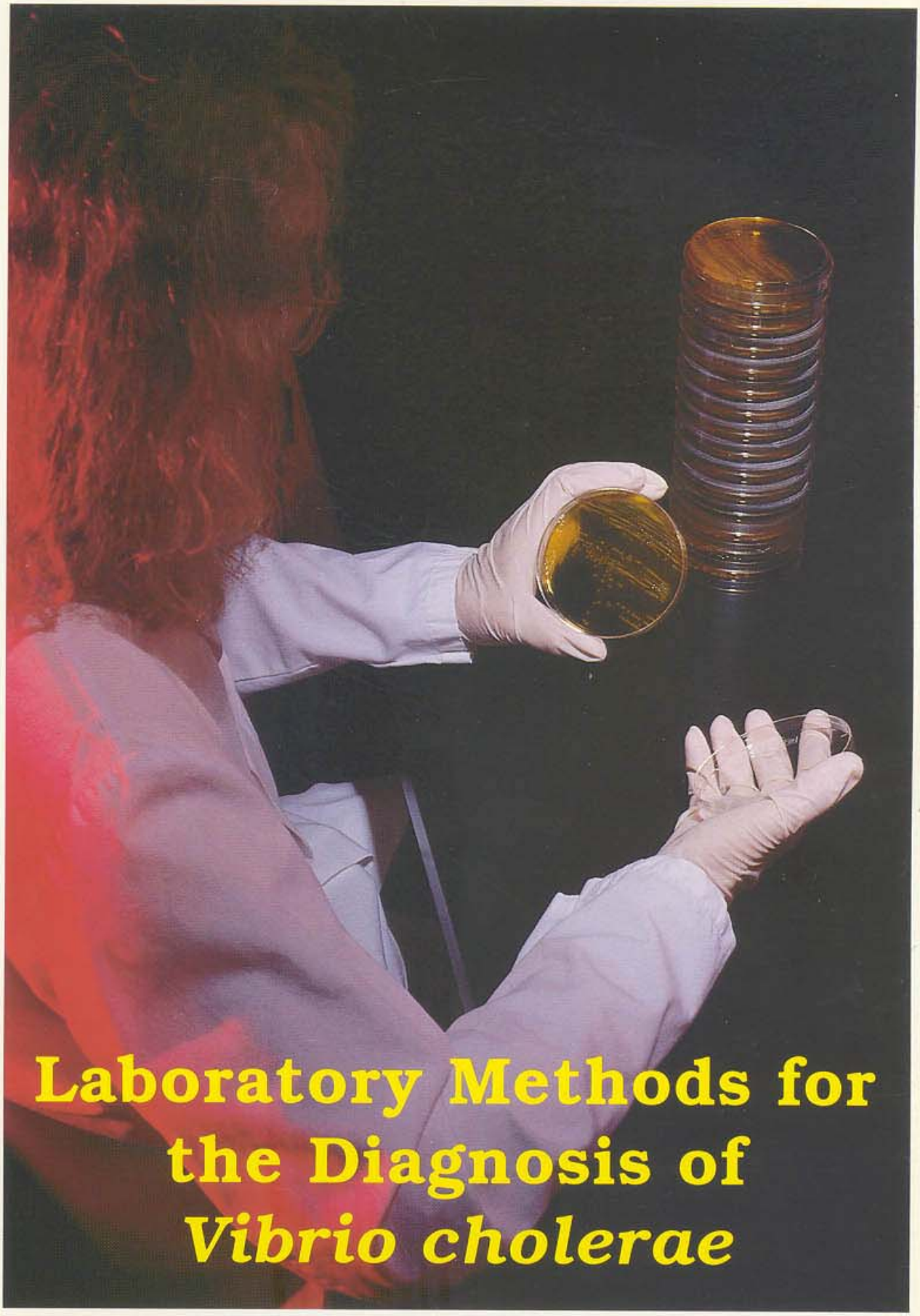

Laboratory Methods for the Diagnosis of *Vibrio cholerae*

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

V. Examination of Food and Environmental Samples

Traditionally, water has been considered to be the most important vehicle for cholera transmission. By 1860, after investigations by John Snow and others, it was apparent that sewage-contaminated water sources, such as municipal water supplies, rivers, streams, or wells, were the principal route of disease transmission. Contact with contaminated food can also spread cholera, although most implicated foods are seafoods. In an epidemic setting, water and food are usually contaminated by *Vibrio cholerae* O1 strains from human feces. Thus, for many years, it was believed that the only reservoir of *V. cholerae* O1 was the human intestine and that survival of the organism in the environment was limited. However, during the past 20 years, investigations in Australia and the United States have yielded evidence that both nontoxigenic and toxin-producing strains of *V. cholerae* O1 may be naturally occurring members of the aquatic ecosystem. These data support the concept that toxigenic and nontoxigenic *V. cholerae* O1 strains may have an environmental reservoir, which would have important implications for efforts to control and eradicate cholera.

