9260 DETECTION OF PATHOGENIC BACTERIA*

9260 A. Introduction

1. General Discussion

One purpose of drinking water and wastewater treatment is to reduce the numbers of viable organisms to acceptable levels, and to remove or inactivate all pathogens capable of causing human disease. Despite the remarkable success of water treatment and sanitation programs in improving public health, sporadic cases and point-source outbreaks of waterborne diseases continue to occur. Water and wastewater may contain a wide variety of bacteria that cause intestinal or extra-intestinal infections. Waterborne pathogens enter human hosts through intact or compromised skin, inhalation, ingestion, aspiration, and direct contact with the mucous membranes of the eye, ear, nose, mouth, and genitals. This section provides an introduction to bacterial agents responsible for diseases transmitted by drinking and recreational waters in the United States.

The Centers for Disease Control and Prevention (CDC) and the U. S. Environmental Protection Agency (USEPA) maintain a collaborative system for collecting and reporting waterborne disease outbreak data for both microbial and chemical agents; however, detection and investigation of waterborne outbreaks is primarily the responsibility of the local, state, and territorial public health departments with voluntary reporting to the CDC.[1] From 1971 to 1998, 691 drinking-waterborne disease outbreaks due to a microbiological, chemical, or unknown etiology were reported in the United States (see Figure 9260:1). In the years 1972 through 1994, 291 outbreaks associated with drinking water were attributed to microorganisms as follows: *Giardia lamblia,* 109 (37.5% of total); *Shigella*, 34 (11.7%); nontyphoidal *Salmonella* serotypes, 13 (4.5%); *Salmonella* serotype Typhi, 5 (1.7%); *Vibrio cholerae,* 2 (0.7%), enterotoxigenic *E. coli*, 1 (0.3%); *E. coli* O157, 1 (0.3%). Community waterborne outbreaks have declined since the mid-1980s (see Figure 9260:1), largely because of the promulgation of more stringent drinking water standards, including the Surface Water Treatment Rule,[2] the Total Coliform Rule,[3,4] and other regulations.[5–7] In addition, many water utilities have made voluntary improvements.

The agents responsible for reported outbreaks are predominantly unidentified, microbial (parasitic, bacterial, or viral), or chemical (Figure 9260:2). Large numbers of parasitic outbreaks in the early 1980s were caused mostly by *Giardia*; these outbreaks were reduced by the implementation of the Surface Water Treatment Rule.[2] Relatively few outbreaks due to viruses have been reported, in part because the detection methodologies have difficulty attributing an outbreak to a specific virus. To better address the occurrence of microbial pathogens in drinking water, the USEPA has issued a Contaminant Candidate List that includes 11 microbes for methods development and potential future regulation.[8,9]

Water contamination and disease transmission may result from conditions generated at overloaded and/or malfunctioning sanitary waste disposal and potable water treatment systems. In addition, common outdoor recreational activities, such as swimming (including pools and hot tubs), wind surfing, and waterskiing, all place humans at risk of waterborne diseases from ingestion or direct contact with contaminated water.[10] Outbreaks of gastroenteritis, pharyngoconjunctivitis, folliculitis, otitis, and pneumonia are associated with these recreational activities. Overcrowded parks and recreational areas contribute to the contamination of surface and groundwater.

Laboratory diagnosis of infectious disease depends on detection or isolation of the etiologic agent or demonstration of antibody response in the patient. Environmental microbiological examinations are conducted for compliance monitoring of the environment, to troubleshoot problems in treatment plants and distribution systems, and in support of epidemiological investigations of disease outbreaks. Ideally, the public health microbiologist can contribute expertise in both clinical and environmental microbiology, thereby facilitating epidemiological investigations.

When testing for pathogens in environmental samples, it is advisable to include analyses for indicator organisms. Currently, coliforms (total coliform, thermotolerant coliform, and *E. coli*) are used as water quality indicators. Fecal streptococci, enterococci, *Clostridium perfringens*, *Bacteroides, Bifidobacterium*, and bacteriophages have been proposed as water quality indicators. No single indicator provides assurance that water is pathogen-free. The choice of monitoring indicator(s) presupposes an understanding of the parameters to be measured and the relationship of the indicator(s) to the pathogen(s). Some bacterial pathogens, such as *Pseudomonas, Aeromonas, Plesiomonas, Yersinia, Vibrio, Legionella*, and *Mycobacterium*, may not correlate with coliform indicators. Traditional bacterial indicators also may not correlate with viruses or parasites in pristine waters or groundwaters, and they may be of limited utility in estuarine and marine waters. Nevertheless, tests for total and fecal bacteria and *E. coli* are useful, because it is rare to isolate bacterial enteric pathogens in the absence of fecal contamination.

Other more general indicators may be of value also for assessing the potential for pathogen contamination and interpreting culture results. Heterotrophic plate count provides information about the total numbers of aerobic organotrophic bacteria and an indication of the total organic composition of the aquatic environment. Physicochemical factors, such as turbidity, pH, salinity, temperature, assimilable organic carbon, dissolved oxygen, biochemical oxygen demand, and ammonia, may provide useful information about contamination or the potential of water to support bacterial growth. For treated waters, chlorine residual should be measured at the sample collection point.

* Approved by Standard Methods Committee, 2007.
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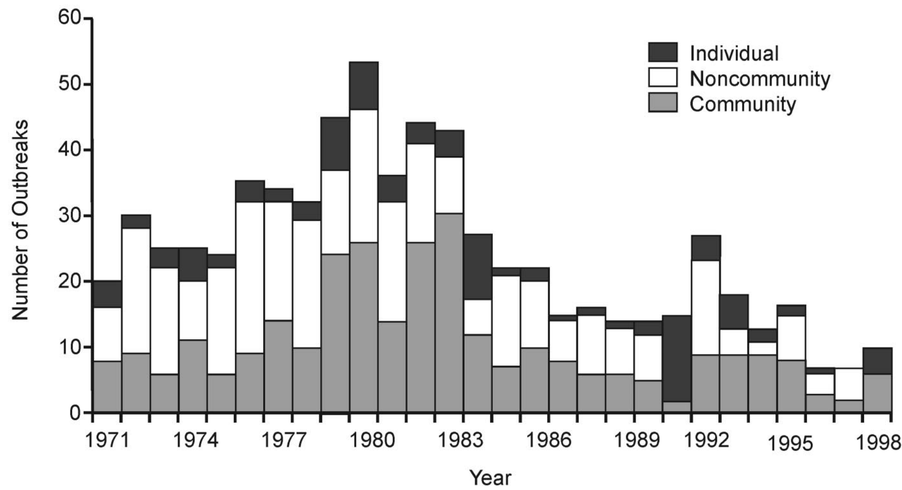

Figure 9260:1. Number of drinking water-related disease outbreaks in the United States, 1971–1998. Individual—private or individual water systems (9% of U.S. population or 24 million users); community—systems that serve >25 users year-round (91% of U.S. population, or 243 million users); noncommunity—systems that serve <25 users and transient water systems, such as restaurants, highway rest areas, and parks (millions of users yearly). Adapted from R.S. BARWICK, D.A. LEVY, G.F. CRAUN, M.J. BEACH & R.L. CALDERON. 2000. Surveillance for waterborne disease outbreaks—United States 1997–1998. *Morbid. Mortal. Week. Rep.* 49 (SS-4):1.

This section contains methods for *Salmonella, Shigella,* diarrheagenic *E. coli, Campylobacter, Vibrio, Leptospira, Legionella, Yersinia entercolitica, Aeromonas,* and *Mycobacterium*. Methods for isolation and enumeration of *P. aeruginosa* are found in Sections 9213E and F. Methods for other pathogens are found elsewhere.[11]

The methods outlined below may be used to analyze samples associated with disease outbreaks, or in other studies concerned with the occurrence of pathogens in water and wastewater. (The methods presented below are not standardized, and may need modification to fit a particular set of circumstances. No single procedure is available for reliable detection of any pathogen or group of pathogens.)

Because the presence of pathogens is intermittent and the survival times in the environment are variable, routine examination of water and wastewater for pathogenic bacteria is not recommended. Even in outbreak situations, the recovery of pathogens from water and wastewater may be limited by lack of facilities, untrained personnel, inadequate methods, and high costs. Despite these constraints, it is important to recover a substantial number of isolates, especially if molecular fingerprinting methods will be used during outbreak investigations.

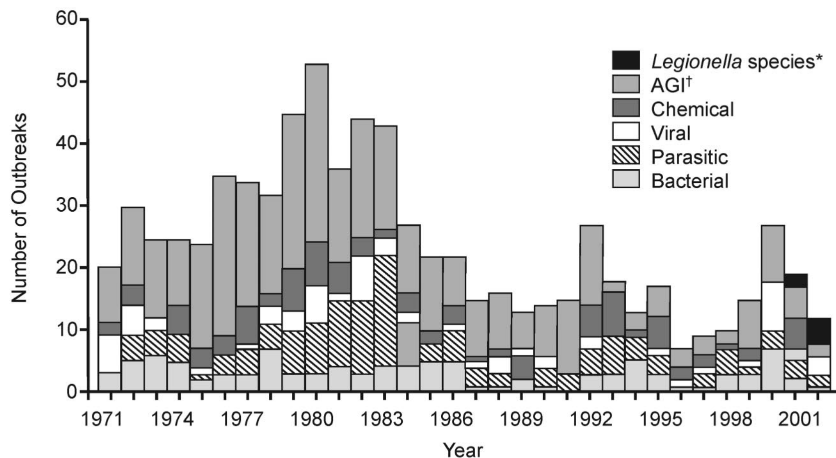

Figure 9260:2. Agents responsible for drinking water-related disease outbreaks. Adapted from BLACKBURN, B.G., G.F. CRAUN, J.S. YODER, V. HILL, R.L. CALDERON, N. CHEN, S.H. LEE, D.A. LEVY & M.J. BEACH. 2004. Surveillance for waterborne-disease outbreaks associated with drinking water—United States, 2001–2002. *Morbid. Mortal. Week. Rep.* 53 (SS-8):23.

A negative result by these methods for pathogenic bacteria does not conclusively prove their absence. The controversial concept of "viable but nonculturable" vs. "injured" organisms should be considered in relation to a negative culture result.[12,13]

All microbes included in this section are human pathogens, and they pose an infectious threat to persons involved in sample collection and laboratory analyses. Biosafety Level 2 precautions are required for all pathogens in this section except the Mycobacteria, which require Biosafety Level 3 precautions.[14]

2. References

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9260 B. *Salmonella*

The genus *Salmonella* has been of concern in water analysis for more than 100 years. Much of the history of water bacteriology evolved around methods to isolate and identify the typhoid bacillus *Salmonella* serotype Typhi (also called *Salmonella enterica* serotype Typhi and *Salmonella typhi*), and the corresponding efforts to reduce the incidence of waterborne typhoid fever. Waterborne outbreaks of typhoid fever and salmonellosis are still being reported in both developing and industrialized countries. From 1972 through 1994, 18 *Salmonella* outbreaks (most were of "non-typhoidal" type) associated with drinking water were reported to CDC for the United States. CDC surveillance data on *Salmonella* isolates in the United States since 1995 are available.*

The genus *Salmonella* is comprised of seven phylogenetic groups [1,2] that are often classified into two species: *Salmonella enterica* and *Salmonella bongori*. The genus includes more than 2500 named serotypes. Most water laboratories need to report only the genus name and the O antigen group, i.e., "*Salmonella* group B" or "*Salmonella* group D." Further study and additional antigenic analysis in public health or reference laboratories can yield a more definitive report.

There is no universally accepted "standard method" for the isolation and identification of *Salmonella* in water, foods, or human clinical specimens. This section is a brief summary of methods for isolating, identifying, and reporting this group of microorganisms. A quantitative procedure (9260B.9) also is included.

Currently available methods used in numerous field investigations demonstrate the presence of *Salmonella* in both fresh and marine water environments; however, the occurrence of *Salmonella* is highly variable. There are limitations and variations in both the sensitivity and selectivity of *Salmonella* isolation procedures, many of which have been adapted from food and clinical microbiology. Thus, a negative result by any of these methods does not prove the absence of *Salmonella* or of other pathogens.

Fluorescent antibody (FA) techniques have been used to detect pathogenic bacteria directly in clinical, food, and water samples. Because equipment and supplies for FA techniques for detecting *Salmonella* are no longer commonly available, this method is not included in this section. Details of the FA method may be found in the 21st Edition of *Standard Methods*.

A newer method for detection after enrichment is polymerase chain reaction (PCR).[3]

* See http://www.cdc.gov/biosafety/publications/bmbl5/.

1. Safety

Strains of *Salmonella* are almost always enteric pathogens. Use normal safety procedures and standard precautions, such as those available from government agencies.† Biosafety Level 2 (BSL 2) is typically specified because aerosol transmission is not usually involved. If isolation of *Salmonella* serotype Typhi is possible, consider additional personal protection, such as gowns, masks, and gloves, because it is a more hazardous laboratory pathogen than the other serotypes. See 9020B.2 (2005) for a discussion of biosafety levels.

Use nonpathogenic and oral vaccine strains of *Salmonella* serotype Typhi‡ in quality control procedures and method-verification studies.

2. Sampling and Concentration

Salmonella and other enteric pathogens isolated from water and other environmental samples usually will be outnumbered by other *Enterobacteriaceae* and other bacteria. Examine a relatively large sample and use enrichment media to maximize the chance of *Salmonella* isolation (see 9260B.4). Consider incubating the sample in noninhibitory media (9260B.3) at 37°C rather than in more toxic enrichment media because enteric pathogens often become "injured" as they survive under less favorable conditions found in drinking and environmental water. These injured cells are more likely to be killed by the toxic components of enrichment media. Small numbers of *Salmonella* cells can often be detected[4,5] with these caveats in mind.

a. Swab technique or "Moore swab": This method has been used to trace typhoid carriers and can be used as a simple method to concentrate other enteric pathogens,[6,7] particularly from rivers, wastewaters, and other flowing waters. Prepare swabs from cheesecloth 23 cm wide, folded five times at 36-cm lengths, and cut lengthwise to within 10 cm from the head into strips approximately 4.5 cm wide. Securely wrap the uncut or folded end of each swab with a wire or coat hanger to secure the swab as it is suspended in water or flowing wastewater. Place the swabs in kraft-type bags and sterilize at 121°C for 15 min. Place swab just below the surface of the sampling location for 1 to 3 d. Longer exposure times apparently do not increase the recovery rate. Gauze pads of similar thickness may be substituted. During sampling, particulate matter and microorganisms attach to the cloth as water passes through and over the swab. After exposure, retrieve the swab, place it in a sterile plastic bag, and send it to the laboratory in an ice chest. Process it as soon as possible, and in all cases, within 6 h. Placing swabs in less inhibitory enrichment media (i.e., media that allow growth of most enteric organisms) before transport may result in overgrowth by competitive organisms that will mask the presence of *Salmonella*. In the laboratory, place the pad or portions of it in pre-enrichment media.

b. Diatomaceous earth technique: Place an absorbent pad (not a membrane filter) on a membrane filter funnel receptacle, assemble funnel, and add 2.5 g sterile diatomaceous earth§ to loosely pack the funnel neck. Apply vacuum and filter 2 L of sample. After filtration, disassemble funnel, divide resulting "plug" of diatomaceous earth and absorbent pad in half aseptically with a sterile spatula (knife edged), and add half of the plug to two enrichment media (see below). Alternatively, place entire plug in a single enrichment medium.

c. Large-volume sampler: Use a filter composed of borosilicate glass microfibers bonded with epoxy resin to examine several liters or more of sample, provided that sample turbidity does not limit filtration.[8] The filter apparatus consists of a 2.5- × 6.4-cm cartridge filter and a filter holder.‖ Sterilize by autoclaving at 121°C for 15 min. Place sterile filter apparatus (connected in series with tubing to a 20-L water bottle reservoir and vacuum pump) in the 20-L sample container appropriately calibrated to measure volume of sample filtered. Apply vacuum and filter an appropriate volume. When filtration is complete, remove filter and place in a selective enrichment medium.

d. Membrane filter technique: To examine low-turbidity water, filter several liters through a sterile 142-mm-diam membrane of 0.45-μm pore size.[9] For turbid waters, precoat the filter: make 1 L of sterile diatomaceous earth suspension (5 g/L reagent-grade water) and filter about 500 mL. Without interrupting filtration, quickly add sample (1 L or more) to remaining suspension and filter. After filtration, place membrane in a sterile blender jar containing 100 mL sterile 0.1% (w/v) peptone water and homogenize at high speed for 1 min. Add entire homogenate to 100 mL double-strength selective enrichment medium. Alternatively, use multiple 47-mm-diam membrane filters to filter the sample. Immerse each membrane aseptically in 50 mL single-strength selective enrichment medium and incubate.

3. Pre-enrichment to Revive Injured Cells

Buffered peptone water was formulated to revive injured *Salmonella* cells found in food and may enhance the yield in water analysis. Pre-enrichment in buffered peptone water,# lactose broth,# universal pre-enrichment broth,# or a similar noninhibitory medium can precede enrichment in one or more inhibitory broths.

4. Enrichment

Although no single enrichment medium is suitable for all *Salmonella* and all conditions, three enrichment media have been widely used: tetrathionate broth, tetrathionate broth with brilliant green, and selenite broth. Direct plating on solid selective media can be done but, because of toxicity to "injured cells," use direct plating only as a supplement to enrichment in broth. Use two or more selective enrichment media in parallel for optimum detection. Elevated incubation temperatures, including 40, 41.5, and 43°C, and the addition of brilliant green dye to media help suppress background growth and may improve *Salmonella* detection. However, these modifications also suppress growth of some serotypes, including *Salmonella* serotype Typhi.

a. Selenite cystine broth: This medium inhibits gram-positive bacteria and many of the other genera of *Enterobacteriaceae* while allowing for recovery of most *Salmonella* serotypes, in-

† See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov. Accessed July 2009.
‡ Available from the American Type Culture Collection, http://www.atcc.org.
§ Celite, World Minerals, Inc., Lompoc, CA, or equivalent.
‖ Balston Type AA filter with Type 90 holder, or equivalent.
Difco, or equivalent.

cluding *Salmonella* serotype Typhi. Optimum incubation time for maximum recovery of *Salmonella* is 48 h at 35 to 37°C. Streak from tubes with turbidity several times during first day and then daily up to 5 d to increase potential recovery of *Salmonella*. To enhance recovery of *Salmonella*, transfer 1 mL selenite broth culture to a fresh tube of selenite broth and incubate and subculture as described above.

b. Selenite broth: This medium allows for optimum recovery of most *Salmonella*, including *Salmonella* serotype Typhi, after 24 h at 35 to 37°C. This increased recovery of *Salmonella* is accompanied by a slight decrease in selectivity when compared to selenite cystine. Most significantly, *E. coli* growth is not inhibited. Adding novobiocin (20 μg/mL) to selenite broth may be useful to inhibit swarming strains of *Proteus*. Streak from tubes with turbidity several times during first day and then daily up to 5 d to increase potential recovery of *Salmonella*. Transfer 1 mL selenite broth culture to a fresh tube of selenite broth and incubate and subculture as described above.

Commercial latex agglutination kits, used to test for the presence of *Salmonella* in tubes of selenite enrichment broth, can be useful as screening tests to help predict enrichment cultures that will be *Salmonella*-positive when subcultured.

c. Tetrathionate broth: When incubated at 35°C for 24 to 48 h, tetrathionate broth inhibits coliforms and gram-positive bacteria, permitting selective enrichment of most *Salmonella*, including serotype Typhi. When incubated for 48 h at 43°C, tetrathionate broth has been reported as more selective for *Salmonella* than selenite-based media. Although this formulation is highly selective, it may not inhibit swarming strains of *Proteus* that can obscure the presence of *Salmonella*. Growth of *Proteus* and *Citrobacter* can be inhibited by the addition of brilliant green. Incubation at 43°C and the addition of brilliant green also will inhibit some serotypes of *Salmonella*, including Typhi. Several commercial products are available with different formulation and modification.

d. Other enrichment broths: Enrichment broths that have proved useful for *Salmonella* isolation include: brilliant green broth, EE broth Mossel, GN broth Hajna, M broth (recommended when immunological assays are being used), Muller Kauffmann tetrathionate broth base, Rappaport-Vassiliadis R10 broth, Rappaport-Vassiliadis soya peptone broth, and TT broth base Hajna (with added iodine and potassium iodide).

5. Immunomagnetic Separation

A recently developed isolation technique, often used in the food industry, is immunomagnetic separation (IMS). Samples that have been incubated for 18 to 24 h in a noninhibitory medium (such as buffered peptone water), and filtered are then reacted with metal beads coated with an antibody for a specific enteric pathogen, such as *Salmonella*. The magnetic beads, with any *Salmonella* cells captured by the antibody, are separated from the enriched sample with a magnet, and then plated on one or more selective media (see 9260B.6) for *Salmonella*. Several manufacturers produce immunomagnetic separation products for *Salmonella* and other pathogens.** An alternative is to purchase uncoated magnetic beads and coat them with antibody that is more specific for the pathogen being sought. In this case, quality control and method validation are necessary to ensure satisfactory performance.

6. Plating Media

Selection of *Salmonella* after enrichment will depend on the plating media chosen and the incubation temperature. All three factors—incubation temperature, enrichment medium, and isolation medium—are interrelated, and no one combination is optimum for recovery of all *Salmonella* serotypes. Method comparisons are encouraged to determine the best combination for a given circumstance.

