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Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera

Centers for Disease Control and Prevention
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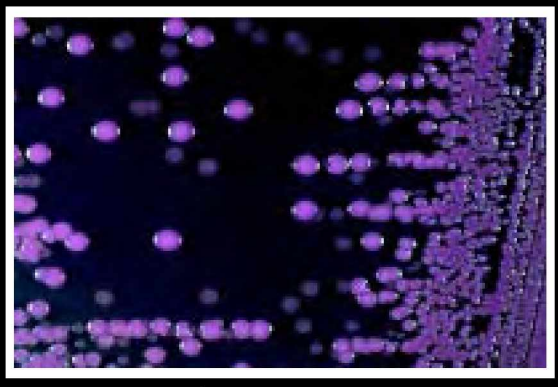

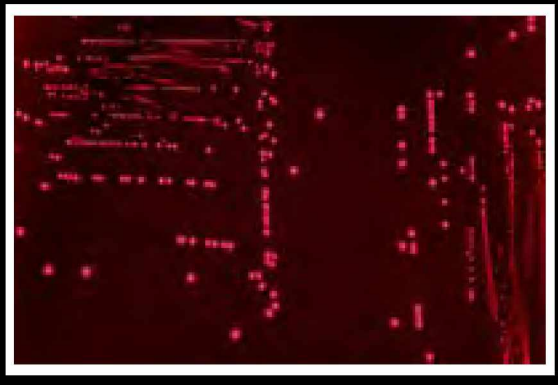

CDC/NCID
CENTERS FOR DISEASE CONTROL AND PREVENTION NATIONAL CENTER FOR INFECTIOUS DISEASES

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This manual was prepared by the National Center for Infectious Diseases (NCID), Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, in cooperation with the World Health Organization Regional Office for Africa, (WHO/AFRO) Harare, Zimbabwe.

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Cover: From top, *Escherichia coli* O157:H7 on sorbitol MacConkey agar, *Vibrio cholerae* O1 on TCBS agar, and *Shigella flexneri* on xylose lysine desoxycholate agar.

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This manual was developed as a result of a joint effort by the World Health Organization Regional Office for Africa, WHO Headquarters, and the Centers for Disease Control and Prevention as part of the activities of the WHO Global Task Force on Cholera Control. In particular, the staff of the project for Improving Preparedness and Response to Cholera and Other Epidemic Diarrhoeal Diseases in Southern Africa have worked closely with many laboratorians and epidemiologists in southern Africa to develop an integrated approach to the laboratory diagnosis of cholera and dysentery upon which this manual is based.

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Introduction

Cholera and dysentery have afflicted humankind for centuries. The epidemics they cause have affected the outcome of wars and the fates of countries. In much of the world, epidemic cholera and dysentery are uncommon, but during the past decade these two diseases have re-emerged as causes of significant morbidity and mortality in many developing countries.

Only a few pathogens cause epidemic diarrhea, although there are many that cause sporadic diarrhea. In developing countries, two etiologic agents are responsible for most epidemic diarrhea: toxigenic *Vibrio cholerae* serogroup O1, which causes watery diarrhea, and *Shigella dysenteriae* serotype 1, which causes bloody diarrhea. Recently, two additional organisms have emerged to cause epidemic diarrhea, *Vibrio cholerae* serogroup O139, which causes watery diarrhea, and *Escherichia coli* serotype O157:H7, which causes bloody diarrhea. The latter is a common agent of diarrhea only in developed countries.

This manual focuses on the epidemiology of these four organisms and the laboratory methods used to identify them and to test their susceptibility to antimicrobial agents in the epidemic setting. The laboratory techniques and study methodology described provide accurate and useful information for the control of epidemics using a minimum of resources. The manual emphasizes coordination of the activities of the microbiologist and the epidemiologist in order to obtain information that can be generalized to develop effective treatment policies for these epidemic diarrheal diseases. It encourages focused studies to determine the organisms causing epidemics and their antimicrobial susceptibility patterns rather than relying on random information that may not accurately represent a situation.

Often the countries that face the challenge of responding to an epidemic are those with the least resources. Therefore, the microbiology laboratory must use its resources wisely in order to have the greatest impact on reducing morbidity and mortality during an epidemic. There may be several ways to reach the end result of identifying the organism causing the outbreak or the epidemic. Often, however, a small added benefit requires a much larger expenditure of materials and time. In this manual this problem is addressed specifically. The procedures described are not new; most have been used for a number of years. However, these procedures were specifically selected for testing specimens from outbreaks rather than for general use in a clinical microbiology laboratory. The selected procedures minimize the materials needed by the laboratory while deriving the most useful information.

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Chapter 1
The Public Health Role of Clinical Laboratories

A. Epidemic Diarrhea

The two most common types of epidemic diarrhea in developing countries are watery diarrhea caused by *Vibrio cholerae* serogroup O1 and bloody diarrhea caused by *Shigella dysenteriae* serotype 1 (Sd1). This chapter presents an overview of these and other organisms that cause epidemic dysentery and cholera. Knowing the epidemiology and clinical presentation of these organisms will aid in understanding the procedures presented in the following chapters.

1. Epidemic cholera

Cholera is a secretory diarrheal disease caused by enterotoxin-producing strains of *V. cholerae*. Although over 150 serogroups of *V. cholerae* have been identified, for decades toxigenic *V. cholerae* serogroup O1 was the only known cause of epidemic cholera. After a large epidemic in Asia in 1992 and 1993, it became clear that toxigenic *V. cholerae* serogroup O139 also could cause epidemics very similar to those caused by *V. cholerae* O1. According to World Health Organization (WHO) guidelines, both *V. cholerae* O1 and O139 are now recognized causes of cholera and should be reported the same way. Isolates of non-O1 and non-O139 *V. cholerae* can cause illness, but they do not pose the public health threat of the O1 and O139 serogroups.

Additional details on the epidemiology, historical background, clinical manifestations and treatment of cholera are presented in Chapter 5.

2. Epidemic dysentery

Dysentery, defined as diarrhea with visible blood, can be caused by many different organisms, including *Shigella* spp., enterohemorrhagic *Escherichia coli* serotype O157:H7, *Campylobacter jejuni*, enteroinvasive *E. coli*, *Salmonella* spp. and, infrequently, *Entamoeba histolytica*. Of these organisms, the only ones known to cause large epidemics are *Shigella dysenteriae* serotype 1 (Sd1), and much less frequently, *E. coli* O157:H7. Additional details on the epidemiology, historical background, clinical manifestations and treatment of Sd1 infection are presented in Chapter 3.

Although uncommon, a species of parasitic ameba, *E. histolytica,* deserves mention. This organism is an occasional cause of dysentery, especially in young adults, but does not cause epidemic disease. Asymptomatic infection with *E. histolytica*, however, is frequent in developing countries, being present in up to 10% of healthy persons. Examination of specimens should be done by a trained microscopist since the organism must be differentiated from nonpathogenic amebae and from white blood cells, which are often mistaken for amebic

trophozoites. In some epidemics of dysentery due to Sd1, *E. histolytica* was also identified and initially thought to be the cause. Because of this incorrect diagnosis, persons with dysentery were treated with anti-amebic drugs, resulting in continued transmission of Sd1 and excess preventable mortality. Finding *E. histolytica* in a bloody stool during an epidemic of dysentery does not indicate that it is the cause of the epidemic, or even that it is the cause of dysentery in an individual patient.

E. coli O157:H7 has caused at least one large outbreak of dysentery in southern Africa. It is suspected to have caused additional outbreaks, but these were not confirmed by microbiologic culture. *E. coli* O157:H7 is included in this manual so that laboratory workers will be familiar with the organism and will be able to identify it if necessary. It may return in the future to cause additional epidemics; laboratories must be prepared to identify it.

Additional details on the epidemiology, historical background, clinical manifestations and treatment of *E. coli* O157:H7 are presented in Chapter 7.

B. Public Health Role of the Laboratory

Clinical laboratories play an especially crucial public health role during epidemics. A laboratory may be the only one in a country that can quickly provide the information needed to develop appropriate treatment policy during an epidemic. In countries with scarce resources, the role of the laboratory is to use those resources to provide the best information for developing treatment policy, rather than to focus on the diagnosis of individual patients. During an epidemic of cholera or dysentery, the laboratory has four primary roles:

- Initial identification of the organism causing the epidemic
- Initial determination of the antimicrobial susceptibility patterns
- Monitoring for changes in antimicrobial susceptibility patterns
- Defining the duration and geographic extent of the epidemic

The World Health Organization (WHO) recommends that countries at risk for epidemics establish an epidemic control committee. Since the laboratory plays an important role in the identification and control of epidemics, a microbiologist should be a part of the epidemic control committee.

1. Initial identification of the organism causing the epidemic

Preparation/laboratory network

In countries at risk for epidemics of dysentery or cholera, the laboratory's first role is to be prepared for an epidemic. This means having the supplies (or ready access to supplies) necessary to identify *V. cholerae* O1/O139 and *Shigella.* Annexes A and B in this manual list laboratory supplies required for isolation, identification, and antimicrobial susceptibility testing. A country-wide public health laboratory network should be established (see Annex C). All countries should have at least one national or central laboratory capable of

identifying *V. cholerae* O1/O139 and *Shigella*, determining antimicrobial susceptibility, and sending isolates to an international reference laboratory (Annex D).

To maintain a laboratory's capability to determine the antimicrobial susceptibility patterns of bacterial pathogens accurately and reproducibly, investments must be made in the infrastructure of the laboratory. These investments include a steady supply of the material resources needed to perform appropriate testing; a trained staff with expertise to conduct the laboratory tests and sufficient time, materials, and supplies to maintain this expertise; and quality control of the staff, supplies, and reagents. Because antimicrobial susceptibility testing is so resource intensive, WHO recommends that this testing be carried out at only one or two laboratories in the country. Peripheral laboratories may perform initial isolation of *Vibrio* spp. or *Shigella* spp., and then refer isolates to the central or national reference laboratory for final confirmation and determination of antimicrobial susceptibility. Peripheral laboratories may also be the sites of focused studies to determine etiologic agents causing an outbreak. First-level laboratories should be supplied with transport medium and the means of sending the specimens to the next level laboratory or to the central laboratory.

Diagnosing epidemics

During a suspected epidemic, the laboratory will determine the organism causing the epidemic and its antimicrobial susceptibilities. An epidemic may be suspected on clinical grounds: for instance, a surveillance system based on clinical diagnosis may note an increase in the number of cases of diarrhea. The laboratory should become involved as soon as possible to identify the causative agent. This underscores the need for good communication between the laboratory, the epidemiologists, and clinicians and other health care workers.

At times, the laboratory may be the first to suspect an epidemic. Laboratory workers may note an increase in the number of stool specimens submitted, an increase in the proportion of stool specimens with blood, or the appearance of a new organism. When a laboratory worker suspects an outbreak or epidemic, he or she should contact the appropriate clinicians and public health authorities as soon as possible.

Once the organism causing the epidemic is identified, it is not necessary to examine a large number of stool specimens. Patients can be treated on the basis of their syndrome.

Diagnosing dysentery epidemics

If an epidemic of dysentery is suspected, the most common cause in most parts of the world is Sd1. During an outbreak or epidemic, Sd1 is likely to be isolated much more frequently than the other organisms that cause dysentery. Therefore, a laboratory should conserve its resources and, according to WHO guidelines, once Sd1 has been confirmed as the cause of an epidemic, patients presenting with dysentery should initially be treated as if they are infected with Sd1. There is no

need for the laboratory to examine the stools of all patients. Rather, it is better to take specimens from a small number of patients during an outbreak or to conduct periodic surveillance for organisms causing dysentery (see below).

If Sd1 is not isolated during a suspected outbreak, the laboratory should test for *E. coli* O157:H7. If neither of these organisms is isolated, arrangements should be made to send specimens to a reference laboratory.

Besides Sd1 and *E. coli* O157:H7, a number of organisms contribute in various proportions to the burden of dysentery in a country. The predominant causes of dysentery will vary by geographic location and time of year. Seasonal peaks occur and may reflect changes in the proportions of the various causative organisms. Laboratories should conduct periodic surveys of the organisms causing dysentery in order to monitor antimicrobial susceptibility patterns and to help clinicians and public health authorities develop rational guidelines for empiric treatment. Procedures for conducting such surveys are described in Annex E.

Diagnosing cholera epidemics

If an epidemic of cholera is suspected, the most common cause is *V. cholerae* O1. If *V. cholerae* O1 is not isolated, the laboratory should test for *V. cholerae* O139. If neither of these organisms is isolated, arrangements should be made to send stool specimens to a reference laboratory.

Infection with *V. cholerae* O139 should be handled and reported in the same manner as that caused by *V. cholerae* O1. The associated diarrheal illness should be called cholera and should be reported as a case of cholera to the appropriate public health authorities.

2. Determining antimicrobial susceptibility patterns of epidemic organisms

Antimicrobial susceptibilities should be determined for the first 30 to 50 isolates identified by the laboratory at the beginning of an epidemic. That number will provide sufficient information to develop antimicrobial treatment policy for the organism. After that, the laboratory should conduct periodic surveys to detect any changes in antimicrobial susceptibility patterns (see Annex E).

The laboratory should not routinely test antimicrobial agents that are not available in the country or antimicrobial agents that are not recommended by WHO as efficacious in the treatment of cholera or dysentery (see Chapters 3 and 5). In addition, if all isolates are resistant to a particular antimicrobial agent during the first round of testing (for example, Sd1 resistance to ampicillin or trimethoprim-sulfamethoxazole), it is probably not useful to test against those agents during future surveys.

Once the organisms are isolated and the antimicrobial susceptibility patterns determined, these results should be transmitted as quickly as possible to the national epidemiologist and to other public health officials. They can then be used to make rational choices for antimicrobial treatment policy.

It is useful to send 10 to 20 of the initial isolates to an international reference laboratory for confirmation of the identification and antimicrobial susceptibility pattern (Annex D).

3. Monitoring for changes in antimicrobial susceptibility

As the epidemic progresses, periodic surveys of 30 to 50 isolates of the epidemic organism should be carried out to detect any changes in the antimicrobial susceptibility pattern of the organism causing the epidemic. These should be conducted every 2 to 6 months, depending on conditions and resources. Any changes should be reported to the national epidemiologist and to other public health officials to modify the antimicrobial treatment policy. If any major changes are noted, it is useful to send isolates to an international reference laboratory for confirmation (Annex D).

4. Defining the duration of the epidemic

The laboratory can help define the end of the epidemic, especially with cholera epidemics. In the course of an epidemic, the number of cases may decrease for several reasons: seasonal variation, transition to an endemic state, or disappearance of cholera from an area. Cholera may nearly disappear in cool seasons, only to reappear in the summer months. The laboratory can assist in determining if the epidemic has actually ended by periodically analyzing stool specimens from patients with acute watery diarrhea. In order for an area to be declared cholera-free by WHO, twice the incubation period (a total of 10 days) must pass without evidence of *V. cholerae* O1/O139. However, because of seasonal variation, surveillance should be maintained for at least 12 months.

Similarly, seasonal variation is seen with epidemic dysentery. The laboratory can periodically analyze stool specimens from patients with dysentery to see if Sd1 is still present in a particular area.

5. Other duties of the laboratory during an epidemic

In addition to the major duties outlined above, the laboratory can support other activities related to the epidemic.

Epidemiologic studies

At times, the laboratory may be asked to provide laboratory support to an epidemiologic study. By combining epidemiologic and laboratory data, studies that examine modes of transmission or risk factors for illness can be more specific and provide better information for the control of the epidemic.

Defining the magnitude of the epidemic and improving surveillance data

Cultures taken from a series of patients that meet the clinical case definition used during an epidemic can determine the predictive value of the definition. Such studies will confirm the accuracy of the case definition used for surveillance purposes and can provide a more accurate picture of the magnitude of the epidemic.

In addition, the laboratory may be called upon to support other activities such as environmental monitoring for *V. cholerae* O1/O139. These requests place additional demands on the resources of the laboratory. Therefore, the microbiologist must be part of the decision-making process to determine whether the laboratory has the capacity to support the particular request and whether it is an appropriate use of the laboratory resources.

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Chapter 2
Collection and Transport of Fecal Specimens

Fecal specimens should be collected in the early stages of any enteric illness, when pathogens are usually present in the stool in highest numbers, and before antibiotic therapy has been started (Table 2-1).

Table 2-1. Collection and transport of specimens for laboratory diagnosis

When to collect	When the patient is having diarrhea, as soon after onset of illness as possible (preferably within 4 days of onset) and before antimicrobial treatment is started.
How much to collect	Rectal swab or swab of fresh stool in transport medium.
Transport medium	Cary-Blair or other suitable transport medium (NOT buffered glycerol saline for *V. cholerae*).
Storage after collection	Refrigerate at 4°C if the specimens will be received by the laboratory within 48 hours, or freeze at -70°C. Fecal specimens from patients with suspected cholera can be transported at ambient temperature and held for longer times if necessary; however, refrigeration is preferred.
Transportation	Seal tubes/containers to prevent leakage; place in waterproof container to protect from wet or dry ice. Ship in insulated box with ice packs, wet ice, or dry ice by overnight delivery.

Stool specimens or rectal swabs should be collected from 10-20 persons who meet the following criteria:

- Currently have watery diarrhea (cholera) or bloody diarrhea (dysentery)
- Had onset of illness less than 4 days before sampling
- Have not received antimicrobial treatment for the diarrheal illness

A. Collection of Stool

Collect stools from patients in clean containers without disinfectant or detergent residue and with tight-fitting, leak-proof lids. Specimens should not be collected from bedpans, as they may contain residual disinfectant or other contaminants. Unpreserved stool should be refrigerated if possible and processed within a maximum of 2 hours after collection. Specimens that cannot be cultured within 2 hours of collection should be placed in transport medium and refrigerated immediately.

1. Placing stool in transport medium

A small amount of stool can be collected by inserting a sterile cotton- or polyester-tipped swab into the stool and rotating it. If mucus and shreds of intestinal epithelium are present, these should be sampled with the swab. Immediately insert the swab into transport medium. (The transport medium should have been chilled for 1 to 2 hours, if possible.) The swab should be pushed completely to the bottom of the tube of transport medium and the top portion of the stick touching the fingers should be broken off and discarded. Replace the screw cap and tighten firmly. Place the tube in a refrigerator or cold box.

2. Collection of rectal swabs

Rectal swabs may be collected as follows: moisten the swab in sterile transport medium, insert through the rectal sphincter 2 to 3 cm (1 to 1.5 inches) and rotate, withdraw and examine to make sure there is some fecal material visible on the swab. Immediately insert the swab into cold transport medium as described in above paragraph. Place the tube in a refrigerator or cold box.

The number of swabs needed will depend on the number of plates to be inoculated. In general, if specimens will be examined for more than one pathogen, at least two stool swabs or rectal swabs should be collected per patient, and both swabs should be inserted into the same tube of transport medium.

Figure 2-1. Cary-Blair semisolid transport medium

3. Transport media

Cary-Blair transport medium

Cary-Blair transport medium can be used to transport many enteric pathogens, including *Shigella*, *Vibrio cholerae*, and *Escherichia coli* O157:H7 (Figure 2-1). Cary-Blair's semisolid consistency provides for ease of transport, and the prepared medium can be stored after preparation at room temperature for up to 1 year. Because of its high pH (8.4), it is the medium of choice for transport and preservation of *V. cholerae*.

Preparation and quality control of Cary-Blair

Prepare according to manufacturer's instructions. [Note: There are several commercially available dehydrated formulations of Cary-Blair. Some require the addition of calcium chloride and some do not. Cary-Blair can also be prepared from individual ingredients.] When Cary-Blair is prepared, it should be dispensed into containers in sufficient volume so that swabs will be covered by at least 4 cm of medium. For example, 5- to 6-ml amounts may be dispensed into 13 x 100-mm screw cap tubes. With the caps loosened, sterilize by steaming (do not autoclave) at 100°C for 15 minutes. Tighten the caps after sterilization. Cary-Blair is quite stable if stored in tightly sealed containers in a cool dark place so that the medium does not dry out. Cary-Blair may be used for up to 1 year as long as there is no loss of volume, contamination, or color change.

Other transport media

Other transport media that are similar to Cary-Blair are Amies' and Stuart's transport media. Both of these are acceptable for *Shigella* and *E. coli* O157:H7, but they are inferior to Cary-Blair for transport of *V. cholerae*.

Alkaline peptone water (APW) may be used to transport *V. cholerae*, but this medium is inferior to Cary-Blair and should be used only when the latter medium is not available. APW should not be used if subculture will be delayed more than 6 hours from the time of collection because other organisms will overgrow vibrios after 6 hours.

Buffered glycerol saline (BGS), a transport medium that is used for *Shigella*, is unsuitable for transport of *V. cholerae*. Additional disadvantages of BGS are that it can be used for only 1 month after it is made and, being a liquid medium, is more likely to leak or spill during transport.

4. Storage of specimens in transport medium

If transport medium has been stored at room temperature, it should be chilled, if possible, for 1 to 2 hours before use. Specimens preserved in transport medium should be refrigerated until processed. If specimens will be kept more than 2 to 3 days before being cultured, it is preferable to freeze them immediately at -70°C. It may be possible to recover pathogens from refrigerated specimens up to 7 days after collection; however, the yield decreases after the first 1 or 2 days. Prompt

plating, refrigeration, or freezing of specimens in Cary-Blair is particularly important for isolation of *Shigella*, which is more fragile than other enteric organisms. Fecal specimens in transport medium collected from patients with cholera need not be refrigerated unless they are likely to be exposed to elevated temperatures (>40°C).

5. Unpreserved specimens

When transport medium is not available, one option for suspect *V. cholerae* specimens is to soak a piece of filter paper, gauze, or cotton in liquid stool and place it into a plastic bag. The bag must be tightly sealed so that the specimen will remain moist and not dry out. Adding several drops of sterile saline to the bag may help prevent drying of the specimen. Refrigeration during transport is desirable but not necessary. This method is not suitable for transport of *Shigella* or *E. coli* O157:H7 specimens and is less effective than transport medium for preserving *V. cholerae* organisms.

B. Preparing Specimens for Shipment

Specimen tubes should be clearly labeled with the specimen number, and if possible, the patient's name and date of collection. Write the numbers on the frosted portion of the specimen tube, using an indelible marker pen. If there is no frosted area, write the information on a piece of first-aid tape and fix this firmly on the specimen container. Patient information should be recorded on a data sheet; one copy should be sent with the specimens and another kept by the sender. A sample data sheet is provided in Annex F.

If a package is to be shipped by air, refer to packaging regulations presented in the publication, *Dangerous Goods Regulations (DGR). International Air Transport Association (IATA).* These regulations are summarized in Chapter 13, "Packing and Shipping of Clinical Specimens and Etiologic Agents." Even if the package will be shipped by other means, these regulations are excellent guidelines for packing all infectious or potentially infectious materials.

1. Refrigerated specimens

Refrigerated specimens should be transported to the laboratory in an insulated box with frozen refrigerant packs or ice. If wet ice is used, place the tubes or containers in waterproof containers such as plastic bags that can be tightly sealed to protect the specimens from the water formed by melting ice.

2. Frozen specimens

Frozen specimens should be transported on dry ice. The following precautions should be observed:

- Place tubes in containers or wrap them in paper to protect them from dry ice. Direct contact with dry ice can crack glass tubes.
- If the specimens are not in leakproof containers, protect them from exposure

to carbon dioxide by sealing the screwcaps with tape or plastic film or by sealing the tubes in a plastic bag. Carbon dioxide will lower the pH of the transport medium and adversely affect the survival of organisms in the specimen.

- Ensure that the cool box is at least one-third full of dry ice. If the specimens are sent by air and more than 2 kg of dry ice is used, special arrangements may be necessary with the airlines. Airlines accept packages with less than 2 kg of dry ice.
- Address the package clearly, including the name and telephone number of the receiving laboratory. Write in large letters: EMERGENCY MEDICAL SPECIMENS; CALL ADDRESSEE ON ARRIVAL; HOLD REFRIGERATED (or "FROZEN" if applicable). Be sure that all applicable labels and forms, such as those required by IATA, are correctly fixed to the outside of the package.

