa, the highest incidence rates of serogroup A meningococcal disease occur in a region of Sub-Saharan Africa extending from Sudan in the east to The Gambia in the west; this region consists of 15 countries comprised of more than 260 million people and has been referred to as the “meningitis belt.” During epidemics, children and young adults are most commonly affected, with attack rates as high as 1,000/100,000 population, or 100 times the rate of sporadic disease. The highest rates of endemic or sporadic disease occur in children less than 2 years of age. In recent years, two major epidemics of meningitis caused by *N. meningitidis* serogroup W135 have also been reported. In 2000, an outbreak of meningococcal disease in Saudi Arabia (which resulted in 253 cases and 70 deaths) was caused by a virulent clone of serogroup W135; this outbreak occurred simultaneously with the annual pilgrimage to Mecca and returning pilgrims disseminated this clone throughout the world, resulting in secondary cases. As of the time of writing of this laboratory manual in mid-2002, a serogroup W135 meningitis epidemic has been reported in Burkina Faso with more than 12,000 cases and 1400 deaths to date.

A quadrivalent polysaccharide vaccine that includes serogroups A, C, Y, and W135 is produced and used in the United States; however, bivalent A and C polysaccharide vaccines are being used in other parts of the world. New meningococcal conjugate vaccines are under development.

Laboratory personnel at risk for exposure to aerosolized *N. meningitidis* should ensure their protective vaccination status remains current and, if possible, work in a biological safety cabinet. Laboratory scientists who manipulate invasive *N. meningitidis* isolates in a manner that could induce aerosolization or droplet formation (including plating, subculturing, and serogrouping) on an open bench top and in the absence of effective protection from droplets or aerosols should consider antimicrobial chemoprophylaxis.

Confirmatory identification of *N. meningitidis*

The following steps are recommended to confirm the identity of cultures that morphologically appear to be *N. meningitidis* (Figure 9). The best results are obtained with day-old cultures. Always check for purity of the growth by performing a Gram stain: *N. meningitidis* is a gram-negative, kidney-bean- or coffee-bean-shaped diplococcus (see Figure 72). When necessary, make subcultures to ensure purity. From growth on a blood agar plate, perform Kovac's oxidase test, and then identify the serogroup with a slide agglutination test. Finally, confirm the results with carbohydrate (*i.e.*, sugar) reactions.

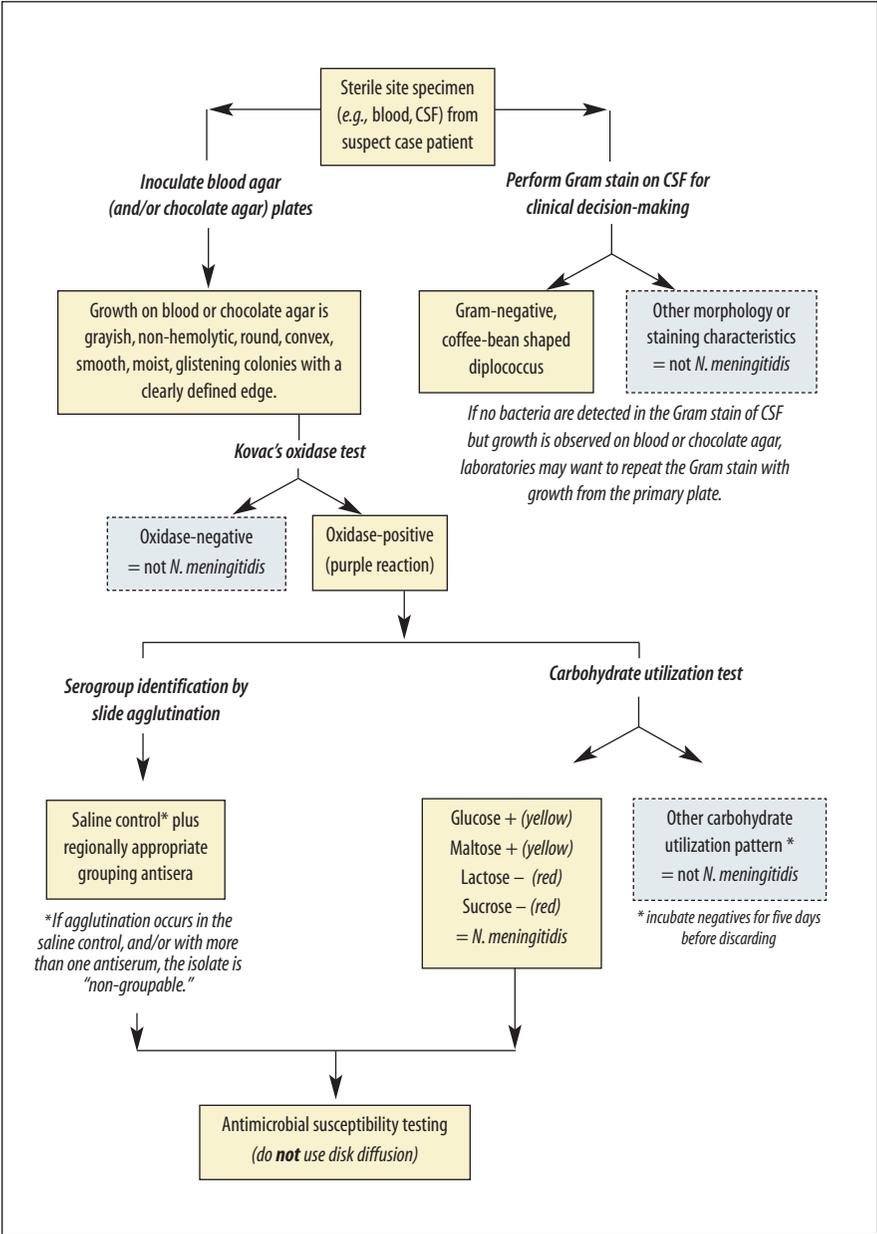
Some laboratorians may be interested in the OMP (*i.e.*, outer membrane protein) subtyping of *N. meningitidis* isolates; these tests may be performed by international reference laboratories.

Kovac's oxidase test for the identification of *N. meningitidis*

The oxidase test determines the presence of cytochrome oxidase. The Kovac's oxidase reagent (1% tetramethyl-*p*-phenylenediamine hydrochloride)⁷ is turned into a purple compound by organisms containing cytochrome *c* as part of their respiratory chain; therefore, an oxidase-positive test will yield a purple reaction. (Instructions for making oxidase reagent are found in Appendix 2.)

⁷ Some laboratories may use a different reagent, Gordon and MacLeod's reagent, (1% [wt/vol] dimethyl-*p*-phenylenediamine dihydrochloride; "dimethyl reagent") to perform the oxidase test. The dimethyl reagent is more stable than the tetramethyl reagent (Kovac's reagent), but the reaction with the dimethyl reagent is slower than that with the tetramethyl reagent. **If the laboratory is using the dimethyl- reagent**, a positive reaction will be indicated by a color change to blue on the filter paper (not purple, as with the tetramethyl reagent), and **with the dimethyl reagent it will take 10 – 30 minutes for a positive reaction to develop.**

FIGURE 9: Flowchart for laboratory identification of *Neisseria meningitidis*



- a) Using a platinum inoculating loop, a disposable plastic loop, or a wooden applicator stick, pick a portion of the colony to be tested and rub it onto a treated strip of filter paper (Figure 10). Do not use a Nichrome loop because it may produce a false-positive reaction.
- b) Positive reactions will develop within 10 seconds in the form of a purple color. Delayed reactions are unlikely with *N. meningitidis*.

The oxidase test aids in the recognition of *N. meningitidis* and other members of the genus *Neisseria*; other, unrelated, bacterial species with cytochrome c in the respiratory chain (e.g., *Pseudomonas aeruginosa* and *H. influenzae*) are also oxidase positive.