Solid media commonly used for *Salmonella* and enteric pathogen detection fall into three broad groups: (*a*) differential media of low selectivity, such as MacConkey agar or EMB agar, that inhibit most gram-positive bacteria but do not inhibit other genera of *Enterobacteriaceae* and many other gram-negative bacteria; (*b*) more selective media containing bile salts or sodium desoxycholate as inhibitors,[10] such as desoxycholate agar or xylose lysine desoxycholate (XLD) agar; and (*c*) media specifically designed to isolate *Salmonella,* such as brilliant green agar, bismuth sulfite agar, and others. Streaking duplicate plates, one heavily inoculated and one lightly inoculated, often aids in recognition of enteric pathogens in the presence of large numbers of interfering organisms.

a. Brilliant green agar: Typical well-isolated *Salmonella* colonies grown on this medium are pinkish white with a red background. *Salmonella* serotype Typhi and a few other serotypes grow poorly because they are inhibited by the concentration of brilliant green dye used. Bacteria that ferment lactose often grow as greenish colonies, but may produce other colors as well. Occasionally, slow lactose-fermenters (*Proteus, Citrobacter)* or non-fermenters (*Pseudomonas*) will produce colonies resembling *Salmonella*. Increasing the agar concentration to 2% may suppress swarming of undesired bacteria. Swarming of *Proteus* also may be reduced by using agar plates that have dried sufficiently to remove surface moisture. If suspect *Salmonella* colonies are not observed after 24 h incubation, reincubate for another 24 h to permit slow-growing or partially inhibited organisms to develop visible colonies. If typical colonies are not observed or if the streak plate is crowded, isolate in pure culture a few colonies for biochemical characterization. Non-lactose-fermenting colonies in close proximity to lactose-fermenting colonies may be missed.

b. Bismuth sulfite agar (Wilson and Blair medium):[11] Many *Salmonella*, including serotype Typhi, grow well on this medium. Examine bismuth sulfite plates after 24 h incubation for suspect colonies; reincubate for 24 h to detect slow-growing strains. Typical colonies of *Salmonella* usually develop a black color, with or without a metallic sheen, and frequently this blackening extends beyond the colony to give a "halo" effect. A few *Salmonella* species will develop a green coloration; therefore, pick some of these colony types when typical colonies are absent. As with brilliant green agar after 48 h incubation, typical colony coloration may be masked by adjacent heavy growth. *Proteus* and other *Enterobacteriaceae* that are H_2S-positive can also appear as black colonies. One disadvantage of this medium is that it must be used within 24 to 36 h of preparation for

** For example, Dynal Biotech (http://www.dynalbiotech.com/) and Matrix Microsciences Ltd. (http://www.matrixmsci.com/).

Salmonella serotype Typhi, and must be refrigerated after preparation for other *Salmonella*.

c. Xylose lysine desoxycholate (XLD) agar: Compared to brilliant green dye, sodium desoxycholate, the selective ingredient in XLD, is only slightly toxic to fastidious *Salmonella,* which grow as black-centered red colonies. *Proteus* and many other *Enterobacteriaceae* grow as yellow colonies. Optimum incubation time is 24 h. If plates are incubated longer, an alkaline reversion and subsequent blackening occur with H_2S-positive genera of *Enterobacteriaceae*, such as *Citrobacter* and *Proteus*.

d. Xylose lysine brilliant green agar: This medium is very good for isolating *Salmonella* from marine samples because the brilliant green inhibits many strains of *Proteus, Enterobacter,* and *Citrobacter*.

e. Other plating media: Several other media that have proved useful in *Salmonella* isolation include: brilliant green agar-modified, brilliant green bile agar-modified, chromogenic substrate plating media,†† desoxycholate citrate agar, Hektoen enteric agar, Rappaport-Vassiliadis Medium with 20 μg/mL novobiocin, SS agar, and XLT-4 agar (with inhibitory surfactant). Check for commercial availability.

7. Screening Tests and Biochemical Identification

There are many approaches to screen for, and identify, colonies as "suspect *Salmonella*"[1,2] and for the other species of *Enterobacteriaceae*, including the other enteric pathogens (Table 9260:I). These approaches include commercial identification kits, commercial *Salmonella*-identification products, screening tests, biochemical and serological identification, fluorescent antibody (FA) screening, genus- and species-specific tests, and molecular methods. These methods are constantly evolving. Complete testing usually will result in a correct identification. Both the sensitivity and specificity of a new method, along with its ease of use, should be considered.

The identification of *Salmonella* only by colony characteristics on selective solid media has obvious limitations. Many other genera and species of *Enterobacteriaceae* can be confused with *Salmonella*;[1] complete biochemical testing will result in the fewest misidentifications.

Table 9260:I lists a series of screening tests that have proved useful for *Salmonella*. Two of the most useful are bacteriophage O1 sensitivity and the reaction with methylumbelliferyl caprylate.‡‡ These are two *Salmonella*-specific tests, and deserve to be evaluated in water analysis because of their high sensitivity and specificity.

Commercially available kits (miniaturized plastic products that contain 20 to 30 biochemical tests and include computer analysis of the results) provide another useful approach[12] to identification and have proved very popular in clinical microbiology laboratories. Their main disadvantage is that many commercial kits include only the genera and species of *Enterobacteriaceae* that occur in human clinical specimens. The omission of organisms that occur in water can lead to misidentifications.

A single-step rapid *Salmonella* test was compared with conventional culture methods for the rapid detection of *Salmonella* in 48 river water samples. The method had a sensitivity of 93% and a specificity of 100%, and was rated as being both rapid and user-friendly.[13]

8. Serological Identification

Serological testing is based on an antigen antibody reaction. The antigen is typically a living or killed *Salmonella* culture that is usually mixed with a commercial rabbit polyclonal antibody that reacts with a surface structure of the bacterium, causing a visible clumping (agglutination). NOTE: It is essential to follow the manufacturer's instructions exactly. Various serological reagents are available and some have been absorbed onto latex or other particles to facilitate recognition of positive reactions. *Salmonella* colonies are first tested in polyvalent antiserum and those that agglutinate strongly are then tested in seven individual sera for O groups A through G. For example, cultures that agglutinate in polyvalent and then only in O group B would be reported "presumptive Group B *Salmonella*." When the culture is confirmed to be *Salmonella* by other methods, a report of "*Salmonella* Group B" can be issued with confidence. Many other *Enterobacteriaceae* (and other bacteria) share antigens with the genus *Salmonella*, so it is essential to do confirmatory testing (Table 9260:I).

Salmonella serotype Typhi cultures are easy to identify because they have characteristic biochemical reactions (Table 9260:I) and typically agglutinate strongly in "Vi" and/or Group D antisera.

Complete serological identification of a *Salmonella* culture is complex and requires determination of O and H antigens and antigen factors; it should be done only by experienced reference laboratories.

9. Quantitative Procedures

The procedure described below is one approach for estimating *Salmonella* density in water samples. Other methods have been described in the literature; a comparative study is strongly recommended to select the best quantitative method for any given application. Modify the following procedure for use with solid or semisolid samples.

Because of the high ratio of coliform bacteria to pathogens, use large samples (1 L or more). Any concentration method in 9260B.2 may be used, but the membrane filter technique (9260B.2*d*) is preferred. After blending the membrane with 100 mL sterile 0.1% (w/v) peptone water, use a quantitative MPN procedure by proportioning homogenate into a five-tube, three-dilution multiple-tube procedure using either selenite cystine, selenite-F, or tetrathionate broth as the selective enrichment medium (see 9260B.4). Incubate for 24 h as required for the enrichment medium used and streak from each tube to brilliant green and xylose lysine desoxycholate agar plates. Incubate for 24 h at 35°C. Select from each plate at least one, and preferably two to three, colonies suspected of being *Salmonella,* confirm their identification as *Salmonella* (see 9260B.7), and then determine serogroup (see 9260B.8). From the combination of *Salmonella* negative and positive tubes, calculate the MPN/1.0 L of original sample (see Section 9221C).

†† CHROMagar™ *Salmonella, Rambach*™ agar, or equivalent.
‡‡ MUCAP™ or equivalent.

TABLE 9260:I. SCREENING TESTS, KEY REACTIONS, AND PROPERTIES OF *SALMONELLA*, *SHIGELLA*, *ESCHERICHIA COLI*, *YERSINIA* AND OTHER *ENTEROBACTERIACEAE**

Organism (Genus, Species or Serotype)	Test Result or Property†
Salmonella	Lactose$^-$, sucrose$^-$, H_2S^+ (strong, whole tube is black), O1 phage$^+$,‡ MUCAP$^+$,§ agglutinates in polyvalent serum,‡ typical colonies on media selective/differential for *Salmonella* (brilliant green agar, SS agar, CHROMagar™ *Salmonella*, Rambach™ agar, etc.), lysed by the *Salmonella* specific bacteriophage O1,‡ often antibiotic resistant
Salmonella serotype Typhi	Fastidious, H_2S^+ (weak, with characteristic blackening pattern), Citrate$^-$, D-xylose$^-$, agglutinates in group D serum and/or Vi serum
Shigella	Nonmotile, lysine$^-$, gas$^-$, agglutinates in polyvalent serum, biochemically inactive, often antibiotic resistant, molecular test: PhoE+§
Shigella dysenteriae	Agglutinates in group A serum, D-mannitol$^-$
Shigella dysenteriae O1	Catalase$^-$, agglutinates in O1 serum, Shiga toxin$^+$
Shigella flexneri	Agglutinates in group B serum, D-mannitol$^+$
Shigella boydii	Agglutinates in group C serum, D-mannitol$^+$
Shigella sonnei	Agglutinates in group D serum, D-mannitol$^+$, ornithine decarboxylase$^+$, lactose$^+$ (delayed), colony variation: smooth to rough
Escherichia coli	Extremely variable biochemically, indole$^+$, MUG$^+$, grows at 44.5°C, sometimes antibiotic resistant, PhoE$^+$ molecular test§
Escherichia coli O157:H7	Colorless colonies on sorbitol-MacConkey agar, MUG$^-$, D-sorbitol$^-$ (or delayed), agglutinates in O157 serum, and H7 serum
Yersinia	Grow on CIN agar, usually more active biochemically at 25°C than 36°C (motile at 25°C, nonmotile at 36°C), urea$^+$
Yersinia enterocolitica, the pathogenic serotypes	CR-MOX$^+$, pyrazinamidase$^-$, salicin$^-$, esculin$^-$, agglutinate in O typing sera: 3; 4, 32; 5, 27; 8; 9; 13a, 13b; 18; 20; or 21
Yersinia enterocolitica O3 (the most common pathogenic serotype)	D-Xylose$^-$, agglutinates in O3 serum, tiny colonies at 24 h on agar plating media
Yersinia enterocolitica, the non-pathogenic serotypes	CR-MOX$^-$, pyrazinamidase$^+$, salicin$^+$, esculin$^+$, do not agglutinate in typing O sera: 3; 4, 32; 5, 27; 8; 9; 13a, 13b; 18; 20; or 21
Citrobacter	Citrate$^+$, lysine decarboxylase$^-$, often grows on CIN agar, strong characteristic odor
Hafnia	Lysed by *Hafnia*-specific bacteriophage,‡ often more active biochemically at 25°C than 36°C
Klebsiella	Mucoid colonies, encapsulated cells, nonmotile, lysine$^+$, very active biochemically, ferment most sugars, VP$^+$, malonate$^+$, resistant to carbenicillin and ampicillin
Enterobacter	Variable biochemically, citrate$^+$, VP$^+$, resistant to cephalothin
Serratia	DNase$^+$, gelatinase$^+$, lipase$^+$, resistant to colistin and cephalothin
Serratia marcescens	L-arabinose$^-$
Serratia, other species	L-arabinose$^-$
Proteus-Providencia-Morganella	Phenylalanine$^+$, tyrosine hydrolysis$^+$, often urea$^+$, resistant to colistin
Proteus	Swarms on blood agar, pungent odor, H_2S^+, gelatin$^+$, lipase$^+$
Proteus mirabilis	Urea$^+$, indole$^-$, ornithine$^+$, maltose$^-$
Proteus vulgaris	Urea$^+$, indole$^+$, ornithine$^-$, maltose$^+$
Providencia	No swarming, H_2S^-, ornithine$^-$, gelatin$^-$, lipase$^-$, urea$^{+ \text{ or } -}$ Very inactive biochemically, no swarming, citrate$^-$, H_2S^-, ornithine$^+$, gelatin$^-$, lipase$^-$
Plesiomonas shigelloides	Oxidase$^+$, lysine$^+$, arginine$^+$, ornithine$^+$, myo-inositol$^+$

* This table gives only the general properties of the genera, species, and serogroups, so there will be exceptions; more details and more precise data, including actual percentages for biochemical test results, have been published.[1] The properties listed for a genus or group of genera generally apply for each of its species, and the properties listed for a species generally apply for each of its serotypes.

† The serological tests refer to slide agglutination in group or individual antisera (O1, O3, etc.) for *Salmonella*, *Shigella*, *Yersinia*, or *Escherichia coli,* respectively.

‡ These are two bacteriophage tests useful for identification.

§ Abbreviations: CIN, cefsulodin-irgasan-novobiocin agar (a plating medium selective for *Yersinia*); CR-MOX, Congo red-magnesium oxalate agar (a differential medium useful for distinguishing pathogenic from nonpathogenic strains of *Yersinia*); MUCAP, 4-methylumbelliferyl caprylate (a genus specific test for *Salmonella*); MUG, 4-methylumbelliferyl-β-D-glucuronidase; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PhoE, a test done by PCR that is sensitive and specific for *E. coli*/*Shigella*; VP, Voges-Proskauer.

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9260 C. (Reserved)

9260 D. (Reserved)

9260 E. *Shigella*

Shigellosis, an acute intestinal infection of humans, can be caused by any of the different species-serotypes of the genus *Shigella* (family *Enterobacteriaceae*). *Shigella* has been of concern in water analysis for more than 100 years. *Shigella* invades the intestinal mucosa, producing dysentery (shigellosis), which is characterized by abdominal pain; tenesmus (straining to produce feces); and bloody diarrhea. The infectious dose for *Shigella* spp. is low; only a few cells may be sufficient. The low infective dose often results in person-to-person transmission and laboratory-acquired infections. When outbreaks occur, they usually are associated with fecal contamination of foods, but waterborne transmission is also important. *Shigella* accounted for 34 of 291 (11.7%) drinking water-related outbreaks that were reported to CDC for the United States during 1972 through 1994.*

The genus *Shigella* has four named species, three of which can be further divided into O antigen groups (serotypes): *S. dysenteriae* (serological group A, O antigen groups 1–15), *S. flexneri* (group B, O groups 1–6), *S. boydii* (group C, O groups 1–20), and *S. sonnei* (group D; with a single O group). The latter is biochemically different from the three other *Shigella* species (see Table 9260:I). *Shigella dysenteriae* O1 is an extremely important

* For subsequent *Shigella* surveillance reports, see http://www.cdc.gov/ncidod/dbmd/phlisdata/shigella.htm. Accessed November 2011.

pathogen in developing countries and frequently causes waterborne outbreaks; *S. sonnei* and *S. flexneri* predominate in most developed countries. Shigellosis is most common among children. Outbreaks with person-to-person transmission have been reported in schools, day-care centers, and institutions providing custodial care. Waterborne outbreaks are associated with the following situations: fecal contamination of private or noncommunity water supplies in which chlorination is inadequate; cross-connections between wastewater and potable water lines; and recreational waters contaminated with feces from cases of shigellosis.

Shigella strains are not unusually resistant to chlorination, and they generally compete poorly with other microorganisms in the aquatic environment. In many types of water their survival time is measured in hours and days, and a survival time of 4 d has been observed in river water. However, if the organic content of the water is very high, survival may be prolonged, depending on extent of fecal pollution, concentration of soluble organic matter, and physical conditions, such as light, temperature, salinity, and pH. Unfortunately, by the time an outbreak is confirmed to be *Shigella* by standard culture-based laboratory methods (2 to 4 d), the organism is unlikely to be found unless there is a continuous source of contamination, such as wastewater seepage or a reservoir of infected individuals who are still shedding the pathogen.

A negative culture result for the presence of *Shigella* in an implicated water supply may be due to nonoptimum sampling location, sample handling, or sample size, or to problems in the sensitivity of isolation and identification methods.

Shigella are normally sought in water samples, either in a search for all *Shigella* species as part of a survey for enteric pathogens in water, or in a search for a particular *Shigella* serotype during the investigation of a specific outbreak. Methods for the quantitative recovery of *Shigella* from the environment are very primitive compared to those for many other organisms. Another difficulty is that *Shigella* and *Escherichia coli* are the "same species" in a phylogenetic sense; this makes selective enrichment for *Shigella*, at the expense of *E. coli*, much more difficult. Classical culture methods that have resulted in isolation of *Shigella* include membrane filtration[1,2] and centrifugation[3,4] with or without subsequent broth enrichment. Recently, the polymerase chain reaction (PCR), immunomagnetic separation, and other methods have shown promise for detection of *Shigella* in environmental samples.[5–7]

1. Safety

All *Shigella* strains are considered enteric pathogens. Use normal safety procedures and standard precautions, such as those available from government agencies.† Biosafety Level 2 (BSL 2) is usually specified because aerosol transmission is not usually involved. However, all *Shigella* strains have high potential for causing laboratory infections. Preferably use additional personal protection, such as gowns, masks, and gloves. In addition, post a sign on each door to the laboratory indicating that *Shigella* is present and listing the necessary precautions for those who enter. Design safety procedures carefully because personnel who perform water analyses may not be accustomed to working with this pathogen. For quality control procedures and method-verification studies, use nonpathogenic strains of *Shigella*, such as those developed for oral vaccines.‡ **CAUTION: Shigella dysenteriae O1 produces a potent toxin and is an extremely hazardous pathogen**. If this organism is being isolated or there is a chance for its isolation, make laboratory managers and staff aware of this danger.

2. Sampling and Concentration

Methods used for sampling and concentration of total coliforms, thermotolerant coliforms, *Escherichia coli*, and *Salmonella* can be used. See membrane filter (9260E.8) and centrifugation (9260E.9) methods below.

3. Enrichment

Choose a selective enrichment medium that has proven successful for isolating *Shigella* from water. Selenite F broth and GN broth have been used most frequently. Enrichment methods that have proved useful in food and clinical microbiology have typically been modified for water analysis, so newer methods in these disciplines also may be considered. Generally, all enrichment broths have been formulated to minimize accumulation of toxic metabolites produced by other *Enterobacteriaceae* and bacteria. Selenite F broth and GN broth have been used successfully to recover shigellae from water and sand.[3,4] Alternatively, use reduced-strength tryptic soy broth adjusted to pH 8.0 (0.15 g tryptic soy broth, added directly to the sample).

During outbreak investigations, the enrichment medium can be made considerably more selective by incorporation of antibiotics. For example, if the outbreak strain is resistant (based on laboratory antibiotic susceptibility studies) to tetracycline and streptomycin at concentrations of 150 μg/mL,[8] these two antibiotics could be incorporated into enrichment broths and plating media. Use careful quality control and method verification to ensure that selective media are not inhibitory to the outbreak strain.

4. Immunomagnetic Separation after Enrichment

See 9260B.5, E.10*b*, and F.4.

5. Plating Media

After incubation, enrichment broths typically are plated on media that are selective and often differential for *Shigella*, such as MacConkey [9221B.4*a*) (2006)], XLD (9260B.6*c*), and SS (*Salmonella Shigella*) agars, with or without antibiotics.

6. Screening Tests and Biochemical Identification

There are many approaches to identifying colonies that are suspected of being *Shigella* (see Table 9260:I). Review also 9260B.7 for other approaches for identifying colonies.

a. Screening on TSI and LIA slants: It has been a common practice to screen suspect colonies by inoculating them into

† See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov.

‡ Available, for example, from the American Type Culture Collection, http://www.atcc.org.

TABLE 9260:II. TYPICAL REACTIONS OF COMMON BACTERIA ON TRIPLE SUGAR IRON (TSI) AND LYSINE IRON AGAR (LIA)

Organism	TSI Reactions			LIA Reactions	
	Slant/butt	Gas	H_2S	Slant/butt	H_2S
Shigella	K/A	–	–	K/A	–
Salmonella	K/A	+	+	K/K	+
Salmonella serotype Typhi	K/A	–	weak+	K/K	- or weak+
Escherichia coli	A/A	+	–	K/K	–
Proteus	K/A	+	+	Red/A	+
Providencia	K/A	+	–	K/A	–
Citrobacter	K/A or A/A	+	+	K/A	+
Enterobacter	A/A	+	–	K/A	–
Yersinia	A/A or K/A	–	–	K/A	–
Plesiomonas (oxidase$^+$)	K/A	–	–	K/K or K/A	–
Aeromonas (oxidase$^+$)	A/A	V	–	K/A	–
Pseudomonas and other nonfermentative bacteria	K/K	–	–	V	V

Abbreviations: K, alkaline; A, acid; +, most strains are positive; −, most strains are negative; V, variable reaction reflecting strain-to-strain variation. TSI determines the fermentation of D-glucose, lactose, and sucrose; gas production during fermentation; and H_2S production from thiosulfate. LIA determines L-lysine decarboxylase and L-lysine deaminase activity; and H_2S production from thiosulfate. The patterns listed are typical for each organism, but there are many exceptions.

triple sugar iron (TSI) agar and lysine iron agar (LIA) slants. *Shigella* strains do not ferment lactose rapidly, are lysine decarboxylase negative, and H_2S negative; their reactions, and those of other common bacteria, on TSI and LIA agar are shown in Table 9260:II. Colonies with typical reactions on these two screening media are then grown on suitable media (as specified by the manufacturer of the serological reagents used), and screened with *Shigella* antisera. However, this is being replaced with more complete testing in clinical and other laboratories.

b. Complete biochemical identification: Biochemical identification can be made with a complete set of 15 to 45 biochemical tests in a wide variety of commercial miniaturized kits or in test tubes of prepared media. These methods are frequently used in state and national reference laboratories. Cultures identified as *Shigella* are then serotyped.