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Chapter 3
Epidemiology of Dysentery Caused by *Shigella*

Epidemic dysentery in developing countries is usually caused by *Shigella dysenteriae* serotype 1 (Sd1). Sd1 is an unusually virulent enteric pathogen that causes endemic or epidemic dysentery with high death rates. It is the most common cause of large-scale, regional outbreaks of dysentery. In recent years, Sd1 has caused epidemic dysentery in Central America, south Asia and central and southern Africa. An epidemic in Central America from 1969 to 1973 was responsible for more than 500,000 cases and 20,000 deaths. The epidemic in central and southern Africa began in 1979, initially affecting eastern Zaire, Rwanda and Burundi. In the early 1990s, epidemic dysentery moved southward, affecting first Zambia, then Malawi, Mozambique, Zimbabwe and southern Africa. A large rise in the number of cases associated with refugee camps was seen in central Africa in 1994.

A. Epidemiology of *Shigella*

The genus *Shigella* is divided into four species: *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*. Each of these species, with the exception of *S. sonnei*, has several serotypes (Table 3-1). In general, *S. sonnei* is more common in developed countries and *S. flexneri* and *S. dysenteriae* are more frequent in developing countries. The proportions of each species vary from country to country. Sd1 differs from the other *Shigella* species in several ways:

- Only Sd1 causes large and prolonged epidemics of dysentery.
- Antimicrobial resistance develops more quickly and occurs more frequently in Sd1 than in other *Shigella* species.
- Infection with Sd1 causes more severe, more prolonged, and more frequently fatal illness than does infection with other *Shigella* species.

Table 3-1. Species and serogroups of *Shigella*

Species	Serogroup designation	Serotypes
S. dysenteriae	Serogroup A	1-13[a,b]
S. flexneri	Serogroup B	1-6
S. boydii	Serogroup C	1-18[b]
S. sonnei	Serogroup D	1

[a] *S. dysenteriae* 1 has special significance since it is unusually virulent and causes endemic or epidemic dysentery with high death rates. Monovalent antiserum (absorbed) is required to identify *S. dysenteriae* 1.

[b] Additional provisional serotypes have been reported but antisera to these new serotypes were not commercially available at the time this manual was printed.

B. Clinical Manifestations

The hallmark of infection with Sd1 is diarrhea with blood (dysentery). *Shigella* causes dysentery by invading and destroying cells that line the large intestine, leading to mucosal ulceration, a hemorrhagic inflammatory exudate and bloody diarrhea. Apart from bloody stools, patients with dysentery often have fever, abdominal cramps and rectal pain. However, the clinical response to infection spans a wide range, from mild to severe diarrhea with or without blood. In almost half of cases, *Shigella* causes acute nonbloody diarrheas that cannot be distinguished clinically from diarrhea caused by other enteric pathogens. Severity of symptoms appears to be dose related. Asymptomatic infections may occur, but not to the extent that they do in *Vibrio cholerae* O1 infections. A chronic carrier state does not occur, although the organisms may be excreted for several weeks. Sd1 infections are most often severe or fatal in young children and in the elderly and malnourished. Although most patients recover without complications within 7 days, persistent diarrhea may occasionally occur.

Infection with Sd1 can be complicated by seizures, sepsis, rectal prolapse, or toxic megacolon. A more frequent complication is the hemolytic-uremic syndrome (HUS), which is characterized by the classic triad of hemolytic anemia, thrombocytopenia and renal failure. HUS may be mild with rapid recovery, or severe, leading to kidney failure and death.

C. Treatment

The mainstay of treatment for Sd1 infection is appropriate antimicrobial therapy, which lessens the risk of serious complications and death. Other supportive measures should be used as well.

The following antimicrobial agents are currently recommended by WHO for treatment of Sd1 infections:

- ampicillin
- trimethoprim-sulfamethoxazole
- nalidixic acid
- pivmecillinam
- ciprofloxacin
- norfloxacin
- enoxacin

The selection of antimicrobial treatment should be based on recent susceptibility testing of Sd1 strains from the area or from nearby areas if Sd1 is new to the area (see Annex E). For developing a treatment policy, the antimicrobial agent chosen should be effective against at least 80% of local Sd1 strains, be given by mouth, be affordable, and be available locally or able to be obtained quickly. Unfortunately, resistance of Sd1 to ampicillin and trimethoprim-sulfamethoxazole has become widespread. Nalidixic acid, formerly used as a "backup" drug to treat resistant shigellosis, is now the drug of choice in most

areas, but resistance to it has appeared in many places. Pivmecillinam (amdinocillin pivoxil) is still effective for most strains of Sd1 but may not be readily available. Fluoroquinolones (i.e., ciprofloxacin, norfloxacin, enoxacin) should be considered only if Sd1 isolates are resistant to nalidixic acid. Fluoroquinolones are often costly and may not be readily available.

Currently, Sd1 strains are often resistant to ampicillin, trimethoprim-sulfamethoxazole, metronidazole, streptomycin, tetracycline, chloramphenicol, and sulfonamides. In addition, although Sd1 may be susceptible to some antimicrobial agents in vitro, the drug may have no documented efficacy in vivo. Examples of such agents are nitrofurans (e.g., nitrofurantoin, furazolidone), aminoglycosides (e.g., gentamicin, kanamycin), first- and second-generation cephalosporins (e.g., cephalexin, cefamandol), and amoxicillin.

Reference

World Health Organization. Guidelines for the control of epidemics due to *Shigella dysenteriae* 1. Geneva: WHO; 1995. Publication no. WHO/CDR/95.4.

Chapter 4
Isolation and Identification of *Shigella*

Isolation and identification of *Shigella* can be greatly enhanced when optimal laboratory media and techniques are employed. The methods presented here are intended to be economical and to offer laboratorians some flexibility in choice of protocol and media. Laboratories that do not have sufficient resources to adopt the methods described in this chapter should consider sending specimens or isolates to other laboratory facilities that routinely perform these procedures.

A. Isolation Methods

Figure 4-1 outlines the procedure for isolation of *Shigella* from fecal specimens. Refer to Annex B for a list of supplies necessary for laboratory identification of *Shigella*.

For optimal isolation of *Shigella*, two different selective media should be used: a general purpose plating medium of low selectivity, such as MacConkey agar (MAC), and a more selective agar medium, such as xylose lysine desoxycholate (XLD) agar. Desoxycholate citrate agar (DCA) and Hektoen enteric (HE) agar are suitable alternatives to XLD agar as media of moderate to high selectivity. **Do not use SS agar as it frequently inhibits the growth of *S. dysenteriae* serotype 1.**

When selective or differential media are incorrectly prepared, the reactions of organisms on those media can be affected. Therefore, it would be helpful to refer to Section D, "Media for isolation and identification of *Shigella*," for a discussion of these media, their preparation, and appropriate quality control strains.

There is no enrichment medium for *Shigella* that consistently provides a greater recovery rate than use of direct plating alone.

1. Inoculation of selective agar

Fecal specimens should be plated as soon as possible after arrival in the laboratory. Selective media may be inoculated with a single drop of liquid stool or fecal suspension. Alternatively, a rectal swab or a fecal swab may be used. If a swab is used to inoculate selective media, an area approximately 2.5 cm (1 inch) in diameter is seeded on the agar plates, after which the plates are streaked for isolation (Figure 4-2). Media of high selectivity such as XLD require more overlapping when streaking than media of low selectivity. When inoculating specimens to a plate for isolation, it is important to use the entire plate to increase the chances of obtaining well-isolated colonies. Incubate the plates for 18 to 24 hours at 35° to 37°C.

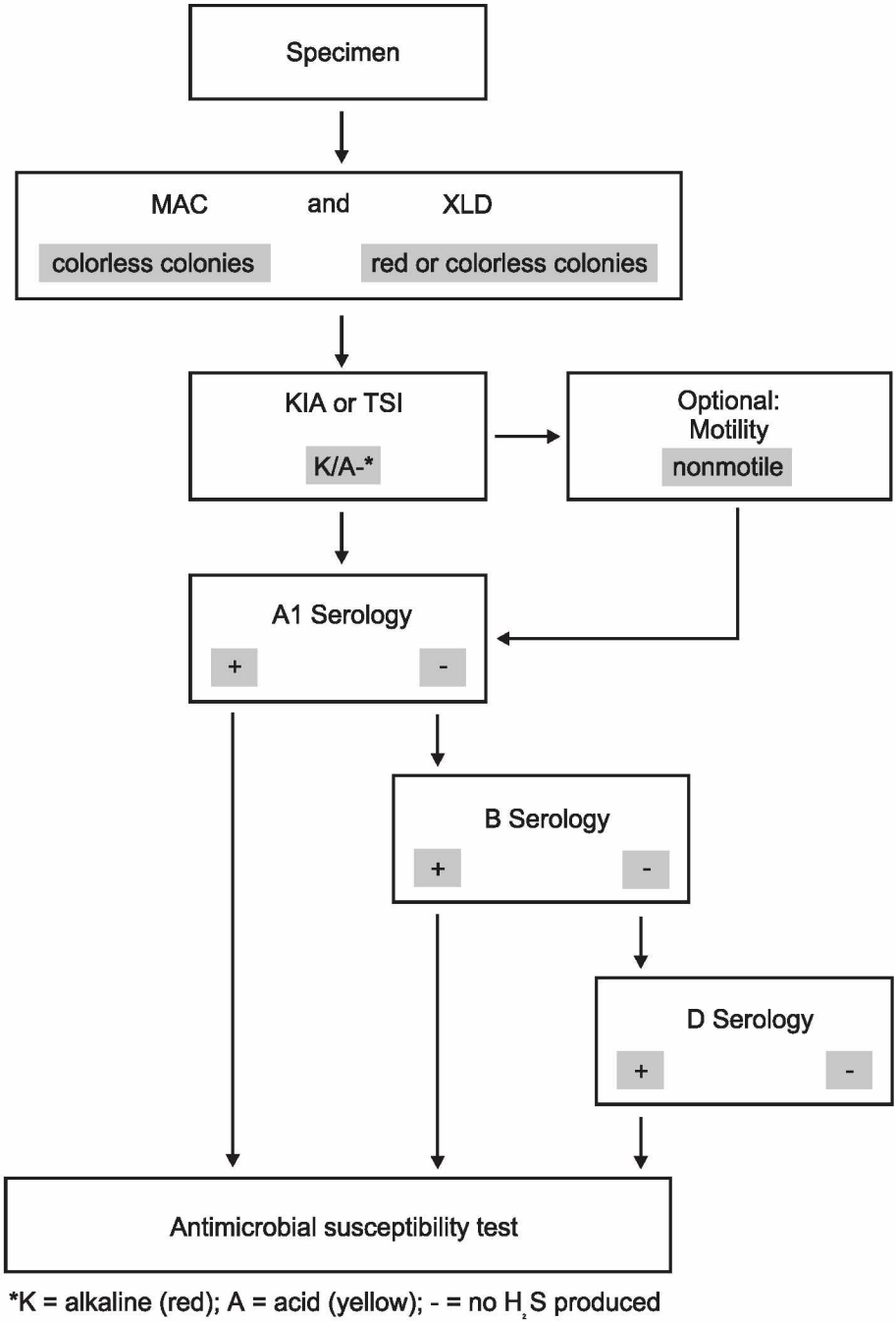

Figure 4-1. Procedure for recovery of *Shigella* from fecal specimens

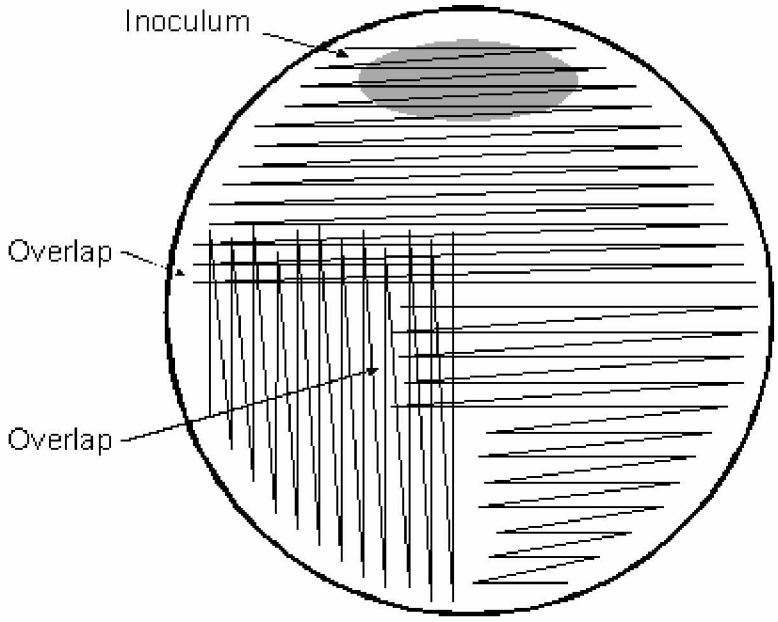

Figure 4-2. Method of streaking plating medium for isolation of *Shigella*

2. Isolation of suspected *Shigella*

After incubation, record the amount and type of growth (e.g., lactose-fermenting or lactose-nonfermenting) on each isolation medium for each patient specimen (a sample worksheet is presented in Figure 4-3). Colonies of *Shigella* on MAC appear as convex, colorless colonies about 2 to 3 mm in diameter. *S. dysenteriae* 1 colonies may be smaller (Table 4-1). *Shigella* colonies on XLD agar are transparent pink or red smooth colonies 1 to 2 mm in diameter. *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike other *Shigella* species. Figures 4-4 to 4-7 show the typical appearance of *Shigella* on XLD and MAC. Select suspect colonies from the MAC and XLD plates and inoculate to appropriate screening media such as Kligler iron agar (KIA) or triple sugar iron agar (TSI).

Table 4-1. Appearance of *Shigella* colonies on selective plating media

Selective agar medium	Color of colonies	Size of colonies
MAC	Colorless	2-3 mm[a,b]
XLD	Red or colorless	1-2 mm[a,c]
DCA	Colorless	2-3 mm[a]
HE	Green	2-3 mm[a]

[a] *S. dysenteriae* 1 colonies may be smaller.
[b] See Section D for discussion of different formulations of commercial dehydrated MacConkey agar and how selectivity is affected for isolation of *Shigella*.
[c] *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike other *Shigella* species.

B. Biochemical Screening Tests

Identification of *Shigella* spp. involves both biochemical and serologic testing. The use of biochemical screening media is usually advisable to avoid wasting antisera. Most laboratories will find KIA (or TSI) to be the single most helpful medium for screening suspected *Shigella* isolates. If an additional test is desired, motility medium can be used to screen isolates before doing serologic testing. Section D in this chapter further describes these media.

1. Kligler iron agar and triple sugar iron agar

To obtain true reactions in KIA or TSI or other biochemical tests, it is necessary to inoculate with a pure culture. Carefully select at least one of each type of well-isolated colony on each plate. Using an inoculating needle, lightly touch only the very center of the colony. Do not take the whole colony or go through the colony and touch the surface of the plate. This is to avoid picking up contaminants that may be on the surface of the agar. If there is doubt that a particular colony is sufficiently isolated from surrounding colonies, purify the suspicious colony by streaking on another agar plate, after which the KIA or TSI slant may be inoculated.

KIA and TSI are inoculated by stabbing the butt and streaking the surface of the slant. After incubation for 18 to 24 hours at 35° to 37°C, the slants are observed for reactions typical of *Shigella*. When incubating most biochemicals, caps should be loosened before placement in the incubator. This is particularly important for KIA and TSI. If the caps are too tight and anaerobic conditions exist, the characteristic reactions of *Shigella* spp. may not occur and a misleading result could be exhibited. It is also important that KIA and TSI be prepared so that the tubes have a deep butt and a long slant (see Section D).

Shigella characteristically produces an alkaline (red) slant and an acid (yellow) butt, little or no gas, and no H_2S (Table 4-2; Figure 4-8). A few strains of *S. flexneri* serotype 6 and very rare strains of *S. boydii* produce gas in KIA or TSI.

Shigella Worksheet

SPECIMEN NUMBER	MEDIA	XYL/ LAC- [a]	XYL/ LAC+ [b]	COLONY	KIA/TSI	OPTIONAL				SLIDE SEROLOGY			IDENTIFICATION
						MOT [c]	UREA	LIA		A1	B	D	
	XLD			X1									
				X2									
				X3									
	MAC			M1									
				M2									
				M3									
	XLD			X1									
				X2									
				X3									
	MAC			M1									
				M2									
				M3									
	XLD			X1									
				X2									
				X3									
	MAC			M1									
				M2									
				M3									

[a]XYL/LAC - = Xylose or lactose negative colonies [b]XYL/LAC + = Xylose or lactose positive colonies [c]MOT = Motility

Figure 4-3. *Shigella* worksheet

Figure 4-4. *S. dysenteriae* 1 colonies on XLD

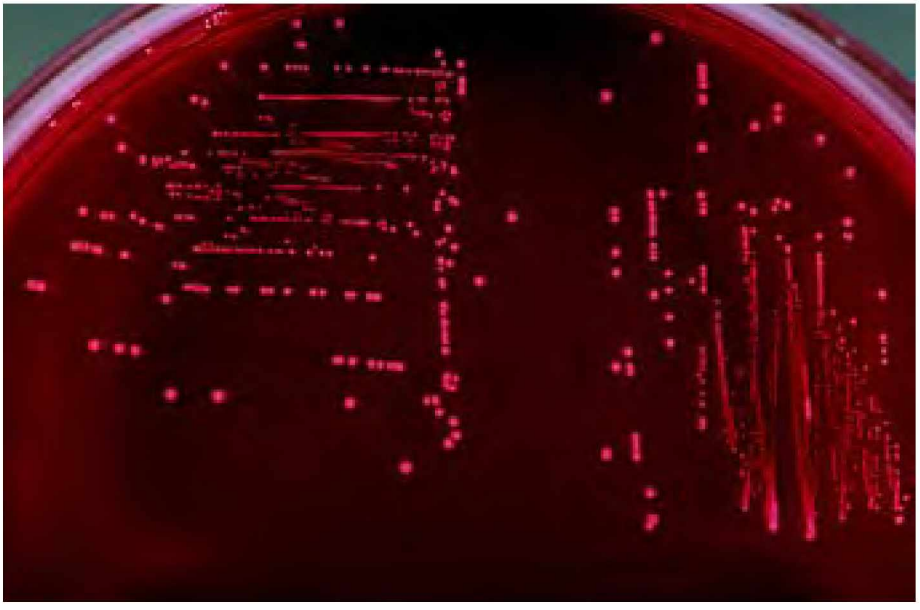

Figure 4-5. *S. flexneri* colonies on XLD

Figure 4-6. *S. flexneri* and *E. coli* colonies on XLD. *S. flexneri* colonies are colorless to red while the *E. coli* colonies are yellow.

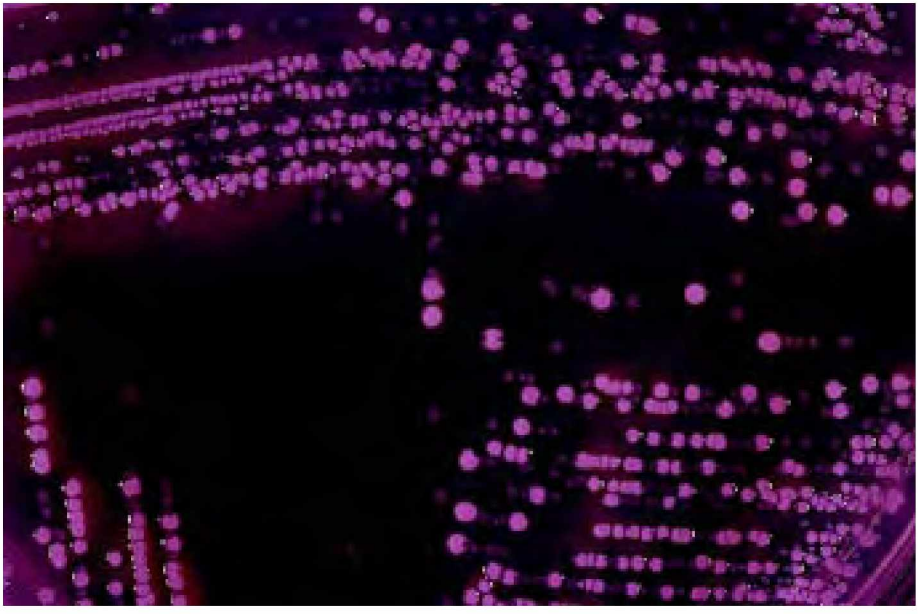

Figure 4-7. *S. flexneri* colonies appear colorless on MAC. *E. coli* colonies are pink to red.

Table 4-2. Reactions of *Shigella* in screening biochemicals

Screening medium	*Shigella* reaction
KIA	K/A, no gas produced (red slant/yellow butt)[a]
TSI	K/A, no gas produced (red slant/yellow butt)[a]
H_2S (on KIA or TSI)	Negative
Motility	Negative
Urea	Negative
Indole	Positive or negative
LIA	K/A (purple slant/yellow butt)[b]

[a] K = alkaline (red); A = acid (yellow); some strains of *S. flexneri* serotype 6 and *S. boydii* produce gas from glucose.

[b] K = alkaline (purple); A = acid (yellow); an alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated. An acid reaction (yellow) in the butt of the medium indicates that lysine was not decarboxylated.

Figure 4-8. Reaction typical of *Shigella* in KIA (alkaline slant and acid butt)

2. Motility agar

Motility agar should be inoculated with a straight inoculating needle, making a single stab about 1 to 2 cm down into the medium. Motility agar may be inoculated with growth from a KIA or TSI that shows a reaction typical of *Shigella*. Alternately, motility agar can be inoculated at the same time as the KIA or TSI slant by using the same inoculating needle without touching the colony again. The motility agar should be inoculated first, after which, the KIA or TSI is inoculated by stabbing the butt first and then streaking the surface of the slant. Do not select a second colony to inoculate the KIA or TSI after the motility agar has been inoculated since it may represent a different organism.

Examine after overnight incubation at 35° to 37°C. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 4-9). Nonmotile organisms do not grow out from the line of inoculation. Motility reactions may be difficult for inexperienced laboratorians to read; therefore reactions should be compared with positive and negative control strains. *Shigella* spp. are always nonmotile (Table 4-2).

The surface of the motility agar should be dry when used. Moisture can cause a nonmotile organism to grow down the sides of the agar creating a haze of growth and appearing to be motile (see Section D).

Sulfide-indole-motility medium is a combination medium that is commercially available in dehydrated form (see Section D). It can be used in place of motility medium.

Figure 4-9. Motility medium showing a nonmotile organism in the left tube and a motile organism in the right tube

3. Additional biochemical screening tests

Other biochemical tests such as urea medium and lysine iron agar may be used for additional screening of isolates before testing with antisera. The value of these should be assessed before using them routinely (Table 4-2, Annex G). These media, their preparation, and suggested quality control strains are described in Section D.

Urea medium

Urea medium screens out urease-producing organisms such as *Klebsiella* and *Proteus*. Urea agar is inoculated heavily over the entire surface of the slant. Loosen caps before incubating overnight at 35° to 37°C. Urease positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color (Figure 4-10). Urease negative organisms do not change the color of the medium, which is a pale yellowish-pink. *Shigella* spp. are always urease negative (Table 4-2).

Lysine iron agar

Lysine iron agar (LIA) is helpful for screening out *Hafnia* spp. and certain *E. coli*, *Proteus*, and *Providencia* strains. LIA should be inoculated by stabbing the butt and streaking the slant. After incubation for 18 to 24 hours at 35° to 37°C, organisms that produce lysine decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the medium and also on the slant (Figure 4-11). H_2S production is indicated by a blackening of the medium. Organisms lacking lysine decarboxylase, produce an alkaline slant (purple) and an acid butt (yellow), no gas, and no H_2S. *Proteus* and *Providencia* spp. will often produce a red slant caused by deamination of the lysine. LIA must be prepared so that the tubes have a deep butt (see Section D).