Identification of the *N. meningitidis* serogroup

Twelve serogroups based on capsular polysaccharides are currently recognized: A, B, C, H, I, K, L, W135, X, Y, Z, and Z' (29E). (**Note:** serogroup D is no longer recognized.) Groups A and C are the common causes of meningitis outbreaks in Africa, but recently outbreaks caused by groups W135 and X have been reported; group B is a cause of endemic meningitis and may also cause outbreaks in some regions of the world (e.g., in Brazil). Grouping antisera are available commercially.

Serogrouping can be expensive, but it is valuable. Serogroup data provides laboratories and public health authorities with the tools to:

- identify outbreaks controllable by a vaccination campaign
- recognize the presence of serogroups causing sporadic disease
- detect the emergence of new outbreak strains (e.g., X or W135).

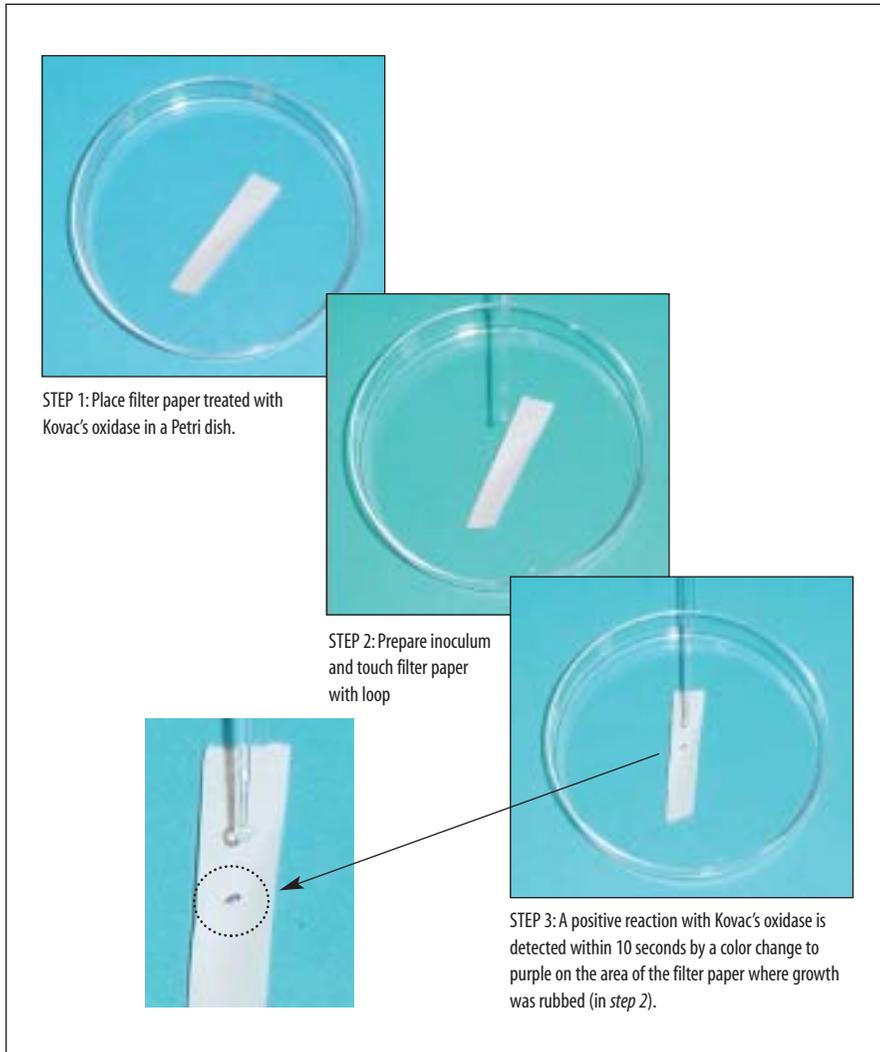
It is therefore essential that high-level reference laboratories have the capacity to isolate, identify and confirm the serogroup of *N. meningitidis* isolates causing sporadic disease as well as those they receive during the course of an outbreak.

Slide agglutination test for serogrouping suspected N. meningitidis isolates

The following methods require both formalinized physiological saline to make the meningococcal suspension and unformalinized physiological saline (or phosphate buffered saline [PBS]) to mix with the antisera. Store antisera in the refrigerator at 4°C when not in immediate use.

- a) Clean a glass slide with alcohol (optional if slides are pre-cleaned). Divide the slides into equal sections (e.g., three 25-mm [1-inch] sections for a 25-mm x 75-mm [1-inch x 3-inch] slide) with a wax pencil or other marker.
- b) Collect a small portion of growth from the surface of an overnight culture on non-selective blood or chocolate agar plate using a sterile inoculating loop.

FIGURE 10: Kovac's oxidase test: a positive reaction on filter paper



Make a moderately milky suspension of the test culture in 250 μl (0.25 ml) of formalinized physiological saline. Vortex the suspension, if possible. If working with only several isolates, it may be more convenient to make the suspension directly on the slide in 10 μl of formalinized physiological saline per droplet.

- **Note:** For safety reasons, it is recommended that formalin-killed meningococcal suspensions rather than saline suspensions of living organisms be used; however, formalin is a carcinogen and must be stored

and handled with great care. (Alternatively, if formalin is not used to kill the meningococci, laboratorians can work under a safety hood.)

- It is not necessary to make a standard suspension for slide serology; however, it should be noted that a “moderately milky suspension” is roughly comparable to a 6 McFarland turbidity standard.
- c) Use a micropipettor or a bacteriologic loop to transfer a drop (5–10 μl) of the cell suspension to the lower portion of each section of the slide prepared in step a of this procedure.⁸
- d) Add a drop of group A antiserum above the drop of suspension in one of the test sections on the slide. In one of the other sections of the slide, add a drop of W135 antiserum below the drop of suspension in that section. For the third section of the slide, use the same method to add a drop of saline below the final drop of suspension.
- **The loop used in the antiserum must not touch either the cell suspension or the other antisera being tested; if it does, it must not be placed back into the source bottle of antiserum.** If the source antiserum is contaminated, a new bottle must be used.
 - **Note:** In Africa, testing with A and W135 antisera (with a saline control to detect nonspecific autoagglutination) should be adequate for serologic characterization of most *N. meningitidis* isolates. Strains reacting negatively with A and W135 antisera should then be tested with other available antisera, particularly C, Y, B, and X.
- e) **Using a separate toothpick (or sterile loop) for each section**, mix each antiserum (and control saline) with its corresponding drop of cell suspension. Avoid contamination across the sections of the slide.
- f) **Gently rock the slide with a back and forth motion** for up to 1 minute. Do not use a circular motion while rocking, as it can cause the mixtures to run together and contaminate each other. After one minute of rocking, observe the three mixed drops and read the slide agglutination reactions under bright light and over a black background (see Figure 2).
- g) **Only strong agglutination reactions (3+ or 4+) are read as positive.** In a strong reaction, all the bacterial cells will clump and the suspension fluid will appear clear (see Figure 11 and Figure 42). When a strain reacts only in one grouping antiserum, it should be recorded as belonging to that serogroup.

⁸ This laboratory manual suggests using a micropipettor or a loop to transfer antiserum from the bottle to the slide (rather than the dropper provided with the bottle of antiserum) because they conserve costly antiserum resources. (Micropipettors permit the precise measurement of antiserum, and the loop method collects only 5–10 μl of antiserum on average; in contrast, the dropper transfers several times this amount in each drop.) Because only 5–10 μl of antisera are required for agglutination reactions to occur using the methods presented in this manual, **using a micropipettor or a loop to transfer antiserum from the bottle to the slide is more cost-effective.**

(For example, an isolate exhibiting a strong agglutination reaction only in group A antiserum would be recorded as '*N. meningitidis*, serogroup A.')