7. Serological Grouping and Complete Serotyping

a. Serological reagents: Cultures that have been identified as *Shigella*, or those with results consistent with *Shigella* on TSI/LIA screenings (Table 9260:II), are tested by slide agglutination with commercial polyvalent and then group-specific antisera. A wide variety of serological reagents have traditionally been available commercially.§ Recently, some manufacturers have formulated tests with antibodies absorbed onto latex particles.‖ Many commercial companies also produce polyvalent grouping antisera and individual O typing sera. Antisera may exhibit problems with both sensitivity and specificity;[9] however, evaluations of commercial products specifically apply only to the individual lot numbers evaluated. Always follow manufacturer's instructions exactly and examine the package insert to determine which *Shigella* serotypes will be detected with each reagent. NOTE: Many other *Enterobacteriaceae* (and other bacteria) share antigens with the genus *Shigella*, so it is essential to do confirmatory biochemical or molecular testing. Refer cultures to a public health reference laboratory for confirmation and additional testing.

b. Testing for a specific Shigella *serotype in water samples:* If a water sample is being analyzed as part of an investigation, in which the species of interest is known (e.g., a recreational lake outbreak due to *S. sonnei)*, test "suspect *Shigella*" colonies with typing sera for that group only. A culture that agglutinates strongly is then confirmed biochemically before being reported.

c. Testing for all Shigella *serotypes in water samples:* If a water sample is being analyzed as part of a general search for all *Shigella* species and serotypes, test "suspect *Shigella*" colonies in each of the *Shigella* polyvalent sera (four to eight, depending on the manufacturer). Test a culture that agglutinates in a polyvalent serum in each dual O sera that is included in the polyvalent. Many state, regional, and national reference laboratories can provide assistance in devising protocols and confirming a water laboratory's identification and serotyping results.

8. Membrane Filter Procedure

This procedure is suitable for low-turbidity potable and surface waters with low concentrations of coliform bacteria. Filter 100-mL to 1-L samples through 0.45-μm pore size membranes and place filters face up on the surface of XLD or MacConkey agar plates; incubate plates at 35°C overnight. Pre-enrichment can be effected by placing the filter on a noninhibitory medium for several hours and then transferring it to the more selective medium; this treatment may result in the growth of cells that have been injured by exposure to the hostile environment of environmental water. Where growth is confluent, remove some

§ Among the sources are BBL, Becton Dickinson Microbiological Systems, Cockeysville, MD; Denka Seiken Co., Ltd., Tokyo, Japan; Difco Laboratories, divisions of Becton Dickinson and Co., Sparks, MD; Murex Diagnostics Ltd., Dartford, U.K.; Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France.

‖ For example, Wellcolex Colour *Shigella*, Murex Diagnostics Ltd., Dartford, U.K.

bacterial growth from the plate and inoculate into GN or Selenite F broth enrichment media; incubate for 6 h and then streak onto MacConkey and XLD plates for colony isolation. Isolated colonies then can be identified or inoculated into screening tests. For screening, pick colorless colonies (i.e., those that are lactose negative) from membrane filters or plates and inoculate slants of TSI and LIA; incubate overnight at 35°C. For interpretation, additional biochemical reactions, and serological grouping, see 9260E.6 and 7.

9. Centrifugation Procedure

This procedure is suitable for surface waters, wastewater, and sediments. Centrifuge 200- to 250-mL water samples at 1520 × *g* for 15 min and pour off all but last 2 mL of supernatant. Resuspend pellet and add 8 mL Selenite F or GN broth. Incubate suspension for 24 h at 35°C. Mix the suspension and inoculate one loopful to each of several MacConkey and XLD plates. Streak plates for isolation and incubate overnight at 35°C. Examine these plates for colorless colonies and pick and then streak suspect colonies onto TSI and LIA slants; incubate at 35°C overnight. For biochemical reactions, and serological grouping, see 9260E.6 and 7.

For solid samples (sediments, soil, sludge, etc.), suspend 10 g sample in 100 mL Selenite F or GN broth and mix thoroughly. Incubate suspension overnight at 35°C. Resuspend sediment and streak one loopful onto each of several MacConkey and XLD agar plates; incubate overnight at 35°C. Pick colorless colonies and streak onto TSI and LIA slants, and proceed as above.

10. Molecular and Research Approaches

a. DNA probe: It is difficult, if not impossible, to devise a plating medium or enrichment broth that selects for *Shigella* at the expense of *E. coli,* which usually outnumber *Shigella* in aquatic specimens. However, all four *Shigella* species contain chromosomal genes, the *ipa* genes (*ipa*B, *ipa*C, and *ipa*D) and plasmid genes (*ipa*H) that are absent on strains of *E. coli* that lack the ability to cause invasive or dysentery-like intestinal infections. This difference between *Shigella* and "generic" *E. coli* offers a unique approach to detect *Shigella*-specific genetic sequences in foods, clinical specimens, or water samples.[7,10–13] Samples that are positive by a molecular method are then cultured to isolate the *Shigella* strain(s) presumably present. Details of a method of enrichment and identification of *Shigella* based on a PCR method are available.[10] An *ipa*C protein-specific monoclonal antibody can be used to detect *Shigella* colonies on nitrocellulose membranes in a colony blot immunoassay.[13] This is an alternative for laboratories without molecular capabilities.

b. Immunomagnetic separations: Another method that shows promise is immunomagnetic separation[14,15] followed by either PCR detection or isolation methods previously described. For example, immunomagnetic particles can be coated with the monoclonal antibody MASFB, which is specific for a common epitope of the O polysaccharides of *S. dysenteriae* type 1 and *S. flexneri*. Cells of these two species attach to the immunomagnetic particles, which are then removed from the sample with a magnet. This or other similar immunomagnetic particles could be added to enrichment cultures to make them much more efficient in detecting *Shigella* strains. Immunomagnetic particles coated with *Shigella* antibodies are not yet commercially available.

c. Other methods: New and novel approaches are constantly being published and evaluated. Methods and procedures described for *E. coli* O157 may provide a useful guide for *Shigella*.

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9260 F. Diarrheagenic *Escherichia coli*

Escherichia coli is a bacterium that has been of concern in water analysis for more than 100 years. *E. coli* is the "type species" of the genus *Escherichia,* which also includes *Escherichia albertii, E. fergusonii*, *E. hermannii*, and *E. vulneris.* A fifth species, *E. blattae,* will probably be removed from the genus. Among microorganisms, *E. coli* is probably the most studied species. Although *E. coli* is a normal inhabitant of the human intestinal tract, some *E. coli* strains cause intestinal infections or attach to intestinal cells and produce enterotoxins.[1–3] The usual result is diarrhea. In this section, these gut pathogens are referred to as "diarrheagenic *E. coli*." This term is more specific,[4] than "pathogenic *E. coli"* because it distinguishes the enteric pathogens from those strains that cause extraintestinal infections because they have evolved in different ways or have acquired different virulence genes. *E. coli* causes a variety of these, such as meningitis, bacteremia, and wound and urinary-tract infections.[4] Specific procedures for the isolation and identification of *E. coli* strains that cause extraintestinal infections can be found in manuals that deal with clinical microbiology.[4]

Diarrheagenic *E. coli* are important causes of intestinal infections worldwide, but particularly in developing countries.[2,3] Many cases of *E. coli* diarrhea are due to foodborne and person-to-person transmission, but waterborne outbreaks also occur.[1,2,5–9] From 1972 through 1994, *E. coli* accounted for only two of 291 (0.7%) drinking water-related outbreaks in the United States that were reported to CDC, one due to an enterotoxin-producing *E. coli* strain and the other to a strain of *E. coli* O157. However, *E. coli* accounted for four of 30 outbreaks associated with recreational water for the years 2001–2002,[9] with three of these attributed to *E. coli* O157:H7.

Four groups of *E. coli* are well established as enteric pathogens:[2,3] Shiga toxin-producing *E. coli* (STEC) [of which enterohemorrhagic *E. coli* (EHEC) is a subgroup], enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enteropathogenic *E. coli* (EPEC). The term *E. coli* O157 is used throughout this section to refer to diarrheagenic members of this group, with or without the H7 antigen. *E. coli* O157 is a member[4] of the STEC group, and is an important pathogen in the United States and many other countries. Because *E. coli* O157 causes life-threatening infections, methods have evolved rapidly for its isolation and identification in human clinical specimens and food, and many commercial reagents are available. These procedures are applicable to water analysis and are covered below.

Other *E. coli* groups have been named[2,3] that are sometimes associated with intestinal infection. They include the enteroaggregative, diffusely adherent, cytotoxic-necrotizing-factor-producing, and cytolethal-distending-toxin-producing *E. coli* groups. These may have a causative role under certain conditions that have not been defined completely. A number of phenotypic properties or specific genes have been found in these different groups and are used in forming operational definitions and as the basis of diagnostic tests.

As described in 9260E.7*b* and *c*, the water laboratory will need to consider two types of situations: a search for all diarrheagenic *E. coli,* or for just one group (e.g., for all enterotoxin-producing *E. coli).* For the typical water laboratory, isolating and identifying diarrheal *E. coli* will be a difficult undertaking because a large number of nonroutine methods are needed to detect one or all of the pathogenicity groups. These methods are best suited for research laboratories and special studies. See 9260F.10 for one molecular solution to this difficult problem. Much easier is the search for a particular *E. coli* strain in the setting of a specific outbreak investigation associated with water. Specific approaches to three outbreak situations are outlined in 9260F.11.

Diarrheagenic *E. coli* often can be differentiated from other *E. coli* on the basis of their O and H antigens but are best defined on the basis of pathogenicity factors or the genes that code for them, such as plasmid-mediated cell invasion, plasmid-mediated colonization and enteroadherence factors, production of several potent cytotoxins, hemolysins, as well as heat-labile and stable enterotoxins.

The general approach for looking for diarrheagenic *E. coli* isolates can be summarized simply: use standard isolation and identification methods for fecal coliforms and *Escherichia coli;* then use special methods to determine if the culture belongs to a diarrheagenic group. Before planning a study to detect diarrheagenic *E. coli* in water, determine the availability of commercial products and reagents.

1. Safety

Diarrheagenic *E. coli* are enteric pathogens; use normal safety procedures and standard precautions, such as those available from government agencies.* Biosafety Level 2 (BSL 2) is usually specified because aerosol transmission usually is not involved. If pathogenic strains of *E. coli* O157 may be isolated, preferably use additional personal protection, such as protective gowns, masks, and gloves. In addition, post a sign on each door to the laboratory indicating that *E. coli* O157 is present and listing the necessary precautions for those who enter. Design safety procedures carefully because personnel who perform water analyses are not accustomed normally to working with this extremely dangerous pathogen. Fatal laboratory infections have occurred with *E. coli* O157, and the infectious dose may be as low as a few cells, as is the case with most *Shigella* strains. This makes it a very hazardous laboratory pathogen. Nonpathogenic and nontoxigenic strains of *E. coli* O157 are available† and should be used in quality control procedures and method-verification studies. **CAUTION: Avoid wild-type and virulent strains whenever possible to protect personnel.**

2. Sampling and Concentration

See sections on coliforms and thermotolerant coliforms (9221 and 9222), and *Salmonella* (9260B); these methods should be generally applicable.

* See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov
† For example, from the American Type Culture Collection, http://www.atcc.org.

3. Enrichment

Methods described for the enrichment of coliforms, thermotolerant coliforms, and *E. coli* are generally applicable for diarrheagenic strains of *E. coli*.

a. Nonselective enrichment: Enriching a water sample in a noninhibitory growth medium before selective enrichment may aid in reviving "injured cells."[10] Several non-selective broth media can be used: heart infusion broth (HIB), peptone water (PW), buffered peptone water (BPW), tryptic soy broth without D-glucose, tryptone soy broth, and others.

In general, avoid media that contain D-glucose (such as tryptic soy broth) because acidic products from glucose fermentation can reduce the pH to very low levels, resulting in death of the desired pathogen. The exception to this generalization may be *E. coli* O157; acid may actually be selective rather than inhibitory.[4]

"Injured cells" of STEC *E. coli* have been resuscitated in the presence of the trihydroxamate siderophore ferrioxamine, a commercial antioxidant, and by an enterobacterial autoinducer.[10]

b. Selective (and differential) enrichment: After enrichment in a nonselective broth, place a portion in one or more of the more selective or selective/differential enrichment broths, such as EC medium with MUG, GN broth, or vancomycin/cefixime/cefsulodin broth.

c. High-temperature enrichment and incubation time: Most strains of *E. coli* (a notable exception is *E. coli* O157) grow and ferment lactose at 44 to 45°C. Many other species of *Enterobacteriaceae* and other organisms are inhibited at this high temperature. Enrichments for *E. coli* have traditionally been at 37°C, but enrichment at 42 or 44.5°C is more selective. Some enrichment procedures have specified 6 to 8 h; others have specified overnight incubation (16 to 24 h).

d. Broths with antibiotics: E. coli strains that cause outbreaks are often antibiotic-resistant. Test the outbreak strain for its antibiotic susceptibility via the standardized single disk method or a commercial product that determines minimum inhibitory concentration for 10 to 25 antibiotics. Based on the outbreak strain's resistance level, incorporate one or more antibiotics into one of the broths listed above to make it highly selective for the outbreak strain being sought (see 9260F.11).

e. Selective enrichment of E. coli *O157:* Broth media can also be made more selective for *E. coli* O157 by adding cefixime and/or potassium tellurite. Cefixime-tellurite (CT) supplement is commercially available‡ and can be incorporated into liquid and solid media. *E. coli* broth supplemented with 20 μg/mL novobiocin, also known as modified EC medium, is commercially available.§ Vancomycin/cefixime/cefsulodin broth also can be used.[11–13]

4. Immunomagnetic Separation after Enrichment

See 9260B.5. Beads coated with antibodies to the O antigen are commercially available for *E. coli* O157, O26, O103, O111, and O145. To prepare immunomagnetic beads for other *E. coli* O groups, purchase commercial O antiserum for the strain being sought and add it to uncoated immunomagnetic beads. The resulting reagent can then be used for immunomagnetic separation after enrichment.

Immunomagnetic separation is being used in both food and water analysis to improve isolation procedures. Several commercial companies describe specific procedures for separation and subsequent identification with their *E. coli* O157 products and give evaluations and references.

5. Plating Media

See the plating media used for total coliforms, fecal coliforms, and *E. coli.* However, no plating medium will select for, or differentiate, diarrheagenic *E. coli* from the nondiarrheagenic *E. coli*. The exception is *E. coli* O157, for which selective and selective/differential media are available.[4,14,15] The population of diarrheagenic *E. coli* in water usually will be only a fraction of the population of nondiarrheagenic strains.

a. MacConkey agar: On this medium, strains of *E. coli* that ferment lactose rapidly will appear as red colonies, usually with precipitated bile around the colony. Although there are many exceptions, colonies with this appearance can be considered "very suspicious as being *E. coli*." Confirmation of this visual identification is required.

b. Other agar media: Nutrient agar with MUG, mTEC agar, violet red bile agar with MUG, and numerous others are commercially available.

c. Plating media for E. coli *O157:* Since most strains of *E. coli* O157 do not ferment D-sorbitol rapidly (within 48 h of incubation) sorbitol-MacConkey agar (SMAC) was developed[15] and is available commercially. This medium has proved useful for isolating *E. coli* O157 from clinical specimens, foods, and water, and can be made more selective by adding cefixime (0.05 mg/L) and potassium tellurite (2.5 mg/L); usually, the resulting medium is referred to as *cefixime-tellurite SMAC*. A few strains of *E. coli* O157 do not grow on this medium.[4] Another plating medium that differentiates *E. coli* O157 from other D-sorbitol-negative organisms‖ can be made more selective by adding potassium tellurite (2.5 mg/L) and/or cefixime (0.025 mg/L) to inhibit *Proteus* strains, or 5 mg/L to inhibit strains of *Pseudomonas* and/or *Aeromonas.*

6. Screening Tests and Biochemical Identification

Sections 9260B and E discussed different approaches to identification to the species level and the use of commercially available products, such as kits. These kits are useful for identification, but many of them will not include tests or information for organisms that normally occur in water, which will limit their application and accuracy for water analysis. *E. coli* and *Shigella* are very closely related in a phylogenetic sense, and belong to the "same species" when most phylogenetic definitions are applied. The *phoE* genetic probe is a sensitive and specific single test for the *E. coli-Shigella* Group that can be performed by reference laboratories.

Diarrheagenic and nondiarrheagenic strains of *E. coli* are generally indistinguishable in their biochemical reactions, but there are two important exceptions. *E. coli* O157 has a unique

‡ Dynal Biotec, or equivalent.
§ Difco, or equivalent.

‖ CHROMagar O157, Dynal Bioscience, or equivalent.

phenotype, and its MUG-negative and D-sorbitol-negative phenotype has been most useful in screening tests. Most invasive strains (EIEC) are nonmotile and negative for lysine decarboxylase and lactose fermentation. This phenotype is in contrast to "gut strains" of *E. coli* but is shared with other "inactive *E. coli*" strains that are not invasive.

Escherichia coli O157:H7 has several unique properties that facilitate its recognition and identification. This microorganism grows as colorless colonies on sorbitol MacConkey agar because it is D-sorbitol negative (or delayed) and is MUG-negative, and will agglutinate in O157 serum and H7 serum.

7. Serological Identification

Although polyclonal antisera have been used for more than 50 years to determine the presence and types of O and H antigens of *E. coli* cultures, monoclonal antibodies and latex agglutination reagents have recently become available for *E. coli* O157 and H7 and a few of the other important serotypes. Check for commercial availability and follow manufacturers' instructions exactly. The procedure below for *E. coli* O157:H7 is a typical example.

a. Serological identification of E. coli *O157 and* E. coli *O157:H7:* Test a pure culture in O157 antiserum or latex beads coated with O157 antiserum. *E. coli* O157 cultures react strongly with the reagent. Complete the manufacturer's confirmatory procedures to ensure that it is O157 and not a cross-reacting O group. Determine that the culture is motile and has the H7 antigen. Determine if the culture produces Shiga toxin (see 9260F.8*a* below). The following are examples of interpretation as to whether the strain is a STEC diarrheagenic *E. coli* O157:

Result	Interpretation: Is it an enteric pathogen?
E. coli O157:H7, Shiga toxin-positive	Yes
E. coli O157:NM (non-motile, no H antigen), Shiga toxin-positive	Yes
E. coli O157:NM (non-motile, no H antigen), Shiga toxin-negative	No
E. coli O157, motile**, but not H7, Shiga toxin-negative	No

b. Screening colonies based on H antigen immobilization test: This test was developed[15] as a quick and simple method to screen hundreds of *E. coli* O157:H7 colonies picked directly from Sorbitol-MacConkey agar plates. Prepare tubes of H7 immobilization medium.[15] Touch a sorbitol-negative (colorless) colony on sorbitol-MacConkey agar and stab it a few millimeters in the top of the tube. Incubate overnight. Cultures of *E. coli* O157:H7 are immobilized and are presumptive positives. Other motile microorganisms will grow throughout the medium. Confirm the presumptive positives with commercial *E. coli* O157:H7 latex reagents.

When commercial latex reagents became available for *E. coli* O157:H7, the H antigen immobilization test was relegated to a secondary role. However, it may be the best and quickest option if hundreds of colonies must be tested in outbreak investigations.

8. Commercial Tests for *E. coli* Toxins

Several commercially available immunoassays make it possible to test water isolates identified as *E. coli* and determine if they produce three important toxins.

a. Immunoassays for Shiga toxin: These products†† have been designed to detect Shiga toxin produced by pure cultures, "colony sweeps," stool specimens, foods, and enrichments. The procedures can be modified to detect Shiga toxin-producing strains in water analysis. Check manufacturer's descriptive materials to determine which Shiga toxins are being detected.

b. Immunoassays for heat-labile enterotoxin: Pure cultures of *E. coli* are grown in the specified medium and then tested for toxin production. Use a reverse passive latex agglutination assay.‡‡

c. Immunoassays for heat-stable enterotoxin: Pure cultures of *E. coli* are grown in the specified medium and tested for toxin production. An enzyme immunoassay for heat-stable enterotoxin is available.§§

9. Other Commercial Assays and Reagents

Several useful listings of commercial products are available.[4,11–13] Because availability is always changing, check current catalogs and Internet sites.

10. Molecular Approaches and DNA-Based Testing

Molecular tests are frequently used to detect diarrheagenic *E. coli*, particularly the two enteropathogenic and enteroinvasive groups, for which there are no simple methods for isolation and identification. Strains of diarrheagenic *E. coli* have genes on the chromosome or on plasmids that code for toxins, colonization factors, bacteriophages, or other factors. Some of these genes are unique to one *E. coli* pathogenic group, but others are shared. Molecular tests have been based on one or more genes but no methodology has as yet emerged as standard.

Several molecular procedures for diarrheagenic *E. coli* in food, with detailed instructions for the assays, have been described.[11–13] These may be useful as a guide for investigations in water.

11. Investigation Methods

Several government and nongovernment organizations have written detailed and rigid procedures for isolating fecal coliforms, *E. coli*, and diarrheagenic *E. coli* from various specimens, particularly food and water. There are advantages and disadvantages to this rigid approach. These rigid procedures can be used as a starting point, with modifications considered for particular situations in water analysis.

** The H antigen of this strain could be determined and a reference laboratory's final report might be: "*Escherichia coli* O157:H14, negative for Shiga toxins I and II."

†† Difco, Dynal Bioscience, or equivalent.
‡‡ VET-RPLA, available from Oxoid, Ogdensburg, NY, or equivalent.
§§ ST EIA kit, Denka Seiken Co. or equivalent.

In contrast to rigid methods, examples of three flexible procedures are given in ¶s *b–d* below for isolating and identifying a specific outbreak strain of *E. coli* during a waterborne outbreak investigation.

a. First steps: Isolate and study the *E. coli* outbreak strain obtained from human diarrhea cases and determine its phenotypic properties. Knowledge of these properties then can be used to develop more sensitive and specific methods for isolating and identifying the outbreak strain. Next, use the outbreak strain and verify that it grows in the media and conditions being considered. This is often called "method validation." Do this before the provisional methods are used to test water samples.

b. Example: Outbreak caused by a Shiga-toxin-producing (STEC) strain of E. coli *O157:H7 that is lactose-positive, D-sorbitol-negative, MUG-negative, and antibiotic-sensitive:* The following are general procedures to consider:

1) Sampling, concentration, pre-enrichment—Use one of the methods previously described.

2) Enrichment—Use one or more of the *E. coli* O157 enrichment broths.

3) Immunomagnetic separation—Test with commercial O157-coated immunomagnetic beads.

4) Plating—Use MacConkey agar, sorbitol MacConkey agar, and CHROMagar O157.

5) Screening—Use *E. coli* O157 latex reagent; test suspicious colonies that grow on the plates.