Shigella spp. are lysine negative and characteristically produce an alkaline (purple) slant and an acid (yellow) butt, no gas, and no H_2S (Table 4-2).

C. Serologic Identification of *Shigella*

Serologic testing is needed for the identification of *Shigella* isolates. The genus *Shigella* is divided into four serogroups, each group consisting of a species that contains distinctive type antigens. The serogroups A, B, C, and D correspond to *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*, respectively. Three of the four, *S. dysenteriae*, *S. flexneri*, and *S. boydii,* are made up of a number of serotypes (see Chapter 3, Table 3-1).

Serologic identification is performed typically by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed, in some cases, by testing with monovalent antisera for specific serotype identification. Monovalent antiserum to *S. dysenteriae* 1 is required to identify this serotype, which is the most frequent cause of severe epidemic dysentery. Once one colony from a plate has been identified as *Shigella*, no further colonies from the same specimen need to be tested.

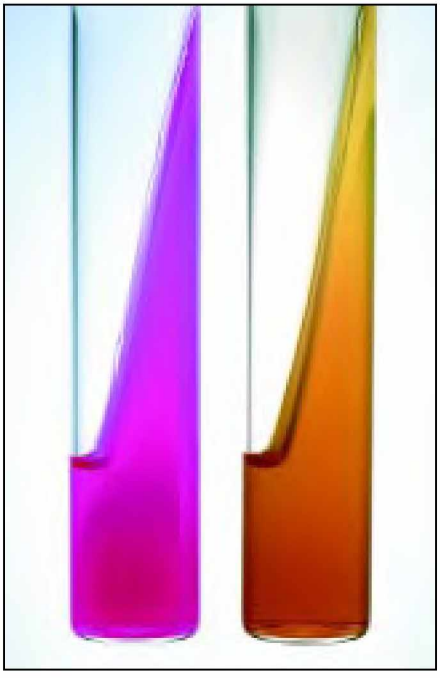

Figure 4-10. A pink color develops in a positive urease reaction (tube on left)

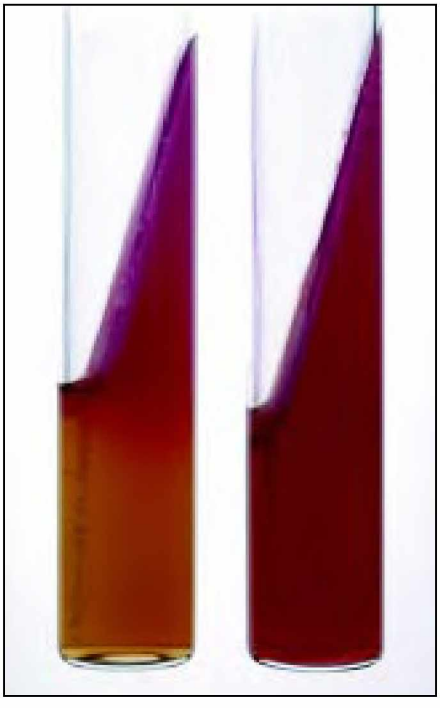

Figure 4-11. Organisms positive for lysine decarboxylase produce a purple color throughout the LIA medium (tube on right). Lysine-negative organisms produce a yellow color (acid) in the butt portion of the tube (tube on left).

Laboratorians should be aware that some *Shigella* commercial antiserum is labeled or packaged differently; for example, *Shigella* polyvalent A, which includes antisera to serotypes 1 through 7, may also be labeled polyvalent A1. Also, monovalent antiserum may be labeled in a way that it may be confused with polyvalent antiserum; for example, monovalent antiserum to *S. dysenteriae* 1 may be labeled "*Shigella* A1" instead of "*S. dysenteriae* serotype 1". When using newly purchased antisera, the laboratorian should read the package insert or check with the manufacturer if the label is not self-explanatory.

1. Slide agglutination

Because *S. dysenteriae* 1 (followed by *S. flexneri* and *S. sonnei*) is the most common agent of epidemic dysentery, isolates that react typically in the screening biochemicals should be screened first with monovalent A1 antiserum, then with polyvalent B antiserum, and finally in polyvalent D antiserum.

Agglutination tests may be carried out in a petri dish or on a clean glass slide. An inoculating loop or needle, sterile applicator stick or toothpick is used to remove a portion of the growth from the surface of KIA, TSI, heart infusion agar (HIA), or other nonselective agar medium. Serologic testing should not be done on growth from selective media such as MAC or XLD because this may give false-negative results. Emulsify the growth in two small drops of physiological saline and mix thoroughly. Add a small drop of antiserum to one of the suspensions. Usually approximately equal volumes of antiserum and growth suspension are mixed, but the volume of suspension may be as much as double the volume of the antiserum. To conserve antiserum, volumes as small as 10 microliters can be used. An inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 4-12). Mix the suspension and antiserum well and then tilt the slide back and forth to observe for agglutination. If the reaction is positive, clumping will appear within 30 seconds to 1 minute (Figure 4-13). Examine the saline suspension carefully to ensure that it is even and does not show clumping due to autoagglutination. If autoagglutination occurs, the culture is termed "rough" and cannot be serotyped.

Cultures that react serologically and show no conflicting results in the biochemical screening tests are reported as positive for *Shigella*. Serologically negative isolates that are biochemically identified as *Shigella* may be sent to a reference laboratory.

2. Quality control of antisera

All lots of antisera should be quality controlled before use. Quality control of antisera is discussed in Chapter 11.

D. Media for Isolation and Identification of *Shigella*

This section contains descriptions of all media mentioned in this chapter and discussions of their characteristics, preparation, and appropriate quality control strains. Each manufacturer's lot number of commercial dehydrated media or each

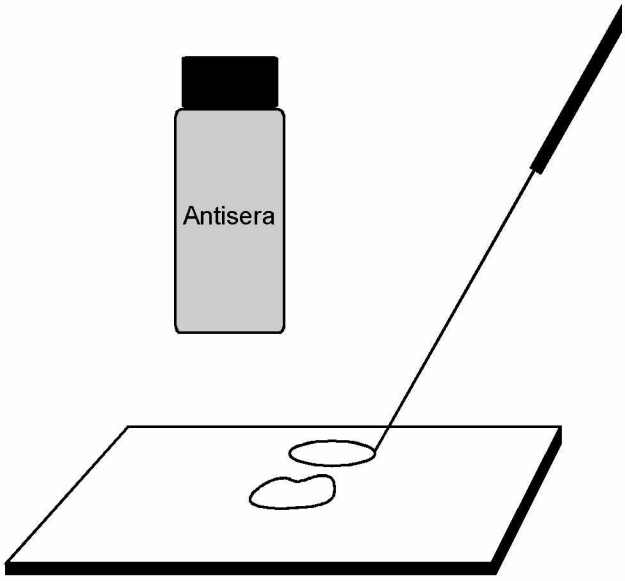

Figure 4-12. A bent loop may be helpful in dispensing small amounts of antiserum for slide agglutination tests.

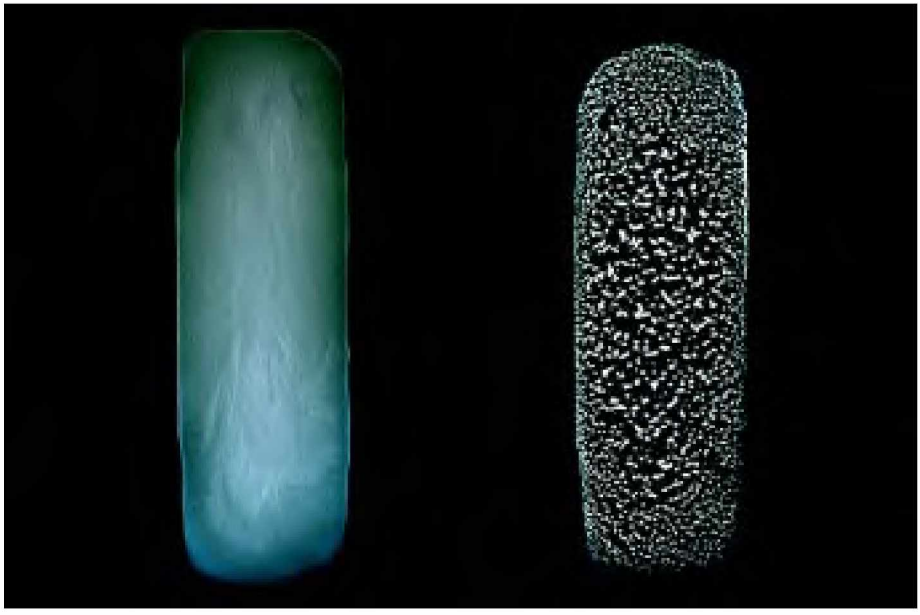

Figure 4-13. *Shigella* antiserum will agglutinate strains of the same serogroup or serotype (right). *Shigella* will not agglutinate when mixed with saline (left).

batch of media prepared from individual ingredients should be quality controlled before use. See Chapter 11 for a description of appropriate quality control methods.

1. Desoxycholate citrate agar

Desoxycholate citrate agar (DCA) is a differential selective plating medium for the isolation of enteric pathogens, particularly *Shigella* and *Salmonella*. Lactose-fermenting organisms produce pink colonies surrounded by a zone of bile precipitation. Colonies of lactose-nonfermenting strains are colorless. Several formulations of DCA, which may vary in selectivity, are available from different manufacturers.

Preparation and quality control

Prepare according to manufacturer's instructions. [Note: It may also be prepared from individual ingredients, but this can result in much greater lot-to-lot variation than when prepared from commercial dehydrated preparations.] DCA medium is very heat-sensitive, and overheating during boiling should be avoided. Do not autoclave. Plates can be stored at 4°C for up to a week.

For quality control of DCA, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics: *E. coli* may be somewhat inhibited, depending on the particular formulation used, but will produce pink colonies surrounded by a zone of precipitated bile; *S. flexneri* and *S. dysenteriae* 1 will produce fair to good growth of colorless colonies.

2. Hektoen enteric agar

Hektoen enteric agar (HE) is a differential selective agar that is useful for isolation of *Salmonella* and *Shigella*. It has an H_2S-indicator system for selecting H_2S-producing *Salmonella*, which produce blue-green colonies with a black center. *Shigella* colonies are green while rapid lactose-fermenters such as *E. coli* are pink to orange with a zone of bile precipitation.

Preparation and quality control

Prepare according to manufacturer's instructions. [Note: Several commercial brands of HE are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.] Heat to boiling to dissolve, but avoid overheating. Do not autoclave. When cool enough to pour, dispense into plates. Plates can be stored at 4°C for up to 1 week.

For quality control of HE, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics: *E. coli* should produce colonies that are pink to orange surrounded by a bile precipitate; *S. flexneri* should produce fair to good growth of green colonies, but *S. dysenteriae* 1 colonies should be smaller.

3. Kligler iron agar and triple sugar iron agar

Kligler iron agar (KIA) and triple sugar iron (TSI) agar are carbohydrate-containing screening media widely used for identification of enteric pathogens, including *Shigella*. Both media differentiate lactose fermenters from nonfermenters and have a hydrogen sulfide indicator. H_2S-producing organisms will cause blackening of the medium in both KIA and TSI.

KIA contains glucose and lactose. Organisms which ferment glucose cause the butt of the tube to become acid (yellow); some also produce gas. Lactose-fermenting organisms will produce an acid (yellow) slant; lactose-nonfermenting organisms will have an alkaline (red) slant.

TSI contains sucrose in addition to the ingredients in KIA. Organisms which ferment either lactose or sucrose will produce an acid (yellow) slant while organisms that ferment neither carbohydrate will have an alkaline (red) slant. As in KIA, glucose-fermenters produce an acid (yellow) reaction in the butt (sometimes with gas produced).

Preparation and quality control

Prepare according to manufacturer's instructions. [Note: There are several commercially available dehydrated formulations of KIA and TSI. These media can also be prepared from individual ingredients, but there may be lot-to-lot variation.] Dispense a quantity of medium in appropriate containers such that the volume of medium is sufficient to give a deep butt and a long slant. For example, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes (leave caps loose), and after autoclaving allow the slants to solidify in a manner such that the medium in the butt of the tube is about 3.5 cm deep and the slant is about 2.5 cm long. Tighten caps and store at 4°C for up to 6 months.

For quality control of KIA or TSI, the following organisms should be adequate for confirmation of biochemical response characteristics: *E. coli* should give an acid slant and butt, with the production of gas but no H_2S; *S. flexneri* should give an alkaline slant, acid butt, without production of gas or H_2S (Figure 4-8); an H_2S-producing *Salmonella* may be used to control this reaction.

4. Lysine iron agar

Organisms that produce lysine decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the medium and also on the slant (Figure 4-11). H_2S production is indicated by a blackening of the medium. Organisms lacking lysine decarboxylase, such as *Shigella*, typically produce an alkaline slant (purple) and an acid butt (yellow) no gas, and no H_2S (Table 4-2). *Proteus* and *Providencia* spp. will often produce a red slant caused by deamination of the lysine. LIA must be prepared so that the volume of medium in the tube is sufficient to give a deep butt. It is important for LIA tubes to have a deep butt because the decarboxylation reaction occurs only in anaerobic conditions.

Preparation and quality control

Prepare medium according manufacturer's instructions on the bottle. [Note: Several companies sell dehydrated LIA. LIA may also be prepared from individual ingredients, but there may be lot-to-lot variation.] Dispense a quantity of medium in appropriate containers such that the volume of medium is sufficient to give a deep butt and a long slant. For example, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes (leave caps loose), and after autoclaving allow the slants to solidify in a manner such that the medium in the butt of the tube is 3.5 cm deep and the slant is 2.5 cm long. Tighten caps and store at 4°C for up to 6 months.

For quality control of LIA, the following organisms may be used: *S. flexneri* should produce an alkaline slant and an acid butt without production of H_2S; an H_2S-producing *Salmonella* strain may be used to control the H_2S reaction and will most likely be lysine-positive and give an alkaline reaction in the butt of the tube.

5. MacConkey agar

MacConkey agar (MAC) is a differential plating medium recommended for use in the isolation and differentiation of lactose-nonfermenting, gram-negative enteric bacteria from lactose-fermenting organisms. Colonies of *Shigella* on MAC appear as convex, colorless colonies about 2 to 3 mm in diameter. *S. dysenteriae* 1 colonies may be smaller.

Several commercial brands of MAC are available. Most manufacturers prepare several formulations of MAC, which may vary in selectivity and thereby affect the isolation of *Shigella*. For example, some formulations of MAC do not contain crystal violet, a selective agent; these types are not as selective and should not be used for isolation of *Shigella*. Oxoid MacConkey Agar No. 3, Difco Bacto MacConkey Agar, and BBL MacConkey Agar are all suitable.

Preparation and quality control

Prepare according to manufacturer's instructions. [Note: MAC can also be prepared from individual ingredients, but this produces much more variable results than a commercial dehydrated formulation.] Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and pour into petri plates. Leave lids ajar for about 20 minutes so that the surface of the agar will dry. Close lids and store at 4°C for up to 1 month. If plates are to be stored for more than a few days, put them in a sealed plastic bag to prevent drying.

For quality control of MAC, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics: *E. coli* should produce pink to red colonies with good to excellent growth; *S. flexneri* should produce colorless colonies with fair to good growth, but *S. dysenteriae* 1 colonies may be smaller.

6. Motility medium

Because *Shigella* spp. are always nonmotile, motility medium is a useful biochemical screening test. Motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation (Figure 4-9). Nonmotile organisms do not grow out from the line of inoculation.

Preparation and quality control

Follow manufacturer's instructions to weigh out and suspend dehydrated medium. [Note: Several commercial dehydrated formulations of motility agar are available. This medium can also be prepared from individual ingredients.] Heat to boiling to make sure medium is completely dissolved. Dispense into tubes or other types of containers, leaving caps loose, and sterilize at 121°C for 15 min. Allow to solidify upright, forming a deep butt with no slant (e.g., about 4 to 5 ml of medium per 13 x 100-mm screw-cap tube). When the medium is solidified and cooled, leave caps loose until the surface of the medium has dried. Tighten caps and store at 4°C for up to 6 months.

For quality control of motility medium, the following organisms may be used: *E. coli* is motile, while *Shigella* spp. are nonmotile. The surface of the medium should be dry when used. If moisture has accumulated in the tube, carefully pour it out before inoculating the tube. Moisture can cause a nonmotile organism to grow down the sides of the agar creating a haze of growth and appearing to be motile.

7. Sulfide-indole-motility medium

Sulfide-indole-motility medium (SIM) is a commercially available combination medium that combines three tests in a single tube: hydrogen sulfide (H_2S) production, indole production, and motility. The indole reaction is not useful for screening suspected *Shigella* isolates because strains vary in their reactions in this test. It is inoculated in the same way as motility agar, by using a needle to stab about 1 to 2 cm down into the medium, and is incubated overnight at 35° to 37°C. The motility reaction is read the same as for motility medium. As in KIA or TSI, H_2S production is indicated by blackening of the medium. Indole production can be tested by either the filter paper method or by adding Kovac's reagent to the tube.

Preparation and quality control

Follow manufacturer's instructions to weigh out and suspend dehydrated medium. Heat to boiling to make sure the medium is completely dissolved. Dispense into tubes and sterilize by autoclaving for 15 minutes at 121°C.

For quality control of SIM medium, the following organisms may be used: *E. coli* is indole positive, H_2S negative, and motility positive; an H_2S-producing *Salmonella* strain may be used to control the H_2S reaction and will most likely be

motile and indole negative; *Shigella* spp. are motility negative and H_2S negative but are variable for the indole reaction.

8. Urea medium

Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color (Figure 4-10). Urease-negative organisms do not change the color of the medium, which is a pale yellowish-pink. *Shigella* spp. are always urease-negative (Table 4-2).

Preparation and quality control

Follow manufacturer's instructions for preparation. [Note: Several commercial brands of urea medium are available, some of which require the preparation of a sterile broth which is added to an autoclaved agar base. Some manufacturers have sterile prepared urea concentrate available for purchase.]

Prepare urea agar base as directed on the bottle. Sterilize at 121°C for 15 min. Cool to 50° to 55°C, then add urea concentrate according to manufacturer's directions. Before adding the urea to the agar base, make sure the agar base is cool since the urea is heat labile. Mix and distribute in sterile tubes. Slant the medium so that a deep butt is formed.

For quality control of urea medium, the following organisms may be used: *Proteus* spp. produce urease; *E. coli* is urease negative.

9. Xylose lysine desoxycholate agar

Xylose lysine desoxycholate agar (XLD) is a selective differential medium suitable for isolation of *Shigella* and *Salmonella* from stool specimens. Differentiation of these two species from nonpathogenic bacteria is accomplished by xylose and lactose fermentation, lysine decarboxylation, and hydrogen sulfide production.

Shigella colonies on XLD agar are transparent pink or red smooth colonies 1 to 2 mm in diameter (Figure 4-5). *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike other *Shigella* species (Figure 4-4). Coliforms appear yellow (4-6). *Salmonella* colonies are usually red with black centers but may be yellow with black centers.

Preparation and quality control

Prepare according to manufacturer's instructions. [Note: Several commercial brands of XLD agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.] Mix thoroughly. Heat with agitation just until the medium boils. **Do not overheat;** overheating when boiling XLD or allowing the medium to cool too long may cause the medium to precipitate. Cool flask under running water until just cool enough to pour. Avoid cooling the medium too long. Pour into petri plates, leaving the lids ajar for about 20 minutes so that the

surface of the agar will dry. Plates can be stored at 4°C for up to a week.

For quality control of XLD, the following organisms should be adequate for confirmation of selective and inhibitory growth characteristics: *S. flexneri* should produce fair to good growth of transparent pink or red smooth colonies that are 1 to 2 mm in diameter; *S. dysenteriae* 1 may produce very small transparent or red colonies; *E. coli* should produce poor to fair growth of yellow colonies.

References

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Chapter 5
Etiology and Epidemiology of Cholera

Isolates of *Vibrio cholerae* serogroup O1 are classified into two biotypes, El Tor and classical, on the basis of several phenotypic characteristics. Currently, the El Tor biotype is responsible for virtually all of the cholera cases throughout the world, and classical isolates are not encountered outside of Bangladesh. In addition *V. cholerae* O1 is classified into two serotypes, Inaba and Ogawa, based on agglutination in antiserum. A possible third serotype, Hikojima, has been described, but it is very rare. During an outbreak or epidemic, it is worth documenting the biotype and serotype of the isolate; however, it is not necessary to know this information to respond appropriately to the epidemic.

Within the O1 and O139 serogroups, the ability to produce cholera toxin (CT) is a major determinant of virulence. In general, isolates of *V. cholerae* O1 or O139 that produce CT are considered fully virulent and capable of causing epidemic cholera (Table 5-1). Most *V. cholerae* isolated during cholera outbreaks will be toxigenic serogroup O1 or O139. However, some isolates of *V. cholerae* O1 do not produce CT and cannot cause epidemic cholera. When these isolates are encountered, they must be considered within their clinical and epidemiologic context. Nontoxigenic isolates may be associated with sporadic diarrheal disease.

A. Historical Background

Cholera is thought to have its ancestral home in the Ganges Delta of the Indian subcontinent. In the nineteenth century, pandemic waves of cholera spread to many parts of the world. In 1961, a massive epidemic began in Southeast Asia; this is now recognized as the beginning of the seventh cholera pandemic. This pandemic was caused by the El Tor biotype of toxigenic *V. cholerae* O1. It spread rapidly through south Asia, the Middle East, and southeastern Europe, reaching Africa by 1970. In January 1991, epidemic cholera appeared in South America in several coastal cities of Peru and spread rapidly to adjoining countries. By the end of 1996, cholera had spread to 21 countries in Latin America, causing over 1 million cases and nearly 12,000 deaths. The number of cholera cases reported elsewhere in the world has also increased in the 1990s. In Africa in the early 1990s, the primary focus of cholera was in southern Africa. However, in the latter part of the decade, the focus moved to west Africa. Overall, more cases were reported from Africa in the 1990s than in a similar time period in previous decades.

Vibrio cholerae serogroup O139

Vibrio cholerae serogroup O139 appeared in India in late 1992. It quickly spread to Bangladesh and other Asian countries, although the rate of spread has slowed after the initial outbreaks. Through 1998, 11 countries have officially

reported transmission of *V. cholerae* O139 to WHO. Imported cases have been reported from the United States and other countries. At this time, *V. cholerae* O139 appears to be confined to Asia.

Table 5-1. Comparison of epidemic- and non-epidemic-associated *V. cholerae* strains

Typing systems	Epidemic-associated	Non-epidemic-associated
Serogroups	O1, O139	Non-O1 (>150 exist)
Biotypes for serogroup O1	Classical and El Tor (not applicable to serogroup O139)	Biotypes are not applicable to non-O1 strains
Serotypes for serogroup O1	Inaba, Ogawa, and Hikojima (not applicable to serogroup O139)	These 3 serotypes are not applicable to non-O1 strains
Toxin production	Produce cholera toxin[a]	Usually do not produce cholera toxin; sometimes produce other toxins

[a] Nontoxigenic O1 strains exist but are rarely associated with epidemics.

The epidemiologic characteristics of the O139 serogroup are similar to those of the O1 serogroup. The isolation and identification characteristics of the O139 serogroup are identical to those of the O1 serogroup except that O139 antiserum is needed for identification. Biotyping tests for *V. cholerae* O1 are not valid for *V. cholerae* O139 or any non-O1/O139 serogroup.