- If a strong agglutination reaction does not occur with the antisera tested:
 - If the isolate is negative in the first two antisera tested (groups A and W135 in Africa) and the saline control, repeat the test with different antisera to identify the serogroup, following steps a through f of this procedure.
- When a strain reacts with more than one antiserum or agglutinates in saline, the strain is categorized as non-groupable. (These results occur rarely with fresh isolates, but they do happen occasionally.) Non-groupable results are characterized by:
 - 1) Autoagglutination in the saline control (“autoagglutinable”).
 - 2) Cross-agglutination with reactions in more than one antiserum (“rough”).
 - 3) No agglutination with either any of the antisera or with the saline control (“non-reactive”).

Report results of *N. meningitidis* serogroup testing back to attending clinicians, as appropriate.

FIGURE 11: Positive and negative agglutination reactions on a slide: grouping antisera and saline control with *Neisseria meningitidis*



When a suspension is mixed with its homologous antiserum, agglutination occurs (*left*). In a negative reaction, as shown with a heterologous antiserum (*center*) or control saline (*right*), the suspension remains smooth and cloudy in appearance.

Carbohydrate utilization by *N. meningitidis*: cystine trypticase agar method

Carbohydrate utilization tests are used to further validate the identification of a strain as *N. meningitidis*. Various carbohydrates are added to the cystine trypticase agar (CTA) base to a final concentration of 1%. To confirm a culture as *N. meningitidis*, a set of four tubes, each containing a sugar (*i.e.*, glucose [dextrose], maltose, lactose, and sucrose) is used. Members of *Neisseria* species produce acid from carbohydrates by oxidation, not fermentation. *N. meningitidis* oxidizes glucose and maltose, but not lactose and sucrose. A phenol red indicator is 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. (Methods for the preparation and quality control of CTA medium are included in Appendix 2.)

- a) With an inoculating needle, collect a small amount of growth from an overnight culture of *N. meningitidis* on blood agar or chocolate agar.
- b) Stab the inoculum several times into the upper 10 mm of medium. Use another sterile needle, or flame the same needle, before inoculating each of the four carbohydrates to be tested.
- c) Fasten caps of tubes **tightly** and place in a 35°C incubator (without CO₂). Incubate for at least 72 hours (and up to 5 days) before discarding as negative.
- d) Development of visible turbidity and a yellow color in the upper portion of the medium indicates growth and the production of acid and is interpreted as a positive test (Figure 12). Although reactions may occur as early as 24 hours after inoculation, some reactions are delayed. If only glucose or maltose or none of the sugars react, continue incubation for up to 5 days before discarding. Occasionally, strains of *N. meningitidis* are encountered that utilize only dextrose or maltose but not both (Table 3).

TABLE 3: Carbohydrate utilization by some species of *Neisseria* and *Moraxella*

Species	Produces acid from ^a			
	Glucose ^b	Maltose	Lactose	Sucrose
<i>N. meningitidis</i>	+	+	–	–
<i>N. gonorrhoeae</i>	(+) ^c	–	–	–
<i>N. sicca</i>	+	+	–	+
<i>N. lactamica</i>	+	+	+	–
<i>M. catarrhalis</i>	–	–	–	–

^a Negative results should not be interpreted prior to 72 hours of incubation in order to avoid false-negative results for delayed acid production reactions.

^b Glucose may be also be referred to as “dextrose”.

^c Strains of *N. gonorrhoeae* that are weak acid producers may appear to be glucose-negative in cystine trypticase agar (CTA) medium.

FIGURE 12: Cystine trypticase agar sugar reactions for acid production from carbohydrates by *Neisseria meningitidis*



Acid is produced by utilization of sugar and causes the CTA medium to turn yellow at or just below the agar surface. For *N. meningitidis*, there is utilization of dextrose and maltose (two tubes on left with yellow color just below the surface) and no utilization of lactose nor sucrose (two tubes on right with solid red color of medium).

Commercial identification kits for *Neisseria*

Several commercial identification systems that use biochemical or enzymatic substrates are available for identification of *Neisseria* species. These systems may occasionally require supplemental tests, and other characteristics, such as microscopic and colony morphology, must be considered; additionally, antimicrobial susceptibility testing cannot be conducted without a confirmed *N. meningitidis* isolate. Generally, each system is self-contained, but addition of one or more reagents to complete certain reactions may be necessary. Follow the manufacturer's instructions precisely when using these kits. For detailed instructions and use of appropriate control strains, also consult the *Clinical Microbiology Procedures Handbook* (see Appendix 15). Rapid sugar utilization test kits may also be used toward the identification of *N. meningitidis*.

Antimicrobial susceptibility testing of *N. meningitidis*

N. meningitidis does not commonly show resistance to many antimicrobial agents. Low-level resistance to penicillin is common in some areas of the world, though the clinical significance of this resistance has not yet been established.

Meningococcal resistance to sulfonamides, rifampicin (also referred to as rifampin), and chloramphenicol has also been described. Chloramphenicol tends to be the empiric drug of choice for treating patients with meningitis caused by *N. meningitidis*; rifampicin and sulfonamides are often used for prophylaxis.

Antimicrobial susceptibility testing of *N. meningitidis* should not be performed by disk diffusion, even though it is the least expensive screen, because results are very difficult to interpret and will not provide data useful for making informed treatment decisions. Two appropriate methods for testing include (1) minimal inhibitory concentration (MIC) determination by broth microdilution, and (2) use of the Etest® strip. The broth microdilution methodology provides laboratorians with quantitative MIC results based on the inhibition of growth of a standard inoculum in standard concentrations (dilutions) of antimicrobial. The Etest® antimicrobial susceptibility test methodology provides laboratorians with semi-quantitative MIC results, because although a standard suspension is used to inoculate a plate, the inoculum is not precisely standardized. Results of the Etest® and conventional MIC testing by broth microdilution are generally comparable.

The broth microdilution procedure can be expensive and challenging to perform and, because of the technical complexity required, countries that do not currently do MIC testing by dilution should utilize the international reference laboratory rather than developing the assay in-country. For laboratories that do not perform MIC testing by dilution methods but still want to perform antimicrobial susceptibility tests on *N. meningitidis* isolates, the Etest® may be a convenient alternative.⁹ The Etest® is easier to quality control and is the focus of this section of the manual, but broth microdilution methodology is included in Appendix 7. Figure 13 shows a sample worksheet for recording antimicrobial susceptibility test results for *N. meningitidis*.

Either 150-mm or 100-mm plates can be used for the Etest®, depending on the number of antimicrobial agents to be tested per isolate. Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and although the manufacturer states that up to six Etest® 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 Etest® strips be used on a 150-mm plate (see Figure 7).

⁹ The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

Minimal inhibitory concentration testing of *N. meningitidis* by Etest® antimicrobial gradient strip

Mueller-Hinton + 5% sheep blood agar is used when testing *N. meningitidis* isolates with the Etest®. Follow the directions on the package insert included with the Etest® strips.

- a) Using a sterile cotton-tip applicator, touch the surface of one to four morphologically similar, isolated colonies grown on a chocolate agar plate incubated in CO₂-enhanced atmosphere (5% CO₂ in a CO₂-incubator or candle-extinction jar) at 35°C for 18–22 hours. Immerse the applicator into a tube containing sterile broth (e.g., Muller-Hinton broth). 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 to form a suspension, being careful not to form froth or bubbles in the suspension when mixing the cells. **This suspension should be used within 15 minutes.**
- b) Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard. If the turbidity of the inoculum is greater than the standard, dilute it with broth to equal the turbidity of the standard. (Figures 51 and 52 in Appendix 2 show how to compare the turbidity of the suspension with the standard and also provide black and white lines as a reading background.)
- c) Immerse a sterile cotton-tipped swab into the adjusted inoculum (prepared in step *b* of this procedure). Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a 15x150-mm Mueller-Hinton + 5% sheep 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 (see Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.
- d) Allow the inoculum to dry on the surface of the plate (which should take approximately 10 minutes). **Be sure the plate is entirely dry before proceeding.** While the plate is drying, remove the Etest® strips from the -20°C freezer, and allow the strips that will be used in the batch of testing to warm to room temperature. Return the antimicrobial gradient strips that will not be used in this batch of testing to the -20°C freezer.
- e) When the surface of the inoculated plate is dry and the Etest® strips are at room temperature, place the antimicrobial gradient strips onto the agar with an Etest® applicator or sterile forceps, as illustrated in Figure 7. 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.) **Once applied, it is important to not move the antimicrobial gradient strips.**

- f) Incubate the plates in an inverted position in a 5% CO₂ atmosphere for 18–22 hours at 35°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 water to the bottom of the incubator or add a dampened paper towel to the candle-extinction jar.