6) Confirmation—Test colonies that agglutinate the O157 latex for the H7 antigen using H7 antisera or H7 latex reagent.

7) Shiga toxin—Test in one of the commercial kits.

8) Report—*E. coli* O157:H7, positive for Shiga toxins 1 and 2.

c. Example: Outbreak caused by invasive (EIEC) strain of E. coli *O124:NM that is lysine-negative, lactose-negative, nonmotile, and resistant to chloramphenicol (MIC of 1048 μg/mL):* The following are general procedures to consider:

1) Sampling, concentration, pre-enrichment—Use one of the methods previously described.

2) Enrichment—Use one or more of the *E. coli* enrichment broths; include enrichment broth(s) with 100 μg/mL chloramphenicol to select for the outbreak strain.

3) Immunomagnetic separation—Use commercial *E. coli* O124 immunomagnetic beads if available; otherwise prepare *E. coli* O124 immunomagnetic beads using commercial *E. coli* O124 antiserum and uncoated immunomagnetic beads.

4) Plating—Use MacConkey agar and MacConkey agar with 100 μg/mL chloramphenicol.

5) Screening—Test lactose-negative colonies that grow on the agar plates in commercial *E. coli* O124 latex reagent (or in commercial *E. coli* O124 antiserum). If commercial *E. coli* O124 latex reagent is not available, prepare it with commercial *E. coli* O124 antiserum and uncoated latex.

6) Confirmation—Confirm colonies that are positives for *E. coli* O124 as being lysine-negative, lactose-negative, nonmotile, and resistant to chloramphenicol (MIC of 1048 μg/mL).

7) Report—*E. coli* O124:NM, lysine-negative, lactose-negative, nonmotile, and resistant to chloramphenicol (MIC of 1048 μg/mL).

8) Reference laboratory—Refer the culture; it can be tested for specific genes-virulence factors that define EIEC.

d. Example: Outbreak caused by an enterotoxin-producing strain (ETEC) of E. coli *O6:H16 that produces both heat-labile and heat-stable enterotoxins (LT^+, ST^+), is lactose-positive, and resistant to tetracycline (MIC of 1048 μg/mL) and ampicillin (MIC of 512 μg/mL):* The following are general procedures to consider:

1) Sampling, concentration, pre-enrichment—Use one of the many methods previously described.

2) Enrichment—Use one or more of the *E. coli* enrichment broths, but also include an enrichment broth(s) with added tetracycline (100 μg/mL) and ampicillin (100 μg/mL) to select for the outbreak strain.

3) Immunomagnetic separation—Prepare *E. coli* O6 immunomagnetic beads using commercial *E. coli* O6 antiserum and uncoated immunomagnetic beads.

4) Plating—Use MacConkey agar and MacConkey agar with tetracycline (100 μg/mL) and ampicillin (100 μg/mL) to select for the outbreak strain.

5) Colony screening—Test suspicious (lactose positive) colonies in *E. coli* O6 latex reagent (use commercial reagent if available, otherwise prepare one by using commercial *E. coli* O6 antisera and uncoated latex). Test with commercial H16 antisera to confirm, or refer to reference laboratory.

As an alternative to screening hundreds of colonies; screen with an H16 immobilization test made with commercial H16 antiserum.[15]

6) Heat-labile enterotoxin and heat-stable enterotoxin—Test with commercial kits.

7) Report—*E. coli* O6:H16, positive for LT and ST, resistant to tetracycline (MIC of 1048 μg/mL) and ampicillin (MIC of 512 μg/mL).

12. Specific Methods

Examples of two specific procedures for *E. coli* O157 are given below.

a. Procedure for E. coli *O157—smaller samples:* The following procedure is a modification of the standard total coliform fermentation technique (9221B) for detecting *E. coli* O157:H7 in water. Inoculate a 100-mL sample into 50 mL 3× lauryl tryptose broth (LTB) and incubate at 35°C for 24 h. Serially dilute the sample, spread plate (0.1 mL) onto sorbitol MacConkey agar and incubate at 35°C for 18 to 24 h. Adding cefixime and tellurite enhances selectivity. EHEC O157:H7 forms colorless colonies because they do not ferment, or are slow fermenters of, sorbitol. Pick ten sorbitol-negative colonies, transfer individually into LTB-MUG (4-methylumbelliferone glucuronide; 0.1 g/L) and incubate at 35°C for 18 to 24 h. *E. coli* O157:H7 ferment lactose, but do not have β-glucuronidase activity to hydrolyze MUG, so cultures will appear gas-positive and will not fluoresce. Assay these for positive glutamate decarboxylase activity and then identify biochemically as *E. coli* [see 9221G.1 (2006)].

b. Procedure for E. coli *O157—larger samples:* Larger volumes of sample also may be examined by the following procedure, modified from a procedure for detecting O157:H7 in food. This procedure has not been tested for use in water analysis; however, it has been used extensively to detect O157:H7 bacteria in apple juice. Centrifuge 200 mL sample at 10 000 × *g* for 10 min. Suspend pellet in 225 mL EHEC enrichment broth (EEB) and incubate at 35°C for 24 h. Spread plate 0.1 mL from EEB and a 1:10 dilution of EEB onto tellurite-cefixime sorbitol MacConkey agar (TC SMAC). Both EEB and TC SMAC contain

antibiotics to reduce growth of normal flora bacteria; therefore, they are best suited for highly contaminated samples. Incubate EEB sample and TC SMAC at 35°C for 18 to 24 h. Observe TC SMAC plates for isolated, colorless colonies. If none are evident, serially dilute the overnight EEB sample and plate onto TC SMAC. Test colorless colonies for positive indole reaction and identify biochemically as *E. coli* before serotyping and analysis for the Shiga toxin or its genes.

See research publication concerning procedures for large-volume concentrations in drinking water.[16]

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9260 G. *Campylobacter*

Campylobacter jejuni is the leading cause of bacterial diarrheal disease, in fact causing more cases than *Salmonella* and *Shigella* combined. The majority of *Campylobacter* infections are sporadic, with few outbreaks.[1] *Campylobacter* infections also have been associated with Guillain-Barre syndrome.[2] Campylobacters are ubiquitous in the environment and throughout the food chain.[1] Poultry,[3] sheep,[4] cattle,[4] and wild birds[5] have all been implicated as reservoirs for *Campylobacter* infection. They have been recovered from water, wastewater, and soil.[6] Peak infections of *Campylobacter* occur in May and September.[1] Outbreaks of *Campylobacter* disease also have been associated with drinking raw milk[1] and private drinking water.[7] In a study of 21 outbreaks of *Campylobacter* infection in England, six were traced to water, five to milk, five to food, and in five the source could not be identified.[8] One difficulty in linking exposure to disease and identifying outbreaks is the long incubation period between exposure and disease: approximately 2 weeks. In addition, it is still difficult to distinguish pathogenic from nonpathogenic *Campylobacter*.[1]

Campylobacter physiology is an important determinant of its presence in water and other environments. These bacteria are microaerophiles and only grow at 3 to 5% oxygen and 2 to 10% CO_2.[9] In the laboratory, campylobacters require a low redox potential for growth and are relatively sensitive to ultraviolet light and desiccation.[6] The presence of campylobacters in streams and rivers is associated with passage through farmland or the introduction of wastewater.[6] Campy-

lobacters have also been recovered from groundwater, an environment consistent with their physiology. The highest numbers of campylobacters in water are found in the winter months [6] and thus do not correlate with the seasonal appearance of disease. Because campylobacters do not grow in surface waters and die off rapidly, their numbers fall rapidly as the distance from the source increases.[6] This fact explains why there was no significant association between *Campylobacter* presence in farmland and water beyond 600 m.[10] Further confounding our understanding of the epidemiology of *Campylobacter* disease is the low infectious dose and the presence of viable but nonculturable campylobacters.[11]

Because of the relatively fastidious requirements for growth, isolation from samples that contain other microorganisms can be increased substantially (e.g., fourfold) by enrichment.

1. Water Collection and Filtration Method

Collect large-volume water samples in sterile 10-L plastic containers. Process samples immediately after collection or store at 4°C and process as soon as possible. Filter one to several liters of the water through a 0.45- or 0.22-μm-pore-size, 47-mm-diam, cellulose nitrate membrane filter. Remove filter and place face down on selective medium plate. Incubate at either 37 or 42°C for up to 5 d.[12] Incubate under microaerobic conditions at either 37 or 42°C for 24 h. Remove filter from the plate and place it face down on another selective medium (see 9260G.2).

For turbid waters, prefilter to remove soil particulates by using a stainless steel filtration device with a 1.5-L reservoir assembled with the following filter sequence. Place a 142-mm, 3.0-μm-pore-size filter on the screen inside reservoir with a 124-mm prefilter on top. In the bottom tubing adapter, place a 47-mm, 1.2-μm filter. Then place filter holders in parallel with a 47-mm, 0.65-μm filter in the upstream filter holder and a 47-mm, 0.45-μm filter in the downstream holder. Add 1 L sample to the reservoir, seal, and apply pressure of about 350 kPa. After filtration, remove the 0.45-μm-pore-size filter, place it on the surface of selective plating medium as described above, and incubate at either 37 or 42°C for 48 h.

2. Isolation

a. Selective media and conditions: Campylobacter isolation requires use of selective media containing antimicrobial agents, microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2), and a 42°C incubation temperature to suppress the growth of most common bacteria.[8] The thermophilic campylobacters (*C. jejuni, C. coli, C. lari*, and *C. upsaliensis*) grow well at 42°C. However, other campylobacters (*C. jejuni* subsp. *doylei* and *C. fetus*) do not grow well at 42°C; incubate plates at both 37 and 42°C for optimal isolation.[12] Microaerobic conditions can be provided by using commercially available systems and equipment.*

Selective media recommended for isolating *Campylobacter* include Skirrow's medium and *Campylobacter* medium, both commercially available. Skirrow's medium contains blood agar base with lysed horse blood, trimethoprim, vancomycin, and polymyxin B. *Campylobacter* medium contains *Brucella* agar base with sheep blood, trimethoprim, vancomycin, polymixin B, amphotericin B, and cephalothin (to which some campylobacters are sensitive). Other media include Butzler's medium, containing thioglycollate agar with sheep blood, bacitracin, novobiocin, cycloheximide, and cefazolin; Preston's medium, containing *Campylobacter* medium base with horse blood, cycloheximide, rifampicin, trimethoprim, and polymyxin B; *Campylobacter* blood-free selective medium; and *Campylobacter* charcoal differential agar.[12]

b. Enrichment media: Several enrichment media, such as *Campylobacter* broth, Campy-thio broth, Gifu anaerobe-modified semisolid medium, and Preston medium, are used to enhance recovery of campylobacters.[13] Add 10 mL water sample to 10 mL *Campylobacter* enrichment broth tubes in duplicate and incubate at 37 and 42°C for 8 h or overnight. Incubation of a water or soil sample in a selective enrichment broth for 4 h at 37°C may be important for recovery of stressed cells of *C. jejuni* that show less tolerance to elevated growth temperatures. Following pre-enrichment, transfer cultures to another incubator at either 37 or 42°C for overnight incubation.[13]

C. jejuni may be induced to a nonculturable state in water, and it is not clear whether pre-enrichment or enrichment will facilitate isolation of these bacteria.[11] Use of a decreased substrate concentration enhances metabolic activity in nonculturable campylobacters from water.[14]

3. Identification

a. Culture examination: Examine *Campylobacter* plates at 24 and 48 h for characteristic colonies, which can range from flat, spreading colonies that cover the entire surface of the plate, to very small, convex, translucent colonies with colony colors ranging from gray to yellowish or pinkish.[12]

b. Microscopy identification: Campylobacter spp. do not stain well by the conventional Gram stain. If safranin is used as a counterstain, apply it for 2 to 3 min; 0.3% carbol fuchsin may be substituted for safranin to improve counterstaining. Even in 24-h cultures, campylobacters appear pleomorphic in stained smears, and cells range from small gram-negative rods and coccoid forms to longer curved, spiral, or S-shaped rods.[12]

c. Motility test: Campylobacters normally are motile by a single polar flagellum at one or both ends. Suspend cells in Mueller-Hinton or nutrient broth and observe darting, tumbling motility using phase contrast microscopy or brightfield microscopy with reduced illumination. Do not use saline or distilled water because they may inhibit motility.[12] Young cells are 0.2 to 0.8 μm wide by 1.5 to 5 μm long, curved or spiral, and motile with darting or corkscrew-like motion.[12]

d. Biochemical tests: Campylobacters are presumptively identified by an absence of growth in air, presence of oxidase and catalase activities, Gram stain, and cell size and morphology.[12]

e. DNA-based identification: Presumptive *Campylobacter* isolates can be identified as *C. jejuni*, *C. coli*, *C. lari,*

* Campy Pak II, BioBag Environmental Chamber or BioBag Type Cfj, Becton Dickenson; Gas Generating Kit System BE56 or Campy Gen, Oxoid; Poly Bag System, Fisher Scientific; or equivalents.

C. hyointestinalis, or *Campylobacter* by single-reaction PCR with different primers for the 16S rRNA gene.[10] PCR-based identification of *C. jejuni* and *C. coli* based on a gene encoding a lipoprotein of the enterochelin transport pathway (*ceu*) also has been developed.[15]

f. Serological identification tests: Kits† for serotyping campylobacters are commercially available. These kits use latex particles coated with polyvalent antibodies for rapid presumptive identification of the thermophilic, enteropathogenic *Campylobacter* species (*C. jejuni, C. coli*, and *C. lari*).

4. Epidemiological Markers

Both phenotypic and genotypic techniques for typing campylobacters have been developed.[1] Phenotypic methods include biotyping, serotyping, and phage typing.[1] A variety of DNA-based (genotypic) molecular techniques have been used to demonstrate the clonal relatedness of *Campylobacter* isolates from patients and environmental sources.[1] Caution must be taken in interpreting results from fingerprinting because instability of fingerprints or profiles of clones has been reported.[16] Multilocus sequence typing (MLS), based on sequences of conserved housekeeping genes, has been used for characterizing populations of different *C. jejuni* isolates.[17] MLS profiles are available for comparison and expansion.‡ Pulsed field gel electrophoresis (PFGE), relying on comparison of large restriction fragments of whole genomes, has led to identification of clonal groups of *C. jejuni* and *C. coli*.[18]

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† Such as Campyslide, BBL Microbiology Systems; Meritec-Campy, Meridian Diagnostics; and Microscreen, Mercia Diagnostics.
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9260 H. *Vibrio*

Vibrio (family *Vibrionaceae*) is a genus that has been of concern in human diseases and water analysis for many years. It now includes more than 60 named species, but only 12 occur in human clinical specimens (Table 9260:III). Eleven of these apparently cause human infections. *Vibrio* species usually cause either diarrhea or extraintestinal infections,[1–3] but some, such as *V. cholerae*, can cause both. Most human infections are related to water exposure, either water itself or through animals, such as fish and shellfish, that live in water. There are several reviews that consider the whole genus and provide many details for isolation and identification.[1–8] Throughout this section, the general term “vibrio” (plural “vibrios”) is defined to be a member of the genera *Vibrio* or *Photobacterium.**

V. cholerae and *V. parahaemolyticus* are well-documented causes of diarrhea, and *V. cholerae* has caused many pandemics

* See http://www.bacterio.cict.fr/index.html for a complete species listing.

TABLE 9260:III. GROWTH OF *VIBRIO* CULTURES ON TCBS AGAR

Organism	Colony appearance on TCBS agar %		Growth-plating Efficiency
	Green	Yellow	
V. cholerae	0*	100*	Good
V. mimicus	100	0	Good
V. parahaemolyticus	99	1	Good
V. alginolyticus	0	100	Good
V. fluvialis	0	100	Good
V. furnissii	0	100	Good
V. hollisae	100	0	Very poor
V. harveyi	0	100	Good
V. damsela	95	5	Reduced at 36°C
V. metschnikovii	0	100	May be reduced
V. cincinnatiensis	0	100	Very poor
V. vulnificus	90†	10†	Good
"Marine vibrios"	Variable	Variable	Variable
Aeromonas and *Enterobacteriaceae*	No growth	No growth	Most strains are totally inhibited

* Percentage of strains that produce green colonies and yellow colonies, respectively.
† The original report describing this species gave the percentage positive for sucrose fermentation as 3%. At the CDC *Vibrio* laboratory, about 15% of the strains have been sucrose positive. The 10% in the table represents a composite value.

of cholera and millions of deaths. It is now divided into three major subgroups: *V. cholerae* O1, *V. cholerae* O139, and *V. cholerae* "non-O1, non-O139" (which comprises hundreds of the remaining numbered serotypes). Any strain of *V. cholerae* may cause diarrhea, but only *V. cholerae* O1 and *V. cholerae* O139 have caused pandemics of cholera.

More recently, *V. fluvialis*, *V. hollisae*, and *V. mimicus* also have been implicated as causative agents of diarrhea, but are less common.[1–3] *V. furnissii*, *V. metschnikovii*, and *V. vulnificus* have been isolated from the feces of patients with diarrhea (particularly after eating raw oysters).[3] Their role as actually causing the diarrhea is unproven, but deserves systematic investigation.[3]

All the *Vibrio* species are primarily aquatic, and the species distribution usually depends on temperature, Na^+ concentration, nutrient content of the water, and the plants and animals present. *Vibrio* species are common in marine and estuarine environments[1–4,6] and on the surfaces and in the intestinal tracts of marine animals. In marine and estuarine environments, vibrios are commonly isolated from sediment, the water column, plankton, and shellfish.[2,4] Seafoods that often harbor *Vibrio* species include bivalve shellfish (oysters, clams, and mussels), crabs, shrimp, and prawns. Vibrios have also been recovered from brackish lakes in the continental United States, and nonhalophilic vibrios have even been isolated from freshwater sources.[2,4,8]

Although all vibrios require Na^+ for growth[1–3] they vary greatly in the minimal amount of Na^+ (almost always expressed as NaCl) they require.[2] They also vary greatly in the amount of NaCl they tolerate (Table 9260:IV). This requirement and tolerance for NaCl has been the basis of many selective broth and agar media.[1–3] *V. cholerae* and *V. mimicus* are defined as "nonhalophilic *Vibrio* species." Although they require small amounts of Na^+ for growth, this requirement is satisfied by the peptones, meat extracts, and similar ingredients found in commercial media.[1–3] All of the other *Vibrio* species are *halophilic*—they do not grow at 36°C in nutrient broth that has no added NaCl in its formulation (see Table 9260:IV). *V. fluvialis*, *V. furnissii*, and *V. metschnikovii* are "moderate halophiles," and grow in nutrient broth with only 0.1% added NaCl.[1–3] Most of the other *Vibrio* species require much more than 0.1% NaCl for growth and can be considered to be the "true halophiles" or "marine vibrios." The NaCl content can be adjusted in designing enrichment broths and agars. A low amount, such as 0.1%, will select against the marine vibrios, but will allow the pathogens to grow. Hundreds of methods have been described for the isolation and identification of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and other *Vibrio* species from clinical specimens, foods, seafood, wastewater, and water, although none of these has yet achieved enough acceptance to be considered a standard method. Several commercial companies produce equipment, supplies, media, and reagents useful in *Vibrio* work. An extensive listing of commercial products and their sources is available.[5]

Newer methods based on DNA probes and PCR are extremely promising as research procedures, but will have limited application for water analysis unless they become available as a ready-to-use commercial kit. The following sections describe methods and approaches that have proved useful and can be considered for a particular situation in water analysis.

1. Safety

See the safety discussions in the previous sections. Use normal safety procedures and standard precautions, such as those available from government agencies.† Because aerosol transmission is not normally involved, Biosafety Level 2 (BSL2) is usually specified. "Oral vaccine" strains of *V. cholerae* are available, and are recommended as a replacement for pathogenic "wild" strains in method verification studies.

† See, for example, http://www.cdc.gov/od/ohs/? and http://www.osha.gov.

TABLE 9260:IV. BIOCHEMICAL TEST RESULTS AND OTHER PROPERTIES OF THE 12 *VIBRIO* SPECIES THAT OCCUR IN HUMAN CLINICAL SPECIMENS

	Percentage Positive for:†											
Test*	*V. cholerae*	*V. mimicus*	*V. metschnikovii*	*V. cincinna-tiensis*	*V. hollisae*	*V. damsela*	*V. fluvialis*	*V. furnissii*	*V. alginolyticus*	*V. parahae-molyticus*	*V. vulnificus-* biogroup[1]	*V. harveyi*
Eight key differential tests:												
Growth in nutrient broth with 0% NaCl*	100	100	0	0	0	0	0	0	0	0	0	0
Growth in nutrient broth with 1% NaCl*	100	100	100	100	99	100	99	99	99	100	99	100
Oxidase production*	100	100	0	100	100	95	100	100	100	100	100	100
Nitrate reduced to nitrite*	99	100	0	100	100	100	100	100	100	100	100	100
Inositol (myo-) fermentation*	0	0	40	100	0	0	0	0	0	0	0	0
Arginine, Moeller's, (1% NaCl)*	0	0	60	0	0	95	93	100	0	0	0	0
Lysine, Moeller's, (1% NaCl)*	99	100	35	57	0	50	0	0	99	100	99	100
Ornithine, Moeller's, (1% NaCl)*	99	99	0	0	0	0	0	0	50	95	55	0
Additional differential tests:												
Indole production (HIB, 1% NaCl)	99	98	20	8	97	0	13	11	85	98	97	100
Methyl red (1% NaCl)	99	99	96	93	0	100	96	100	75	80	80	100
Voges-Proskauer (1% NaCl; Barritt*)*	75	9	96	0	0	95	0	0	95	0	0	50
Citrate, Simmons	97	99	75	21	0	0	93	100	1	3	75	0
H_2S on TSI	0	0	0	0	0	0	0	0	0	0	0	0
Urea hydrolysis	0	1	0	0	0	0	0	0	0	15	1	0
Phenylalanine deaminase	0	0	0	0	0	0	0	0	1	1	35	NG
Motility, (36°C)	99	98	74	86	0	25	70	89	99	99	99	0
Gelatin hydrolysis, (1% NaCl, 22°C)	90	65	65	0	0	6	85	86	90	95	75	0
KCN test (percentage that grow)	10	2	0	0	0	5	65	89	15	20	1	0
Malonate utilization	1	0	0	0	0	0	0	11	0	0	0	0
D-Glucose, acid production	100	100	100	100	100	100	100	100	100	100	100	50
D-Glucose, gas production	0	0	0	0	0	10	0	100	0	0	0	0
Acid production from:												
D-Adonito[1]	0	0	0	0	0	0	0	0	1	0	0	0
L-Arabinose*	0	1	0	100	97	0	93	100	1	80	0	0
D-Arabitol*	0	0	0	0	0	0	65	89	0	0	0	0
Cellobiose*	8	0	9	100	0	0	30	11	3	5	99	50
Dulcitol	0	0	0	0	0	0	0	0	0	3	0	0
Erythritol	0	0	0	0	0	0	0	0	0	0	0	0
D-Galactose	90	82	45	100	100	90	96	100	20	92	96	0
Glycero[1]	30	13	100	100	0	0	7	55	80	50	1	0
Lactose*	7	21	50	0	0	0	3	0	0	1	85	0
Maltose*	99	99	100	100	0	100	100	100	100	99	100	100
D-Mannitol*	99	99	96	100	0	0	97	100	100	100	45	50
D-Mannose	78	99	100	100	100	100	100	100	99	100	98	50
Melibiose	1	0	0	7	0	0	3	11	1	1	0	0
α-Methyl-d-glucoside	0	0	25	57	0	5	0	0	1	0	0	0
Raffinose	0	0	0	0	0	0	0	11	0	0	0	0
L-Rhamnose	0	0	0	0	0	0	0	45	0	1	0	0
Salicin*	1	0	9	100	0	0	0	0	4	1	95	0
D-Sorbitol	1	0	45	0	0	0	3	0	1	1	0	0
Sucrose*	100	0	100	100	0	5	100	100	99	1	15	50
Trehalose	99	94	100	100	0	86	100	100	100	99	100	50

TABLE 9260:IV. CONT.