Like other members of the *Vibrionaceae*, *V. cholerae* O1 can survive in aquatic environments for extended periods and is considered by many to be an indigenous species in estuarine and brackish waters. Various biological and physiochemical factors, such as nutrient content, salinity, temperature, and pH, may influence the growth, survival, and distribution of *V. cholerae* in aquatic environments. The survival time of *V. cholerae* in water may extend from hours to months. The ability of *V. cholerae* to produce chitinase may also contribute to its survival in estuarine environments where plankton and other chitin-containing marine life are plentiful. *V. cholerae* O1 was able to bind to diverse plankton species collected from Bangladesh, where cholera is endemic. Other aquatic biota, such as water hyacinths from Bangladesh waters, have also been shown to be colonized by *V. cholerae* and to promote its growth. This relationship with plankton may be an important aspect of the ecology of *V. cholerae* O1 in cholera-endemic regions of Bangladesh, and this is supported by the fact that seasonal plankton blooms accompany cholera epidemics in Bangladesh. However, although natural bodies of water probably serve as both environmental reservoirs and a means of transmission to humans, attempts to isolate *V. cholerae* from lakes, rivers, streams, and ponds in areas with epidemic disease have not always been successful.

A. Transport of Specimens

Because *V. cholerae* survives better in specimens held at 4°C than in frozen samples, specimens should be held refrigerated by placing them in an insulated box with frozen refrigerant packs (these may be commercial or homemade). If specimens are refrigerated with wet ice instead of refrigerant packs, water from the melting ice should not seep into the speci-

mens or leak from the container. This can be avoided by placing the specimen containers in waterproof plastic bags that can be tightly sealed. Submersion of samples in ice should also be avoided to prevent partial freezing of the samples.

B. Selection of Isolation Methods for Environmental Samples

The selection of the isolation method depends on the type of sample to be cultured. Samples from marine and estuarine environments may contain numerous other *Vibrio* species that grow as well as *V. cholerae* in alkaline peptone water (APW) and on thiosulfate citrate bile salts sucrose agar (TCBS). These samples should be diluted in 10-fold increments to 10^{-3} to reduce the numbers of competing microorganisms. Incubation of APW at an elevated temperature (42°C) inhibits the growth of some competitors, particularly other vibrios that do not grow as well at that temperature. The isolation of *V. cholerae* O1 from estuarine or marine samples may therefore be enhanced because the primary competitors are inhibited at this temperature. If laboratory resources permit, a duplicate set of dilutions may be prepared to be incubated at 42°C. In contrast, specimens such as fresh water or sewage effluent, which contain relatively fewer vibrios and vibrio-like organisms, do not usually require dilution before culturing or incubation at 42°C.

C. Isolation of *V. cholerae* from Sewage

Surveillance using the Moore swab method is a practical and effective technique for detecting *V. cholerae* in sewage (Figure V-1). The swabs can be easily assembled, and when suspended in the intake lines of a municipal sewage system, they can detect *V. cholerae* infections in areas where surveillance of diarrheal illness has failed to detect cholera. The sensitivity of the technique appears not to be dependent on the distance of the source of infection from the sampling site. Using this method to sample all major intake lines of a community sewage system is a simple way to determine whether *V. cholerae* O1 infections are occurring in an area. The assembly, placement, and subsequent handling of Moore swabs for *V. cholerae* sewage surveillance are described below.

Materials (for 10 specimens)

- 10 Moore swabs
- 100 yards nylon fishing line (25 lb test or higher)
- 1 insulated cooler and frozen refrigerant packs for transport
- 10 securely closing suitable containers (500-1000 ml)
- 10 pairs latex gloves
- 3-5 liters of alkaline peptone water (see Chapter XI for formula)