After incubation, an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **Quality control results must be reviewed before reading and interpreting the Etest® MIC.** MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® 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 and isolated colonies. Figure 8 presents a reading guide for the Etest®,¹⁰ and shows drug-related effects, technical and handling effects, organism-related effects, and resistance mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but also include increments between those standard values. The standard values (see Table 27 in Appendix 7) 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 graduations on the Etest® strip might be 0.094 µg/ml; however, the reported MIC would be 0.125µg/ml.

Quality control for antimicrobial susceptibility testing of *N. meningitidis*

To verify that antimicrobial susceptibility test results are accurate, it is important to include at least one control organism. It is of note here that NCCLS¹¹ does not publish MIC ranges specific to *N. meningitidis*; however, the Centers for Disease Control and Prevention (CDC, United States of America) recommends that if antimicrobial susceptibility testing of *N. meningitidis* is going to be performed, then a banked control strain for fastidious organisms (*S. pneumoniae* ATCC 49619) should be used for quality control. The NCCLS MIC ranges for quality control testing of *S. pneumoniae* ATCC 49619 with the antimicrobial agents penicillin, rifampicin, and sulfonamides are included in Table 4. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

¹⁰ AB Biodisk also maintains a website with an Etest® reading guide: <http://www.abbiodisk.com>.

¹¹ Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

TABLE 4: Minimal inhibitory concentration (MIC) ranges for quality control of *Neisseria meningitidis* antimicrobial susceptibility testing

QC strain for <i>N. meningitidis</i> ^a	Penicillin MIC Range ^b	Rifampicin (Rifampin) MIC Range ^b	Trimethoprim-sulfamethoxazole MIC Range ^b	Chloramphenicol MIC Range ^b
<i>S. pneumoniae</i> ATCC 49619	0.25 – 1 µg/ml	0.015 – 0.06 µg/ml	0.12/2.4 – 1/19 µg/ml	2 – 8 µg/ml

^a Source: Dr. F. Tenover, Centers for Disease Control and Prevention, Atlanta, Georgia, USA: 2002.
^b 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 USA.

Resistance to antimicrobials other than penicillins and rifampicin is not commonly detected in *N. meningitidis*; however, laboratories, clinicians, and other public health practitioners may be interested in performing annual screens of isolates in storage. (Appendix 11 provides methods for how to preserve and store meningococcal isolates.) Periodic, non-routine surveillance for characteristics such as β-lactamase production, and ceftriaxone, chloramphenicol and fluoroquinolone resistance will help provide information to public health agencies and international reference laboratories regarding the emergence of new *N. meningitidis* strains of clinical and public health concern.

Antimicrobial susceptibility tests are affected by variations in media, inoculum size, incubation time, temperature, and other 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 for trimethoprim-sulfamethoxazole (cotrimoxazole); organisms may then appear to be resistant to these drugs when in fact they are not. If the depth of the agar in the plate is not 3–4 mm or the pH 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. (**Do not attempt to adjust the pH of this Mueller-Hinton agar** even if it is out of range; see Appendix 2)

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, if 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 disks even when the isolates being tested are susceptible.

Reading and interpreting the Etests®

Read the MIC at the point where the zone of inhibition intersects the MIC scale on the strip, as illustrated in Figure 8. Record the quality control results first. If zones

produced by the control strain are out of the expected ranges (Table 4), the laboratorian should consider possible sources of error. If all antimicrobial agents are in control, read the test MICs. Note any trailing endpoints.

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, agar depth, storage, time, and others), quality control practices must be followed carefully.

Although NCCLS has not defined standardized breakpoints for the interpretation of an *N. meningitidis* isolate as susceptible or not, the MICs obtained by antimicrobial susceptibility testing methods as described in this document can still be used. Just as a laboratory might assess antimicrobial susceptibility for the very many other organisms for which no NCCLS breakpoints have been defined, laboratorians and clinicians should consider the site of infection in conjunction with the dose and pharmacokinetics of the antimicrobial agent to determine how much drug reaches the site of the infection. This information should then be compared to the MIC value to determine if the concentration of drug available is at least four times greater than the MIC. If the concentration of drug available is ≥ 4 times the MIC, the organism may be considered susceptible; if not, it is resistant.

Data for decision-making

Once the laboratory has confirmed the identification and serogroup (and antimicrobial susceptibility patterns, if appropriate) of *N. meningitidis* isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- Immunization should be considered if a *N. meningitidis* vaccine serotype is a major cause of local invasive disease.
- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be 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.

Streptococcus pneumoniae

CONFIRMATORY IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

Streptococcus pneumoniae is a common agent of lower and upper respiratory diseases, such as pneumonia, meningitis and acute otitis media (middle ear infections), affecting children and adults worldwide. This bacterial pathogen is the cause of approximately 40% of acute otitis media. Although acute otitis media and other upper respiratory tract infections do not commonly progress to invasive disease they do contribute significantly to the burden and cost of pneumococcal disease. Meningitis in infants, young children and the elderly is often caused by *S. pneumoniae*. Persons who have sickle cell disease, anatomic asplenia, or are immunocompromised also have increased susceptibility to *S. pneumoniae* infection. Pneumococcal meningitis is the most severe presentation of disease, but most illnesses and deaths result from pneumococcal pneumonia. Pneumococcal polysaccharide vaccine has been available for preventing invasive disease in the elderly and in persons with chronic illnesses that may impair their natural immunity to pneumococcal disease; however, this vaccine is not effective in children <2 years of age. In contrast to polysaccharide vaccines, conjugate vaccines are effective in young children. A pneumococcal conjugate vaccine covering seven serotypes that most commonly cause bacteremia in children in the United States (and some other industrialized nations) was approved for clinical use in 2000; research on vaccine formulations containing serotypes more common in developing countries is underway.

S. pneumoniae is frequently carried in the throat without causing disease. On occasion, public health investigations call for studies on the prevalence of *S. pneumoniae* carriage. For this research, samples may be collected using nasopharyngeal (NP) swabs; methodology for collection and isolation with NP swabs is included in Appendix 5. Antimicrobial susceptibility testing on isolates should be performed as presented in this chapter.

Confirmatory identification of *S. pneumoniae*

S. pneumoniae are gram-positive diplococci or chains of cocci (see Figure 73). On blood agar and chocolate agar plates, *S. pneumoniae* colonies appear small, greyish and mucoid (*i.e.*, watery), and are surrounded by a greenish zone of alpha-hemolysis (α -hemolysis).

Colonies of pneumococci and α -hemolytic viridans streptococci each appear raised when young; however, after 24–48 hours, the center of pneumococcal colonies becomes depressed, whereas viridans streptococcal colonies retain their raised appearance (Figure 14). A 3x hand-lens or a microscope (30x–50x) can therefore be a useful aid in differentiating pneumococci from α -hemolytic viridans streptococci. Laboratory differentiation between *S. pneumoniae* and viridans streptococci is accomplished by optochin and bile solubility testing: pneumococci are susceptible to optochin and bile-soluble, while viridans streptococci are not. Commercially available slide agglutination tests can also be used for identification of pneumococci. For optimal results plates for pneumococcal identification assays should be incubated in a 5% CO₂ atmosphere.

A flowchart diagram of the laboratory identification of *S. pneumoniae* is included in Figure 15. Presumptive identification of *S. pneumoniae* is made by determining the susceptibility of the strain to optochin (*i.e.*, ethylhydrocupreine). The bile solubility test is also used for identification of *S. pneumoniae*, particularly when results of the optochin susceptibility test are ambiguous.