Test*	Percentage Positive for:†											
	V. cholerae	*V. mimicus*	*V. metschnikovii*	*V. cincinna-tiensis*	*V. hollisae*	*V. damsela*	*V. fluvialis*	*V. furnissii*	*V. alginolyticus*	*V. parahae-molyticus*	*V. vulnificus-* biogroup[1]	*V. harveyi*
D-Xylose	0	0	0	43	0	0	0	0	0	0	0	0
Mucate-acid production	1	0	0	0	0	0	0	0	0	0	0	0
Tartrate-Jordan	75	12	35	0	65	0	35	22	95	93	84	50
Esculin hydrolysis	0	0	60	0	0	0	8	0	3	1	40	0
Acetate utilization	92	78	25	14	0	0	70	65	0	1	7	0
DNase (25°C)	93	55	50	79	0	75	100	100	95	92	50	100
Lipase*	92	17	100	36	0	0	90	89	85	90	92	0
ONPG Test*	94	90	50	86	0	0	40	35	0	5	75	0
Yellow pigment (25°C)	0	0	0	0	0	0	0	0	0	0	0	0
Tyrosine clearing	13	30	5	0	3	0	65	45	70	77	75	0
Growth in nutrient broth with:												
6% NaCl*	53	49	78	100	83	95	96	100	100	99	65	100
8% NaCl*	1	0	44	62	0	0	71	78	94	80	0	0
10% NaCl*	0	0	4	0	0	0	4	0	69	2	0	0
12% NaCl*	0	0	0	0	0	0	0	0	17	1	0	0
Swarming (marine agar, 25°C)	−	−	−	−	−	−	−	−	+	+	−	100
String test*	100	100	100	80	100	80	100	100	91	64	100	100
O129, zone of inhibition‡	99	95	90	25	40	90	31	0	19	20	98	100
Polymyxin B, % with any zone of inhibition	22	88	100	92	100	85	100	89	63	54	3	100

* Test is recommended as part of the routine set for *Vibrio* identification. 1% NaCl in parentheses indicates 1% NaCl has been added to the standard media to enhance growth; HIB, heart infusion broth; the Barritt reagent for the Voges-Proskauer test contains α-naphthol for greater sensitivity; TSI, triple sugar iron agar; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; the positive string test indicates cell lysis in the presence of a 0.5% sodium desoxycholate solution.
† The number gives the percentage positive after 48 h of incubation at 36°C (Unless other conditions are specified). Most of the positive reactions occur during the first 24 hours. NG (no growth) means that the organism does not grow, probably because the NaCl concentration is too low.
‡ Disk content = 150 μg.

Vibrio vulnificus causes serious, sometimes fatal wound infections. If this organism is being worked with, or there is a chance that it will be isolated from water samples, use disposable gloves to minimize contact with hands, particularly if the skin is not intact.

2. Sampling and Concentration

Methods used for coliforms, thermotolerant coliforms, *E. coli*, and *Salmonella* are generally applicable. The "Moore Swab" technique also has been particularly useful in water analysis.[9] Swabs can be placed in sewer pipes to detect and then trace cholera cases (*V. cholerae* O1). Vibrios in water[1–4] are often attached to particulate matter, such as plankton with chitin shells (e.g., copepods), algae, and similar microenvironments. If these particulates are filtered out to permit a greater sample volume for analysis, also culture the filter.

3. Enrichment

a. Nonselective enrichment: Enriching a water sample in a noninhibitory growth medium before selective enrichment may be helpful. It is possible that "injured" or "stressed" cells can be revived by pre-enrichment in special media.[10]

Several nonselective broth media can be used, including alkaline peptone water (see ¶ *c* below), peptone water (PW), buffered peptone water (BPW), heart infusion broth (HIB), and marine broth. In general, it is advisable to avoid media that contain D-glucose (such as tryptic soy broth) because acidic end products from glucose fermentation can reduce the pH to very low levels, resulting in rapid death of the desired *Vibrio* species.

b. Selective enrichment: There are many ways to select for one or more pathogenic *Vibrio* species at the expense of other vibrios and nonvibrios. Some of these include: raising the pH or incubation temperature, incorporating chemicals or antibiotics to reduce undesired vibrios and other organisms, and incorporating a sugar or organic compound used only by the desired *Vibrio* species.[1]

c. Enrichment media:

1) *Alkaline peptone water:* This is perhaps the most used broth to enrich *Vibrio cholerae* and other *Vibrio* species. *Vibrio* species typically grow better than other organisms at the high pH (8.5 to 9.0) of this medium, and also tend to concentrate at the aerobic surface (meniscus) of the liquid, often forming a pellicle. Culturing is done from the surface for this reason. Enrichment in alkaline peptone water usually is followed by plating the liquid's surface onto Thiosulfate Citrate Bile Salts (TCBS) agar and/or other media selective for *Vibrio*.

Alkaline peptone water is not a single medium. Most formulations typically contain 0.5 to 1% NaCl, which allows the growth of both pathogenic and environmental *Vibrio* species. The type of peptone used in the medium also has varied widely depending on several factors, including local availability. The final pH also has varied in different formulations. The formula given below,‡ with 0.5% NaCl, has been used in many laboratory procedures and epidemiological investigations. Others formulations may be equally effective.

Peptone§	10 g
Sodium chloride, NaCl	5 g
Sodium hydroxide, NaOH, 1*N*	~6 mL
Water	~994 mL

Dissolve peptone and sodium chloride in the water. Insert a pH electrode and add 1*N* NaOH dropwise until pH has risen to 8.4; about 6 mL will be required. Final volume will be 1000 mL. Dispense and autoclave at 121°C for 15 min. The final medium will be clear and amber-colored.

2) *Alkaline peptone water—saltless:*[1]‖ The content of NaCl in alkaline peptone water can be adjusted to be selective for different *Vibrio* groups. If no NaCl is added, the medium will select for the nonhalophilic species *V. cholerae* and *V. mimicus* at the expense of the other species, which are all halophilic.

Prepare as with alkaline peptone water, ¶ 1) above, but omit the 5 g NaCl. While this medium has no added NaCl, the peptone formulation contains enough Na^+ for *V. cholerae* and *V. mimicus* to grow.

3) *Alkaline peptone water—0.1% NaCl:* This medium is selective for the moderate halophilic *Vibrio* species, because the NaCl content is too low for the true halophilic species to grow.

Prepare as described for alkaline peptone water, ¶ 1) above, but add 1 g, rather than 5 g, of NaCl.

4*) Other alkaline peptone waters:* The NaCl content can be adjusted based on the NaCl tolerance of a particular outbreak strain or *Vibrio* species being sought (Table 9260:IV) to make the medium more selective. For example, alkaline peptone water with 8% NaCl would select for *V. alginolyticus* at the expense of less tolerant species. Similarly, other selective agents could be added based on the phenotypic properties of a specific strain being sought.

d. Procedures for Vibrio *species:*

1) *V. cholerae*

a) Enrichment—Use alkaline peptone water—saltless and a second enrichment medium such as alkaline peptone water or alkaline peptone water—saltless with added colistin or Polymyxin B.[1–3] Incubate 6 to 8 h at 36°C.

b) Tentative identification—After incubation, plate a loopful from the surface onto TCBS agar and sheep blood agar. Test yellow colonies on TCBS agar and typical colonies on sheep blood agar (many will be strongly hemolytic) with commercial latex for *V. cholerae* O1. Confirm the identification for those that agglutinate and test them for the production of cholera toxin.

In successful enrichments of water samples, *V. cholerae* O1 may be present in high enough numbers to allow its immediate detection before subculture. If enough *V. cholerae* O1 antigen is present, it will agglutinate latex coated with antibodies to the O1 antigen. Add a drop of the enrichment culture to a drop of *V. cholerae* O1 latex. Agglutination is a presumptive positive; confirm by culture.

A second method for direct detection in enrichments is the microscopic immobilization test, which shows a rapid loss of motility of *V. cholerae* O1 cells in the presence of commercial *V. cholerae* O1 serum as observed with a microscope.

For tentative identification of *V. cholerae* O139 after enrichment but before subculture, use commercial antisera or latex for *V. cholerae* O139 instead of the O1 reagents in both assays described in the preceding two paragraphs.

2) *V. parahaemolyticus*—Use salt Polymyxin broth# and alkaline peptone water.

3) *V. vulnificus*—Use alkaline peptone water–0.1% NaCl and alkaline peptone water–0.1% NaCl with added colistin or Polymyxin B.[1,3]

4) *V. fluvialis and V. furnissii*—Use a basal salts medium, such as BM medium of Baumann and Baumann[1], but keep the NaCl concentration at 0.1% by substituting K^+ salts for Na^+ salts. Add D-galacturonate as the sole source of carbon and energy, which will select for these two species.[1]

5) *V. cincinnatiensis*—Prepare enrichment as described in ¶ 4) above, but substitute myo-inositol as the sole source of carbon and energy to select for this species.[1]

4. Immunomagnetic Separation after Enrichment

See immunomagnetic separation of *Salmonella* (9260B.5) and diarrheagenic *E. coli* (9260F.4). Immunomagnetic separation should greatly improve the yield of a particular strain after enrichment.[11,12] Purchase commercial O (or K) antiserum for the strain being sought (such as *V. cholerae* O1 or *V. parahaemolyticus* O3:K6) and add it to uncoated immunomagnetic beads according to manufacturer's directions. Use reagent for immunomagnetic separation after enrichment.

Few sera are commercially available to assist in preparing antibody-coated beads for the other *Vibrio* species. Check for availability from reference and research laboratories and with commercial laboratories that prepare custom antisera with an antigen supplied by the customer.

5. Plating Media

a. General guidance: Marine agar** is a nonselective medium, and essentially all *Vibrio* strains will grow on it. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Table 9260:III) is commercially available†† and is extremely useful for isolating *V. cholerae* and *V. parahaemolyticus* from water as well as from human clinical specimens.[1–4] Cultures of *Vibrio* grow well on sheep blood agar and may be beta hemolytic (*V. cholerae* non-O1 and some *V. cholerae* O1 strains of the El Tor biotype),

‡ CDC Medium 1494 of *Vibrio* Reference Laboratory, Centers for Disease Control and Prevention, Atlanta, GA.
§ Bacto™, or equivalent.
‖ CDC Medium 1495 of *Vibrio* Reference Laboratory, Centers for Disease Control and Prevention, Atlanta, GA.

Nissui Co., or equivalent.
** BD Biosciences, or equivalent.
†† BD Biosciences, Oxoid, Eiken, or equivalent.

alpha hemolytic (*V. vulnificus* and many others), or nonhemolytic.[3] *Vibrio* strains usually grow on MacConkey agar (sometimes with a reduced plating efficiency) and will appear as colorless (lactose-negative) colonies. *Vibrio* cultures often do not grow well on more selective "enteric plating media." Oxidase testing[1–3] appears to be a cost-efficient method for detecting *Vibrio* isolates from clinical specimens and water and can be conducted on colonies grown on blood agar and on lactose-negative colonies on selective media. Most vibrios are oxidase-positive; however, lactose-positive colonies tested directly from selective media, such as MacConkey agar, are often oxidase-negative, (i.e., a false negative reaction). Test individual colonies for oxidase production, or add reagent to an area of growth on the plate.

b. TCBS agar minus sucrose, plus a different sugar(s): Sucrose is the sugar in commercial formulations of TCBS agar. Prepare agar from the original ingredients[1] but omit sucrose and add 5 to 10 g/L of one or more other sugars. This modified medium will be differential or selective because vibrio species will have different reactions depending on whether they can ferment the sugar compound added to the medium.[1–3] For example, add D-galacturonate for *V. fluvialis* and *V. furnissii* because most other vibrio species do not ferment this compound (Table 9260:IV), or add myo-inositol for *V. cincinnatiensis.*

c. Other selective-differential agar media: Many media are commercially available, and formulas for some are published elsewhere. Alternative media for *V. parahaemolyticus* are *V. parahaemolyticus* sucrose agar (VPSA)[13] and a chromogenic agar‡‡; media for *V. vulnificus* are modified cellobiose polymyxin colistin agar (mCPC),[13] cellobiose-colistin agar (CC agar),[13] and *Vibrio vulnificus* agar (VVA).[13]

6. Biochemical Identification

Only 11 *Vibrio* species cause human infections, and their identification is not difficult if the key tests (1 through 3) or all the tests listed in Table 9260:IV are done. For *Vibrio* identification with standard tube tests, add NaCl to a final concentration of 1% for several biochemical test media because some commercial media formulations do not include NaCl. If this is not done, halophilic *Vibrio* species will not grow or will grow poorly and give negative reactions in tests that should be positive. Fortunately, commercial media for most of the biochemical tests are formulated to contain 0.5 to 1.0% NaCl.

a. Identification of pathogenic Vibrio *species:* The most common *Vibrio* species that require identification are *V. cholerae* (O1, O139, non-O1, non-O139), *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. fluvialis*, and *V. mimicus*.

In the investigation of cholera outbreaks there is no need to do a large number of biochemical tests to confirm a culture as *V. cholerae*. Agglutination in *V. cholerae* O1 or O139 serum is diagnostic; confirm by biochemical testing for the first few isolates. Phenotypically, *V. cholerae* O139 is almost identical to *V. cholerae* O1 (the El Tor biotype), and is identified by its agglutination in O139 serum. Another possible differential characteristic is susceptibility to the vibriostatic compound O-129. *V. cholerae* O139 strains are usually O-129-resistant, whereas most O1 isolates are sensitive.

Strains identified as *V. cholerae* that do not agglutinate in O1 or O139 sera are identified as *V. cholerae* non-O1, non-O139. Further serotyping would yield a more precise identification, but complete serotyping is done by only a few reference laboratories. The test for Na^+ requirement differentiates *V. cholerae* from the halophilic *Vibrio* species (Table 9260:IV), and sucrose fermentation differentiates it from its close relative *V. mimicus.*

Strains of *V. parahaemolyticus* are usually typical in their biochemical reactions (Table 9260:IV). *V. alginolyticus* is biochemically similar to *V. parahaemolyticus*, but it usually swarms, is Voges-Proskauer-positive, and grows in higher concentrations of NaCl (Table 9260:IV).

V. vulnificus strains grow well on blood agar and TCBS agar. Most strains are sucrose-negative and green on TCBS agar, but occasional strains are sucrose-positive and produce yellow colonies. *V. vulnificus* is unique among *Vibrio* species because it ferments lactose, salicin, and cellobiose and is also ONPG–positive. It has no zone of inhibition or a small zone around colistin, but large zones around ampicillin and carbenicillin. *V. vulnificus* biogroups 2 and 3 have been described[1–3] and are difficult to identify without doing a complete set of biochemical tests.

V. fluvialis and *V. furnissii* are often confused with *Aeromonas,*[1–3] because all three are usually arginine-dihydrolase-positive and are biochemically similar. In contrast to strains of *Aeromonas*, *V. fluvialis*, and *V. furnissii* are slightly halophilic and will grow in nutrient broth only if NaCl is added. Phenotypically, *Vibrio furnissii* is almost identical to *Vibrio fluvialis*, and gas production in glucose is the key differential test.[1–3]

Key points of identification for other species are as follows: *V. hollisae* strains are fastidious. They grow on blood agar, but not on MacConkey or TCBS agar. Strains are halophilic, triple decarboxylase-negative, poorly motile, and have a characteristic fermentation pattern. Strains also have a unique antibiogram,[1–3] with very large zones around all antibiotics, including penicillin. *V. damsela* has a unique biochemical profile and resistance pattern,[1–3] which make identification easy. *V. metschnikovii* is unique among the pathogenic *Vibrio* species because it is oxidase-negative and does not reduce nitrate to nitrite. *V. cincinnatiensis* ferments myo-inositol. *V. harveyi* is biochemically distinct and is resistant to ampicillin, carbenicillin, and colistin.[1–3]

b. Identification with commercial systems: Consult manufacturer's list of the *Vibrio* species included in product's database, published evaluations of the product, and the formula of the product's suspending medium and tests to determine if the Na^+ content is sufficient. A recent study[14] evaluated six commercial identification products for their accuracy in identifying pathogenic *Vibrio* species, and found some problems with each product. One of the most common errors of commercial systems is that they misidentify cultures of *Aeromonas* as *Vibrio fluvialis*/*V. furnissii* and *vice versa*, because these three organisms are very similar biochemically. A good check on a commercial system's identification of a culture is to test one of the halophilic vibrio species and determine its oxidase reaction, growth in nutrient broth with 1% NaCl and without NaCl, and growth on TCBS agar. Disagreements in any of these key properties warn of a possible misidentification.

c. Identification of water and seafood Vibrio *isolates:* This can be extremely difficult because over 60 species of *Vibrio*, *Photobacterium* and their relatives must be considered.[1–4] Do the

‡‡ CHROMagar Vibrio, CHROMagar, Paris, France, or equivalent.

key tests (1 through 3) or all of the tests in Table 9260:IV and compare the isolate's profile with each species. If there is a perfect match, the isolate is most likely correctly identified. If several tests are in disagreement, there is the danger that the isolate is not one of the "12 clinical species." Molecular tests and 16S rRNA sequencing[2] may prove good alternatives to phenotypic methods for this complex group of organisms, but currently this is a research rather than a routine test; optimally, send the isolate to a commercial laboratory for definitive identification.

7. Serological Identification

The availability of commercial antisera will determine the procedures that are practical. Several polyclonal antisera and latex agglutination reagents are available for *V. cholerae* O1 and O139.§§ Rapid and complete agglutination is a strong presumptive positive, and can be followed by toxin testing. Biochemical confirmation will reduce the chance of a false positive, and should be done for at least a few positive cultures. Commercial antisera are also available for *V. parahaemolyticus*|| || but rarely for the other *Vibrio* species. Reference laboratories may be willing to furnish some of their reference sera they have made for *V. vulnificus*, *V. fluvialis*, *V. furnissii*, and other species.

8. Toxin Assays

Commercial kits are now available to test for cholera toxin and the thermostable direct hemolysin (TDH) of *V. parahaemolyticus.*

a. Commercial immunoassay for cholera toxin: Grow pure cultures of *V. cholerae* or *V. mimicus* in specified media and test for cholera toxin production in a reverse passive latex agglutination assay (VET-RPLA).## PCR is another method. Typical reports:

- *V. cholerae*, positive for cholera toxin
- *V. mimicus*, positive for cholera toxin

This assay also detects the heat labile enterotoxin of *E. coli*, which is structurally similar to cholera toxin.

b. Commercial immunoassay for heat-labile enterotoxin and heat-stable enterotoxin of V. cholerae *and* V. mimicus: Grow the culture in special media. Test in one of the commercial products previously described for *E. coli* (see 9260F.8). Typical reports:

- *V. cholerae*, non O1-O139, positive for heat-labile enterotoxin, negative for heat-stable enterotoxin
- *V. mimicus*, negative for heat-labile enterotoxin, positive for heat-stable enterotoxin

c. Commercial immunoassay for the thermostable direct hemolysin (TDH) of V. parahaemolyticus: Strains of *V. parahaemolyticus* that contain the *tdh* gene produce this toxin and are enteric pathogens; strains that lack the *tdh* gene do not produce this toxin. Strains of *V. parahaemolyticus* that contain the *trh* gene and produce the TRH toxin are also potential enteric pathogens. No commercial assays are available to determine the presence of the *tdh* gene or its toxin, and *trh*-positive strains are rarer. *V. parahaemolyticus* is a very common inhabitant of water and the surfaces and intestines of fish and shellfish. However, most strains isolated from these and other environmental samples will be negative for both TDH and TRH, and thus are not considered enteric pathogens.

Grow the strain of *V. parahaemolyticus* in a special medium. Test the supernatant for *tdh* in a commercial kit that uses an immunological assay.##

d. Screening colonies of V. parahaemolyticus *on Wagatsuma agar to detect strains that are* TDH-*positive ("Kanagawa positive"):* This method has an advantage over that of ¶ *a* above if hundreds of *V. parahaemolyticus* colonies must be screened for toxin production. Prepare commercial Wagatsuma agar*** and add washed red blood cells as specified. Spot-inoculate the plates with individual colonies suspected as being *V. parahaemolyticus*. Read for hemolysis around the colonies. Hemolytic colonies are referred to as "Kanagawa-positive." Use method of ¶ *c* above to confirm that Kanagawa-positive colonies really produce the thermostable direct hemolysin.