B. Clinical Manifestations

Cholera is a secretory diarrheal disease. The enterotoxin produced by *V. cholerae* O1 and O139 causes a massive outpouring of fluid and electrolytes into the bowel. This rapidly leads to profuse watery diarrhea, loss of circulation and blood volume, metabolic acidosis, potassium depletion, and ultimately vascular collapse and death. In severe cases, purging diarrhea can rapidly cause the loss of 10% or more of the body's weight, with attendant hypovolemic shock and death; however, 75% or more of initial infections with *V. cholerae* O1 or O139 may be asymptomatic, depending on the infecting dose. Of the 25% of persons with symptomatic infections, most have mild illness. Approximately 5% of patients have moderate illness that requires medical attention but not hospitalization. In only about 2% of patients does the illness progress to life-threatening "cholera gravis." Persons with blood type O are more likely to develop severe cholera than those with other blood types.

C. Treatment

Successful treatment of cholera patients depends on rapid replacement of fluid and electrolyte losses. With proper treatment, mortality is less than 1% of reported cases. Fluids and electrolytes can be replaced rapidly through either oral or intravenous routes. Intravenous therapy is required for patients who are in profound shock or cannot drink.

Antimicrobial therapy is helpful, although not essential, in treating cholera patients. Antimicrobial agents reduce the duration of illness, the volume of stool, and the duration of shedding of vibrios in the feces. When antimicrobial agents are used, it is essential to choose one to which the organism is susceptible. Antimicrobial agents recommended by WHO for treating cholera patients include tetracycline, doxycycline, furazolidone, trimethoprim-sulfamethoxazole, erythromycin, or chloramphenicol. Ciprofloxacin and norfloxacin are also effective. Because antimicrobial resistance has been a growing problem in many parts of the world, the susceptibility of *V. cholerae* O1 strains to antimicrobial agents should be determined at the beginning of an epidemic and be monitored periodically (see Annexes C and E).

For *V. cholerae*, the results of disk diffusion tests for ampicillin, sulfonamides, tetracycline, and trimethoprim-sulfamethoxazole (i.e., percentage of susceptible, intermediate, and resistant) correlate well with the minimum inhibitory concentration (MIC) results determined by broth microdilution. Disk diffusion tests should not be used for doxycycline and erythromycin because the results for these drugs are frequently inaccurate for *V. cholerae* O1 and O139 strains. However, the tetracycline disk test can be used to predict the likely susceptibility of isolates to doxycycline. Additional details on antimicrobial susceptibility testing are given in Chapter 9.

D. Epidemiology

When cholera first appears in epidemic form in an unexposed population, it can affect all age groups. In contrast, in areas with high rates of endemic disease, most of the adult population have gained some degree of natural immunity because of illness or repeated asymptomatic infections. In this setting, the disease occurs primarily in young children, who are exposed to the organism for the first time, and in the elderly, who have lower gastric acid production and waning immunity. The poor are at greatest risk because they often lack safe water supplies, are unable to maintain proper hygiene within the home, and may depend on street vendors or other unregulated sources for food and drink.

Numerous investigations have linked cholera transmission to drinking water drawn from shallow wells, rivers or streams, and even to bottled water and ice. Food is the other important means of cholera transmission. Seafood has repeatedly been a source of cholera, particularly raw or undercooked shellfish harvested from sewage-contaminated beds or from environments where *V. cholerae* O1 occurs naturally. Although *V. cholerae* O1 and O139 are easily killed by drying, sunlight, and acidity, they grow well on a variety of moist

alkaline foods from which other competing organisms have been eliminated by previous cooking. Cooked rice is an excellent growth medium, as are lentils, millet, and other cooked grains and legumes with neutral pH. Fruits and vegetables grown in sewage and eaten without cooking or other decontaminating procedures are potential vehicles of cholera transmission. Freezing foods or drinks does not prevent cholera transmission.

Person-to-person spread through direct contact, as by shaking hands or touching or by taking care of a patient, has not been shown to occur. Outbreaks on crowded hospital wards are likely to be due to contaminated food or water. Likewise, outbreaks following the funeral of a cholera patient have been caused by eating contaminated foods served at the wake, often prepared by the same persons who prepared the body for burial.

E. Cholera Vaccine

During the past 15 years, considerable progress has been made in the development of new oral vaccines against cholera. Two oral cholera vaccines, which have been evaluated with volunteers from industrialized countries and in regions with endemic cholera, are commercially available in several countries: a killed whole-cell *V. cholerae* O1 in combination with purified recombinant B subunit of cholera toxin (WC/rBS); and an attenuated live oral cholera vaccine, containing the genetically manipulated *V. cholerae* O1 strain CVD 103-HgR. The appearance of *V. cholerae* O139 has redirected efforts to develop an effective and practical cholera vaccine. None of the currently available vaccines is effective against this strain.

References

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Chapter 6
Isolation and Identification of *Vibrio cholerae* Serogroups O1 and O139

Isolation and identification of *V. cholerae* serogroups O1 and O139 can be greatly enhanced when optimal laboratory media and techniques are employed. The methods presented here are intended to be economical and to offer laboratorians some flexibility in choice of protocol and media. Laboratories that do not have sufficient resources to adopt the methods described in this chapter should consider sending the specimens or isolates to other laboratory facilities that routinely perform these procedures.

A. Isolation Methods

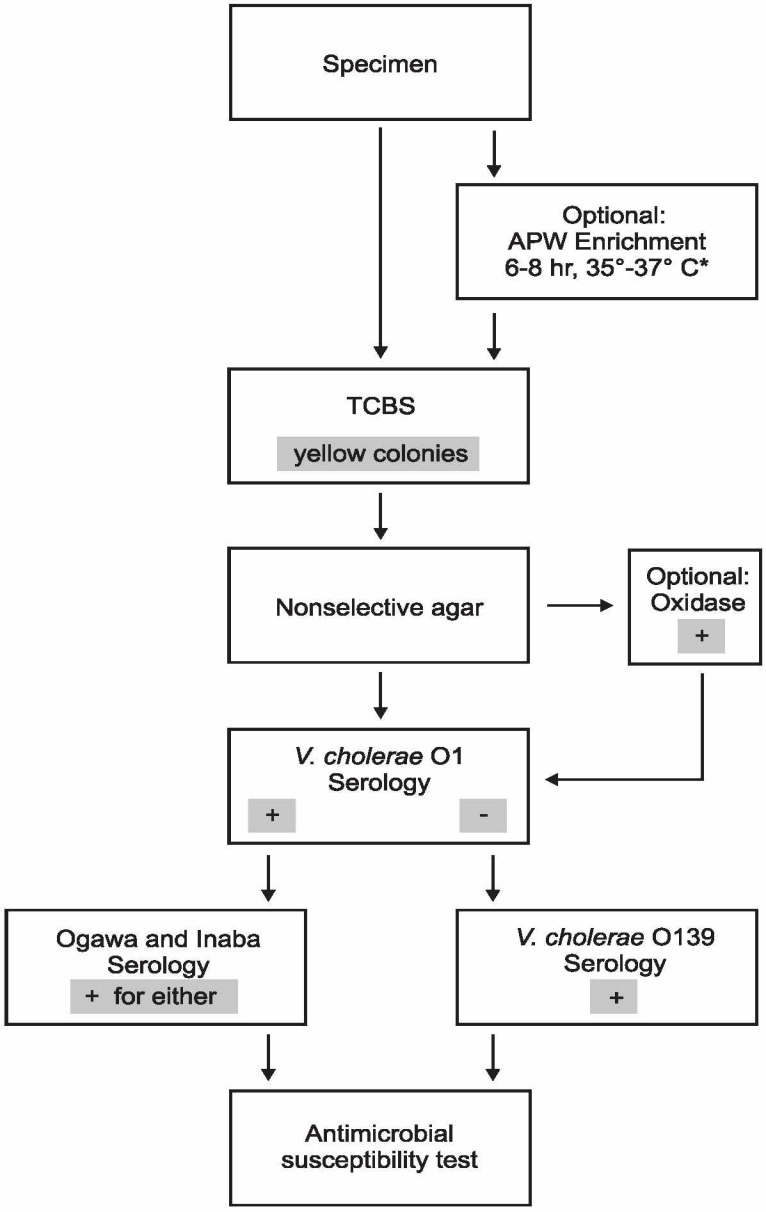
Before 1992, of the more than 150 serogroups of *Vibrio cholerae* that have been reported, only the O1 serogroup was associated with epidemic and pandemic cholera. However in late 1992 and early 1993, large outbreaks of cholera due to a newly described serogroup, O139, were reported in India and Bangladesh. This strain, like serogroup O1 *V. cholerae*, produces cholera enterotoxin. Because the cultural and biochemical characteristics of these two serogroups are identical, the isolation and identification methods described below apply to both O1 and O139. Both serogroups must be identified using O-group-specific antisera. Annex A lists diagnostic supplies necessary for laboratory confirmation and antimicrobial susceptibility testing of *V. cholerae*.

Although *V. cholerae* will grow on a variety of commonly used agar media, isolation from fecal specimens is more easily accomplished with specialized media. Alkaline peptone water (APW) is recommended as an enrichment broth, and thiosulfate citrate bile salts sucrose agar (TCBS) is the selective agar medium of choice (Figure 6-1). In certain instances (for example, when the patient is in very early stages of illness), it may not be necessary to enrich specimens or use selective plating media. However, enrichment broth and a selective plating medium should always be used with convalescent patients, suspected asymptomatic infections, environmental specimens, and whenever high numbers of competing organisms are likely to be present in the specimen.

Refer to Section C, "Media and Reagents for *V. cholerae*," before preparing any of these media because incorrect preparation can affect the reactions of organisms in these tests. Chapter 11 discusses methods for quality control of selective media and antisera.

1. Enrichment in alkaline peptone water

Enrichment in APW can enhance the isolation of *V. cholerae* when few organisms are present, as in specimens from convalescent patients and asymptomatic carriers. *Vibrio* spp. grow very rapidly in APW, and at 6 to 8 hours will be present in greater numbers than non-*Vibrio* organisms.



* If the APW cannot be streaked after 6-8 hours of incubation, subculture at 18 hours to a fresh tube of APW; incubate for 6-8 hours, and then streak to TCBS

Figure 6-1. Procedure for recovery of *Vibrio cholerae* O1 and O139 from fecal specimens

APW can be inoculated with liquid stool, fecal suspension, or a rectal swab. The stool inoculum should not exceed 10% of the volume of the broth. Incubate the tube with the cap loosened at 35° to 37°C for 6 to 8 hours. After incubation, subculture to TCBS should be made with one to two loopfuls of APW from the surface and topmost portion of the broth, since vibrios preferentially grow in this area. Do not shake or mix the tube before subculturing. If the broth cannot be plated after 6 to 8 hours of incubation, subculture a loopful at 18 hours to a fresh tube of APW. Subculture this second tube to TCBS agar after 6 to 8 hours of incubation (Figure 6-1).

2. Isolation from TCBS selective agar

TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective (see Section C). Growth on this medium is not suitable for direct testing with *V. cholerae* antisera.

Inoculation of TCBS

Figure 6-1 outlines the procedure for isolation of *V. cholerae* from fecal specimens. Inoculate the TCBS plate as described in Chapter 4 (Figure 4-2). After 18 to 24 hours' incubation at 35° to 37°C, the amount and type of growth (e.g., sucrose-fermenting or sucrose-nonfermenting) on the TCBS plate should be recorded on data sheets (Figure 6-2). Colonies suspicious for *V. cholerae* will appear on TCBS agar as yellow, shiny colonies, 2 to 4 mm in diameter (Figure 6-3). The yellow color is caused by the fermentation of sucrose in the medium. Sucrose-nonfermenting organisms, such as *V. parahaemolyticus,* produce green to blue-green colonies.

Isolation of suspected V. cholerae

Carefully select at least one of each type of sucrose-fermenting colony from the TCBS plate to inoculate a heart infusion agar (HIA) slant or another nonselective medium. Do not use nutrient agar because it has no added salt and does not allow optimal growth of *V. cholerae.* Using an inoculating needle, lightly touch only the very center of the colony. Do not take the whole colony or go through the colony and touch the surface of the plate. This is to avoid picking up contaminants that may be on the surface of the agar. If there is doubt that a particular colony is sufficiently isolated from surrounding colonies, purify the suspicious colony by streaking on another agar plate.

Incubate the HIA slants at 35° to 37°C for up to 24 hours; however, there may be sufficient growth at 6 hours for serologic testing to be done. Slide serology with polyvalent O1 and O139 antisera is sufficient for a presumptive identification (see section B below for a description of serologic identification).

Vibrio cholerae **Worksheet**

SPECIMEN NUMBER	MEDIA	SUC + [a]	SUC - [b]	COLONY	OPTIONAL				SLIDE SEROLOGY				IDENTIFICATION
					OXIDASE TEST	STRING TEST	GRAM STAIN OR WET MOUNT		PV O1	INABA	OGAWA	O139	
	DIRECT TCBS			T1									
				T2									
				T3									
	APW-TCBS			AT1									
				AT2									
				AT3									
	DIRECT TCBS			T1									
				T2									
				T3									
	APW-TCBS			AT1									
				AT2									
				AT3									
	DIRECT TCBS			T1									
				T2									
				T3									
	APW-TCBS			AT1									
				AT2									
				AT3									

[a]SUC + = Sucrose-positive colonies [b]SUC - = Sucrose-negative colonies

Figure 6-2. *Vibrio cholerae* worksheet

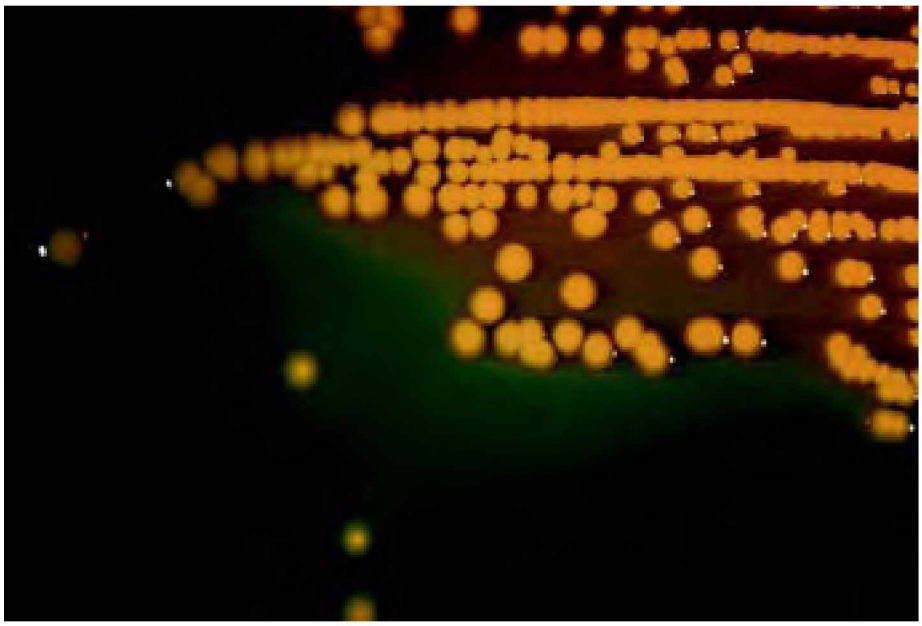


Figure 6-3. Growth of *V. cholerae* on TCBS

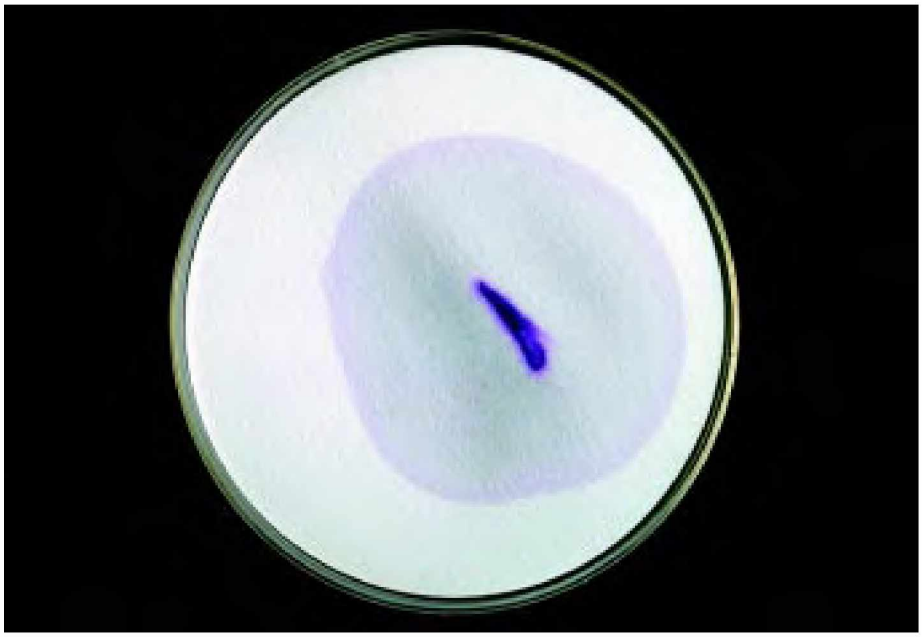


Figure 6-4. A positive oxidase test (shown here) results in the development of a dark purple color within 10 seconds. *V. cholerae* is oxidase positive.

3. Screening tests for suspected *V. cholerae* isolates

Generally for suspected *V. cholerae* isolates from fecal specimens, screening with biochemical tests prior to testing with O1 and O139 antisera is not necessary. However, if the supply of antisera is limited, the oxidase test may be useful for additional screening of isolates before testing with antisera.

Oxidase test

Conduct the oxidase test with fresh growth from an HIA slant or any non-carbohydrate-containing medium. Do not use growth from TCBS agar because it may yield either false-negative or false-positive results. Place 2 to 3 drops of oxidase reagent (1% *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) on a piece of filter paper in a petri dish. Smear the culture across the wet paper with a platinum (not nichrome) loop, a sterile wooden applicator stick or toothpick. In a positive reaction, the bacterial growth becomes dark purple immediately (Figure 6-4). Oxidase-negative organisms will remain colorless or will turn purple after 10 seconds. Color development after 10 seconds should be disregarded. Positive and negative controls should be tested at the same time. Organisms of the genera *Vibrio* (including *V cholerae*, Table 6-1), *Neisseria*, *Campylobacter*, *Aeromonas*, *Plesiomonas*, *Pseudomonas*, and *Alcaligenes* are all oxidase positive; all *Enterobacteriaceae* are oxidase negative.

Table 6-1. Reactions of *V. cholerae* in screening tests

Screening test	*Vibrio cholerae* reactions
Oxidase test	Positive
String test	Positive
KIA	K/A, no gas produced (red slant/yellow butt)[a]
TSI	A/A, no gas produced (yellow slant/yellow butt)[a]
LIA	K/K, no gas produced (purple slant/purple butt)[a,b]
Gram stain	Small, gram-negative curved rods
Wet mount	Small, curved rods with darting motility

[a] K = alkaline; A = acid

[b] An alkaline reaction (purple) in the butt of the medium indicates that lysine was decarboxylated. An acid reaction (yellow) in the butt of the medium indicates that lysine was not decarboxylated.

Additional biochemical screening tests

The string reaction, Kligler iron agar (KIA) or triple sugar iron agar (TSI), lysine iron agar (LIA), Gram stain, and wet mount for motility are other possible tests that may be used for additional screening of isolates before testing with antisera (Table 6-1). The value of these tests should be assessed to determine their usefulness before they are applied routinely. See Section C for instructions on preparation of media and appropriate quality control strains.

String test

The string test, using fresh growth from nonselective agar, is useful for ruling out non-*Vibrio* spp., particularly *Aeromonas* spp. The string test may be performed on a glass microscope slide or plastic petri dish by suspending 18- to 24-hour growth from HIA or other noninhibitory medium in a drop of 0.5% aqueous solution of sodium deoxycholate. If the result is positive, the bacterial cells will be lysed by the sodium deoxycholate, the suspension will lose turbidity, and DNA will be released from the lysed cells, causing the mixture to become viscous. A mucoid "string" is formed when an inoculating loop is drawn slowly away from the suspension (Figure 6-5). *V. cholerae* (Table 6-1) strains are positive, whereas *Aeromonas* strains are usually negative. Other *Vibrio* spp. may give a positive or weak string test reaction.

Figure 6-5. A positive string test with *V. cholerae*

Kligler iron agar and triple sugar iron agar

Kligler iron agar (KIA) and triple sugar iron agar (TSI) can be used to rule out *Pseudomonas* spp. and certain *Enterobacteriaceae*. The reactions of *V. cholerae* on KIA, which contains glucose and lactose, are similar to those of lactose-nonfermenting *Enterobacteriaceae* (alkaline (red) slant, acid (yellow) butt, no gas, no H_2S) (see Table 6-1 and Figure 6-6). However, on TSI, *V. cholerae*

strains produce an acid (yellow) slant and acid (yellow) butt, no gas, and no H_2S.

KIA or TSI slants are inoculated by stabbing the butt and streaking the surface of the medium. Incubate the slants at 35° to 37°C and examine after 18 to 24 hours. Caps on all tubes of biochemicals should be loosened before incubation, but this is particularly important for KIA or TSI slants. If the caps are too tight and anaerobic conditions exist in the KIA or TSI tube, an inappropriate reaction will occur and the characteristic reactions of *V. cholerae* may not be exhibited.

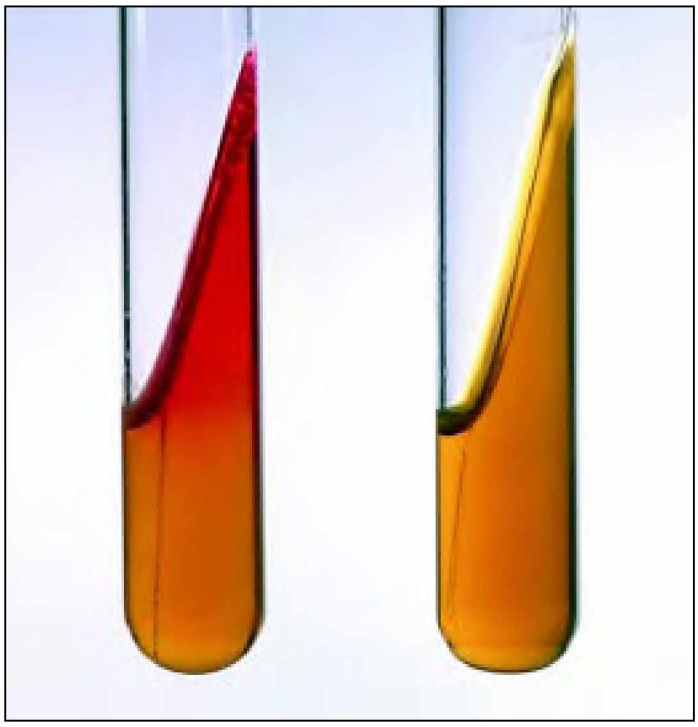


Figure 6-6. Reactions of *V. cholerae* in Kligler iron agar (left) and triple sugar iron agar (right)

Lysine iron agar

LIA is helpful for screening out *Aeromonas* and certain *Vibrio* spp., which, unlike *V. cholerae*, do not decarboxylate lysine. LIA is inoculated by stabbing the butt and streaking the slant. After incubation for 18 to 24 hours at 35° to 37°C, examine the LIA slants for reactions typical of *V. cholerae*. Organisms that produce lysine decarboxylase in LIA cause an alkaline reaction (purple color) in the butt of the tube (see Chapter 4, Figure 4-11). Organisms without the enzyme

produce a yellow color (acid) in the butt portion of the tube. H_2S production is indicated by a blackening of the medium. The LIA reaction for *V. cholerae* is typically an alkaline slant (purple), alkaline butt (purple), no gas, and no H_2S (Table 6-1). *Proteus* and *Providencia* spp. will often produce a red slant caused by deamination of the lysine.

It is important that KIA, TSI, and LIA be prepared so the tubes have a deep butt and a long slant. If the butt is not deep enough, misleading reactions may occur in these media. In LIA, the decarboxylation of lysine occurs only in anaerobic conditions and a false-negative reaction may result from insufficient medium in the tube (Section C).