Optochin susceptibility test

The optochin susceptibility test is performed with a 6-mm, 5- μ g optochin disk,¹² and is used to differentiate between *S. pneumoniae* and viridans streptococci. Optochin-susceptible strains can be identified as *S. pneumoniae*.

Performance of the optochin susceptibility test

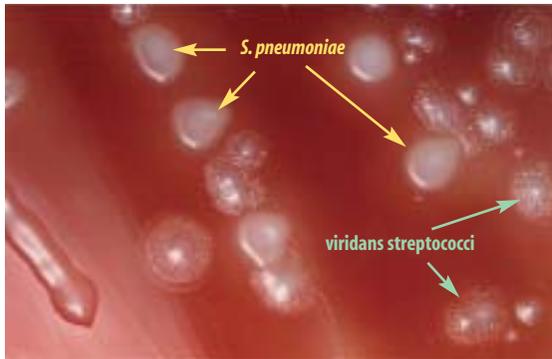
- a) Touch the suspect α -hemolytic colony with a sterile bacteriological loop and streak for isolation onto a blood agar plate in a straight line. Several strains can be tested on the same plate at once, streaked in parallel lines and properly labeled.
- b) Aseptically place an optochin or “P” disk with a diameter of 6 mm (and containing 5 μ g of ethylhydrocupreine) on the streak of inoculum, near the end where the wire loop was first placed. Because the inoculum is streaked in a straight line, three to four colonies may be tested on the same plate (Figure 16).

¹² The results and interpretation of the optochin susceptibility test presented in this document are appropriate for the 6-mm, 5- μ g optochin disk (“P”-disk), although different size disks (and possibly optochin concentrations) are available for purchase. When using optochin disks with different size and/or concentration parameters, follow the manufacturer’s instructions for interpretation.

FIGURE 14: A properly streaked blood agar plate with pneumococci and viridans streptococci



Note how growth is heavy where streaking began on the left and then thins to individual colonies.



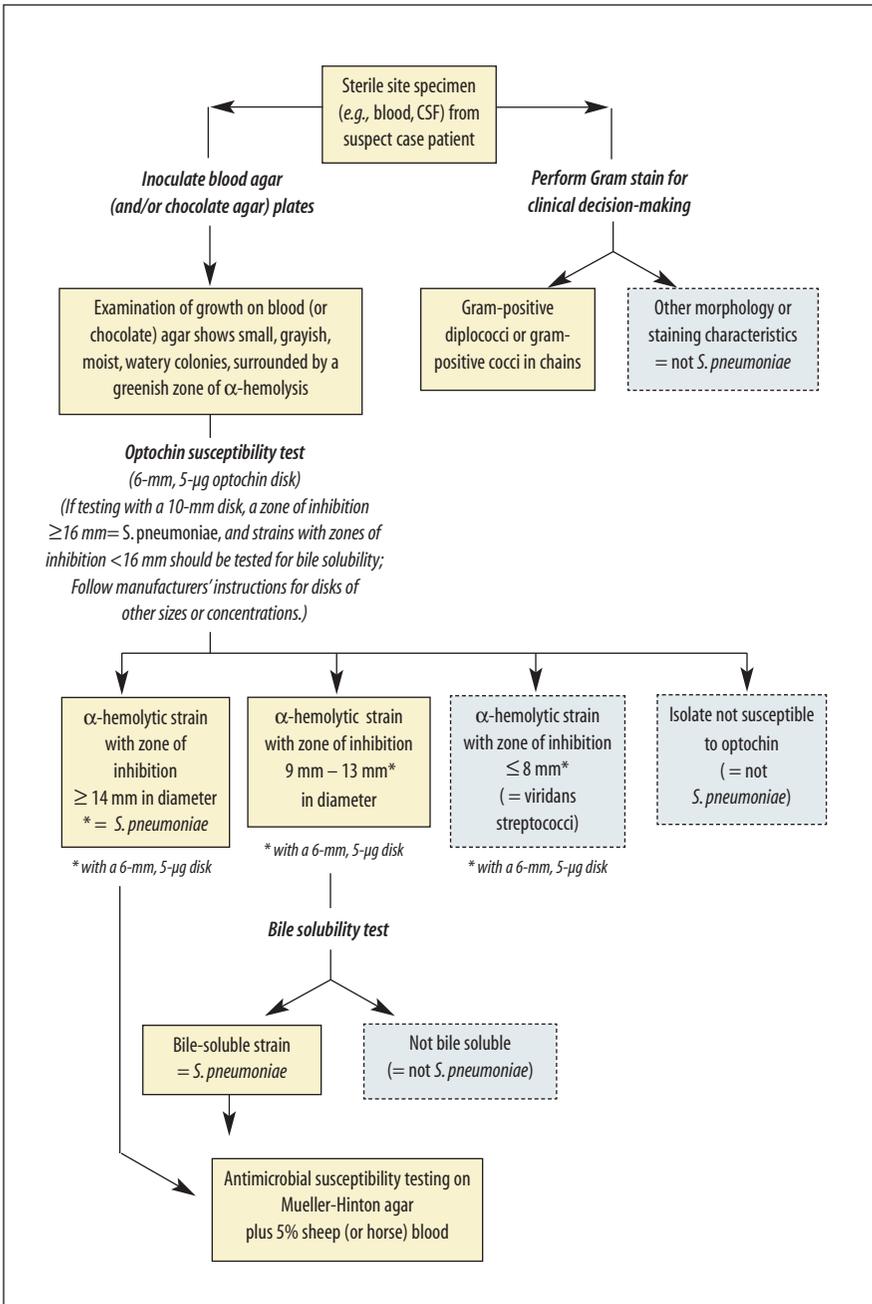
The *S. pneumoniae* has a depressed center (yellow arrows) at 24 – 48 hours incubation, whereas the viridans streptococci retain a raised center (black arrows).

- c) Incubate the plates in a CO₂-incubator or candle-jar at 35°C for 18–24 hours.
- d) Read, record, and interpret the results.

Reading and interpreting the optochin susceptibility test results

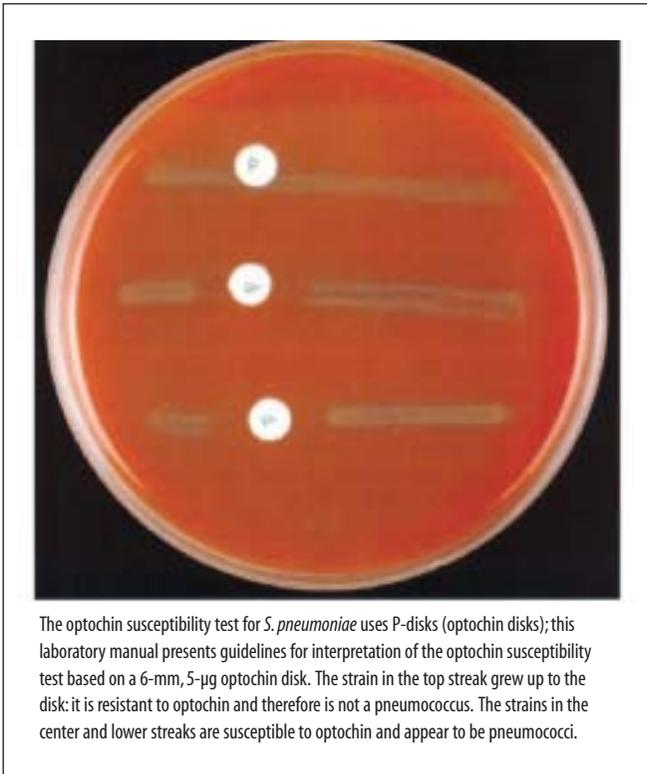
In Figure 16, the strain in the top streak is resistant to optochin and, therefore, is not a pneumococcus. The strains in the center and lower streaks are susceptible to optochin and appear to be pneumococci.