9. Molecular Approaches and DNA-based Testing

DNA-based testing and molecular approaches have been described for many of the *Vibrio* species[1–5,7,8,11] and include methods for identification of species, such as 16S rRNA sequencing, strain typing such as pulsed-field gel electrophoresis (PFGE), PCR and determining toxins and virulence factors. Detailed instructions for several molecular procedures for *Vibrio* species in food are available.[5,7] Genetic sequences most likely to be of interest in vibrio work include:

- *V. cholerae*—the cholera toxin gene *ctx*, genes that code for the O1 and O139 antigen.
- *V. parahaemolyticus*—*tdh* and *trh* genes and perhaps the *tlh* gene for pathogenicity, and the urease plasmid.
- *V. vulnificus*—the cytotoxin hemolysis gene *vvhA* for identification of the species. Detection of this gene[7] has proved extremely useful in identifying environmental isolates of this species.

These molecular methods are evolving very rapidly. Consult current literature for technical details such as primer sequences, reaction conditions, and detection methods.

10. Investigation Methods

Many of the methods described in 9260H.1–9 allow for flexibility. However, several detailed and specific procedures have been described to isolate and identify pathogenic *Vibrio* species in food and water. It is helpful to compare general and specific methods before deciding on the best approach for a particular situation in water analysis. Some procedures listed below give all the technical details for their methods describing: enrichment-isolation, media-reagents, immunomagnetic separation, screening, identification, toxin testing, and PCR or other molecular methods. These include procedures for *Vibrio* species,[15] *V. cholerae* O1,[7,16] *V. parahaemolyticus,*[7,11] and *V. vulnificus.*[7]

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§§ BD Biosciences, Denka Seiken, Columbia Diagnostics, or equivalent.
|| || Denka Seiken, Nichimen, or equivalent.
Denka Seiken, distributed by Oxoid Inc., or equivalent.

*** Kyoto Pharmaceutical, or equivalent.

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9260 I. *Leptospira*

Leptospira spp. are motile, aerobic spirochetes that require fatty acids for growth.[1] Serum or polysorbate enrichments must be incorporated into artificial media, and some pathogenic strains may require CO_2 on initial isolation. Leptospires are divided into two groups based on their pathogenicity and growth characteristics. The pathogenic leptospires make up the Interrogens Complex; they have an optimal growth temperature of 28 to 30°C and grow over a pH range from 5.2 to 7.7. Saprophytic leptospires are assigned to the Biflexa Complex; they prefer a growth temperature between 5 and 10°C below pathogenic strains, grow in the presence of 8-azaguanine at 225 μg/mL, and fail to form spherical forms in 1*M* NaCl.

Since 1998, the phenotypic serological classification of the genus has been replaced with genotypic classification, resulting in recognition of 10 genomospecies. The new genomospecies for *L. interrogans* and *L. biflexa* do not correspond to the earlier phenotypic and serological characteristics of these species, thus creating difficulty for laboratories unable to perform genotypic identification. However, while reclassification of *Leptospira* spp. based upon genomospecies is taxonomically correct, the older phenospecies and serovars are much more practical in clinical microbiology and epidemiology and probably will be used until genotypic classification systems are readily available and widely used.[2] Leptospires prefer alkaline conditions, and they persist longest in warm, moist environments protected from sunlight. Under favorable temperature and pH conditions, leptospires survive for 3 to 5 d in damp soil and up to 10 d in natural waters. They also survive for 12 to 14 h in undiluted wastewater, up to 3 d in aerated wastewater, and up to 4 weeks in sterile tapwater at pH 7. Nonpathogenic leptospires are ubiquitous and have been isolated from municipal water supplies.[3] Pathogenic leptospires usually require an animal host and do not survive and propagate in the environment.

Leptospirosis is a worldwide zoonotic disease of wild animals.[4] Reservoirs of leptospires in wildlife include deer, foxes, raccoons, skunks, opossums, muskrats, and rodents. Domestic animals harboring leptospires include horses, cattle, goats, pigs, and sheep. Dogs may become infected but not cats. Humans are incidental hosts.

Humans acquire leptospirosis (Weil's disease) directly from animals, and from occupational or recreational exposure to urine-contaminated water or environmental surfaces.[5] Rats and other rodents are the most important reservoir for humans.[6] Occupational and recreational activities placing people in contact with animal urine are the primary risk factors for acquiring leptospirosis. The highest prevalence occurs in tropical and subtropical regions where environmental survival is greatest.[7] Increased human exposure is associated with rainfall resulting in flooding. Triathletes,[8] military personnel,[9] and eco-challenge participants (survivors)[10] are at increased risk of infection.

Swimming, kayaking, and other water sports,[10,11] travel to tropical areas with occupational or recreational exposure to surface waters,[12] and natural disasters that affect sewer systems and runoff[13,14] increase risk of the disease. Urine from rats, cows, pigs, and dogs has been implicated in surface water contamination leading to outbreaks. Outbreaks of leptospirosis associated with drinking water are unusual, and are invariably caused by contamination of domestic water reservoirs with urine of infected rodents.[15] Drinking water outbreaks have been reported from urine contamination of water, for example, fountains, holding tanks, and wells.[2]

Leptospirosis ranges from mild nonspecific febrile illnesses to severe or fatal renal, hepatic, or meningeal disease.[16,17] Leptospires enter the blood stream through imperfections in the skin, through mucous membranes, or by ingestion of contaminated water. Urine of infected animals and humans may contain 10^6 to 10^8 microorganisms/mL and leptospires may be shed into the environment up to 3 months after clinical recovery from disease.

Diagnosis of disease in animals and humans usually is based upon serology, darkfield examination of urine sediments, examination of histopathological stains, or culture of the organism from urine or tissues. Recently, polymerase chain reaction (PCR) methods have been introduced for diagnosis and typing of leptospires.[18,19] PCR also has been used to differentiate pathogenic from nonpathogenic leptospires.[20–23] PCR methods are insensitive for detection of leptospires in environmental samples and they suffer from the inability to differentiate between living and dead organisms. Because serovar information has epidemiological and public health significance, culture and serotyping are currently preferred to molecular detection methods.[2]

While leptospirosis remains relatively common in tropical regions of the world, only 40 to 120 cases/year have been reported in the United States over the past 30 years. Leptospirosis was dropped from the list of notifiable diseases in 1995. Only one outbreak has been reported in the continental United States since 1995.[8]

Leptospires are recovered from environmental sources with great difficulty.[24–27] Because both saprophytic and pathogenic strains of leptospires may be recovered from environmental samples, their presence has no public health significance apart from an epidemiological context.

1. Sample Collection

Collect water samples of 100 mL to 1 L in sterile containers. Transport samples to the laboratory at ambient temperature within 72 h of collection. Multiple samples from each sample site usually are required for successful isolation because finding leptospires in 10 to 20% of samples of surface waters receiving farm runoff is considered a high yield. Leptospires find their ecological niche at the interface between sediment and shallow water. Gently agitate the water to bring some of the sediment to the surface of shallow bodies of water to improve the probability of recovering organisms.[28]

2. Sample Processing

Centrifuge a portion of a water sample at $5000 \times g$ for 10 min. Examine sediment for leptospires by darkfield microscopy; skill and experience are required to differentiate artifacts from leptospires. Their presence indicates that conditions are favorable for leptospire survival, but does not differentiate saprophytic from pathogenic forms. In the laboratory, thoroughly mix soil samples with three volumes of sterile deionized water and let coarse particulate material settle by gravity. Process remaining suspension as a water sample. *Leptospira* can pass through 0.22-μm membrane filters (¶ *a* below); this ability has been exploited to separate them from other bacteria in environmental samples and in mixed cultures. Similarly, guinea pig inoculation (¶ *b* below) has been used as a biological filter for isolation of leptospires from contaminated samples.

a. Filtration method: Filter surface water samples through filter paper* to remove coarse debris before membrane filtration. Occasionally, samples may have to be passed through a series of prefilters of decreasing pore sizes (8-μm, 4-μm, 1-μm, 0.65-μm, and 0.45-μm) to prevent clogging of the final 0.22-μm filter.

b. Animal inoculation method: Filter water through a 0.45-μm membrane filter and inoculate 1 to 3 mL intraperitoneally into weanling guinea pigs. After 3 to 6 d, inject a small amount of sterile saline and withdraw fluid for darkfield examination. If leptospires are seen, perform a cardiac puncture to obtain blood for inoculation of culture media. If no leptospira are seen by darkfield examination, record rectal temperatures daily until a fever spike indicates infection, then repeat the darkfield examination of peritoneal fluid for leptospires. Exsanguinate guinea pigs at 4 weeks and save serum for serological tests. Culture blood, kidney, and brain of guinea pigs with serological evidence of infection. Details of the method are described elsewhere.[29]

3. Culture

Cultures of environmental samples usually will be contaminated with other bacteria unless the samples are filtered through a 0.22-μm membrane filter before inoculation. Filtration also may be used to isolate leptospires from mixed cultures by direct filtration or another method.[30] Unless sample filtration is used in conjunction with selective media or animal inoculation, a culture contamination rate of 60 to 80% is not uncommon. The amount of sample cultured will depend on the amount of particulate material in the sample. Generally, culture sample volumes from a few drops to 3.5 mL.

a. Culture media: Pathogenic leptospires have been cultured in liquid, semisolid, and solid media, but not all pathogenic strains will grow on solid media. Solid media have been used to purify mixed cultures and to detect hemolysin production.[31,32] Optimal pH of culture media is 7.2 to 7.4 and optimal incubation temperature is 30°C. Keep glassware free of detergent residues because leptospires are sensitive to detergents [see Section 9020B.5*a*2) (2005)]. When using serum enrichments in culture media, use serum free of antibody to leptospires. Bovine serum albumin shows manufacturer and lot variations; test new batches for their ability to support growth of leptospires.

Modifications of the Ellinghausen-McMullough formulation (EMJH) that incorporate bovine serum albumin fraction V and polysorbates are used as serum replacements.[33–36] EMJH base is available commercially. Neomycin is used in culture media at concentrations between 5 and 25 μg/mL to inhibit competing

* Whatman No. 1, or equivalent.

microflora, but it may be toxic to some strains.[37] 5-Fluorouracil is used at 100 or 200 μg/mL in culture media, but it is toxic also for some strains, particularly at concentrations above 100 μg/mL.[38]

b. Culture methods:

1) Direct culture method—To recover leptospires from surface waters, place a few drops of water in EMJH liquid medium and incubate overnight at 30°C. Filter inoculated medium through a 0.22-μm membrane filter into a sterile tube and reincubate at 30°C for up to 6 weeks.

2) Dilution method—When samples may contain reasonable numbers of organisms in the presence of inhibitors or competing microflora, prepare 10-fold dilutions in duplicate and inoculate 0.1 mL undiluted sample and each dilution into EMHJ medium. One tube of each pair may be made selective by addition of a single 30-μg neomycin antimicrobial susceptibility disk to the media before incubation. Incubate cultures at 20 to 30°C for up to 4 months.

3) Animal inoculation method—Add 1 to 2 drops of heart blood from infected guinea pigs to each of three to five tubes of EMJH medium. Incubate cultures at 20°C for up to 4 months.

c. Culture examination: Leptospires usually are detected in cultures of environmental samples within 7 to 14 d; however, incubate and examine cultures weekly for up to 4 months before discarding them as negative. Observe tubes for a lightly turbid ring of growth just below the surface of the medium. This band of maximum turbidity at the zone of optimal oxygen tension is referred to as Dinger's ring. Remove a drop of the culture weekly for darkfield examination and prepare subcultures if motile leptospires are observed. Generally, saprophytic leptospires grow at lower temperatures, and form rings closer to the surface of culture media than pathogenic serovars. Cultures remain viable in semisolid media for at least 8 weeks at room temperature. Cultures may be maintained by repeated subculture, by lyophilization, or by freezing at –70°C.[29]

4. Identification

The biochemical tests previously thought to differentiate between pathogenic and saprophytic serovars do not reliably predict pathogenicity of leptospires, and they are not recommended. *Leptospira* are identified to serogroup by the microscopic agglutination test using reference antisera. Identification to serovar requires use of adsorbed antisera that are available only in reference laboratories. More than 200 serotypes of *Leptospira* are known.

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9260 J. *Legionella*

Legionella were first isolated and identified as part of the investigation of respiratory illness in persons attending an American Legion convention in Philadelphia in 1976.[1,2] This highly publicized investigation documented 239 cases and 34 deaths due to a previously unrecognized cause of pneumonia. It was later shown that this disease occurs when sufficient numbers of legionellae are aerosolized from colonized water sources and subsequently inhaled by a susceptible host.[3] The bacteria are associated with two forms of respiratory illness, collectively referred to as legionellosis.[4,5] Legionnaires' disease is the pneumonic and more severe form of legionellosis. The other form of respiratory illness is named Pontiac fever after the first documented outbreak, which occurred at a health department in Pontiac, Michigan.[4] Pontiac fever is a less severe, self-limited illness. Possible explanations for the manifestation of these two disease syndromes caused by the same bacteria include the inability of some legionellae to multiply in human tissue (for a variety of reasons, including virulence, host range, or viability of the bacteria) and differences in host susceptibility.[6–8] Community-based pneumonia incidence studies have estimated that there are between 8000 and 18 000 cases of legionellosis annually in the United States, approximately 25-fold higher than the number of cases annually reported to the Centers for Disease Control and Prevention.[9] The majority of cases of legionellosis are sporadic, with only about 4% outbreak-related.[9] The sources of community-acquired cases are difficult to identify, partly because of the ubiquitous nature of the bacterium. Although the organisms are relatively common in the environment, they cause disease infrequently.[10] It is generally accepted that in order to cause disease legionellae must, first, be present in an environmental reservoir, then amplify from low to high concentrations, and be disseminated to susceptible hosts.[11] This model explains the epidemiology of legionellosis as it is used to develop prevention strategies.

Bacteria of the genus *Legionella* are gram-negative, aerobic, rod-shaped bacteria. Cells are 0.3 to 0.9 by 1 to 20 μm and motile, with one or more polar or lateral flagella.[12] Legionellae use amino acids as their carbon and energy sources and do not oxidize or ferment carbohydrates. Currently, there are 49 species comprising 71 distinct serogroups in the genus *Legionella.*[13-15] Species identification and differentiation are performed serologically, although antisera for many species and serogroups are not available commercially.[12] A single species of *Legionella*, *L. pneumophila*, causes approximately 90% of all documented cases of legionellosis.[16] Although there are now 15 serogroups of *L. pneumophila*, 82% of all legionellosis cases are caused by *L. pneumophila* serogroup 1. Approximately half of the species of Legionellae have been associated with human disease. It is likely that most of the legionellae can cause human disease under the appropriate conditions; however, these infections are infrequently reported because they are rare and there is a lack of diagnostic reagents. Some unidentified legionellae cannot be grown on routine *Legionella* media; these organisms have been given the acronym LLAPs (*Legionella*-like amoebal pathogens) because they have been detected through their ability to grow intracellularly in protozoan cells.[17]

Water is the major reservoir for legionellae, and the bacteria are found in freshwater environments worldwide.[18] Legionellae have been detected in as many as 40% of freshwater environments by culture and in up to 80% of freshwater sites tested by polymerase chain reaction (PCR).[14] Several outbreaks of legionellosis have been associated with construction, and it was originally believed that the bacteria could survive and be transmitted to humans via soil. However, legionellae do not survive in dry environments and these outbreaks are more likely the result of massive descalement of plumbing systems due to changes in water pressure during construction.[19,20]

Initially, it was difficult to explain the pervasiveness of legionellae in aquatic environments because these bacteria are fastidious and require an unusual combination of nutrients in bacteriologic medium. These levels of nutrients would rarely be found in aquatic environments and, if present, would serve only to amplify faster growing bacteria that would compete with the legionellae. However, the nutrients required by legionellae represent the need for an intracellular environment, not soluble nutrients commonly found in fresh water. Legionellae survive in aquatic and, possibly, in some soil environments as intracellular parasites of free-living protozoa.[21,22] They infect the protozoa by use of a novel IV secretion system and use the same mechanism to infect and multiply within human macrophages.[23] Protozoa play a crucial role in the ecology of legionellae and this interaction is key to the development of successful prevention strategies. To understand the ecology of legionellae, these bacteria must be considered in the context of their microbial community, not as independent inhabitants of freshwater environments.

Inhalation of legionellae in aerosolized droplets is the primary means of transmission for legionellosis.[3] These aerosolized droplets must be of a respirable size (1 to 5 μm). No person-to-person transmission of Legionnaires' disease has been documented. A number of devices have been implicated as sources of aerosol transmission of legionellae.

These sources are of two general types: those producing aerosols of contaminated potable water, such as showers, faucets, decorative fountains, ultrasonic mist machines, humidifiers, and respiratory therapy equipment, and those producing aerosols of nonpotable water, such as cooling towers, evaporative condensers, hot tubs, and whirlpool spas. Meaningful identification of sources of transmission requires a multidisciplinary approach including epidemiology, molecular epidemiology, and microbiologic techniques including water and, rarely, air sampling.[3]

Detection of legionellae in an environmental source is not necessarily evidence of the potential for disease. As previously stated, legionellae are ubiquitous and could be isolated from 60% of buildings tested in a recent study.[24] The relationship between the presence of the bacterium in the environment and frequency of resulting disease remains poorly defined. Monitoring of building water systems is warranted to identify the source of an outbreak of legionellosis or to evaluate the efficacy of biocides or prevention measures. Monitoring may be warranted in special settings where people are highly susceptible to illness due to *Legionella* infection, such as an organ transplant ward within a hospital.[25]

1. Collection of Samples

Determine on an individual basis the number and types of sites to be tested to detect legionellae. A published sampling protocol[26] can serve as a prototype for identifying sites to be sampled. Generally, consider any water source that may be aerosolized a potential source for the transmission of legionellae. The bacteria are rarely found in municipal water supplies and tend to colonize plumbing systems and point-of-use devices. To colonize a system, the bacteria must multiply, and this requires temperatures above 25°C.[12] Therefore, legionellae are most commonly found in hot-water systems. These bacteria do not survive drying, and so condensate from air-conditioning equipment, which frequently evaporates, is not a likely source.[19]

When sampling for legionellae collect both water samples and swabs of point-of-use devices or system surfaces.[27] Collection of at least 1 L of water allows concentration of the sample if necessary. If the water source has recently been treated with chlorine or bromine, 1 mL of 10% sodium thiosulfate may be added to each 1-L sample to neutralize the disinfectant.

Swabs allow sampling of biofilms, which frequently contain legionellae. These can be taken from various points within plumbing systems or from surfaces of basins of cooling towers or spas. Take swabs of faucet aerators and showerheads in conjunction with water samples from these sites, with the aerator or showerhead removed. The swabs can be streaked directly onto an agar plate or submerged in a small volume of water taken at the same time to prevent drying during transportation to the laboratory.

Transport all samples at ambient temperature to the laboratory in insulated coolers as protection against extreme heat or cold. Refrigerate samples that will not be processed within 24 to 48 h from the time of collection.

2. Pretreatment of Water Samples

The method selected for processing water samples depends on the expected degree of total bacterial contamination in a particular sample. Potable waters generally have low bacterial concentrations and are either cultured directly or concentrated to detect legionellae. Nonpotable waters, such as those from cooling towers, generally do not require concentration because of their high bacterial concentrations.

Samples may be concentrated 10-fold or more by using either filtration or centrifugation. Filtration is used more frequently, although either procedure can be used successfully.[27,28] Filter concentrate water in a biological safety cabinet using 0.2-μm-pore-size polycarbonate filters. Polycarbonate membranes allow suspended particles to collect on the filter surface without being trapped as they are in matrix-type filters. Resuspend the filter membrane into a volume of the sterile water and vortex for 30 s. Concentrate samples by centrifugation at $1000 \times g$ for 10 min, removing all but 10 mL of the supernatant, and vortex.[28]

Use a selective procedure to reduce the number of non-*Legionella* bacteria before culturing some water samples with high total bacterial concentrations. Non-legionellae bacteria can be killed selectively by either acid pretreatment or brief exposure to higher temperatures.[29,30] Legionellae are more resistant to lower pH and brief exposures to higher temperatures than many other freshwater bacteria. For acid pretreatment, mix the sample and incubate with an acid buffer (pH 2.2) for 3 to 30 min.[29] The sample is neutralized by the buffer within buffered charcoal yeast extract (BCYE) agar and therefore must be spread on the agar plate at the end of the period of incubation with the acid buffer. For heat pretreatment, incubate 10 mL sample in a 50°C water bath for 30 min.[30]

If amoebae are present, intracellular legionellae numbers can be increased by "heat enrichment" or incubation of specimens at 35°C. This can improve recovery of legionellae by up to 30%.[31] However, this procedure requires a considerable length of time before results can be obtained and may not be practical in many situations. Heat enrichment relies on autochthonous protozoa to amplify undetectable levels of legionellae. Portions of samples are incubated at 35°C and cultured after 2 to 6 weeks.

TABLE 9260:V. COMPONENTS AND SUPPLEMENTS OF BCYE AGAR FOR CULTURING LEGIONELLAE FROM THE ENVIRONMENT

Component	Concentration	Purpose
Charcoal	2.0 g/L	Base component
Yeast extract	10.0 g/L	Base component
ACES* buffer	10.0 g/L	Base component
Ferric pyrophosphate	0.25 g/L	Base component
L-cysteine	0.4 g/L	Base component
Potassium”-ketoglutarate	1.0 g/L	Base component
Agar	17.0 g/L	Base component
Glycine	3.0 g/L	Selective agent
Polymyxin B	50-100 U/mL	Selective agent (gram negative)
Vancomycin or cefamandole	1-5 g or 4 mg/L	Selective agent (gram positive)
Anisomycin or cycloheximide	80 μg/mL (for either)	Selective agent (fungal)
Bromocresol blue	10 mg/L	Indicator dye
Bromocresol purple	10 mg/L	Indicator dye
Bovine serum albumin	10 g/L	Supplement for some fasitidious Legionellae

* N-(2-Acetamido)-2-aminothanesulfonic acid.