Gram stain

Examining overnight growth from an HIA slant by Gram stain will demonstrate typical small, curved gram-negative rods (Table 6-1). Staining with crystal violet only is a more rapid technique and will still demonstrate the cell morphology typical of *Vibrio* spp.

Wet mount

Dark-field and phase-contrast microscopy have been used for screening suspected isolates of *V. cholerae*. With these techniques, saline suspensions are microscopically examined for the presence of organisms with typical small, curved rods and darting ("shooting star") motility (Table 6-1).

B. Serologic Identification of *V. cholerae* O1 and O139

1. Presumptive identification using O1 and O139 antisera

For slide agglutination testing with polyvalent O1 or O139 antisera, fresh growth of suspected *V. cholerae* from a nonselective agar medium should be used. Using growth from TCBS agar may result in false-negative reactions. Usually after 5 to 6 hours of incubation, growth on the surface of the slant is sufficient to perform slide serology with antisera; if not, incubate for a longer period. If the isolate does not agglutinate in O1 antiserum, test in O139 antiserum. If it is positive in the polyvalent O1 or in the O139 antiserum, it may be reported as presumptive *V. cholerae* O1 or O139. Presumptive *V. cholerae* O1 isolates should be tested in monovalent Ogawa and Inaba antisera (see below). Once one colony from a plate has been identified as *V. cholerae* O1 or O139, no further colonies from the same plate need to be tested.

2. Confirmation of *V. cholerae* O1 using Inaba and Ogawa antisera

The O1 serogroup of *V. cholerae* has been further divided into three serotypes, Inaba, Ogawa, and Hikojima (very rare). Serotype identification is based on agglutination in monovalent antisera to type-specific O antigens (see Table 6-2). A positive reaction in either Inaba or Ogawa antiserum is sufficient to confirm the identification of a *V. cholerae* O1 isolate. Isolates that agglutinate weakly or slowly with serogroup O1 antiserum but do not agglutinate with either Inaba or

Ogawa antiserum are not considered to be serogroup O1. Identifying these antigens is valid only with serogroup O1 isolates. For this reason, Inaba and Ogawa antisera should never be used with strains that are negative with polyvalent O1 antiserum.

Strains of one serotype frequently produce slow or weak agglutination in antiserum to the other serotype, depending on how well the serotype-specific antisera have been absorbed. For this reason, agglutination reactions with Inaba and Ogawa antisera should be examined simultaneously, and the strongest and most rapid reaction should be used to identify the serotype. With adequately absorbed antisera, strains that agglutinate very strongly and equally with both the Ogawa and Inaba antisera are rarely, if ever, encountered. If such reactions are suspected, the strains should be referred to a reference laboratory for further examination and may be referred to as "possible serotype Hikojima."

Refer to Chapter 11 for a discussion on quality control of antisera.

Table 6-2. Serotypes of *V. cholerae* serogroup O1

	Agglutination in absorbed antiserum	
V. cholerae O1 serotype	Ogawa antiserum	Inaba antiserum
Ogawa	+	-
Inaba	-	+
Hikojima	+	+

3. Slide agglutination procedures

Agglutination tests for *V. cholerae* somatic O antigens may be carried out in a petri dish or on a clean glass slide. Use an inoculating loop or needle, sterile applicator stick, or toothpick to remove a portion of the growth from the surface of HIA, KIA, TSI, or other nonselective agar medium. Emulsify the growth in two small drops of physiological saline and mix thoroughly. Add a small drop of antiserum to one of the suspensions. Usually approximately equal volumes of antiserum and growth suspension are mixed, but the volume of suspension may be as much as double the volume of the antiserum. To conserve antiserum, volumes as small as 10 microliters (0.01 ml) can be used. An inoculating loop may be used to dispense small amounts of antisera if micropipettors are not available (Figure 4-12). Mix the suspension and antiserum well and then tilt the slide back and forth to observe for agglutination. If the reaction is positive, clumping will appear within 30 seconds to 1 minute. Examine the saline suspension carefully to ensure that it does not show clumping due to autoagglutination. If autoagglutination occurs, the culture is termed "rough" and cannot be serotyped.

4. Confirmation of *V. cholerae* O139

A suspected *V. cholerae* isolate that reacts in O139 antiserum but not in polyvalent O1 antiserum should be sent to a reference laboratory. Confirmation of *V. cholerae* O139 includes testing for production of cholera enterotoxin and verification of the O139 antigen. No serotypes have been identified in the O139 serogroup.

C. Media and Reagents for *V. cholerae*

1. Alkline peptone water

[Note: There are several different published formulations for this medium.]

Peptone	10.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Add ingredients to the water and adjust to pH 8.5 with 3 N NaOH solution. Distribute and autoclave at 121°C for 15 minutes. Store at 4°C for up to 6 months making sure caps are tightly closed to prevent a drop in pH or evaporation.

When inoculated into APW for quality control, *V. cholerae* O1 should show good growth at 6 to 8 hours.

2. Kligler iron agar and triple sugar iron agar

[Note: There are several commercially available dehydrated formulations of KIA and TSI. These media can also be prepared from individual ingredients but there may be lot-to-lot variation.]

Prepare according to manufacturer's instructions. Dispense a quantity of medium in appropriate containers such that the volume of medium is sufficient to give a deep butt and a long slant. For example, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes (leave caps loose), and after autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is 3 cm deep and the slant is 2 cm long. Tighten caps and store at 4°C for up to 6 months.

Each new lot should be quality controlled before use. *E. coli* should give an acid slant and butt, with production of gas but no H_2S. *S. flexneri* should give an alkaline slant, acid butt, without production of gas or H_2S [Note: some *S. flexneri* 6 strains produce gas].

3. Lysine iron agar

[Note: Several companies sell dehydrated LIA. LIA may also be prepared from individual ingredients but there may be lot-to-lot variation.]

Prepare medium according to manufacturer's instructions on the bottle. Dispense a quantity of medium in appropriate containers such that the volume of

medium is sufficient to give a deep butt and a long slant. For example, dispense 6.5 ml of medium into 16 x 125-mm screw-cap tubes (leave caps loose), and after autoclaving, allow the slants to solidify in a manner such that the medium in the butt of the tube is about 3 cm deep and the slant is about 2 cm long. When the agar is cooled and solidified, tighten caps and store at 4°C for up to 6 months.

Each new lot of dehydrated medium should be quality controlled before use. *S. flexneri* should produce an alkaline slant and an acid butt without production of H_2S. H_2S-producing *Salmonella* strains should produce an alkaline slant and an alkaline butt with blackening of the medium due to H_2S. *V. cholerae* strains are lysine-positive and will produce an alkaline reaction in the butt of the LIA.

4. Oxidase reagent

N,N,N′,N′-Tetramethyl-*p*-phenylenediamine dihydrochloride	0.05 g
Distilled water	5.0 ml

Dissolve the reagent in purified water (do not heat to dissolve). Prepare fresh daily.

Positive and negative controls should be tested every time the reagent is prepared. *V. cholerae* is oxidase positive; *E. coli* is oxidase negative.

5. Sodium deoxycholate reagent (0.5%) for string test

Sodium deoxycholate	0.5 g
Sterile distilled water	100.0 ml

Add sterile distilled water to sodium deoxycholate and mix well. Store at room temperature for up to 6 months.

Each new batch should be quality controlled before use. A *V. cholerae* O1 strain should be used as positive control. E. *coli* may be used as a negative control.

6. Thiosulfate citrate bile salts sucrose agar

[Note: Several commercial brands of thiosulfate citrate bile salts sucrose agar (TCBS) agar are available. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.]

Follow manufacturer's instructions to weigh out and suspend the dehydrated medium. Heat with agitation. Medium should be completely dissolved. Cool agar in a water bath until cool enough to pour (50° to 55°C). Pour into petri plates, leaving lids ajar about 20 minutes so that the surface of the agar will dry. Close lids and store at 4°C for up to 1 week.

Each new lot should be quality controlled before use since TCBS is subject to lot-to-lot and brand-to-brand variations in selectivity. *V. cholerae* O1 should

show good growth of yellow colonies. *E. coli* should have none to poor growth of translucent colonies.

References

World Health Organization. Manual for the laboratory investigations of acute enteric infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.

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Centers for Disease Control and Prevention. Laboratory methods for the diagnosis of *Vibrio cholerae.* Atlanta, Georgia: CDC; 1994.

Kay, BA, Bopp CA, Wells JG. Isolation and identification of *Vibrio cholerae* O1 from fecal specimens. In: Wachsmuth IK, Blake PA, and Olsvik O., ed. *Vibrio cholerae* and cholera: molecular to global perspectives. Washington, DC: ASM Press; 1994: 3-26. Worksheet

Chapter 7
Epidemiology of *Escherichia coli* Serotype O157:H7

Escherichia coli O157:H7 is a recently recognized pathogen that causes a dysentery-like illness. The disease is typically a bloody diarrhea, often without prominent fever, that can be complicated by hemolytic uremic syndrome. It is primarily found in developed countries. Only one confirmed outbreak has occurred in a developing country–in Swaziland in 1992 affecting 20,000 persons. Other outbreaks have been thought to occur but have not been confirmed.

The major modes of transmission are through undercooked beef, unpasteurized milk, and foods that have come in contact with materials of animal origin. Waterborne outbreaks have been reported, as have outbreaks associated with swimming in contaminated lakes.

The organism produces toxins similar to those produced by *Shigella dysenteriae* serotype 1. Treatment with antimicrobial agents has not been demonstrated to be useful in improving the course or outcome of infection with *E. coli* O157:H7. In fact, treating with some agents may actually worsen the outcome. Since no treatment is recommended, it is not necessary to test the antimicrobial susceptibility of isolates of *E. coli* O157:H7.

Laboratories should be familiar with this organism and should periodically look for it in stools from patients with bloody diarrhea. It is not necessary to examine every stool submitted to the laboratory for *E. coli* O157:H7, but it should be considered in outbreaks of dysentery in which *Shigella* spp. are not isolated from the stools of patients with bloody diarrhea. Laboratory supplies required for diagnosis of *E. coli* O157:H7 are listed in Annex H.

Reference

World Health Organization. Prevention and control of enterohemorrhagic *Escherichia coli* (EHEC) infections. Report of a WHO Consultation. Geneva, Switzerland, 28 April-1 May 1997. WHO/FSF/FOS/97.6.

Chapter 8
Isolation and Identification of *Escherichia coli* Serotype O157:H7

Isolation and identification of *Escherichia coli* serotype O157:H7 can be greatly enhanced when optimal laboratory media and techniques are employed. The methods presented here are intended to be economical and to offer laboratorians some flexibility in choice of protocol and media. Laboratories that do not have sufficient resources to adopt the methods described below should consider sending specimens or isolates to other laboratory facilities that routinely perform these procedures. Laboratory supplies required for diagnosis of *E. coli* O157:H7 are listed in Annex H.

A. Isolation and Identification Methods

E. coli O157:H7 rapidly ferments lactose and is indistinguishable from most other *E. coli* serotypes on traditional lactose-containing media. However, unlike approximately 80% of other *E. coli*, nearly all isolates of *E. coli* O157:H7 ferment D-sorbitol slowly, or not at all. Sorbitol-MacConkey (SMAC) agar was developed to take advantage of this characteristic by substituting the carbohydrate sorbitol for lactose in MacConkey agar, and it is the medium of choice for isolation of *E. coli* O157:H7. Sorbitol-negative colonies will appear colorless on SMAC (Figure 8-1).

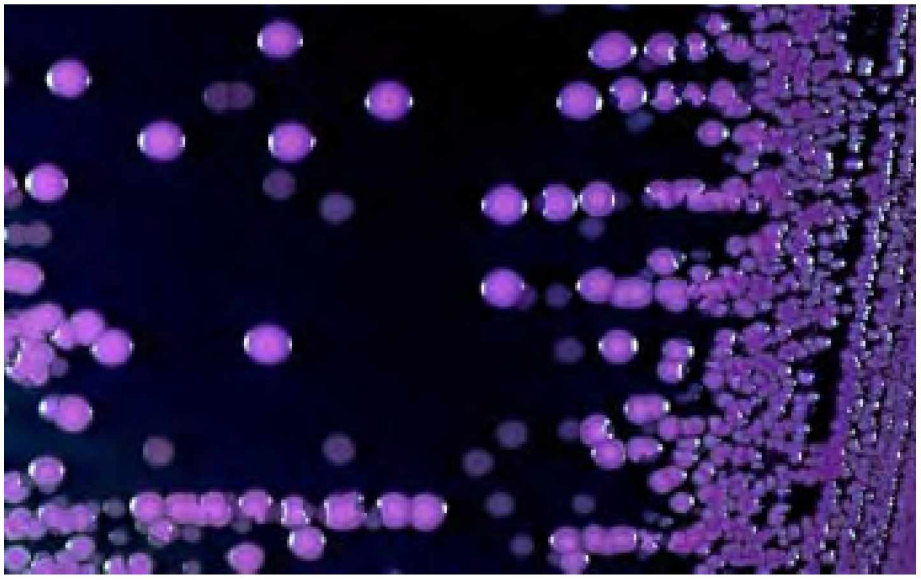


Figure 8-1. *E. coli* O157:H7 colonies are colorless on SMAC. Non-O157 *E. coli* colonies are pink.

Enrichment for *E. coli* O157:H7 is not usually necessary for isolation of the organism from acutely ill patients.

Figure 8-2 illustrates the procedure for recovery of *E. coli* O157:H7 from fecal specimens. SMAC is inoculated as described in Chapter 4 (Figure 4-2). Incubate 18 to 24 hours at 35° to 37°C. After 18 to 24 hours' incubation, the amount and type of growth (e.g., sorbitol-positive or sorbitol-negative) on SMAC should be recorded on data sheets for each patient specimen (Figure 8-3). Colonies suspicious of *E. coli* O157:H7 will appear colorless and about 2 to 3 mm in diameter (Figure 8-1).

Test sorbitol-negative colonies selected from SMAC with *E. coli* O157 antiserum or latex reagents (O157 antibody-coated latex and control latex) according to the procedures recommended by the manufacturer. Suspected colonies may be tested with antisera directly from the SMAC plate or subcultured to a nonselective medium (HIA, for example) and tested the next day. This provides more growth on which to perform the agglutination assay (however, some manufacturers of O157 latex reagents recommend testing only colonies taken directly from the plate). If colonies are tested directly from the plate, a colony that is positive in O157 latex reagent should also be transferred to another medium for subsequent testing. Once one colony from a plate has been identified as O157-positive, no further colonies from the same plate need to be tested.

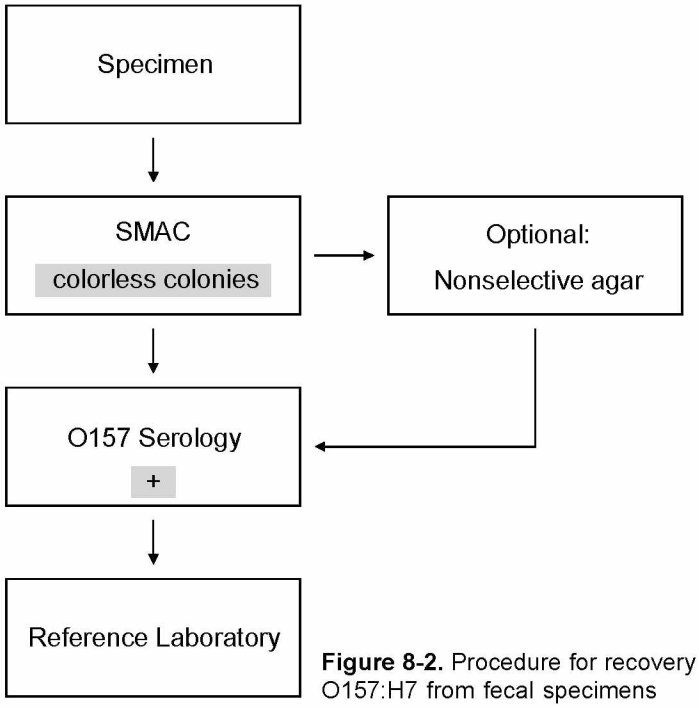


Figure 8-2. Procedure for recovery of *E. coli* O157:H7 from fecal specimens

***Escherichia coli* O157:H7 Worksheet**

SPECIMEN NUMBER	MEDIA	SORBITOL -	SORBITOL +	COLONY	SEROLOGY		BIOCHEMICAL ID *E. coli* YES/NO	PRESUMPTIVE IDENTIFICATION
					O157 LATEX	CONTROL LATEX		
	SMAC			SM1				
				SM2				
				SM3				
	SMAC			SM1				
				SM2				
				SM3				
	SMAC			SM1				
				SM2				
				SM3				
	SMAC			SM1				
				SM2				
				SM3				
	SMAC			SM1				
				SM2				
				SM3				
	SMAC			SM1				
				SM2				
				SM3				

Figure 8-3. *Escherichia coli* O157:H7 worksheet

If O157 latex reagent is used, it is important to test any positive colonies in the latex control reagent also; this is because some sorbitol-nonfermenting organisms will react nonspecifically with latex. The manufacturers of these kits recommend that strains reacting in both the antigen-specific and control latex reagents be heated and retested. However, in a study that used this procedure, none of the nonspecifically reacting strains were subsequently identified as *E. coli* O157:H7.

Isolates that are O157 positive should be sent to a reference laboratory for confirmation. The reference laboratory should identify isolates biochemically as *E. coli* because strains of several species cross-react with O157 antiserum. Identification of the H7 flagellar antigen is also required for confirmation. Isolates that are nonmotile or that are negative for the H7 antigen should be tested for production of Shiga toxins to identify pathogenic strains.

It is not necessary to test *E. coli* O157:H7 isolates for susceptibility to antimicrobial agents (see Chapter 7).

B. Preparation and Quality Control of Sorbitol-MacConkey agar

Prepare according to the manufacturer's instructions. [Note: Several brands of SMAC are available commercially. This medium can also be prepared from individual ingredients, but results may be much more variable than with a commercial dehydrated formulation.] Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and pour into petri plates. Leave lids ajar for about 20 minutes so that the surface of the agar will dry. Close lids and store at 4°C for up to 1 month. If medium is to be stored for more than a few days, put plates in a sealed plastic bag to prevent drying. Each new lot should be quality controlled before use.

E. coli should produce good to excellent growth of pink to red colonies. *E. coli* O157:H7 should produce colorless colonies.

References

Strockbine NA, Wells JG, Bopp CA, Barrett TJ. Overview of detection and subtyping methods. In: Kaper JB, O'Brien AD, ed. *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. Washington, DC: ASM Press; 1998: 331-356.

Bopp CA, Brenner FW, Wells JG, Strockbine NA. *Escherichia, Shigella,* and *Salmonella.* In: Murray PR, Pfaller MA, Tenover FC, Baron EJ, Yolken RH, ed. Manual of clinical microbiology, 7th ed. Washington, DC: ASM Press; 1999: 459-474.

Chapter 9
Antimicrobial Susceptibility Testing (Agar Disk Diffusion Method)

The disk diffusion method presented in this chapter has been carefully standardized by the National Committee for Clinical Laboratory Standards (NCCLS) and if performed precisely according to the protocol below, will provide data that can reliably predict the in vivo effectiveness of the drug in question. However, any deviation from the method may invalidate the results. For this reason, if laboratories lack the resources to perform the disk diffusion test exactly as described, they should forward isolates to other laboratories for susceptibility testing.

A. Considerations for Antimicrobial Susceptibility Testing

As antimicrobial resistance increases in many parts of the world, it becomes increasingly important to monitor the antimicrobial susceptibility of *Shigella* and *Vibrio cholerae* O1 and O139. However, because antimicrobial therapy for *Escherichia coli* O157:H7 infection has not been demonstrated to be efficacious or safe, except for cases of cystitis and pyelonephritis, determination of the antimicrobial susceptibility pattern is usually not meaningful.

See Chapters 3 and 5 for a discussion of appropriate antimicrobial agents for treatment of dysentery and cholera. Testing *Shigella*, *V. cholerae*, and *E. coli* O157:H7 against certain drugs may yield misleading results when in vitro results do not correlate with in vivo activity. *Shigella* spp., for instance, are usually susceptible to aminoglycosides (e.g., gentamicin, kanamycin) in the disk diffusion test, but treatment with these drugs is often not effective. Some special considerations for susceptibility testing of *V. cholerae* are discussed in section B below. Antimicrobial agents suggested for use in susceptibility testing of *Shigella* and *V. cholerae* are listed in Table 9-1.

B. Procedure for Agar Disk Diffusion

Figure 9-1 summarizes the disk diffusion method of susceptibility testing. Laboratory supplies required for *Shigella* and *V cholerae* disk diffusion testing are listed in Annexes A and B.

1. Mueller-Hinton susceptibility test agar

Mueller-Hinton agar medium is the only susceptibility test medium that has been validated by NCCLS. Mueller-Hinton agar should always be used for disk diffusion susceptibility testing, according to NCCLS and international guidelines. Because the way Mueller-Hinton is prepared can affect disk diffusion test results, it is very important to refer to Section C below for instructions on preparation and quality control of this medium.

Table 9-1. Antimicrobial agents suggested for use in susceptibility testing of *Shigella* and *V. cholerae* O1 and O139

Agents for *Shigella*	Agents for *V. cholerae*
Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole
Chloramphenicol	Chloramphenicol
Ampicillin	Furazolidone
Nalidixic acid[a]	Tetracycline[b]

[a] If resistant to nalidixic acid, test with ciprofloxacin.
[b] The results from the tetracycline disk are used to predict susceptibility to doxycycline also.

2. McFarland turbidity standard

A McFarland 0.5 standard should be prepared and quality controlled prior to beginning susceptibility testing (see Section C). If tightly sealed to prevent evaporation and stored in the dark, the standard can be stored for up to 6 months. The McFarland standard is used to adjust the turbidity of the inoculum for the susceptibility test.

3. Preparation of inoculum

Each culture to be tested should be streaked onto a noninhibitory agar medium (blood agar, brain heart infusion agar, or tryptone soy agar) to obtain isolated colonies. After incubation at 35°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline (see Section C) or nonselective broth (Mueller-Hinton broth, heart infusion broth, or tryptone soy broth) and vortex thoroughly. The bacterial suspension should then be compared to the 0.5 McFarland standard. This comparison can be made more easily if the tubes are viewed against a sheet of white paper on which sharp black lines are drawn (see Figures 9-2 and 9-3). The turbidity standard should be agitated on a vortex mixer immediately prior to use. If the bacterial suspension does not appear to be the same density as the McFarland 0.5, the turbidity can be reduced by adding sterile saline or broth or increased by adding more bacterial growth.

Alternatively, the growth method may be used to prepare the inoculum. Four or five colonies are picked from overnight growth on agar and inoculated into broth (Mueller-Hinton broth, heart infusion broth, or tryptone soy broth). Incubate the broth at 35°C until turbid, and then adjust the turbidity to the proper density.