FIGURE 15: Flowchart for laboratory identification of *Streptococcus pneumoniae*



- α -hemolytic strains with a zone of inhibition of growth greater than 14 mm in diameter are pneumococci.
(If using a 10-mm, 5- μ g disk, α -hemolytic isolates with a zone of inhibition of growth >16 mm in diameter are considered susceptible to optochin and, therefore, are pneumococci.)
- α -hemolytic strains with no zones of inhibition are viridans streptococci.
- **α -hemolytic strains with zones of inhibition ranging between 9 mm and 13 mm should be tested for bile solubility** for further characterization and identification.
(If using a 10-mm disk, α -hemolytic isolates with a zone of inhibition of growth <16 mm should be tested for bile solubility.)

FIGURE 16: Optochin susceptibility test for identification of *Streptococcus pneumoniae*



Bile solubility test

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

Tube method for the performance of the bile solubility test

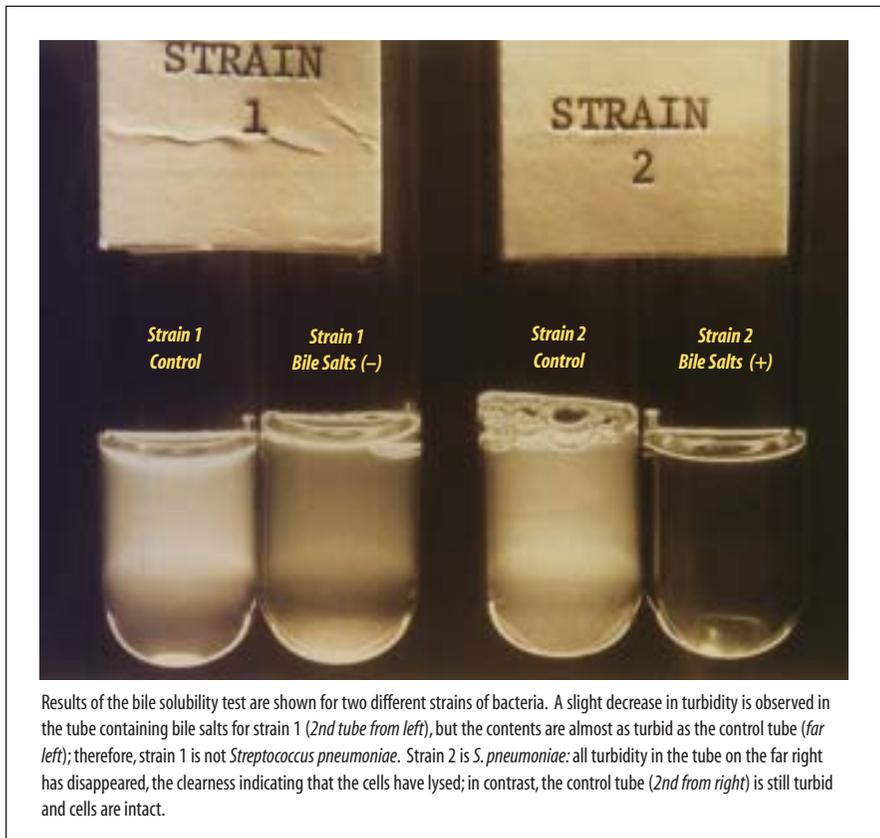
Two tubes are required for bile solubility testing of each suspect strain of *S. pneumoniae*.

- a) Take a loop of the suspect strain from fresh growth on a blood agar plate and prepare a bacterial cell suspension in 0.5 ml of sterile saline. The suspension of bacterial cells should be cloudy, similar to that of a 0.5 or 1.0 McFarland turbidity standard. (Preparation of a McFarland turbidity standard is described in Appendix 2.)
 - If growth on the optochin test plate is sufficient, the suspension can be made with the bacterial cells collected from the specific streak of suspect *S. pneumoniae*.
 - When there is insufficient growth to make a suspension of the proper density in 0.5 ml of sterile saline, inoculate a blood agar plate with the suspect growth and incubate overnight (*i.e.*, for 18 – 24 hours at 35°C in a CO₂-enriched atmosphere) to prepare a fresh culture.
- b) Divide the suspension into two equal amounts (*i.e.*, 0.25 ml per tube). Add 0.25 ml of saline to one tube and 0.25 ml of 2% sodium desoxycholate (bile salts) to the other.
 - To make a 2% concentration of bile salts, add 0.2 g of sodium desoxycholate to 10 ml of saline.
- c) Shake the tubes gently and incubate them at 35° – 37°C for up to 2 hours.
- d) Examine the tubes periodically for lysis of cells in the tube containing the bile salts. A clearing of the tube, or a loss in turbidity, is a positive result (Figure 17).
 - Strains that yield clearing of the suspension in tube in the bile solubility test should be reported as “bile soluble.”
 - Strains for which the turbidity in the tube remains the same as that in the saline control tube is reported as negative for bile solubility (or “bile insoluble” or “bile resistant”).

Plate method for the performance of the bile solubility test

In place of the tube test for bile insolubility, a laboratorian may perform the bile solubility test using the plate method. **A freshly prepared culture** of the suspect organism must be used for this test.

FIGURE 17: Positive and negative results of the bile solubility test



- a) Place a drop of 10% sodium desoxycholate solution directly on a colony of the suspect pneumococcal strain to be tested.
 - (To prepare the 10% solution of bile salts, add 1 g of sodium desoxycholate bile salts to 10 ml of sterile saline.)
- b) Keep the plate at room temperature (*i.e.*, 72°–75°C) or place it face up (*i.e.*, agar-side up) and on a level surface in an ambient air incubator (*i.e.*, **not** a CO₂-incubator) at 35°C for approximately 15 minutes (or until the 10% bile salt reagent dries).
 - *Optional*: instead of leaving the plate out at room temperature, laboratorians may choose to put the plate top-side (*i.e.*, agar-side) up on a level surface in an ambient air incubator (*i.e.*, **not** a CO₂-incubator) at 35°C until the reagent dries (approximately 10–15 minutes).
- c) When the reagent dropped on the suspect colony is dry, read, record, and interpret the results.

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

Interpretation of the combined optochin and bile solubility tests for pneumococcal identification

The following summary of results of the optochin and bile-solubility tests is commonly used to accurately and conveniently identify *S. pneumoniae* (i.e., pneumococcus).

- A strain exhibiting a zone of inhibition by optochin ≥ 14 mm (with a 6-mm, 5- μg disk) is a pneumococcus.
- A strain exhibiting a smaller but definite zone of inhibition by optochin (9–13 mm with a 6-mm, 5-mg disk) **and** that is also bile soluble is a pneumococcus.

The following summary of results of the optochin and bile-solubility tests should be interpreted as negative for *S. pneumoniae* (and positive for viridans streptococci).

- A strain with a small zone of inhibition by optochin (≤ 8 mm with a 6-mm, 5- μg disk) that is not bile soluble is not a pneumococcus. (The colonies can be identified as viridans streptococci.)
- Strains with no zones of inhibition by optochin are not pneumococci. (The colonies can be identified as viridans streptococci.)

Commercial test kits for identification (slide agglutination test)

Commercially available slide agglutination tests (e.g., Slidex Pneumo-kit® and the Pneumoslide™) can also help identify colony growth from blood agar plates as *S. pneumoniae*. Follow the manufacturer's instructions precisely when using these and any other commercial tests.

If a colony appears to be *S. pneumoniae* on the basis of morphology and susceptibility to optochin, but it has a negative bile solubility test, slide agglutination tests can assist with identification of the isolate. A positive slide agglutination test should be interpreted as a possible *S. pneumoniae* isolate, whereas a negative slide agglutination reaction in conjunction with the positive optochin and negative bile solubility would indicate the isolate is not *S. pneumoniae*.