Source: FIELDS, B. 2002. Legionellae and Legionnaires' disease. *In* Hurst, C.L., R.L. Crawford, G.R. Knudsen, M.J. McInerney & L.D. Stetzenbach, eds. Manual of Environmental Microbiology, 2nd ed. American Soc. Microbiology, ASM Press, Washington, D.C.

3. Culture Media

The medium currently used for the culture of legionellae is buffered charcoal yeast extract (BCYE) agar;[32–34] its most widely used form is supplemented with alpha-ketoglutarate.[33,34] Table 9260:V lists the primary components of BCYE agar and the supplements added for various purposes.[32,35–37]

Culture of environmental samples requires the use of selective and nonselective media in conjunction with the previously described selection procedures. Most laboratories use multiple plates for each sample, including a BCYE agar plate, a BCYE agar plate containing three antimicrobial agents, and a BCYE agar plate containing the three antimicrobial agents plus glycine (Table 9260:V). These media can be prepared with indicator dyes, which impart a color specific for certain species of *Legionella*.[36] Although the majority of *Legionella* spp. grow readily on BCYE agar, some require supplementation with bovine serum albumin to enhance growth. *L. micdadei* and several strains of *Legionella bozemanii* show a preference for BCYE with 1.0% albumin.[35] Inoculate all agar plates with 0.1 mL of sample by the spread plate technique and incubate at 35°C in a humidified 2.5% CO_2 atmosphere or candle extinction jar.

4. Identification of Legionellae Colonies

Colonies of legionellae require approximately 72 h to appear on BCYE agar and may require 7 d or longer. Ideally, examine plates after 4 d incubation and again before discarding them after 7 to 10 d incubation. Examine plates with a dissecting microscope and a light source to detect bacterial colonies resembling legionellae. After approximately 4 d of incubation, these colonies are 2 to 4 mm in diameter, convex, and round with entire edges. The center of the colony is usually a bright white with a textured appearance that has been described as “cut-glass like” or speckled. The white center of the colony is often bordered with blue, purple, green, or red iridescence. Some species of legionellae produce colonies that exhibit blue-white or red autofluorescence.[30] The primary isolation plates can be examined with long-wave UV light to detect these autofluorescent colonies.

Colonies resembling legionellae can be presumptively identified on the basis of their requirement for L-cysteine by subculture on blood agar or BCYE agar without L-cysteine. Subcultured colonies that grow on BCYE agar, but not on blood agar or BCYE without L-cysteine, are presumed to be legionellae. Legionellae are relatively inert in many biochemical test media, so these tests are of limited value in their identification. Definitive identification is usually made by using a direct fluorescent antibody (DFA) or slide agglutination test with specific antisera.[27] Fatty acid analysis and DNA hybridization[12] are other identification techniques.

5. Nonculture Methods for Detection of Legionellae

Several nonculture methods have been developed to detect legionellae in environmental samples and offer the potential of greatly increased sensitivity. These nonculture methods include detection of the organisms with specific antisera by DFA staining and procedures to detect nucleic acids of legionellae using polymerase chain reaction (PCR). However, culture remains the method of choice for detecting legionellae, primarily because nonculture methods cannot provide information regarding the viability of the bacteria.

The use of DFA to detect legionellae is limited by the number of specific antisera that can be used. Because no antisera specifically react with all *Legionella* species, a different antiserum must be used for each species or serogroup. Reports on the sensitivity and specificity of DFA testing of environmental specimens vary greatly, with most studies indicating that the test is relatively insensitive and nonspecific.[38]

The use of PCR for detecting nucleic acids of legionellae in the environment has proved to be valuable in some investigations of outbreaks of legionellosis.[7] A number of *Legionella* genes, including 5S rRNA, 16S rRNA, and *mip* genes, have been

used as targets for PCR.[39,40] Use of PCR to detect legionellae in the environment has indicated that up to 80% of fresh waters are positive while only 20 to 40% are positive by culture.[41,42] This discrepancy could be due to the presence of nonviable or injured organisms, nonspecific reactions with unrelated organisms, or the presence of related organisms, such as *Legionella*–like amoebal pathogens, that cannot be detected by conventional techniques used for legionellae.

Most investigations of epidemic legionellosis have used culture to detect legionellae in the environment; thus, most epidemiologically relevant information about legionellosis is based on direct culture data. Interpret results from non-culture-based methods cautiously.

6. Subtyping Techniques

Molecular subtyping procedures, as well as epidemiologic evidence, are required to associate an environmental isolate of *Legionella* with a clinical isolate from a patient. *L. pneumophila* serogroup 1 (Lp1), which accounts for most cases of legionellosis, can be divided into a number of subtypes, indicating that this is a fairly heterogeneous serogroup.[43] Identification of the bacterium, even to the serogroup level, is not sufficient to implicate an environmental isolate as the source of disease.

The variety of strains and distribution of Lp1 necessitate more elaborate subtyping procedures to discriminate within these bacteria. Several groups of monoclonal antibodies have been developed for this purpose.[44] An international panel of seven monoclonal antibodies was proposed in 1986;[44,45] use of these monoclonal antibodies has identified 10 type strains within Lp1. Although much information has been gained through the use of this panel, several of the cell lines have been lost and most of these reagents are no longer available.

DNA fingerprinting techniques provide a level of discrimination similar to the use of monoclonal antibodies; these techniques are complementary.[43] Techniques used to discriminate between isolates of legionellae include restriction fragment length polymorphism analysis, plasmid analyses, electrophoretic alloenzyme typing, RNA/DNA probing of DNA digests, pulsed-field gel electrophoresis, and arbitrarily primed PCR.[43,46,47] Amplified fragment length polymorphism (AFLP) has been used since the late 1990s.[48,49] A study using a standardized protocol demonstrated that the AFLP was highly reproducible and epidemiologically concordant with good discrimination. The method has been adopted as the first standardized typing method for the investigation of travel-associated Legionnaires' disease in Europe.[50] Currently a number of laboratories are investigating the use of multi-locus sequence typing (MLST) as the next generation typing method for legionellae.[51] This method involves comparison of nucleic acid sequence data for a limited number of well-characterized bacterial genes. Given the advances in nucleic acid sequence technology and bioinformatics, it would appear that sequence-based typing systems eventually will replace other molecular typing methods.

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9260 K. *Yersinia enterocolitica*

The genus *Yersinia* comprises gram-negative coccobacilli, of which three species—*Y. pestis* (the plague bacillus), *Y. pseudotuberculosis*, and *Y. enterocolitica*—are well-known human pathogens. However, not all strains of *Y. enterocolitica* are capable of causing human intestinal infections. Primarily on the basis of biochemical reactions, *Y. enterocolitica* has been classified[1] into six biogroups that have distinct patterns of serogroup designations, human pathogenic potential, and ecologic and geographic distribution (Table 9260:VI). Isolates belonging to biogroup 1A (positive for salicin fermentation, esculin-hydrolysis, and pyrazinamidase production) are thought to be incapable of causing human intestinal infections, while isolates negative for these traits are more likely to cause intestinal infections, which include enterocolitis, mesenteric adenitis, or terminal ileitis.

Yersinia enterocolitica is a gram-negative bacterium that can cause acute intestinal infection and can be found in cold or temperate U.S. climates.[2,3] It is widespread in nature and occurs in the gastrointestinal tract of numerous animal hosts, including mammals associated with aquatic habitats, avian species, and cold-blooded species.[4] From these reservoirs, terrestrial and aquatic ecosystems may become contaminated and human infections may ensue. Some animal hosts (e.g., domestic dogs and cats and farm animals) carrying pathogenic *Y. enterocolitica* are likely to come in contact with humans either directly or via ingestion (e.g., pork).[5,6]

Y. enterocolitica can grow at temperatures as low as 4°C with a generation time of 3.5 to 4.5 h if at least trace amounts of organic nitrogen are present.[3] Most environmental strains of *Y. enterocolitica* and the closely related species—*Y. kristensenii, Y. frederiksenii,* and *Y. intermedia*—lack the virulence factors to cause intestinal infections, but they cause, or are associated with, extra-intestinal infections, most frequently soft-tissue infections. Disease outbreaks associated with *Y. enterocolitica* have been associated with environmental sources.[7–9] Some strains lacking classic virulence markers also may be associated with disease.[7–10]

Y. enterocolitica has become recognized worldwide as an important human pathogen. In several countries, it is nearly as common as *Salmonella* and *Campylobacter* as a leading cause of acute or chronic intestinal infection.[11] *Y. enterocolitica* usually is associated with sporadic cases of intestinal infection in the United States. Epidemiologic investigations suggest that the predominant pathogenic serotype isolated in the United States has been changing.[10,12] *Y. enterocolitica* serogroup O3 has replaced O8 as the most common serogroup recovered from intestinal and systemic human infections, reflecting the same pattern seen in other parts of the world.[10,11] The emergence of serogroup O3 *Y. enterocolitica* infection was first noted in New York in 1983[12] and numbers of cases have increased steadily,[6,10] while serogroup O8 has become rare in the United States.

Yersinia strains have been isolated from untreated surface and ground waters in the Pacific Northwest, New York, and other regions of North America, with the highest isolations occurring during the colder months.[13–16] Concentrations have ranged from 3 to 7900 CFU/100 mL; the tests did not discriminate between pathogenic and nonpathogenic strains. Neither enteropathogenic or nonenteropathogenic *Yersinia* strains correlate with levels of total and fecal coliforms or total plate count bacteria.[15] There is also little information on *Yersinia* survival in natural waters and water treatment processes. Two incidents of waterborne gastroenteritis[3,13] and septicemia[2] caused by *Yersinia* have been documented.

In studies of *Y. enterocolitica* in chlorinated–dechlorinated secondary effluent and receiving (river) water, the organism was isolated in 27% of effluent samples, 9% of upstream samples, and 36% of downstream samples.[17] However, determination of biogroup status, and hence virulence potential of the isolates, were not assessed. Mean total and thermotolerant coliform reductions in effluent chlorination were 99.93 and 99.95%, respectively. In a survey of untreated and treated (chlorination or filtration plus chlorination) drinking water supplies, *Y. enterocolitica* was found in 14.0 and 5.7% of the samples, respectively.[15] Further, of all the water samples containing less than 2.2 coliforms/100 mL, 15.9% were *Yersinia*-positive. *Y. enterocolitica* isolation did not correlate with the presence of total or thermotolerant coliforms in this study. Another study also confirmed that *E. coli* is not a good indicator for the presence of *Yersinia* in water and that *Y. enterocolitica* O3 strains harboring a virulence plasmid have enhanced resistance to chlorine compared to nonvirulent strains.[18]

Yersinia could be an important drinking water pathogen because of its widespread occurrence, its persistence in natural and

TABLE 9260:VI. ASSOCIATION OF *YERSINIA ENTEROCOLITICA* WITH BIOGROUP, SEROGROUP, ECOLOGIC, AND GEOGRAPHIC DISTRIBUTION

Probable Cause of Intestinal Human Infection	Biogroup	Serogroup(s)	Ecologic/Geographic
Yes	1B	O8; O4; O13a,13b; O18; O20; O21	Environment, pig (O8) United States, Japan, Europe, The Netherlands (O8-like)
Yes	2	O9; O5, 27	Pig, Europe (O9), United States (O5, 27), Japan (O5, 27), Sweden, The Netherlands
Yes	3	O1, 2, 3; O5, 27	Chinchilla (O1, 2, 3), Pig (O5, 27)
Yes	4	O3	Pig, Europe, United States, Japan, South Africa, Scandinavia, Canada, The Netherlands
Yes	5	O2, 3	Hare, Europe
No*	1A	O5; O6, 30; O7, 8; O18, O46, nontypable	Environment, pig, food, water, animal and human feces, global

* May cause extra-intestinal infections

SOURCE: WAUTERS, G., K. KANDOLO & M. JANSSENS. 1987. Revised biogrouping schema of *Yersinia enterocolitica. Contrib. Microbiol. Immunol.* 9:14.

treated waters (at least in some geographic areas), the existence of animal reservoirs, the evidence for possible waterborne outbreaks, and the lack of definitive data on its reduction via treatment processes.

1. Enrichment and Isolation with Selective Media

Yersinia-selective agar, first proposed in 1979,[19] is the medium of choice for the isolation of *Y. enterocolitica* and other *Yersinia* species. The powdered medium, known as "*Yersinia* selective agar base" or "Cefsulodin Irgasan® Novobiocin (CIN) agar base," contains three agents (sodium desoxycholate, crystal violet, and triclosan) that inhibit gram-positive bacteria and many gram-negative bacteria. Because they are heat-sensitive, two other selective agents (cefsulodin and novobiocin) have been formulated separately as a supplement to be added to the prepared agar base. These agents inhibit most other *Enterobacteriaceae* and other enteric bacteria. Most *Yersinia* strains are resistant to the five inhibitory ingredients and will grow on the agar; most enteric bacteria are inhibited, with the exception of some strains of *Aeromonas*, *Citrobacter*, *Serratia*, and *Enterobacter*. These other organisms are easily differentiated by screening tests, biochemical reaction, serological screening, or other methods (see Table 9260:I). The agar also contains D-mannitol as the differential test; most strains of *Yersinia* ferment it rapidly and turn pink-red in the presence of the medium's pH indicator neutral red.

a. Double-strength Yersinia*-selective broth:* Reconstitute powdered *Yersinia* selective agar base* as described by the manufacturer, but use 500 mL rather than 1 L water. Let the insoluble agar settle. Pour off the clear liquid. If desired, filter the liquid through a coarse filter paper to remove any remaining agar particles; however, these should not be detrimental to most enrichment procedures. Autoclave and cool to room temperature. Add one vial *Yersinia* antimicrobic supplement.*

b. Enrichment procedure: Combine equal volumes of water sample and double-strength broth and incubate at 36 or 25°C. Subculture the enrichment at 24 and 48 h to agar (¶ *c* below). Consider subculturing the enrichment to other plating media, such as MacConkey agar, Congo red-magnesium oxalate (CR-MOX) agar (to determine pathogenic serotypes of *Y. enterocolitica*), and MacConkey agar base with added D-xylose to recognize the xylose-negative *Y. enterocolitica* O3, which is the most common pathogenic serotype in human infection.[20]

c. Growth on Yersinia*-selective agar:* Prepare agar, including supplement, according to manufacturer's directions, or purchase in disposable petri dishes. This medium can be inoculated with a small volume of a water sample, a membrane filter, an enrichment culture (see above) or a pure culture that is a "suspect" *Yersinia*. Incubation can be at 25 or 36°C; the latter will result in faster growth and larger colonies. At 24 h, cultures of *Y. enterocolitica* typically appear translucent or translucent with dark pink centers. At 48 h, they appear dark pink with a translucent border and may be surrounded by precipitated bile. Other *Yersinia* species grow well and in a similar manner.

* Difco, or equivalent.

2. Membrane Filter Method

A membrane filter method for enumerating and isolating *Yersinia enterocolitica* with mYE medium is available.[21] This method may be used for examining large volumes of low-turbidity water and for presumptively identifying the organism without transferring colonies to multiple confirmatory media.

Filter sample through a 0.45-μm membrane filter and place filter on a cellulose pad saturated with mYE recovery broth. Incubate for 48 h at 25°C. Aseptically transfer the membrane to a lysine-arginine agar substrate and incubate anaerobically at 35°C. After 1 h, puncture a hole in the membrane next to each yellow to yellow-orange colony with a needle, transfer the membrane to a urease-saturated absorbent pad, and incubate at 25°C for 5 to 10 min. Immediately count all distinctly green or deep bluish-purple colonies next to punctures. The green or bluish colonies are sorbitol-positive, lysine- and arginine-negative, and urease-positive. They may be presumptively identified as *Y. enterocolitica* or a closely related *Yersinia* species. Additional biochemical testing will be necessary to determine related species and biogroups (Table 9260:VII). Reasonably simple tests have been described to screen isolates for pathogenicity, and these tests correlate with the most common *Y. enterocolitica* serogroups.[1,22]

3. Identification

Strains of *Yersinia* are distinct from other enteric bacteria (see Table 9260:I), which makes it easy to identify a strain to the genus level (i.e., *Yersinia* species). Identification within the genus is more difficult, and commercial identification products may give incorrect identifications. Similarly, identification of *Y. enterocolitica* can be difficult because the other *Yersinia* species are so similar in their biochemical reactions. *Y. enterocolitica* O3, the most common and important serotype, does not ferment D-xylose, so this sugar is a useful screening test (see Table 9260:I). Cultures of *Yersinia* typically are more active biochemically at 25°C than 36°C; thus a test for definitive identification should be incubated at the lower temperature. Then compare the results to an identification chart that contains the reactions of all the species.[23]

4. Determination of the O antigen

Commercial antisera are available to serotype the most common and important pathogenic serotypes of *Y. enterocolitica;*† check for current availability. Sera for groups 1–6 of *Yersinia pseudotuberculosis* are also available.‡ Subculture colonies from plating media or enrichments, incubate, then test culture for agglutination in each antiserum. See manufacturer's instructions for details. See 9260B.8, E.7, and F.7 and 12 as a guide. Report the O antigen along with the species and biogroup (Table 9260:VI) (i.e., *Y. enterocolitica* O3, Biogroup 4). Refer cultures to a reference laboratory if they do not react in commercial sera but have properties of the enteric pathogens.

† Denka Seiken, distributed by Oxoid, www.oxoid.com/us; Statens Serum Institut, www.ssi.dk; and others.
‡ Denka Seiken, or equivalent.

TABLE 9260:VII. DEFINITION OF THE SIX BIOGROUPS OF *YERSINIA ENTEROCOLITICA* BASED ON REACTIONS AT 25°C

Test	Result for Biogroup*					
	1A	1B	2	3	4	5
Pyrazinamidase (48 h)	+	−	−	−	−	−
Salicin fermentation (24 h)	+	−	−	−	−	−
Esculin hydrolysis (24 h)	+	−	−	−	−	−
Tween 80 esterase (lipase)	+	+	−	−	−	−
Indole production (48 h)	+	+	(+)	−	−	−
D-Xylose fermentation	+	+	+	+	−	−†
Ornithine decarboxylase	+	+	+	+	+	−
Voges-Proskauer	+	+	+	+	+	−
Nitrate reduction to nitrite	+	+	+	+	+	−
D-Sorbitol fermentation	+	+	+	+	+	−
D-Sorbose fermentation	+	+	+	+	+	−
Sucrose fermentation	+	+	+	+	+	−
Trehalose fermentation	+	+	+	+	+	−
DNA'ase	−	−	−	−	+	+

Composite reactions[23] from the biotyping schemas proposed by Niléhn,[22] Wauters,[1] and the subsequent revision by Wauters.[1] Variable reactions are excluded in the operational definitions of the biogroups. The length of incubation period is specified if it is critical. The incubation time for a final reaction has varied depending on the particular laboratory and test.
* + = positive; (+) = weak positive; − = negative
† Some positive strains occur.

These commercial *Y. enterocolitica* antisera could also be used to coat magnetic particles, yielding a specific immunomagnetic separation reagent that would be useful in isolation. See 9260B.5 and F.4 and 11 as a guide.

5. Differentiation of Enteropathogenic Strains

Identify the culture to species, then use screening tests (see Table 9260:I), biotyping results (Table 9260:VII), and the culture's O antigen to determine whether it is likely to have the capacity to cause intestinal infections. In addition, if the culture produced tiny red colonies on CR-MOX agar (see Table 9260:I) it contains the *Yersinia* virulence plasmid and is probably an enteric pathogen. This plasmid is rapidly lost during enrichment and culturing.

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9260 L. *Aeromonas*

1. Introduction

Aeromonas spp. are natural inhabitants of aquatic environments worldwide. These gram-negative, non-spore-forming facultatively anaerobic, glucose-fermenting organisms have been isolated from groundwater, treated drinking water, surface waters, wastewater, sludge, and sediment. Their populations are seasonal in all natural waters, with the highest numbers present in warmer months. Aeromonads cause serious diseases of aquatic animals and represent an economic threat to the aquaculture industry. Some species among the motile aeromonads have emerged as a potential microbial threat to human populations, especially the immunocompromised.[1]

As a result of recent taxonomic studies, *Aeromonas* bacteria have been removed from the family *Vibrionaceae* and established as a genus of the new family *Aeromonadaceae*. The genus *Aeromonas* comprises 22 phenospecies and 18 genomospecies, three of which are unnamed.[2] Phenotypic characterization of genomospecies has advanced via incorporation of nontraditional substrates into biochemical identification schema. Environmental microbiologists usually combine all motile, mesophilic aeromonads into the *Aeromonas hydrophila* complex, or simply report isolates as *A. hydrophila*. These practices obscure understanding of the medical and public health significance of aeromonads isolated from clinical specimens, environmental samples, and public water supplies; identification of *Aeromonas* isolates according to established taxonomic principles is preferable.[3] *A. hydrophila* (HG-1), *A. caviae* (HG-4), *A. veronii* (HG-8), *A. jandaei* (HG-9), *A. schubertii* (HG-12), and *A. trota* (HG-14) are most frequently associated with clinical specimens.[2]

Although no U.S. outbreaks of aeromonad-related gastroenteritis have been attributed to public drinking water supplies to date, this does not mean that no connection exists. The epidemiologic association between ingestion of untreated well water and subsequent *Aeromonas* gastrointestinal illness has been widely documented. Numerous cases and outbreak investigations of water- and food-transmitted illnesses associated with aeromonads have been reported.[4] Outbreaks of gastroenteritis associated with aeromonads have occurred in custodial care institutions, nursing homes, and day-care centers. *Aeromonas* contamination of drinking water has been associated with travelers' diarrhea.[5]

For many years, *Aeromonas* have been considered nuisance microorganisms by environmental microbiologists because they were reported to interfere with coliform multiple tube fermentation (MTF) methods. While aeromonads comprise 12% of bacteria isolated from drinking water by presence–absence methods, no data have demonstrated inhibition of coliform organisms by aeromonads in drinking water. Slight turbidity of laurel-typtose broth (LTB) tubes, with or without a small bubble of gas in the inverted tube, is suggestive of aeromonads. When the MTF method is used for drinking water samples, cultures producing turbidity at 35°C that remain clear at 44.5°C are suggestive of aeromonads. The presence of aeromonads can be verified by subculturing a loopful of turbid broth to a MacConkey plate and screening colorless colonies for gelatinase and oxidase production. No data are available to support invalidation of coliform MTF tests based on turbidity of tubes in the absence of gas production.