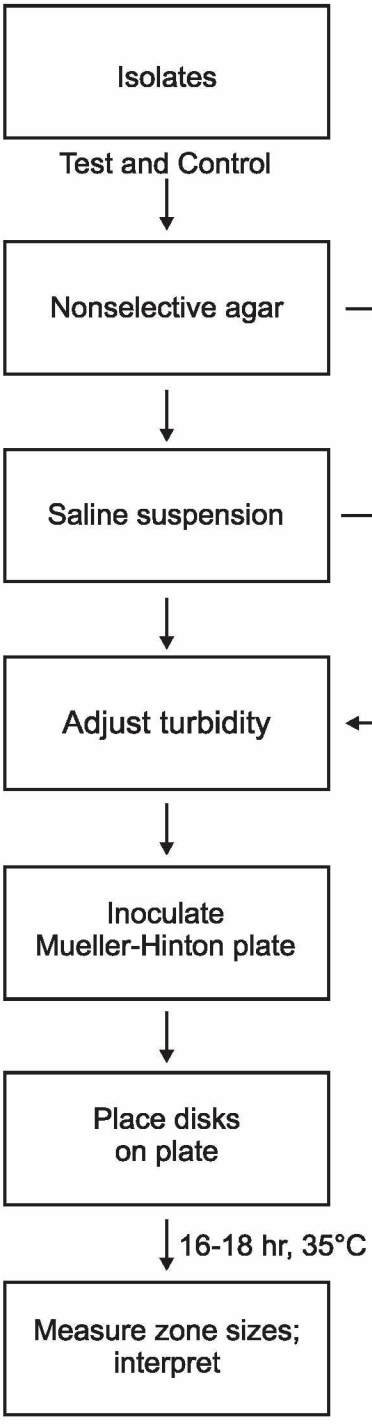


Figure 9-1. Procedure for disk diffusion antimicrobial susceptibility testing

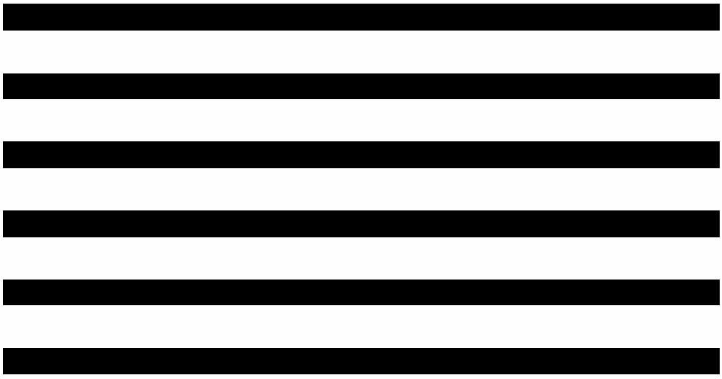


Figure 9-2. Background lines for viewing turbidity of inoculum

Figure 9-3. Comparison of McFarland 0.5 with inoculum suspension. From left to right, the tubes are the McFarland 0.5 standard, *E. coli* ATCC 25922 adjusted to the 0.5 McFarland turbidity, and uninoculated saline.

4. Inoculation procedure

Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of the medium three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum (Figure 9-4). Finally, swab all around the edge of the agar surface.

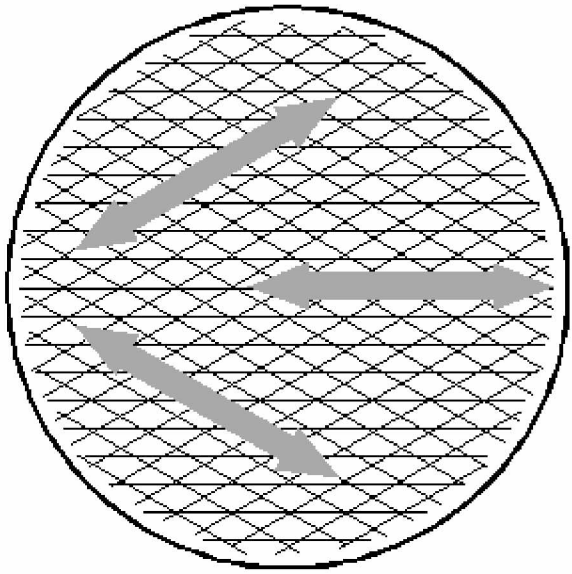


Figure 9-4. The Mueller-Hinton plate should be swabbed over the entire surface of the medium three times, rotating the plate 60 degrees after each application.

5. Antimicrobial disks

The working supply of antimicrobial disks should be stored in the refrigerator (4°C). Upon removal of the disks from the refrigerator, the package containing the cartridges should be left unopened at room temperature for approximately 1 hour to allow the temperature to equilibrate. This reduces the amount of condensation on the disks. If a disk-dispensing apparatus is used, it should have a tight-fitting cover, be stored in the refrigerator, and be allowed to warm to room temperature before using.

Table 9-2. Zone size interpretative standards for *Enterobacteriaceae* for selected antimicrobial disks

Antimicrobial agent	Disk potency (µg)	Zone diameter (mm)			Zone diameter limits (mm) for *E. coli* ATCC 25922
		Resistant	Intermediate	Susceptible	
Chloramphenicol[a,b]	30	≤12	13-17	≥18	21-27
Ampicillin[a]	10	≤13	14-16	≥17	16-22
Furazolidone[c] for *V. cholerae*	100	<18	–	≥18	22-26
Trimethoprim-sulfamethoxazole[a]	1.25/ 23.75	≤10	11-15	≥16	24-32
Tetracycline[a]	30	≤14	15-18	≥19	18-25
Ciprofloxacin[a, d]	5	≤15	16-20	≥21	30-40
Nalidixic acid[a]	30	≤13	14-18	≥19	22-28
Nalidixic acid[c] for *V. cholerae*	30	<19	–	≥19	

[a]Source: National Committee on Clinical Laboratory Standards (NCCLS), 1998.
[b]Use these interpretive standards with caution as the disk diffusion test may misclassify many organisms (high minor error rate)
[c]Proposed interpretive criteria based on multi-laboratory studies. Criteria have not been established for *V. cholerae* by NCCLS.
[d]These zone sizes are valid for interpreting disk diffusion results for *Shigella* and *Enterobacteriaceae*. However, zone sizes for *V. cholerae* have not been established by NCCLS.

Apply the antimicrobial disks to the plates as soon as possible, but no longer than 15 minutes after inoculation. Place the disks individually with sterile forceps or with a mechanical dispensing apparatus, and then gently press down onto the agar. In general, place no more than 12 disks on a 150-mm plate and no more than 4 disks on a 100-mm plate. This prevents overlapping of the zones of inhibition and possible error in measurement. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

6. Recording and interpreting results

After the disks are placed on the plate, invert the plate and incubate at 35°C for 16 to 18 hours. After incubation, measure the diameter of the zones of complete inhibition (including the diameter of the disk) (Figure 9-5) and record it in millimeters (Figures 9-6, 9-7). The measurements can be made with a ruler on the undersurface of the plate without opening the lid. With sulfonamides and trimethoprim-sulfamethoxazole, a slight amount of growth may occur within the inhibition zone. In this instance, slight growth (80% inhibition) should be ignored and the zone diameter should be measured to the margin of heavy growth. The zones of growth inhibition should be compared with the zone-size interpretative table (see Table 9-2), and recorded as susceptible, intermediate, or resistant to each drug tested.

Colonies growing within the clear zone of inhibition may represent resistant variants or a mixed inoculum. The distance from the colony(ies) closest to the disk to the center of the disk should be measured and then doubled to obtain a diameter. The diameter of the outer clear zone should be recorded as well and an interpretation recorded for each diameter. The colony(ies) inside the zone should be picked, re-isolated, re-identified, and retested in the disk diffusion test to confirm the previous results. The presence of colonies within a zone of inhibition may predict eventual resistance to that agent.

7. Quality control

To verify that susceptibility test results are accurate, it is important to include at least one control organism (ATCC 25922 is the *E. coli* control strain used when testing *Enterobacteriaceae* and *V. cholerae*) with each test. Zone diameters obtained for ATCC 25922 should be compared with NCCLS published limits (see Table 9-2 for diameters of the zones of inhibition for ATCC 25922). If zones produced by the control strain are out of the expected ranges, the laboratorian should consider possible sources of error.

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. Organisms may appear to be resistant to these drugs

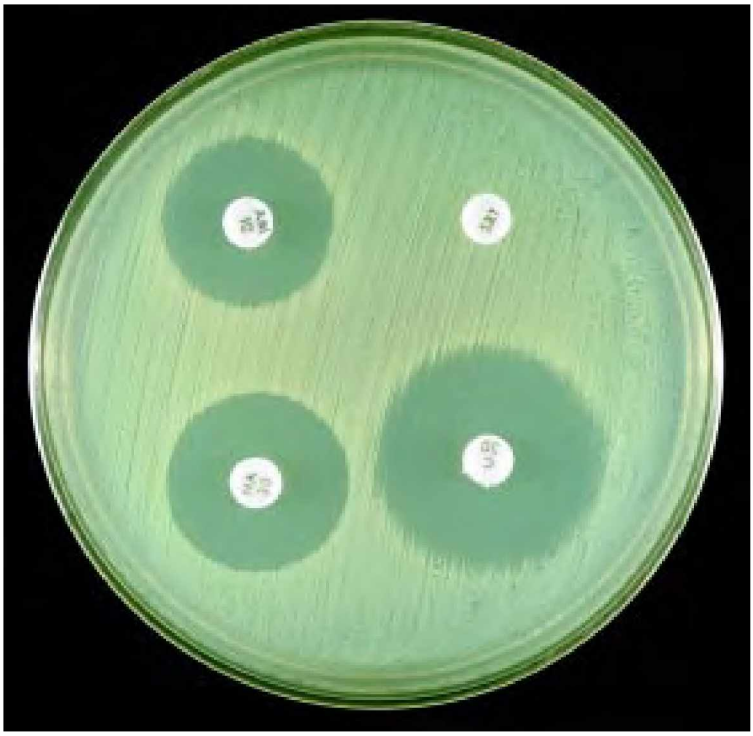


Figure 9-5. Results of the disk diffusion assay. This *Shigella* isolate is resistant to trimethoprim-sulfamethoxazole and is growing up to the disk (SXT), the zone of which is recorded as 6 mm.

when in fact they are not. If the depth of the agar in the plate is not 3 to 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.

If the inoculum is not a pure culture or does not contain a concentration of bacteria that approximates the McFarland standard, the 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 testing susceptible isolates.

If antimicrobial disks are not stored properly or are used beyond the stated expiration date, their potency may decrease; this will usually be indicated by a decrease in the size of the inhibition zone around the control strain.

Vibrio cholerae **Antimicrobial Susceptibility Results**

DATE TESTED:					
SPECIMEN NUMBER	ORGANISM	TETRACYCLINE	FURAZOLIDONE	TRIMETHOPRIM/SULFA	CHLORAMPHENICOL
ATCC 25922					

Figure 9-6. Sample worksheet for recording disk diffusion susceptibility results for *V. cholerae* O1 or O139

Shigella Antimicrobial Susceptibility Results

DATE TESTED:						
SPECIMEN NUMBER	ORGANISM	TRIMETHOPRIM/SULFA	CHLORAMPHENICOL	AMPICILLIN	NALIDIXIC ACID	CIPROFLOXACIN (optional)
ATCC 25922						

Figure 9-7. Sample worksheet for recording disk diffusion susceptibility results for *Shigella* isolates

As mentioned above, testing some bacteria against certain antimicrobial agents may yield misleading results because these in vitro results do not necessarily correlate with in vivo activity. Examples include narrow- and expanded-spectrum cephalosporins and aminoglycosides (e.g., gentamicin) tested against *Shigella* spp. (see Chapter 3), and erythromycin tested against *V. cholerae* (see section C below).

C. Special Considerations for Susceptibility Testing of *V. cholerae*

Although the disk diffusion technique is the most commonly used method for antimicrobial susceptibility testing, zone size interpretive criteria for *V. cholerae* O1 and O139 have been established only for ampicillin, chloramphenicol, sulfonamides, tetracycline and trimethoprim-sulfamethoxazole. It has been determined that disk diffusion results are not accurate for *V. cholerae* when testing erythromycin and doxycycline, and these agents should not be tested by this method. The results from the tetracycline disk should be used to predict susceptibility to doxycycline. If susceptible to tetracycline, the strain will be susceptible to doxycycline. At this time there is no in vitro method to accurately determine susceptibility to erythromycin.

The reliability of disk diffusion results for other antimicrobial agents, including ciprofloxacin, furazolidone and nalidixic acid, has not been validated. Until interpretive criteria have been established for *V. cholerae,* disk diffusion may be used to screen for resistance to ciprofloxacin, using interpretive criteria for the *Enterobacteriaceae* as tentative zone size standards. Tentative breakpoints have been proposed for testing furazolidone and nalidixic acid with *V. cholerae* (see Table 9-2). When screening with the disk diffusion method for these agents, results should be interpreted with caution. If zone sizes for these drugs fall within the intermediate range, the organism should be considered possibly resistant.

D. Preparation and Quality Control of Media and Reagents

1. Mueller-Hinton agar

[Note: Several commercial formulations of Mueller-Hinton agar are available. This medium should not be prepared from individual ingredients because this can diminish the quality. Commercial dehydrated Mueller-Hinton is carefully quality controlled before being released for sale.]

Follow manufacturer's instructions to prepare medium. After autoclaving, cool medium to 50°C. Measure 60 to 70 ml of medium per plate into 15 x 150-mm plates, or measure 25 to 30 ml per plate into 15 x 100-mm plates. Agar should be poured into flat-bottom glass or plastic petri dishes on a level pouring surface to a uniform depth of 4 mm. Using more or less agar will affect the susceptibility results. Agar deeper than 4 mm may cause false-resistance results, whereas agar less than 4 mm deep may be associated with a false-susceptibility report.

Freshly prepared plates may be used the same day or stored in a refrigerator (2° to 8°C) for up to 2 weeks. If plates are not used within 7 days of preparation, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is on the surface, plates should be placed in an incubator (35° to 37°C) until the moisture evaporates (usually 10 to 30 min). Do not leave lids ajar because the medium is easily contaminated.

Each new lot should be quality controlled before use by testing the *E. coli* ATCC 25922 and/or *Staphylococcus aureus* ATCC 25923 standard strains. These standard strains are used with every test run for *Enterobacteriaceae* and gram-positive aerobes, respectively. The pH of each new lot of Mueller-Hinton should be between 7.2 to 7.4. If outside this range, the pH medium should not be adjusted by the addition of acid or base; the batch of plates should be discarded and a new batch of plates prepared. If the pH for every batch is too high or low, the entire lot of dehydrated medium may have to be returned to the manufacturer as unsatisfactory.

2. Turbidity standards (McFarland)

McFarland 0.5 turbidity standards are available from various manufacturers. Alternately, the 0.5 McFarland may be prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 1% (vol/vol) sulfuric acid. The turbidity standard is then aliquoted into test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland standard tubes with wax, Parafilm, or some other means to prevent evaporation. McFarland standards may be stored for up to 6 months in the dark at room temperature (22° to 25°C). Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube.

The accuracy of the density of a prepared McFarland standard should be checked by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at a wavelength of 625 nm should be 0.08 to 0.1. Alternately, the accuracy of the McFarland standard may be verified by adjusting a suspension of a control strain (e.g., *E. coli* ATCC 25922) to the same turbidity, preparing serial 10-fold dilutions, and then performing plate counts (see Figure 9-8). The adjusted suspension should give a count of 10^8 colony forming units/ml.

3. Physiological saline

NaCl	8.5 g
Distilled water	1 liter

Dissolve NaCl in water, heating if necessary. May be sterilized by autoclaving or membrane filtration. Store at ambient temperature for up to 6 months with caps tightened to prevent evaporation.

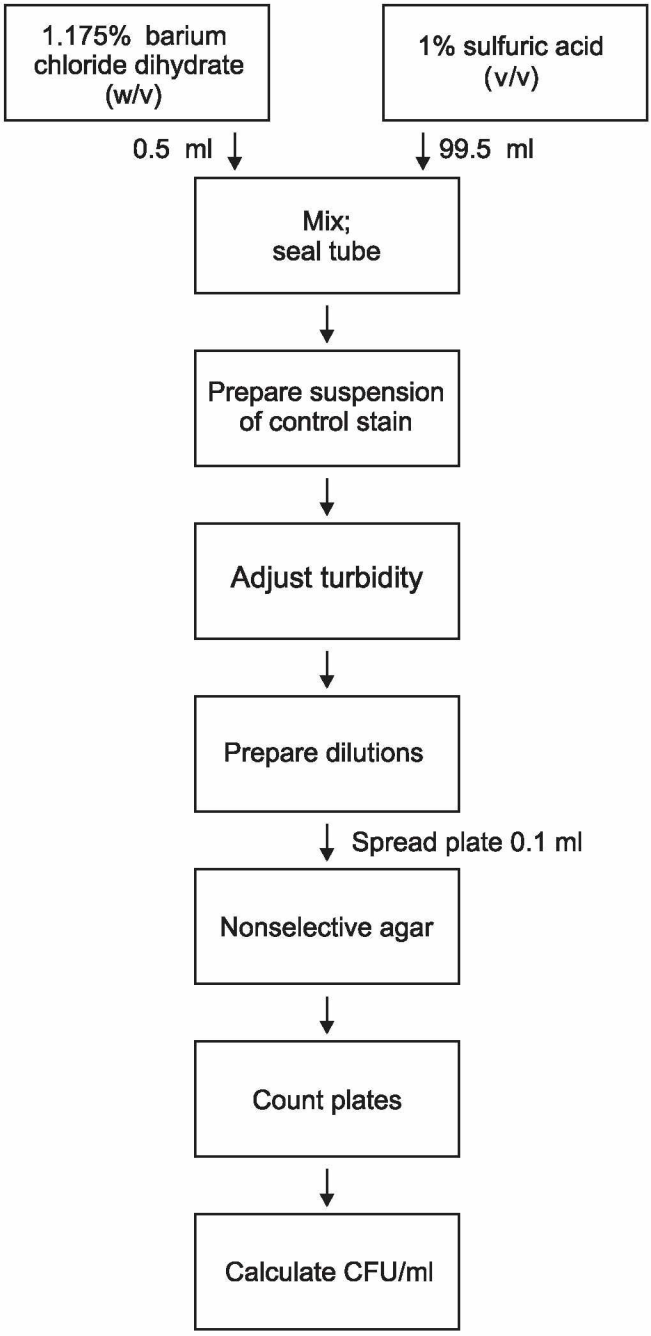


Figure 9-8. Procedure for preparation and quality control of the McFarland 0.5 standard

References

Jorgensen JH, Turnidge JD, Washington JA. Antibacterial susceptibility tests: dilution and disk diffusion methods. In: Murray PR, Pfaller MA, Tenover FC, Baron EJ, Yolken RH, ed. Manual of clinical microbiology, 7th ed.. Washington, DC: ASM Press; 1999:1526-1543.

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing; ninth informational supplement. Wayne, Pennsylvania: NCCLS; 1999: document M100-S9, Vol. 19. No. 1, Table 2I.

Chapter 10
Storage of Isolates

Shigella, Vibrio cholerae, or *Escherichia coli* serotype O157:H7 will usually remain viable for several days on solid medium held at ambient temperature (22° to 25°C) unless the medium dries out or becomes acidic. However, if cultures are to be maintained for longer than a few days, they should be appropriately prepared for storage. Selection of a storage method depends on the length of time the organisms are to be held and the laboratory equipment and facilities available.

A. Short-term Storage

Blood agar base (BAB), tryptone soy agar (TSA), and heart infusion agar (HIA) are examples of good storage media for enteric organisms. Carbohydrate-containing media (e.g., Kligler iron agar or triple sugar iron agar) should not be used because acidic byproducts of metabolism quickly reduce viability. BAB, TSA, and HIA all contain salt, which enhances growth of *V. cholerae*. Nutrient agar should not be used for growth or storage of *V. cholerae* since it has no added salt.

When preparing storage medium, while the tubes are still hot after autoclaving, place them in a slanted position to provide a short slant and deep butt (2 to 3 cm). To inoculate, stab the inoculating needle to the butt of the medium once or twice, then streak the slant. Incubate overnight at 35° to 37°C. Seal the tube with cork stoppers that have been soaked in hot paraffin or treated in some other way to provide a tight seal. Store cultures at 22° to 25°C in the dark.

Sterile mineral oil may also be used to prevent drying of slants. Add sufficient sterile mineral oil to cover the slants to 1 cm above the top of the agar. Subculture when needed by scraping growth from the slant; there is no need to remove mineral oil to subculture. Strains maintained in pure culture in this manner will usually survive for several years.

B. Long-term Storage

Bacterial cultures may be stored frozen or lyophilized in a variety of suspending media formulated for that purpose. There are many formulations of suspending medium, but in general, skim milk, serum-based media, or polyvinylpyrrolidone (PVP) medium is used for lyophilization, and skim milk, blood, or a rich buffered broth such as tryptone soy broth with 15% to 20% reagent grade glycerol is used for freezing.

Frozen storage (ultralow freezer, -70°C; or liquid nitrogen freezer, -196°C)

Isolates may be stored indefinitely if they are maintained frozen at -70°C or below. Storage at -20°C is not recommended because some organisms will lose viability at this temperature.

- Inoculate a TSA or HIA slant (or other noninhibitory, salt-containing growth medium) and incubate overnight at 35° to 37°C.
- Harvest cells from the slant and make a suspension in freezing medium.
- Dispense suspension into cryovials (freezing vials specially designed for use at very low temperatures). **Caution**: Do not use glass ampoules for freezing in liquid nitrogen because they can explode upon removal from the freezer.
- Prepare an alcohol and dry ice bath by placing dry ice (frozen CO_2) in a leakproof metal container large enough to hold a metal culture rack, and add enough ethyl alcohol to submerge about half of the cryovial. Rapidly freeze the suspension by placing the sealed vials in the dry ice bath until frozen. Transfer the frozen vials to a freezer. If there is no dry ice available, a container of alcohol may be placed in the freezer overnight and then used to quick-freeze vials.

Recovery of cultures from frozen storage

- Place frozen cultures from the freezer on dry ice or into an alcohol and dry ice bath and transfer to a laboratory safety cabinet or to a clean area if a cabinet is not available.
- Using a sterile loop, scrape the topmost portion of the culture and transfer to growth medium, being careful not to contaminate the top or inside of the vial.
- Reclose vial before the contents completely thaw, and return vial to the freezer. With careful technique, transfers can be successfully made from the same vial several times.

Lyophilization

Most organisms may be successfully stored after lyophilization (freeze-drying). Freeze-drying involves the removal of water from frozen bacterial suspensions by sublimation under reduced pressure. Freeze-dried cultures are best maintained at 4°C.

Chapter 11
Quality Control of Media and Reagents

Each laboratory must ensure adequate control of the media and reagents it uses. Quality control includes the selection of satisfactory raw materials, the preparation of media according to approved formulations or specific manufacturer's instructions, and the use of well-characterized reference strains to check prepared media.

A. Quality Control of Media

1. Considerations for quality control of media

Each batch of medium prepared from individual ingredients or each different manufacturer's lot number of dehydrated medium should be tested for one or more of the following characteristics, as appropriate:

- Sterility
- Ability to support growth of the target pathogen(s)
- Ability to produce appropriate biochemical reactions

Sterility

One tube or plate from each autoclaved or filter-sterilized batch of medium should be incubated overnight at 35° to 37°C and examined for contaminants.

Ability to support growth of the target organism(s)

- For selective media: use at least one strain to test for ability to support growth of the target pathogen (e.g., for MacConkey agar (MAC), a *Shigella* strain such as *S. flexneri*); it should also be noted if this strain produces the appropriate biochemical reactions/color on the test medium (see below).

Ability to produce appropriate biochemical reactions

- For selective media: use at least one pathogen and one nonpathogen to test for the medium's ability to differentiate target organisms from competitors (e.g., for MAC, a lactose-nonfermenting organism such as *S. flexneri* and a lactose-fermenting organism such as *E. coli*).
- For biochemical media: use at least one organism that will produce a positive reaction and at least one organism that will produce a negative reaction (e.g., for urea medium, a urease-positive organism such as *Proteus* and a urease-negative organism such as *E. coli*).

2. Methods for quality control of media

When testing for ability to support growth, to avoid using too heavy an inoculum, prepare a dilute suspension to inoculate the medium. A small inoculum will

give greater assurance that the medium is adequate for recovery of a small number of organisms from a clinical specimen. An example of a protocol for quality control of media is given here:

- The control strain is inoculated to nonselective broth (e.g., tryptone soy broth) and grown up overnight.
- To prepare a standardized inoculum for testing selective and inhibitory media, make a 1:10 dilution of the overnight nonselective broth culture. If testing nonselective media, prepare an additional 1:10 dilution (to give a 1:100 dilution of the broth).
- One tube or plate of each medium should be inoculated with the standardized inoculum of the control strain(s). When testing selective plating media, a nonselective plating medium such as heart infusion agar should be inoculated at the same time for comparison purposes.
- Using a loopful of the 1:10 or 1:100 dilution prepared above (use a calibrated loop, if available) inoculate media to be tested, streaking for isolation on plating media. The same loop should be used for all quality control of all media; it is more important to use the same inoculating loop every time than it is to use a calibrated loop.