Identification of the *S. pneumoniae* serotype

Serotyping of pneumococci is not usually necessary for a clinical response. However, in some situations or settings (e.g., studies focusing on evaluation

of vaccine efficacy), it will be appropriate to type these isolates. Methods for serotyping and Quellung typing are included in Appendix 6.

Antimicrobial susceptibility testing of *S. pneumoniae*

The results of antimicrobial susceptibility tests will be used to help make recommendations for clinical treatment. There are a variety of methods by which one can determine the antimicrobial susceptibility of a bacterial pathogen, commonly including disk diffusion, testing by agar dilution or broth microdilution, and testing by antimicrobial gradient agar diffusion (e.g., with the Etest® strip). The disk diffusion method presented in this chapter is a modification of the Kirby-Bauer technique that has been carefully standardized by NCCLS;¹³ 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. This section describes the optimal media, inoculum, antimicrobial agents to test, incubation conditions, and interpretation of results for *S. pneumoniae*.

The disk diffusion method gives valid data for only certain antibiotics, so this laboratory manual recommends use of the Etest® to gather data about the minimal inhibitory concentration (MIC) of antimicrobial agents.¹⁴ MIC testing can also be done by dilution; however 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 should utilize the international 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.)

This laboratory manual describes antimicrobial susceptibility testing of *S. pneumoniae* by the disk diffusion method and by the Etest® antimicrobial gradient strip method. (Figure 18 is a sample worksheet for recording results of the antimicrobial susceptibility tests.) Although disk diffusion will provide information for most antimicrobial agents regarding interpretation of a strain as susceptible, intermediate, or resistant, the Etest® 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 on-going basis.**

¹³ Formerly known as the National Committee on Clinical Laboratory Standards (and now known solely by the acronym), NCCLS is an international, interdisciplinary, nonprofit educational organization that develops updated consensus standards and guidelines for the healthcare community on an annual basis.

¹⁴ The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

FIGURE 18: Sample form for recording antimicrobial susceptibility test results for *Streptococcus pneumoniae*

Date of Testing: ____/____/____ Test performed by: _____		Interpretation of susceptibility: S = susceptible I = intermediate R = resistant				
Specimen number	Meningitis isolate? ^a	Organism	Chloramphenicol	Trimethoprim-sulfamethoxazole	Oxacillin ^b (disk) or (Penicillin) (MIC)	(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
ATCC 49619	N/A	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

^a if an *S. pneumoniae* isolate is from a meningitis patient, the breakpoints for interpretation of the MIC may differ from those for isolates from other sites.
^b if an oxacillin disk yields a zone diameter <20 mm for *S. pneumoniae*, MIC testing to a specific penicillin must be done in order to interpret the susceptibility.

Reviewed by: _____ Date of Report: ____/____/____

Note: After 20-24 hours of incubation, check the results for the quality control (QC) strains 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 5.)

Quality control of antimicrobial susceptibility testing of *S. pneumoniae*

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. *S. pneumoniae* ATCC 49619 is the NCCLS control strain to use when performing antimicrobial susceptibility testing on *S. pneumoniae* isolates.

Inhibition zone diameters obtained for the control strain should be compared with NCCLS published limits, which are included in Table 5. If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

- **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. (**Note:** Do not attempt to adjust the pH of the Mueller-Hinton agar test medium if it is outside the range; see Appendix 2.)
- **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 trimethoprim-sulfamethoxazole disks.

Quality control (“QC”) 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 antimicrobial susceptibility test medium and every time a new lot of disks is used.

Antimicrobial susceptibility testing by disk diffusion

Antimicrobial susceptibility can be determined using the disk diffusion method; however, disk diffusion antimicrobial susceptibility testing is generally not performed on meningitis isolates. This laboratory manual describes the optimal media, inoculum, antimicrobial agents to be tested, incubation conditions, and interpretation of results.

- 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.
- The 1- μ g oxacillin disk is recommended for predicting the susceptibility of *S. pneumoniae* to penicillin because penicillin disks do not provide reproducible results. Interpretations of the oxacillin disk diffusion test are generalizable across the β -lactam drugs for *S. pneumoniae*.
 - **It is only possible to conclude if a strain is susceptible to penicillin based on the oxacillin screen, and not if it is resistant to penicillin. If the zone of inhibition around the oxacillin disk is less than 20 mm, additional MIC testing (e.g., by Etest®) must be performed to assess whether the isolate is resistant or susceptible to penicillin.**
- A 30- μ g chloramphenicol disk is used for detecting resistance to chloramphenicol.
- A 25- μ g trimethoprim-sulfamethoxazole (cotrimoxazole) disk (*i.e.*, a disk comprised of 1.25 mg trimethoprim plus 23.75 mg sulfamethoxazole) is used for detecting trimethoprim-sulfamethoxazole resistance in *S. pneumoniae*. **The Mueller-Hinton agar used for this test should be thymidine free to obtain accurate results with trimethoprim-sulfamethoxazole.**

Methods for antimicrobial susceptibility testing by disk diffusion

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. (Preparation of a McFarland turbidity standard and plate count methods are described in Appendix 2.)

- a) Suspend viable colonies from an overnight sheep blood or 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 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 (see Figures 51 and 52, Appendix 2). If the density is too heavy, the suspension should be diluted with additional suspending medium (*i.e.*, saline or broth). If the density is too light, additional bacteria should be added to the suspension.
- c) When the proper density is achieved, dip a cotton or dacron 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.
- d) 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 (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 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.)
- f) After the plate is dry, place the antimicrobial disks on the plates (as shown in Figure 6). Use sterile forceps to place the disks on the Mueller Hinton agar and tap them gently to ensure they adhere 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) Incubate the plates in an inverted position in a 5% CO₂ atmosphere for 20–24 hours at 35°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 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 QC strains are included in Table 5.
- h) After overnight incubation, measure the diameter of each zone of inhibition with a ruler or calipers. 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 (see 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.

TABLE 5: Antimicrobial susceptibility test breakpoints and quality control ranges for *Streptococcus pneumoniae*

Antimicrobial agent	Disk potency	Diameter of zone of inhibition (mm) and equivalent MIC breakpoint (µg/ml) ^a			NCCLS QC strain <i>S. pneumoniae</i> ATCC 49619
		Susceptible	Intermediate	Resistant	
Chloramphenicol	30 µg	≥ 21 mm (≤ 4 µg/ml)	~ ~	≤ 20 mm (≥ 8 µg/ml)	23 – 27 mm (2 – 8 µg/ml)
Trimethoprim-sulfamethoxazole (cotrimoxazole)	1.25 / 23.75 µg	≥ 19 mm (≤ 0.5 – 9.5 µg/ml)	16 – 18 mm (1/19 – 2/38 µg/ml)	≤ 15 mm (≥ 4/76 µg/ml)	20 – 28 mm (0.12/2.4 – 1/19 µg/ml)
Oxacillin ^b	1 µg Disk diffusion ONLY	≥ 20 mm ^b	** ^b	** ^b	≤ 12mm ^c
Penicillin ^b	MIC ONLY	(≤ 0.06 µg/ml)	(0.12 – 1 µg/ml)	(≥ 2 µg/ml)	(0.25 – 1 µg/ml)
Ceftriaxone ^{d,e}	MIC ONLY				
	Non-meningitis isolate MIC	(≤ 1 µg/ml)	(2 µg/ml)	(≥ 4 µg/ml)	(0.03 – 0.12 µg/ml)
Cefotaxime ^{d,e}	MIC ONLY				
	Non-meningitis isolate MIC	(≤ 1 µg/ml)	(2 µg/ml)	(≥ 4 µg/ml)	(0.03 – 0.12 µg/ml)
	Meningitis isolate MIC	(≤ 0.5 µg/ml)	(1 µg/ml)	(≥ 2 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 Oxacillin should only be tested by disk diffusion. If the zone is <20 mm, an isolate cannot be reported as susceptible, intermediate or resistant, and MIC testing must be conducted for an appropriate penicillin (or other β-lactam) drug.