The ecology of mesophilic aeromonads in aquatic environments, including water treatment plants and distribution systems, has been reviewed.[6] The Netherlands and the Province of Quebec have established drinking water standards for *Aeromonas* at 20 CFU/100 mL for water leaving the treatment plant and 200 CFU/100 mL for distribution system water. Canada has established an *Aeromonas* Maximum Contamination Limit (MCL) of 0 (zero) for bottled water. A resuscitation method for recovery of aeromonads in bottled water has been published.[7]

The ability to isolate, enumerate, and identify aeromonads from water and wastewater sources is important because of their role in causing human and animal disease, their ability to colonize treatment plants and distribution systems, and their presence and distribution as alternative indicators of the trophic state of waters. The diversity of aeromonads in drinking water plants and distribution systems was shown by several investigators.[8–10]

Many media and methods have been proposed for the isolation and enumeration of aeromonads.[11,12] The methods presented below represent a compromise, because no single enrichment method, isolation medium, or enumeration method is capable of recovering all aeromonads present in a water sample. The methods were chosen on the basis of reproducibility of results, objectivity of interpretation, availability of materials, and specificity of the method for detecting aeromonads in the presence of other heterotrophic bacteria. Consult the literature for additional methods for use in special circumstances.[13]

2. Sample Collection

Collect water samples in sterile screw-capped glass or plastic bottles or plastic bags.* Sample volumes of 200 mL to 1 L are sufficient for most analyses. For chlorinated waters, add sodium thiosulfate (see Section 9060A.2). The potentially toxic effect of heavy metals is neutralized by adding EDTA (see Section 9060A.2).

Transport samples to the laboratory at 2 to 8°C within 8 h. Samples for presence–absence analyses may be transported at ambient temperatures within 24 h. Grab samples are most common. Moore swabs (see 9260B.2*a*) have been used for wastewater sampling, and Spira bottles have been used for tapwater sampling.[13] Both of these methods are used with enrichment in 1% alkaline peptone water (APW), pH 8.6.[13] Place sediment and sludge samples in bottles or bags and submit in same way as water samples.

3. Enrichment Methods

Do not use enrichment methods for ecological studies because the predominant strain(s) will overgrow other organisms. Reserve enrichments for presence–absence tests for aeromonads in drinking water, foods, stools, or for monitoring aeromonad populations in wastewater or marine environments, where organisms may be present in low numbers or require resuscitation due to injury from exposure to inimical agents or hostile physical environments. For isolation of aeromonads from clear water samples, filter through 0.45-μm membrane filters, place filters in a bottle with 10 mL APW, incubate overnight at 35°C, and inoculate to plating media for isolation. Optimally, to sample clear water intended for drinking, filter a volume of water through a mini-capsule filter,† decant residual water from inlet, plug ends with sterile rubber stoppers, and fill filter with APW, pH 8.6, through syringe port. Incubate filter at 35°C for 6 h or overnight and streak loopfuls of broth onto selective and differential plating media.[13]

* WhirlPak™, ZipLoc™, or equivalent.
† Gelman 12123 or equivalent.

4. Enumeration Methods

a. Spread plates: Enumerate samples expected to contain predominantly aeromonads in high numbers (sludge, sediments, wastewater effluents, polluted surface waters, etc.) directly by spreading 0.1-mL portions of decimal dilutions on ampicillin dextrin agar (ADA) plates.[14–16] Incubate plates at 35°C overnight and count bright yellow colonies 1 to 1.5 mm in diameter. Presumptively identify colonies using the screening methods below.

b. Membrane filtration: Enumerate aeromonads in drinking water samples or other low-turbidity waters by using membrane filtration procedures with ADA medium[15] and incubating aerobically overnight at 35°C. Filter sample volumes equivalent to 1, 10, and 100 mL. To achieve a countable plate (1 to 30 colonies), prepare decimal dilutions when aeromonads are present in high numbers. Count bright yellow colonies, 1 to 1.5 mm in diameter, and pick to screening media.

A recent modification[17] of the membrane filter method incorporates vancomycin into ADA to inhibit *Bacillus* species, which produce yellow colonies that could have been counted as presumptive *Aeromonas* spp.[16–18] This method has been validated and used in surveys to characterize the presence of *Aeromonas* spp. in distribution system water in the United States.

c. Multiple-tube fermentation tests (MTF): Multiple-tube fermentation tests using APW, pH 8.6, or trypticase soy broth (TSB) containing ampicillin at 30 μg/mL (TSB30) have been applied to foods; however, they have not been used for enumeration of aeromonads in water samples. Some aeromonads are sensitive to ampicillin and will not grow in TSB30 medium. ADA without agar has been used to enumerate aeromonads in drinking water.[8] Use MTF methods only for clean samples, such as groundwater or treated drinking water samples, because the effects of competing microflora present in surface waters on recovery of aeromonads in broth media has not been studied adequately. Similarly, the correlation between MTF population estimates and other enumeration methods has not been examined adequately for matrices other than foods.

5. Screening Tests

Pick 3 to 10 colonies resembling aeromonads on differential and selective plating media or membrane filters and stab-inoculate into deeps of Kaper's multi-test medium[18] or one tube each of triple sugar iron (TSI) agar and lysine iron agar (LIA). Incubate cultures at 30°C for 24 h. Perform a spot oxidase test on growth taken from the LIA slant. Do not test for oxidase on growth from TSI slants, MacConkey agar, or other selective or differential media, because acid production interferes with the oxidase reaction. Reactions of enteric bacteria on TSI and LIA media are shown in Table 9260:VIII. When Kaper's medium is used instead of TSI/LIA slants, colonies may be picked and inoculated onto sheep blood agar plates; incubate at 35°C overnight to provide growth for the oxidase test and to record hemolysin production. Cultures are identified presumptively using Kaper's medium according to the characteristics shown in Table 9260:IX. When using the membrane filter method with vancomycin, test presumptive yellow colonies for oxidase production, trehalose fermentation, and indole production according to the procedures described in the method.[17]

TABLE 9260:VIII REACTIONS OF ENTERIC BACTERIA ON TSI AND LIA MEDIA

Organism	TSI Reactions*	LIA Reactions*
Shigella	K/A–	K/A–
Salmonella	K/Ag+	K/K+
Escherichia	A/Ag–	K/A–
Proteus	A/Ag+ or K/Ag+	R/A+
Citrobacter	A/Ag+	K/A+
Enterobacter	A/Ag–	K/A–
Aeromonas	A/A–	K/A–
Yersinia	A/A– or K/A–	K/A–
Klebsiella	A/Ag–	K/A–

* Fermentation reactions = slant/butt; H_2S production = + or −; K = alkaline, A = acid, R = red (deaminase reaction); g = gas produced.

If species identification is desirable, submit presumptively identified *Aeromonas* cultures to a reference laboratory. Cultures with potential public health or regulatory significance may be subtyped using various molecular methods to determine clonality for outbreak investigations and troubleshooting of treatment plant or distribution system problems.[19,20]

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TABLE 9260:IX. REACTIONS OF *AEROMONAS* AND ENTERIC BACTERIA ON KAPER'S MEDIUM

Organism	Fermentation Pattern*	Motility	H_2S	Indole
Aeromonas hydrophila	K/A	+	−	+
Klebsiella pneumoniae	A/A	−	−	−
Klebsiella oxytoca	A/A	−	−	+
Escherichia coli	K/K or K/A	+ or −	−	+
Salmonella spp.	K/K, K/A, A/K or A/A	+	+	−
Enterobacter spp.	K/K, K/N or N/N	+	−	−
Proteus spp.	R/K or R/A	+	+ or −	+
Yersinia enterocolitica	K/K, K/N or N/N	−	−	+ or −
Citrobacter spp.	K/K or K/A	+	+	−
Serratia spp.	K/K, K/N or N/N	+	−	−

* K = alkaline; A = acid; N = neutral; R = red (deamination reaction).

9260 M. *Mycobacterium*

The genus *Mycobacterium* comprises more than 70 characterized species that are nonmotile non-spore-forming, aerobic, acid-fast bacilli measuring 0.2 to 0.6 × 1 to 10 μm. They are conveniently separated into slow-growing (>7 d for colony formation) and rapid-growing (<7 d for colony formation) species. Many species are capable of causing disease in humans.[1] The two most important pathogens in this group include *Mycobacterium tuberculosis* and *Mycobacterium leprae,* the causative agents of tuberculosis and Hansen's disease (leprosy), respectively. Recently there has been an increase in the incidence of disease caused by opportunistic mycobacteria (also called non-tuberculosis mycobacteria), probably related to the increasing numbers of elderly[2] and immunocompromised patients.[3] In the genus *Mycobacterium*, the most important environmental opportunistic pathogens include *M. avium* and *M. intracellulare, M. kansasii, M. marinum, M. xenopi*, and *M. simiae*. A number of the rapidly growing mycobacterial species (e.g., *M. fortuitum, M. chelonae*, and *M. abscessus*) have been found in cases of nosocomial infections.[4] Some of the common hosts and environmental reservoirs of *Mycobacteria* are shown in Table 9260:X.

Because of the complex nature of the cell wall, which is rich in lipids and therefore has a hydrophobic surface, these microorganisms are impermeable and resistant to many common disinfectants. The ability of the cell walls to retain dyes (i.e., the acid-fast property) is due to their high lipid content. As a result of their hydrophobicity, mycobacteria are present in biofilms on surfaces, such as pipes, and are readily aerosolized from water.[5] Because of disinfectant (e.g., chlorine) resistance, several members of this genus have become important waterborne pathogens in the elderly[2] and immunocompromised population.[3] Their relative slow growth is due, in part, to their impermeability. Some species, such as *M. avium-intracellulare*, require from 3 to 8 weeks to form colonies on culture media.

Because *Mycobacterium avium* and *Mycobacterium intracellulare* exhibit overlapping properties, speciation is extremely difficult. Because of their close relatedness, these two pathogens are grouped together and called the *M. avium* complex (MAC). Organisms from this group are ubiquitous in the environment and have been isolated from potable water systems,[5] including those in hospitals[4–6] as well as from soil and dairy products.[7] MAC causes a chronic pulmonary disease in immunocompetent hosts, including the elderly, that is clinically and pathologically indistinguishable from tuberculosis.[2,8] MAC also causes disseminated disease in immunocompromised hosts.[3] In children less than 5 years old, *M. avium* causes cervical lymphadenitis.[9] The primary routes of transmission are via ingestion and inhalation of mycobacterial-laden aerosols or dusts.

TABLE 9260:X. MYCOBACTERIA OF WATERBORNE OR UNKNOWN ORIGIN

Mycobacterium species	Environmental Contaminant	Reservoir
M. kansasii	Rarely	Water, swine, cattle
M. marinum	Rarely	Fish, water
M. simiae	No	Primates, possibly water
M. scrofulaceum	Possibly	Soil, water, foodstuffs
M. szulgai	No	Unknown
M. avium-intracellulare	Possibly	Soil, water, swine, cattle, birds
M. xenopi	Possibly	Water
M. ulcerans	No	Unknown
M. fortuitum	Yes	Soil, water, animals, marine life
M. chelonae	Yes	Soil, water, animals, marine life

1. Sample Collection and Concentration

Mycobacteria typically constitute a minority of the microflora in waters and require sample concentration. Collect water samples in sterile 1-L polypropylene containers. For water samples containing disinfectants (e.g., chlorine) add 1 mL 10% (w/v) sodium thiosulfate solution per liter of water collected. Transport samples to laboratory immediately after collection. If samples cannot be analyzed immediately, store at 4°C and begin analysis within 24 h of sampling.

To sample biofilms, scrape material from a defined area of any surface (e.g., pipe or water meter) and suspend it in 1 to 10 mL sterile water. Because of the aggregation of mycobacterial cells due to hydrophobicity, disrupt biofilm suspensions by vortexing with or without 1-mm glass beads or by treatment with a sterilized tissue homogenizer.

2. Screening Water Samples by Direct Fluorescent Assay

Before committing the sample to lengthy culture incubation, survey for acid-fast bacteria by using a combination solution of auramine-rhodamine (A-R) fluorescent dye.* Auramine and rhodamine nonspecifically bind to mycolic acids and resist decolorization by acid alcohol.[10]

Filter a minimum of 500 mL finished water, or 100 mL source water (depending on turbidity), through a sterile 0.45-μm-porosity, 47-mm-diam black filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube and add 5 mL of buffered dilution water. Resuspend organisms from filter by vortexing for 2 min. Aspirate suspension and aseptically transfer to a sterile 15-mL polypropylene centrifuge tube. Centrifuge suspension at 5000 × *g* for 10 min and discard all but about 0.5 mL of supernatant. Vortex to resuspend pellet. Transfer 100 mL of the concentrate to a clean glass slide and air-dry and heat-fix at 60 to 70°C for 2 h or overnight. Stain the smear with A-R (15 min), then decolorize with acid-alcohol for 2 to 3 min, and rinse with deionized water. Next, apply secondary potassium permanganate counterstain (no longer than 2 to 4 min), rinse, and let air-dry. Examine smear at 100 and 400× with a microscope fitted with a BG-12 or 5113 primary filter with an OG-1 barrier filter. Acid-fast organisms will stain yellow-orange on a black background. To confirm for acid-fastness, apply a traditional acid-fast stain (Ziehl-Nielsen with Kenyon modification) directly to the prepared smear following the A-R stain.

* Catalog #40-090, Remel, Lenexa, KS, or equivalent.

For wastewater or highly turbid source waters, collect a 10-mL subsample and transfer to a sterile polypropylene 15-mL tube. Centrifuge at 5000 × *g* for 10 min and discard all but about 0.5 mL of supernatant. Follow slide preparation procedure and staining as above.

3. Sample Decontamination and Culture Methods

Some mycobacteria form colonies on laboratory media only after 7 d incubation at 37°C. Therefore, eliminate from the sample naturally occurring microorganisms that can outcompete and overgrow the mycobacteria. Various isolation and identification methods have been described for the recovery of mycobacteria, especially in the hospital environment.[11–13] Selective decontamination of the sample concentrate is required for the selection of mycobacteria before culturing. In addition, the matrix may affect the success of the recovery of mycobacteria; for example, decontamination is not required for many drinking water samples, while decontamination usually is required for biofilm samples.[5] However, all decontamination methods also reduce the number and, hence, the recovery of mycobacteria.[14] Several methods are detailed below for recovering mycobacteria from water samples [¶s *a*1)–4) below] and from biofilms (¶ *b* below).

a. Methods for water: Determine which of the four methods below performs best with the matrix to be examined.

1) Centrifuge 500-mL water sample (5000 × *g* for 20 min), discard the supernatant, and suspend the pellet in 1 mL sterile water. For decontamination, add 1 mL 1*M* NaOH and immediately centrifuge (5000 × *g* for 20 min), discard supernatant, suspend pellet in 1 mL of phosphate-buffered saline (PBS) at pH 7.4, and spread 0.1-mL samples on Middlebrook 7H10 agar medium.† Use thick plates (30 to 35 mL per plate) and seal plates with thermoplastic self-sealing film‡ to prevent drying of the medium during the long incubation (3 to 8 weeks). Incubate at any temperature between 15 and 45°C and examine plates for the appearance of small (0.2-mm-diam), transparent or yellow-pigmented colonies. The M7H10 agar medium allows detection of the small, transparent mycobacterial colonies. Although other microorganisms are killed, only 5% of mycobacteria survive sample decomtamination.[14] For gentler decontamination, suspend pellet in 0.005% (w/v) cetylpyridinium chloride (CPC) and leave at room temperature for 24 h.[15] Centrifuge (5000 × *g* for 20 min) to pellet the cells, suspend in 1 mL PBS and spread 0.1 mL on M7H10 agar medium.

2) Filter 500-mL water sample through a sterile 0.45-μm-porosity, 47-mm-diam filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube. Add 5 mL sterile distilled water and resuspend organisms off the filter by shaking with two 5-mm glass beads for 1 h on a mechanical shaker.[11] Add a 3% sodium lauryl sulfate and 1% NaOH solution, then incubate on the bench for 10 min.[11,13] Pellet cells by centrifugation (5000 × *g* for 20 min), discard supernatant, suspend cells in 5 mL PBS to wash cells free of detergent and base, and pellet cells again. Suspend cells in 1 mL sterile distilled water and spread portions of this suspension onto M7H10 agar medium.

3) Filter 500-mL water sample through sterile 0.45-μm-porosity, 47-mm-diam filter. Aseptically transfer filter to a sterile polypropylene 50-mL tube. Add 5 mL sterile distilled water and resuspend organisms off the filter by shaking with glass beads for 5 min on a mechanical shaker. Add 10 mL 1*M* NaOH for 20 min followed by centrifugation at 5000 × *g* at 4°C for 20 min. Discard supernatant and add 5 mL 5% aqueous oxalic acid for 20 min. Re-centrifuge, discard supernatant, and add 30 mL sterile distilled water to neutralize. Centrifuge again and resuspend in 0.7 mL distilled water.[11] Spread 0.1 mL on M7H10 agar medium.

4) Add 20 mL 0.04% (w/v) cetylpridinium chloride (CPC) to 500-mL water sample and leave at room temperature for approximately 24 h. Filter sample and wash filter with 500 mL sterile water.[15]A study of decontamination methods for the isolation of mycobacteria from drinking water samples found a CPC concentration of 0.005% (w/v) to yield the highest isolation rate and lowest contamination rate for the water examined.[15]

b. Method for biofilms: Obtain a section of pipe or a water meter. Expose the pipe or meter surface and scrape a defined area (4 cm^2) with a sterile rubber policeman or spatula. Transfer the material to 1 to 10 mL sterile water. For a small-diameter pipe section, measure length and interior diameter of pipe and use a sterilized pipet brush to collect the biofilm. Disperse biofilm suspension by vortexing with 1-mm-diam glass beads or with a tissue homogenizer. Add CPC to a final concentration of 0.005% (w/v) and leave on bench for 24 h. Pellet the cells by centrifugation (5000 × *g* for 20 min), discard supernatant liquid, and suspend pellet in 1 mL water. Spread 0.1 mL on the surface of M7H10 agar medium and incubate at 15 to 45°C.

4. Selective Growth

Culture all samples in duplicate. After sample decontamination, either spread 0.1-mL portions of the concentrates or use sterile forceps to place filters on selective media. For environmental samples, use M7H10 agar medium. Pour thick plates (30 to 35 mL/15 × 100 mm petri dish) to prevent drying during the prolonged incubation. If fungal contamination is a problem, add 100 μg cycloheximide/mL or increase concentration of malachite green to 5 μg/mL. Although the common egg-based media (e.g., Lowenstein-Jensen medium) have been used successfully to isolate mycobacteria from patient samples, it is difficult to detect the small transparent mycobacterial colonies and obtain quantitative results on those media. Seal plates with plastic film§ or place plates in humid chambers or gas-permeable bags to prevent dehydration and incubate at 37°C. Additional plates also can be incubated at any temperature between 15 and 45°C in a humidified chamber to detect mycobacteria that grow optimally at lower or higher temperatures; for example, *M. avium* and *M. xenopi* grow at 45°C. Examine plates or slants periodically during a 3- to 8-week incubation period. Count suspect colonies (acid-fast coccobacilli), streak for isolation on M7H10 agar medium, and subculture to a tube of M7H9 broth.‖ After 5 d, remove subsamples and stain with Ziehl-Nielsen stain with Kenyon modification.

† BBL Microbiology Systems, Cockeysville, MD, or equivalent.
‡ Parafilm®, or equivalent.
§ Parafilm®, or equivalent.
‖ BBL Microbiology Systems, Cockeysville, MD, or equivalent.

TABLE 9260:XI. PHENOTYPIC CHARACTERISTICS OF CLINICALLY SIGNIFICANT ENVIRONMENTAL MYCOBACTERIA*

Species	Growth Rate	Pigmentation	Urease	Nitrate Reduction	Hydrolysis of Polyoxyethylene Sorbitan Monooleate†
M. kansasii	S	P	±	+	+
M. marinum	S	P	+	−	+
M. simiae	S	P	±	−	−
M. scrofulaceum	S	S	±	−	−
M. szulgai	S	S/P	+	+	±
M. xenopi	S	S	−	−	−
M. avium-intracellulare	S	N	−	−	−
M. ulcerans	S	N	±	−	−
M. fortuitum	R	N	+	+	±
M. chelonae	R	N	+	−	±

* S = slow (3 to 8 weeks), R = rapid (7 d or longer), P = photochromogenic, S = scotochromogenic, N = nonphotochromogenic, S/P = scotochromogenic at 37°C and photochromogenic at 24°C.
† Tween 80®.

5. Identification

Identification of acid-fast isolates can be conducted by genetic or phenotypic tests.

a. Genetic tests: One genetic test involves PCR amplification of the mycobacterial *hsp-65* heat-shock protein gene followed by restriction endonuclease digestion (with either *Bst*EII or *Hae*III) and identification of species-specific fragments.[16,17] For a few mycobacteria (e.g., *M. avium* and *M. intracellulare*), DNA probe-based tests are available;# these are based on hybridization between 16S rRNA genes and species-specific oligonucleotide probes.

b. Phenotypic tests: These include cultural, biochemical, and enzymatic tests (Table 9260:XI), as well as identification of mycolic acid profiles by HPLC[18] cellular fatty acids by GC.[19] Although phenotypic tests have been the standard for species identification, there are several inherent problems in this approach. First, because initial identification of mycobacteria can take 3 to 8 weeks, observing biochemical changes entails additional time for the isolates (especially those of slowly growing mycobacteria) to metabolize specific substrates or to exhibit certain characteristics. Second, phenotypic traits subject to variation depend on the growth medium and prior growth conditions.

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