3. Sources of quality control strains

Suitable quality control strains may be obtained in several different ways. A laboratory may use strains isolated from clinical specimens or quality assurance specimens, provided the strains have been well characterized by all available methods (e.g., biochemical, morphologic, serologic, molecular). Many laboratories purchase quality control strains from official culture collections, such as the National Collection of Type Cultures (Public Health Laboratory Service, London NW9, England) or the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852). Quality control strains also may be purchased from commercial companies such as Lab M (Topley House, 52 Wash Lane, Bury, BL9 6AU, England).

B. Quality Control of Reagents

As with all other products used in testing, reagents, either purchased or prepared in the laboratory, should be clearly marked to indicate the date on which they were first opened and the expiration date, if appropriate. Each reagent should be tested to make sure the expected reactions are obtained. If the reagent is a rare, expensive, or difficult-to-obtain product such as diagnostic antiserum, it does not necessarily have to be discarded on the expiration date. If satisfactory sensitivity and specificity can still be verified by normal quality control procedures, the laboratory may indicate on the vial label the date of verification of quality of the reagent. All reagents should be tested for quality at intervals established by each laboratory to ensure that no deterioration has occurred.

Slide agglutination method for quality control of antisera

For quality control of antiserum, two or more control strains (one positive and one negative) should be used to test its agglutination characteristics. The results of all reactions should be recorded. Following is an example of a typical quality control procedure.

- Place a drop (about 0.05 ml) of each antiserum on a slide or plate. Also, place a drop of 0.85% saline on each slide or plate to test each antigen for roughness or autoagglutination.
- Prepare a densely turbid suspension (McFarland 2 or 3; see Table 11-1) of each control isolate in normal saline with growth aseptically harvested from an 18- to 24-hour culture from nonselective agar (for example, heart infusion agar or tryptone soy agar).
- Add one drop of the antigen suspension to the antiserum and the saline. Mix thoroughly with an applicator stick, glass rod, or inoculating loop. Rock the slide back and forth for 1 minute.
- Read the agglutination reaction over a light box or an indirect light source with a dark background. The saline control must be negative for agglutination for the test to be valid.

The degree of agglutination should be read and recorded as follows:

Percent agglutination	Record reaction as:
100	4+
75	3+
50	2+
25	1+
0	negative

C. Advantages of Centralized Acquisition of Media and Reagents

There are several benefits of centralizing acquisition of media and reagents in the national reference laboratory or Ministry of Health:

- Large amounts of a single lot of medium or reagent can be purchased and subsequently divided into smaller aliquots for distribution to provincial/district laboratories. This may be more cost effective (i.e., discount for larger orders, lower shipping costs, less waste because of product going past expiration date).
- Quality control can be performed in the central laboratory, avoiding duplication of effort among provincial and district laboratories. An unsatisfactory medium or reagent may then be returned to the manufacturer before the lot is distributed to other laboratories.
- The standardization of methods among laboratories at all levels is facilitated by use of single lots of media.

Table 11-1. Composition of McFarland turbidity standards

Turbidity standard no.	Barium chloride dihydrate (1.175 %)	Sulfuric acid (1%)	Corresponding approximate density of bacteria/ml
0.5	0.5 ml	99.5 ml	1×10^8
1	0.1 ml	9.9 ml	3×10^8
2	0.2 ml	9.8 ml	6×10^8
3	0.3 ml	9.7 ml	9×10^8
4	0.4 ml	9.6 ml	12×10^8
5	0.5 ml	9.5 ml	15×10^8
6	0.6 ml	9.4 ml	18×10^8
7	0.7 ml	9.3 ml	21×10^8
8	0.8 ml	9.2 ml	24×10^8
9	0.9 ml	9.1 ml	27×10^8
10	1.0 ml	9.0 ml	30×10^8

Chapter 12
Standard Safety Practices in the Microbiology Laboratory

Laboratorians working with infectious agents are subject to laboratory-acquired infections through accidents or unrecognized incidents. The degree of hazard depends upon the virulence of the biological agent concerned and host resistance. Laboratory-acquired infections occur when microorganisms are inadvertently ingested, inhaled, or introduced into the tissues. The primary laboratory hazard associated with enteric pathogens such as *Shigella* and *E. coli* O157:H7 is accidental ingestion. Biosafety Level 2 (BSL-2) practices are suitable for work involving these agents, which are a moderate potential hazard to personnel and the environment. BSL-2 requirements:

- Laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists;
- Access to the laboratory is limited when work is being conducted;
- Extreme precautions are taken with contaminated sharp items;
- Certain procedures in which infectious aerosols or splashes may be created are conducted using protective clothing and equipment.

A. Standard Microbiological Safety Practices

The safety guidelines listed below apply to all microbiology laboratories regardless of biosafety level.

Limiting access to laboratory

Biohazard signs or stickers should be posted near all laboratory doors and on all equipment (incubators, hoods, refrigerators, freezers) used for laboratory work. Children under 12 years of age and pets are not allowed in laboratory areas. All laboratories should be locked when not in use. All freezers and refrigerators located in corridors should be locked.

Handwashing

Each laboratory should contain a sink for handwashing. Frequent handwashing is one of the most effective procedures for avoiding laboratory-acquired infections. Hands should be washed with an appropriate germicidal soap before exiting the laboratory or after handling infectious materials.

Eating

Eating, drinking, and smoking are not permitted in the work areas. Food must be stored and eaten outside of the work area in designated areas used for that purpose only. Do not lay personal articles such as handbags or eyeglasses on the workstations.

Mouth pipetting

Mouth pipetting should be strictly prohibited in the laboratory. Rubber bulbs or mechanical devices should be used.

Sharps

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

Aerosols

Perform all procedures carefully to minimize the creation of splashes or aerosols. Techniques that tend to produce aerosols should be avoided. Cool inoculating wires and loops by holding them still in the air for 5 to 10 seconds before touching colonies or clinical material. Loops containing infectious material should be dried in the hot air above the burner before flaming. Vortexing and centrifugation should be done in closed containers. Gauze should be used to remove the tops on blood specimens and should be placed around the top of blood culture bottles to minimize aerosol production during removal of the needle. Needles should never be cut or removed from the syringe before autoclaving. All body fluids should be centrifuged in carriers with safety caps only.

When procedures with a high potential for creating infectious aerosols are conducted or when there is a risk of splashing or spraying the face with infectious or other hazardous materials, laboratory work should be conducted in a safety cabinet or with face protection (goggles, mask, face shield or other splatter guards). Procedures that pose a risk may include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of infectious materials whose internal pressures may be different from ambient pressures, inoculating animals intranasally, and harvesting infected tissues from animals or eggs. Face protection should also be used when working with high concentrations or large volumes of infectious agents.

Decontaminating bench tops and other surfaces

Bench tops should be wiped with a disinfectant (a phenolic disinfectant, 1% sodium hypochlorite, or 70% alcohol) routinely after working with infectious agents or clinical specimens or after spills, splashes, or other contamination by infectious materials. Solutions of disinfectants should be maintained at the work station (see *Disinfectants* below).

Disposal of contaminated materials

All discarded plates, tubes, clinical samples or other contaminated materials are to be placed in disposal containers at each bench. Special disposal boxes must be used for sharps such as syringes or broken glass to minimize the risk of injury. Avoid overfilling such containers. Containers of contaminated material should be carefully transported to the autoclave room and autoclaved before disposal.

Autoclaving

An autoclave must be available for the BSL-2/3 laboratory and must be operated only by personnel who have been properly trained in its use. To verify that each autoclave is working properly, spore strips or other biological indicators designed to test for efficiency of sterilization should be included in autoclave loads on a regular basis. Each autoclave load should be monitored with temperature-sensitive tape, thermograph, or other means (e.g., biological indicators).

General laboratory policies

All areas of the laboratory must be kept clean and orderly. Dirt, dust, crowding, or clutter is a safety hazard and is not consistent with acceptable biological research. Floors should be kept clean and free of unnecessary clutter. They should be washed with a germicidal solution on a regular basis and after any spills of infectious material have occurred.

Refrigerators and freezers

Refrigerators and freezers should be regularly inspected for the presence of broken vials or tubes containing infectious agents. Wear gloves and proper attire when removing and discarding broken material. Refrigerators and freezers should be regularly cleaned with a disinfectant and defrosted to prevent possible contamination and temperature failure.

Fire prevention

Keep burners away from lamps and flammable materials. Bulk flammable material must be stored in the safety cabinet. Small amounts of these materials, such as ethyl acetate, ethyl alcohol, and methanol, can be stored in safety containers. Turn off burners when not in use. Know the location of fire extinguishers, fire blankets, and showers. Fire safety instructions and evacuation routes should be posted.

B. Special Practices

Transport of biohazardous materials

Transport of biohazardous materials from one building to another increases the risk of breakage and spills. If transport is necessary, the primary infectious agent container (regardless of size) must be placed in an unbreakable second container that can be sealed (e.g., screw-top tube, plastic bag).

Disinfectants

Organisms may have different susceptibilities to various disinfectants. As a surface disinfectant, 70% alcohol is generally effective for the *Enterobacteriaceae*, but other organisms are more resistant. However, 70% alcohol is not the disinfectant of choice for decontaminating spills. Phenolic disinfectants, although expensive, are usually effective against many organisms. Always read disinfectant labels for manufacturers' recommendations for dilution and for exposure times for efficacy, especially before use on BSL-3 organisms such as *Mycobacterium tuberculosis*. A good general disinfectant is a 1:100 (1%) dilution of household bleach in water; at this dilution, bleach can be used for wiping surfaces of benches, hoods and other equipment. A 1:10 (10%) dilution of bleach is corrosive and will pit stainless steel and should not be used routinely; however, it may be used to clean up spills of cultured or concentrated infectious material where heavy contamination has occurred. **Dilutions of sodium hypochlorite should be made daily from a stock solution.**

Decontamination of spills

The following procedure is recommended for decontaminating spills. Isolate the area to prevent anyone from entering. Wear gloves and protective clothing (gown or lab coat; mask if the spill may contain a respiratory agent or if the agent is unknown). Absorb or cover the spill with disposable towels. Saturate the towels with an appropriately diluted intermediate or high level disinfectant (e.g., a phenolic formulation or household bleach). Place disinfectant-soaked towels over the area and leave them in place for at least 15 minutes before removing and discarding them. Wipe area using clean disinfectant-soaked towels and allow area to air dry. Place all disposable materials used to decontaminate the spill into a biohazard container. Handle the material in the same manner as other infectious waste.

Accidents

All injuries or unusual incidents should be reported immediately to the supervisor. When cuts or puncture wounds from potentially infected needles or glassware occur, the affected area should be promptly washed with disinfectant soap and water. In the event of a centrifuge accident in which safety carriers have not been used, other personnel in the area should be warned immediately and the area isolated to prevent anyone from entering.

C. Protective Clothing and Equipment

Laboratory coats

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

Gloves

Regardless of the type of infectious material, gloves should be worn when performing potentially hazardous procedures (e.g., slide agglutination) in which there is a risk of splashing or skin contamination or when the laboratory worker has cuts or broken skin on his or her hands. Gloves should always be worn when handling clinical specimens, body fluids, and tissues from humans and animals. These tissues should be assumed to be positive for hepatitis B virus, HIV, other bloodborne pathogens, or *Mycobacterium tuberculosis*. Gloves must be removed when contaminated by splashing or spills or when work with infectious materials is completed. Gloves should not be worn outside the laboratory. Do not use the telephone or open doors with gloves that have been used in laboratory procedures. Dispose of all used gloves by discarding them with other disposable materials and autoclaving. Hands should be washed immediately after removing gloves.

Barrier precautions

Clinical specimens, body fluids, and tissues from humans and animals should be assumed to be positive for hepatitis B virus, HIV, other bloodborne pathogens, or *Mycobacterium tuberculosis.* These materials should be handled in a safety cabinet or using other barrier precautions such as goggles, mask, face shield or other splatter guards whenever there is a potential for creating an aerosol.

References

Centers for Disease Control and Prevention, National Institutes of Health. Biosafety in microbiological and biomedical laboratories. Washington, DC: U.S. Government Printing Office; 1999: stock no. 017-040-00547-4.

World Health Organization. Laboratory biosafety manual, 2nd edition. Geneva: WHO; 1993: ISBN 92 4 154450 3.

Chapter 13
Packing and Shipping of Clinical Specimens and Etiologic Agents

A. Preparation for Transport of Infectious Specimens and Cultures

Transport of clinical specimens and etiologic agents should be done with care to minimize the hazard to humans or the environment and also to protect the viability of suspected pathogens. Transport of infectious items by public or commercial delivery systems may be subject to local or national regulations.

If possible, send specimens so that they will arrive during working hours to ensure proper handling and prompt plating of the specimens. Inform the receiving laboratory as soon as possible that the specimens are coming, preferably before sending the specimens.

Depending on local conditions, within-country transport may be by ground or by air. If specimens are sent by a messenger, the messenger must know the location of the laboratory and the appropriate person to contact. The sender should identify the fastest and most reliable way of transport in advance, whether it be by bicycle, motorcycle, car, ambulance or public transport, and should make sure that adequate funds are available to reimburse costs for fuel or public transport. For longer distances, the fastest transport service may be air freight or expedited delivery service. Since the ice packs or dry ice will last only 24 to 48 hours, arrangements should be made for immediate collection at the receiving airport. When the specimens are shipped by air, the following information should be communicated immediately to the receiving laboratory: the air bill number, the flight number, and the times and dates of departure and arrival of the flight.

B. Transport and Shipment of Cultures and Specimens

1. Regulatory organizations

The United Nations Committee of Experts on the Transport of Dangerous Goods is continually developing recommendations for the safe transport of dangerous goods. The International Civil Aviation Organization (ICAO) has used these recommendations as the basis for developing regulations for the safe transportation of dangerous goods by air. The regulations of the International Air Transport Association (IATA) contain all the requirements of the ICAO Technical Instructions for the Safe Transport of Dangerous Goods. However, IATA has included additional requirements that are more restrictive than those of ICAO. Member airlines of the IATA have adopted the use of the IATA regulations governing dangerous goods, and shippers must comply with these regulations in addition to any applicable regulations of the state of origin, transit, or destination.

The shipment of infectious agents or diagnostic specimens by air must comply with local, national and international regulations. International air transport regulations may be found in the IATA publication entitled, *Dangerous Goods Regulations*. This reference is published annually in January, and frequently the regulations change from year to year. A copy of the IATA regulations in English, Spanish, French, or German may be obtained from one of the regional offices listed below.

Orders from the Americas, Europe, Africa, and the Middle East:

Customer Service Representative
International Air Transport Association
800 Place Victoria
P.O. Box 113
Montreal, Quebec
CANADA H4Z 1M1
Telephone: 1-514-390-6726
FAX: 1-514-874-9659
Teletype: YMQTPXB

Orders from Asia, Australasia, and the Pacific:

Customer Service Representative
International Air Transport Association
77 Robinson Rd.
No. 05-00 SIA Bldg.
SINGAPORE 068896
Telephone: +65-438-4555
FAX: +65-438-4666
Telex: RS 24200 TMS Ref: TM 2883
Cable: IATAIATA
Teletype: SINPSXB

Internet Orders:

sales@iata.org

Internet Information:

www.iata.org

2. Shipping regulations for infectious substances and clinical/ diagnostic specimens

In general, all packages that are being shipped by air via commercial and cargo carriers such as Federal Express and passenger aircraft are affected by the IATA regulations. These regulations are outlined below as an example of acceptable packaging procedures for infectious materials. However, because they may not reflect current national or IATA requirements for packaging and labeling for

infectious substances, anyone packaging isolates or infectious specimens should consult the appropriate national regulations and the current edition of `Dangerous Goods Regulations` before packing and shipping infectious substances by any means of transport.

Definition of infectious substances

Infectious substances are defined as substances known to contain, or reasonably expected to contain, pathogens. Pathogens are microorganisms (including bacteria, viruses, rickettsia, parasites, fungi) or recombinant microorganisms (hybrid or mutant) that are known or reasonably expected to cause infectious disease in humans or animals.

Classification of clinical/diagnostic specimens

- Specimens (human, animal, food, environmental, etc.) known or reasonably expected to contain pathogens are now to be classified as infectious substances. When these specimens are transported/shipped for any purpose, including initial or confirmatory testing for the presence of pathogens, they are to be packaged and shipped as infectious substances (see below).
- Specimens that have a relatively low probability of containing pathogens are to be classified as clinical/diagnostic specimens. When these specimens are transported/shipped for the purpose of routine screening tests or initial diagnosis for other than the presence of pathogens, they are to be packaged and shipped as clinical/diagnostic specimens.
- Those specimens known not to contain pathogens are to be packaged and shipped as nonrestricted, i.e., packaging and shipping is not regulated. They are to be packaged in watertight primary containers and leakproof secondary containers.

Unless it has been specifically determined, i.e., by testing, that a clinical/diagnostic specimen does not contain a pathogen(s), it is considered to fall within the categories either of those specimens known or reasonably expected to contain pathogens or those specimens that have a relatively low probability of containing pathogens.

Guidelines for packaging and labeling infectious substances

Persons who ship infectious agents or diagnostic specimens must comply with all local and international regulations pertaining to the packaging and handling of these items. They must ensure that specimens arrive at their destination in good condition and that they present no hazard to persons or animals during shipment.

The inner packaging must include the following:

- An inner watertight primary container that is glass, metal, or plastic and has a leakproof seal. **Petri plates should not be shipped.**
- A watertight, impact-resistant secondary container
- Absorbent material between the primary container and the secondary

container. If multiple primary containers are placed in a single secondary packaging, they must be wrapped individually to ensure that contact between them is prevented. The absorbing material, such as cotton wool, must be sufficient to absorb the entire contents of all primary containers.
- An itemized list of contents between the secondary packaging and the outer packaging

Multiple primary receptacles placed in a single secondary packaging must be wrapped individually, or for infectious substances transported in liquid nitrogen, separated and supported to ensure that contact between them is prevented. The absorbing material must be sufficient to absorb the entire contents of all primary receptacles.

The outer packaging must meet the following requirements:

- Be of sufficient strength to adequately protect and contain the contents
- Be at least 100 mm (4 inches) in its smallest overall external dimension
- Be durably and legibly marked on the outside with the address and telephone number of the consignee. A biohazard warning label must be affixed to the outside of the outer container, and must bear the inscription, "Infectious substance. In case of damage or leakage immediately notify public health authority." Packaging for infectious substances must be marked with United Nations specification markings denoting that the packaging has been tested and certified for shipping infectious substances.

Figure 13-1 illustrates these packaging recommendations.

Guidelines for packaging and labeling clinical/diagnostic specimens not for microbiologic examination

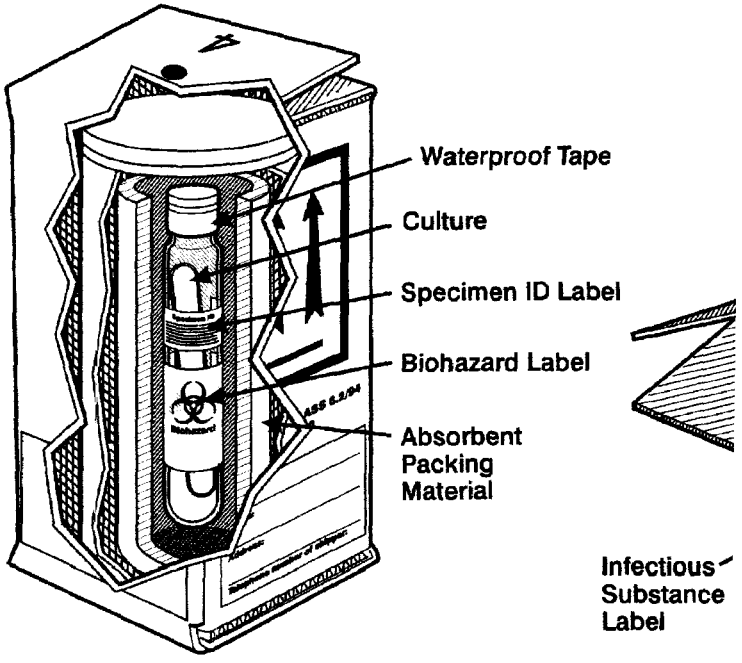
Clinical specimens with a low probability of containing an infectious agent that are not being transported for examination for the presence of pathogens must be packaged as follows:

- Be "triple packaged" as described above for infectious agents
- Be in packaging that will not leak after a 4-foot drop test
- Have a "Clinical Specimens" label affixed to the outside of the outer container
- If being shipped by air, bear the following statement, "Contents not restricted, packed in compliance with IATA packing instruction 650."

Figure 13-2 illustrates these packaging recommendations.

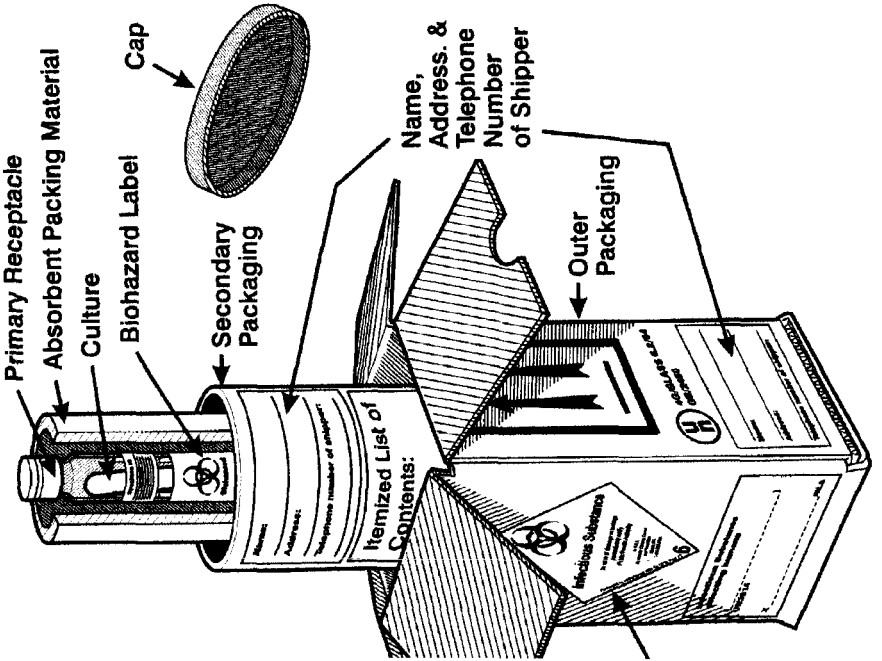
Reference

International Air Transport Association. 1999. Annual publication. Dangerous goods regulations. Montreal, Quebec, Canada: IATA Publications Office.