^c "Deterioration in oxacillin disk content is best assessed with QC organism *Staphylococcus aureus* ATCC 25923, with an acceptable zone diameter of 18 to 24 mm" [NCCLS 2002].

^d Penicillin, ceftriaxone, and cefotaxime should only be tested by a method that will provide an MIC; it is presented in this table as a follow-up for an equivocal oxacillin disk diffusion test (i.e., oxacillin zone of inhibition <20 mm). Perform MIC testing on the specific penicillin (or other β-lactam) drug that would be used to treat.

^e Ceftriaxone and cefotaxime have separate interpretive MIC breakpoints for meningitis and non-meningitis isolates.

- i) Interpret the antimicrobial susceptibility of the test strain (and check that results for the QC strain *S. pneumoniae* ATCC 49619 are within the acceptable control range) by comparing the results to the NCCLS standard zone sizes (Table 5).

The Etest® for minimal inhibitory concentration testing of *S. pneumoniae*

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 and oxacillin (a penicillin agent) 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 minimal inhibitory concentration (MIC) testing of penicillin or any other beta-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 the international reference laboratory rather than developing the assay in-country. The World Health Organization (WHO) recommends that only one laboratory in a resource-limited region perform antimicrobial susceptibility testing; however, in countries where MIC testing is done at more than one laboratory, standardization and quality control should be conducted at each laboratory in accordance with the standardized guidelines presented in this manual.

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. Methods for preservation and storage of isolates are presented in Appendix 11, and detailed methods for transport of isolates according to international regulations are presented in Appendix 12.

With increasing antimicrobial resistance testing being performed outside of international reference laboratories, the Etest® serves as a test method that is both convenient and reliable.¹⁵ The Etest® requires less technical expertise than MIC testing by dilution methods, but it gives comparable results. **Etest® strips must be consistently stored in a freezer at -20°C.**

The Etest® is 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 (µg/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.¹⁶

The continuous concentration gradient of stabilized, dried antibiotic is equivalent to 15 log₂ dilutions by a conventional reference MIC procedure as suggested by the NCCLS. The Etest® has been compared and evaluated beside both the agar and broth dilution susceptibility testing methods recommended by the NCCLS. Authoritative reports indicate that an (approximately) 85% – 100% correlation exists between the accepted conventional MIC determinations and the MIC determined by the Etest® procedure for a variety of organism-drug combinations

¹⁵ The Etest® can be expensive; contact the manufacturer (AB BIODISK) to inquire about discounts available for laboratories in resource-poor regions (see Appendix 13).

¹⁶ Antimicrobial susceptibility testing with an antimicrobial gradient strip such as the Etest® can be considered to be a semi-quantitative method (because although the suspension used to inoculate a plate for Etest® is standardized, the inoculum itself is not standardized). However, results are generally comparable to quantitative results of standard broth microdilution or agar dilution MIC tests.

(see, e.g., Jorgensen *et al.* [1994] and Barry *et al.* [1996] in Appendix 15). Some studies have cited Etest® MICs as approximately one dilution higher than MICs determined by standard dilution methods.

Although this manual serves as a general guide to use of the Etest® antimicrobial gradient strip, **always follow the manufacturer's directions for use of the Etest®**, as certain antibiotic-bacteria (“drug-bug”) combinations have special testing requirements. For example, macrolides (e.g., azithromycin, erythromycin) should be tested in a normal atmosphere, not with CO₂.

Methods for performing antimicrobial susceptibility testing of *S. pneumoniae* with the Etest®

The manufacturer of the Etest® indicates that when testing *S. pneumoniae*, the Mueller-Hinton agar test medium can be supplemented with either sheep or horse blood; however, it may be easier to interpret results on medium prepared with sheep blood (except when testing susceptibility to trimethoprim-sulfamethoxazole, in which case sheep blood should not be used as a supplement) [CDC, unpublished data]. 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 Etest® (except when testing for susceptibility to **trimethoprim-sulfamethoxazole, in which case horse blood should be used** in place of sheep blood). Either 150-mm or 100-mm plates can be used, depending on the number of Etests® used per sample (Figure 7). Two different Etest® antimicrobial strips can be placed in opposite gradient directions on a 100-mm plate, and note that although the manufacturer states that up to six Etest® 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 Etest® strips be used on a 150-mm plate.

- a) Suspend viable colonies from an overnight blood agar plate into a broth tube 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. **This suspension should be used within 15 minutes.**
- b) Dip a cotton swab into the bacterial suspension. Press the swab on the side of the tube to drain excess fluid. Inoculate the entire surface of the agar plate three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure confluent growth of the bacteria (Figure 34). Use a single swab of inoculum, and do not return the swab to the broth after each rotation.
- c) 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 Etest® strips from the -20°C freezer, and allow the strips that will be used in the batch of testing to warm to room temperature. Return the strips that will not be used in this batch of testing to the -20°C freezer.

- d) Place the Etest® strips onto the dried, inoculated agar plate with an Etest® applicator or sterile forceps (Figure 7.) 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.) **Once applied, do not move the antimicrobial gradient strips.**
- e) Incubate the plates in an inverted position in a CO₂-enriched atmosphere (2%–5% CO₂) for 20–24 hours at 35°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.
- f) After incubation, there will be an ellipse of bacterial growth will have formed on the plate around the strip and the Etest® can be read. **It is important to review quality control results before reading and interpreting the Etest® MIC.**

MICs are read from the intersection of the ellipse-formed zone of inhibition with the value printed on the Etest® 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 and isolated colonies. Figure 8 presents a reading guide for the Etest®,¹⁷ and shows drug-related effects, technical and handling effects, organism-related effects and resistance-mechanism-related effects.

- The graduation marks on the Etest® strip correspond to the standard concentrations for the agar dilution method, but the marks also represent increments between those standard values. The standard values (Table 27 in Appendix 7) are used for interpretation and reporting of antimicrobial susceptibility test results. It is advised that if the MIC appears to be an inter-dilutional value, 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 a *S. pneumoniae* isolate to penicillin, an MIC recorded from the graduations on the Etest® strip might be 0.094 µg/ml; however, the reported MIC would be 0.125µg/ml, and the organism would be interpreted as being intermediate to penicillin.

Breakpoints follow the NCCLS guidelines, **unless exceptions made by the manufacturer are provided in the package insert.** NCCLS breakpoints for *S. pneumoniae*–antimicrobial combinations are included in Table 5.

¹⁷ AB Biodisk also maintains a website with an Etest® reading guide: <http://www.abbiobdisk.com>.

Surveillance for emerging pneumococcal resistance

Laboratories may wish to help detect the emergence of new strains of pathogens by testing isolates against a panel of drugs in which reduced susceptibility is not expected to be found. This could be done, for example, on a sampling of preserved isolates in storage on an annual basis. Methods for preservation and long-term storage of isolates can be found in Appendix 11.

Antimicrobials of interest could include (but are not necessarily limited to): tetracycline, erythromycin, clindamycin, rifampin, ceftriaxone, amoxicillin, ciprofloxacin and vancomycin. Appropriate zone sizes can be found in NCCLS documents, which are updated regularly. Laboratorians should notify a reference laboratory of any isolates observed to have rare characteristics of non-susceptibility; for example, as of early 2002, no pneumococcus has exhibited decreased susceptibility to vancomycin [NCCLS 2002]. A list of international reference laboratories is included in Appendix 14.

Data for decision-making

Once the laboratory has antimicrobial susceptibility patterns of *S. pneumoniae* isolates, the information should be reported promptly to public health officials. Factors to consider in the development of a treatment policy include:

- The antimicrobial agent chosen should be affordable.
- The antimicrobial agent chosen should be available locally (or be 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. Information on pneumococcal antimicrobial resistance, together with data on the major pneumococcal serotypes responsible for disease, may become increasingly valuable to public health officials in the future, as new formulations of multivalent pneumococcal conjugate vaccines become available for global use.¹⁸

¹⁸ The Vaccine Alliance maintains information on these sorts of activities on its website: www.vaccinealliance.org.