Cross Section of Proper Packing

Figure 13-1. Packing and labeling of infectious substances


Primary Receptacle
Absorbent Packing Material
Culture
Biohazard Label
Cap
Secondary Packaging
Name, Address, & Telephone Number of Shipper
Outer Packaging
Itemized List of Contents:
Infectious Substance

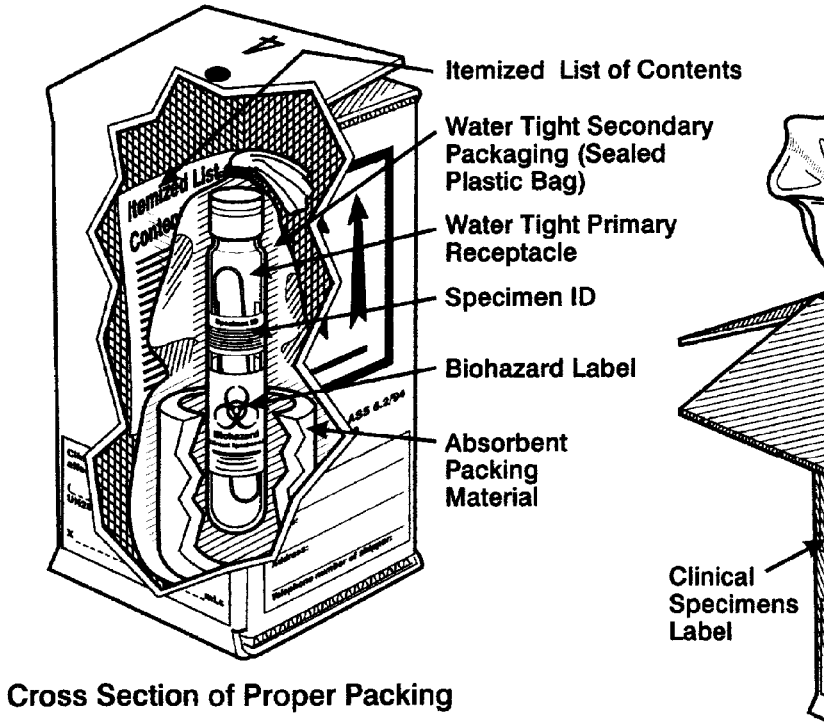
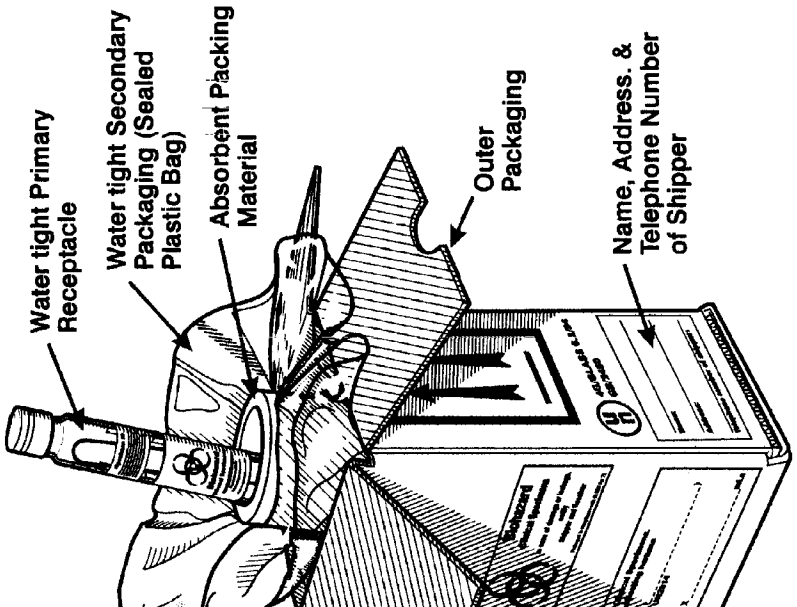


Figure 13-2. Packing and labeling of clinical specimens


Water tight Primary Receptacle
Water tight Secondary Packaging (Sealed Plastic Bag)
Absorbent Packing Material
Outer Packaging
Name, Address, & Telephone Number of Shipper

Annex A
Diagnostic Supplies Needed for 1 Year for Laboratory Confirmation of Outbreaks and for Laboratory-Based Surveillance for *Vibrio cholerae* O1/O139 Antimicrobial Susceptibility

Assumptions:

- Supply each district with materials to collect and transport 50 specimens
- Supply each regional laboratory with materials to process 100 specimens
- Supply each national reference laboratory with materials to confirm 500 isolates

District Level

Supplies needed for each district

50 cotton swabs
50 bottles or tubes of Cary-Blair (or other) transport medium
Transport for specimens to regional laboratory

Regional Level

Supplies needed for each regional laboratory

100 sterile cotton or polyester swabs
500 g Cary-Blair medium
500 g TCBS medium
25 g sodium desoxycholate
Glass slides for serologic testing and string test
5 g *N,N,N′N′*-tetramethyl-D-phenylenediamine dihydrochloride (oxidase reagent)
Filter paper for oxidase test
Sterile wooden sticks or platinum inoculating loops for oxidase test
500 g nonselective agar* (e.g., tryptone soy agar, heart infusion agar)
4 x 2 ml polyvalent *V. cholerae* O1 diagnostic antiserum
500 g Bacto-peptone medium
500 g NaCl
NaOH
pH paper or pH meter
500 petri dishes (9 cm)
1000 test tubes (e.g., 13 x 100 mm or 16 x 125 mm)
Transport for specimens to reference laboratory
Materials and postage for production and dissemination of reports

*Do not use nutrient agar because some formulations have no added salt and do not allow optimal growth of *V. cholerae*.

National Reference Laboratory

Supplies needed by each national reference laboratory for confirmation

5 x 100 sterile cotton or polyester swabs
5 x 500 g Cary-Blair medium
5 x 500 g TCBS medium
5 x 25 g sodium desoxycholate
Glass slides for serologic testing and string test
5 x 5 g *N,N,N′N′*-tetramethyl-D-phenylenediamine dihydrochloride (oxidase reagent)
Filter paper
Sterile wooden sticks or platinum inoculating loops
5 x 500 g nonselective agar* (e.g., tryptone soy agar, heart infusion agar)
20 x 2 ml polyvalent *V. cholerae* O1 diagnostic antiserum
5 x 2 ml *V. cholerae* O139 diagnostic antiserum
5 x 2 ml *V. cholerae* O1 serotype Ogawa diagnostic antiserum
5 x 2 ml *V. cholerae* O1 serotype Inaba diagnostic antiserum
5 x 500 g Bacto-peptone medium
5 x 500 g NaCl
NaOH
pH paper or pH meter
5 x 500 petri dishes (9 cm)
5 x 1000 test tubes (e.g., 13 x 100 mm or 16 x 125 mm)

Antimicrobial susceptibility test supplies (disk diffusion method)

5 x 500 g Mueller-Hinton agar
200 disks of the following antibiotics
- Trimethoprim-sulfamethoxazole
- Chloramphenicol
- Furazolidone (if furazolidone is being considered for use in cholera treatment)
- Tetracycline

Control strain *Escherichia coli* ATCC 25922
0.5 McFarland turbidity standard
Sterile cotton swabs
Sterile saline
Forceps and 95% alcohol for flaming
Zone size criteria chart
Materials and postage for production and dissemination of reports

*Do not use nutrient agar because some formulations have no added salt and do not allow optimal growth of *V. cholerae.*

Annex B
Supplies Needed for Laboratory Identification of *Shigella dysenteriae* 1 During an Outbreak

Assumptions:

- Supply each district with materials to collect and transport 50 specimens
- Supply each regional laboratory with materials to process 100 specimens
- Supply each national reference laboratory with materials to confirm 500 isolates

District Level (Materials to collect and transport 50 specimens)

Supplies needed for each district

100 cotton swabs
50 bottles or tubes of Cary-Blair or other transport medium
Transport for specimens to regional laboratory

Regional Level (Materials to process 100 specimens)

Supplies needed for each regional laboratory

200 sterile cotton or polyester swabs
100 bottles or tubes of Cary-Blair (or other) transport medium
500 g XLD medium
500 g MacConkey medium
500 g Kligler iron agar
500 g motility agar
500 g nonselective agar (e.g., tryptone soy agar, heart infusion agar)
Diagnostic antisera:
  4 x 2 ml monovalent *S. dysenteriae* serotype 1 (not Group A polyvalent)
  2 x 2 ml polyvalent *S. flexneri* (Group B)
  2 ml polyvalent *S. sonnei* (Group D)
Glass slides for serologic testing
500 disposable petri dishes (9 cm)
1000 disposable test tubes (e.g., 13 x 100 mm or 16 x 125 mm)
Transport for specimens to reference laboratory
Materials and postage for production and dissemination of reports

National Reference Laboratory (Materials to confirm 500 isolates)

Supplies needed by each national reference laboratory for confirmation

500 sterile cotton or polyester swabs
5 x 500 g Cary-Blair medium or other transport medium
5 x 500 g XLD medium
5 x 500 g MacConkey medium
3 x 500 g Kligler iron agar
3 x 500 g motility agar
3 x 500 g nonselective agar (e.g., tryptone soy agar, heart infusion agar)
Diagnostic antisera:
- 20 x 2 ml monovalent *S. dysenteriae* serotype 1 (not Group A polyvalent)
- 10 x 2 ml polyvalent *S. flexneri* (Group B)
- 5 x 2 ml polyvalent *S. sonnei* (Group D)

Glass slides for serologic testing
5 x 500 disposable petri dishes (9 cm)
5 x 1000 disposable test tubes (e.g., 13 x 100 mm or 16 x 125 mm)

Antimicrobial susceptibility test supplies for 100 Shigella *isolates*

2 x 500 g Mueller-Hinton Agar
200 disposable petri dishes (9 cm)
200 disks of the following antibiotics:
- Trimethoprim/sulfamethoxazole
- Chloramphenicol
- Ampicillin
- Nalidixic acid
- Ciprofloxacin (1 cartridge only)

Control strain *Escherichia coli* ATCC 25922
0.5 McFarland turbidity standard
Sterile cotton swabs
Sterile saline
Forceps and 95% alcohol for flaming
Zone size criteria chart
Materials and postage for production and dissemination of reports

Annex C
Guidelines for Establishing a Public Health Laboratory Network for Cholera Control

Purpose

- To establish a routine system for confirming the presence of *Vibrio cholerae* O1 and O139.
- To monitor the antimicrobial susceptibility patterns of *V. cholerae* O1 isolates from throughout the country.
- To provide feedback to guide development of appropriate antimicrobial treatment policies for cholera.

Overview

When outbreaks of a cholera-like illness occur, there is a need for accurate data to confirm the presence of *V. cholerae* O1. In addition, data about the antimicrobial susceptibility patterns of *V. cholerae* O1 isolates from throughout the country are needed to develop an effective antimicrobial treatment policy. Following is an outline of a system involving regional and reference laboratories to carry out these activities. Laboratories at different levels have corresponding degrees of responsibility for collection and transport of specimens, identification and confirmation of isolates, and feedback of the results to the appropriate levels. The roles and responsibilities of each level are outlined below, along with the basic supplies needed to carry out these activities. A full listing of the supplies needed for a 1-year period can be found in Annex A.

A. Surveillance

The laboratory-based surveillance will consist of two parts:

- Initial confirmation of the outbreak
- Ongoing surveillance for antimicrobial susceptibility of the *V. cholerae* isolates.

1. Initial confirmation of the outbreak

In areas currently not experiencing a cholera outbreak, cholera should be suspected if a patient older than 5 years develops severe dehydration or dies from acute watery diarrhea, or if there is a sudden increase in the daily number of patients with acute watery diarrhea. If such events are noted, 5 to 10 stool specimens should be sent to the regional laboratory for confirmation. Specific instructions for collecting and transporting stools can be found in the WHO "Guidelines for Cholera Control" and in Chapter 2 in this manual. A stool specimen data sheet to send with the specimens is found in Annex F.

Once the outbreak is confirmed, it is not necessary to collect specimens from additional patients for diagnosis. The diagnosis for treatment purposes can be made on clinical criteria. Collecting and processing an excessive number of stool specimens can quickly deplete scarce laboratory resources.

In areas where cholera is known to be present, confirmation of additional outbreaks is not necessary.

2. Surveillance for antimicrobial susceptibility of *Vibrio cholerae* isolates

Every 3 months, the regional laboratories in areas that are affected by cholera should each send 10 to 20 *V. cholerae* isolates to the national reference laboratory for susceptibility testing. The affected districts in the region should each send sufficient specimens for the regional laboratory to achieve this number. The regional laboratory should send these isolates to the reference laboratory for confirmation. A representative sample of isolates from each reference laboratory (a total of 10 to 20) should periodically be sent to an international reference laboratory for confirmation of the antimicrobial susceptibility pattern and possibly for additional studies, such as subtyping by ribotyping, pulsed-field gel electrophoresis or other molecular studies. Arrangements can be made through WHO for sending these isolates to an international reference laboratory on a regular basis.

B. Roles of District, Regional, and Reference Laboratories

1. District level

When an outbreak begins in a district, it should be confirmed by collecting 5 to 10 stool specimens and sending them to the regional laboratory for confirmation of the presence of *V. cholerae.* The basic materials needed at the district level to collect the stool specimens are as follows:

- Cary-Blair (or other) transport medium in tubes
- Sterile swabs

Each district should have sufficient supplies to send 50 stool specimens to the regional laboratory. In addition, the district will need to develop a rapid and reliable means of sending the specimens to the regional laboratory.

2. Regional level

The regional laboratory receives the specimen from the district and performs the initial isolation of *V. cholerae.* Each region should have sufficient supplies to identify at least 100 isolates of *V. cholerae* O1 and to send the isolates to the national reference laboratory for additional testing. The basic materials needed at the regional level are as follows:

- Thiosulfate citrate bile salts sucrose agar (TCBS) medium
- Ingredients to prepare alkaline peptone water
- Polyvalent O1 *V. cholerae* diagnostic antisera

- Heart infusion agar (HIA) or other nonselective medium
- Petri dishes
- Tubes for transport (HIA used as transport medium for isolates)

3. Reference level

The regional laboratory sends isolates to a national reference laboratory for confirmation and antimicrobial susceptibility testing. Each reference laboratory will need sufficient materials to confirm at least 500 isolates sent from the regional level throughout the year. The basic materials needed are as follows:

- TCBS medium
- Ingredients to prepare alkaline peptone water
- Polyvalent O1 and O139 group *V. cholerae* diagnostic antiserum
- Monovalent Ogawa diagnostic antiserum
- Monovalent Inaba diagnostic antiserum
- HIA or other nonselective medium
- Petri dishes
- Tubes for transport and storage of isolates (HIA medium used for this)
- Supplies for antimicrobial susceptibility testing (see Annex B)

4. Referral to international reference laboratories

As part of the laboratory-based surveillance process, isolates should periodically be sent to an international reference laboratory for confirmation of the antimicrobial susceptibility patterns. This is especially important if strains exhibit a new or unusual antimicrobial susceptibility pattern. Arrangements can be made through WHO for sending such specimens to an international reference laboratory and to provide for the rapid feedback of the results.

5. Feedback of Results

Regional laboratory to district, confirmation of outbreak

When the regional laboratory confirms the presence of *V. cholerae* O1 in stool specimens received from the district, it should contact the district as quickly as possible to inform the health authorities that *V. cholerae* O1 has been identified from the district.

Reference laboratories to regional laboratories

The reference laboratory should regularly communicate the results of the studies carried out on isolates submitted from the regional laboratory. The results should be sent to the regional laboratory and to the Ministry of Health. This includes the results of isolates sent both for confirmation of outbreaks and for the routine surveillance for *V. cholerae* carried out every 3 months. These results can serve as an internal quality control for the regional laboratories. In addition, every 3 months summaries of the results from all national reference laboratories should be distributed to the regional laboratories and appropriate persons in the Ministry of Health for further distribution to all relevant parties.

International reference laboratory to reference laboratory

The international reference laboratory should provide timely feedback of results to the national reference laboratory that is coordinating the shipping of the specimens. These results will be shared with the other reference laboratories and will serve as an external quality control for identification of *V. cholerae* O1 and O139 and for determining the antimicrobial susceptibility of the these strains.

6. Additional components for network

This system could be expanded to include other bacterial pathogens, such as those causing dysentery. For instance, periodic surveillance of isolates from patients presenting with bloody diarrhea could be done to determine the prevalence of various organisms causing dysentery and their antimicrobial susceptibility patterns.

Annex D
International Reference Laboratories

WHO Collaborating Centre for Research, Training, and Control in Diarrhoeal Diseases
International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B)
G.P.O. Box 128
Dhaka 100
BANGLADESH

WHO Collaborating Centre for Diarrhoeal Diseases Research and Training
National Institute of Cholera and Enteric Diseases
P-33, CIT Road Scheme XM
Beliaghata
P.O. Box 177
Calcutta 700 016
INDIA

WHO Collaborating Centre for Phage-Typing and Resistance of Enterobacteria
Central Public Health Laboratory
61 Colindale Avenue
London NW9 5HT
UK

International *Escherichia* and *Klebsiella* Centre (WHO)
Department of Clinical Microbiology
Statens Seruminstitut
Artillerivej 5, 2300 Copenhagen S
DENMARK

WHO Collaborating Centre for *Shigella*
National Reference Laboratory for *Escherichia coli* and *Shigella*
Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS C03
Atlanta, GA 30333
USA

WHO Collaborating Centre for Global Monitoring of Antimicrobial Resistant Bacteria
Nosocomial Pathogens Laboratory Branch
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS G-08
Atlanta, GA 30333
USA

National Reference Laboratory for *Vibrio cholerae* O1 and O139
Epidemic Investigations and Surveillance Laboratory
Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
1600 Clifton Rd., N.E., MS C03
Atlanta, GA 30333
USA

Annex E
Designing a Survey to Examine Antimicrobial Susceptibility of Organisms Causing Epidemic Diarrhea

Rationale

In many places where cholera and epidemic dysentery caused by *Shigella dysenteriae* serotype 1 occur, laboratory resources are scarce. In addition, the characteristics of the patient population may make it necessary to begin treatment and provide a full treatment course of an antimicrobial agent when the patient is first seen. The clinician cannot wait for test results. Often the test results and final antimicrobial susceptibility pattern take up to a week to determine.

One method of overcoming these shortcomings of laboratory testing of individual patients is to design and carry out a survey to determine the organisms causing epidemic diarrhea and their susceptibility patterns, use the information to choose an appropriate antimicrobial agent for treatment, then develop a treatment policy based on the syndrome (i.e., dysentery or watery diarrhea). This method will conserve resources and improve the case management of diarrhea.

Below is a basic outline of how to carry out such a survey that can be adapted to local conditions. It is important for the laboratory and epidemiology departments to work together on studies of this sort. Doing so fosters cooperation, shares the workload, and brings in additional expertise.

Methods

Location

Do the survey in locations that are representative of the population. Include some urban and some rural health centers. The sites chosen should be easily accessible so that specimens can be quickly transported to the laboratory doing the survey. In addition, they should have sufficient patients with diarrhea to allow the health care worker to collect 10-20 specimens in a few days.

Timing

If possible, the survey should be done at the beginning of the cholera or dysentery season when there are adequate numbers of patients with diarrheal disease at clinics and when the information gained will help establish treatment policies and drug purchase for the coming year.

How many patients

Enough patients should be sampled to provide 40 to 50 isolates. Isolation rates range from 25% to 75%. Thus 100 patients is a reasonable target. Select enough sites with high enough patient flow to reach this target in 1 to 2 weeks.

Logistics

Patients should be selected systematically, such as the first 5 patients in the morning; every third patient; or, in the case of clinics with fewer patients, all patients presenting with bloody diarrhea or watery diarrhea that particular day. The number of specimens collected should not overwhelm the laboratory.

If the survey is being carried out for dysentery, patients should, if possible, currently have diarrhea with visible blood, been ill for fewer than 4 days, and not have received an antimicrobial agent before a stool specimen is collected. Patients should be given a cup to collect a stool sample. Examine the stool for blood. If blood is visible, take a swab of the stool and place it in refrigerated (4°C) transport medium (see Chapter 2 for instructions on transport of specimens). Dispose of the stool cup so as to minimize the chance of infecting other persons.

If the survey is being carried out for presumptive cholera, the patients should have acute watery diarrhea with illness for fewer than 4 days, and should not have received an antimicrobial agent before a stool specimen is collected. For cholera, it is preferable to focus on adults and children over age 2 (younger children have many other causes of watery diarrhea that would reduce the yield of *V. cholerae* O1).

Transport the specimens to the laboratory. Examine specimens and test the antimicrobial susceptibilities of *S. dysenteriae* 1 and *V. cholerae* O1/O139 isolated (see Annexes A and B for necessary laboratory supplies).

What to Do with Results

Share the results with other health workers in the country, especially those involved in developing treatment policies or purchasing drugs. If the country has a health bulletin, use it to publish and disseminate the results. It is helpful to share the results with neighboring countries and with the country WHO office, or with the WHO Inter-country or Regional Office, so that they can be easily and quickly shared with the other countries in the area.

What to Do with the Isolates

Keep the isolates if possible. Methods to do so are described in Chapter 10, "Storage of Isolates." Any unusual isolates or those with novel antimicrobial susceptibility patterns should be sent to a national or international reference laboratory for confirmation.

Annex F
Stool Specimen Data Sheet for Epidemic Diarrhea

STOOL SPECIMEN DATA SHEET – EPIDEMIC DIARRHEA

Country _____ Region _____

District _____ Village/Town _____

Specimen Number	Date collected	Date of illness onset	Name	Age	Sex *(M/F)*	Blood in stool? *Yes / No*	Appear-ance*	Taken antibiotics *Yes / No* * *

* Formed (F); Soft (S); Watery (W); Bloody-mucus (BM)
**Type of antibiotic, dose and number of days taken.

Collected by: Name & Title _____

Transmit results to: Name & Title _____

Address _____

Phone/Fax/ Telex _____

Annex G
Most Frequently Encountered Reactions in Screening Biochemicals[a]

Test	Organism				
	Shigella	*Escherichia coli*	*Salmonella*	*Salmonella* ser. Typhi	*Vibrio cholerae*
Kligler iron agar[b]	K/A-	A/AG-	K/AG+	K/A(+)	K/A-
Triple sugar iron agar[b]	K/A-	A/AG-	K/AG+	K/A(+)	A/A-
Lysine iron agar[b]	K/A-	K/K -	K/K+	K/K(+)	K/K-
Lysine decarboxylation[c,d]	-	+	+	+	+
Motility[c]	-	+	+	+	+
Urea hydrolysis[c]	-	-	-	-	-
Indole production[c,d]	+ or -	+	-	-	+
Oxidase production[c]	-	-	-	-	+

[a] For each of these organisms, variable reactions may occur.
[b] Reactions expressed as "slant/butt"; K = alkaline; A = acid; G = gas produced; + = hydrogen sulfide (H_2S) produced; (+) = weakly positive for H_2S production; - = no H_2S produced.
[c] + = positive reaction; - = negative reaction.
[d] For *V. cholerae*, 1% salt (NaCl) added to biochemical formulation.

References

World Health Organization. Manual for the laboratory investigations of acute enteric infections. Geneva: World Health Organization, 1987; publication no. WHO/CDD/83.3 rev 1.

Bopp CA, Brenner FW, Wells JG, Strockbine NA. *Escherichia, Shigella,* and *Salmonella.* In: Murray PR, Pfaller MA, Tenover FC, Baron EJ, Yolken RH, ed. Manual of Clinical Microbiology, 7th ed. Washington, DC: ASM Press; 1999: 459-474.

Centers for Disease Control and Prevention. Laboratory methods for the diagnosis of *Vibrio cholerae.* Atlanta: CDC; 1994.

Annex H
Diagnostic Laboratory Supplies for Isolation and Presumptive Identification of *Escherichia coli* O157:H7 During an Outbreak (Sufficient for 100 Specimens)

100 sterile cotton or polyester swabs
500 g Cary-Blair or other transport medium
500 g sorbitol MacConkey agar
500 g nonselective agar (e.g., tryptone soy agar, heart infusion agar)
O157 latex agglutination kit for 100 tests
200 petri dishes (9 cm)
200 test tubes (e.g., 13 x 100 mm or 16 x 125 mm)

Suggested Citation

Centers for Disease Control and Prevention. Laboratory Methods for the Diagnosis of Epidemic Dysentery and Cholera. Atlanta, Georgia: CDC, 1999.

Additional copies of this manual can be obtained from:

Foodborne and Diarrheal Diseases Laboratory Section
Centers for Disease Control and Prevention
Mailstop C03
1600 Clifton Road, N.E.
Atlanta, GA 30333 USA
Fax 404-639-3333