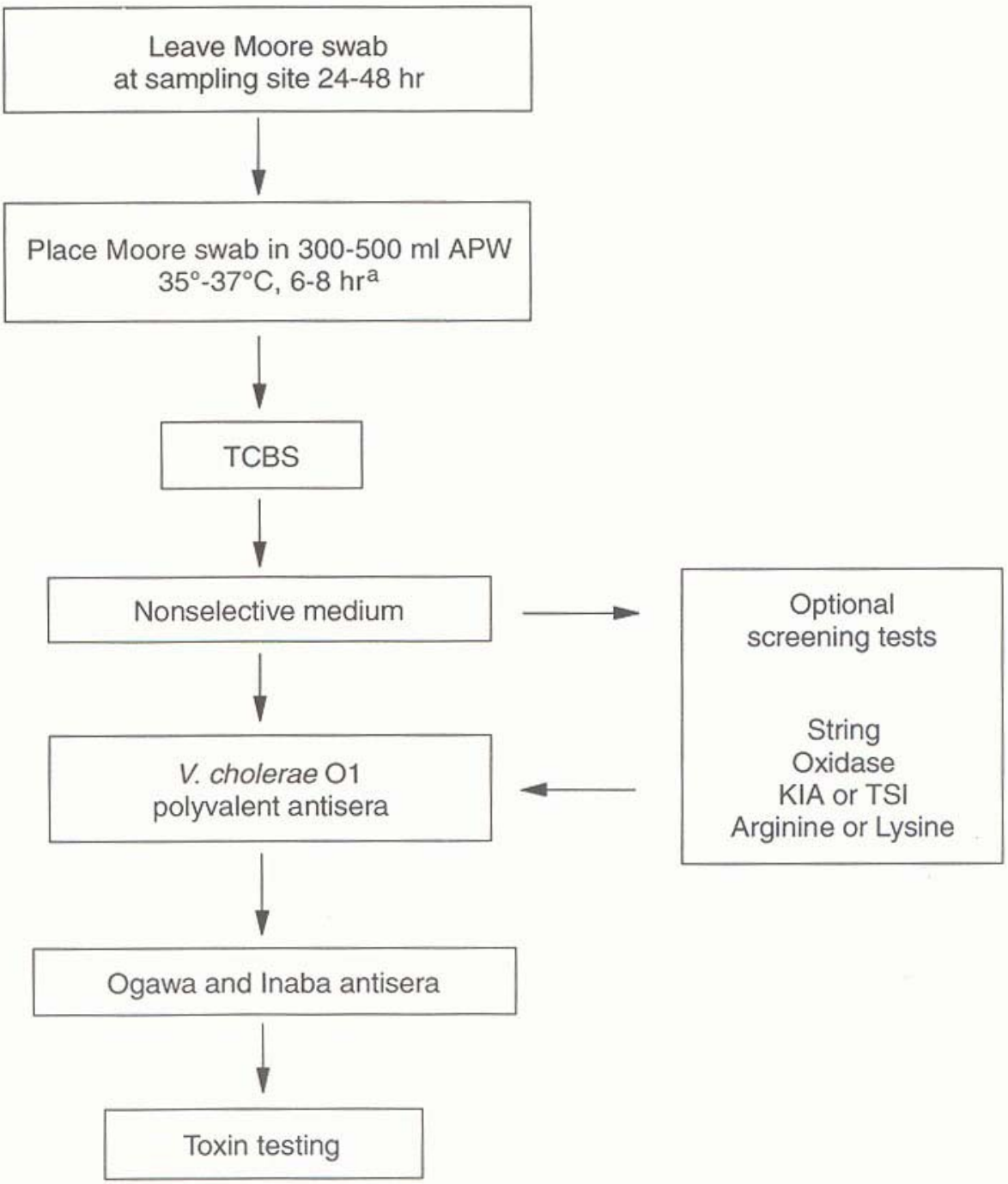

Figure V-1. Moore swab technique for recovering *Vibrio cholerae* O1 from sewage or water

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

Assembly of Moore swabs

Moore swabs can be made by cutting pieces of cotton gauze 4 feet long by 6 inches wide (120 cm by 15 cm), folding or rolling the gauze lengthwise several times, and firmly tying the center with fishing line. Sterilization by wrapping in heavy paper and autoclaving before use is optional (Figure V-2).

Placement of Moore swabs

For effective surveillance, place Moore swabs in all main intake lines at the sewage plant or other central locations in the sewage system. The site for swab placement must be carefully evaluated for conditions that could inhibit the recovery of the *V. cholerae* organism. Placement must be upstream of any septic waste dump sites or partially treated recycled sewage to avoid possible contamination with toxic waste. Suspend the swab in the sewage to be tested. In the case of intake lines accessible only by manhole, a piece of wire should be attached to the end of the line to prevent cutting the line when the manhole cover is replaced. The swab should be left in place for 24 to 48 hours.

Collection of specimens

Wear latex gloves and change between specimens to prevent cross-contamination. When Moore swabs, including their attachment line, are removed from the sampling site, they should be placed in securely-closing containers of a suitable size. Label the containers with collection site and

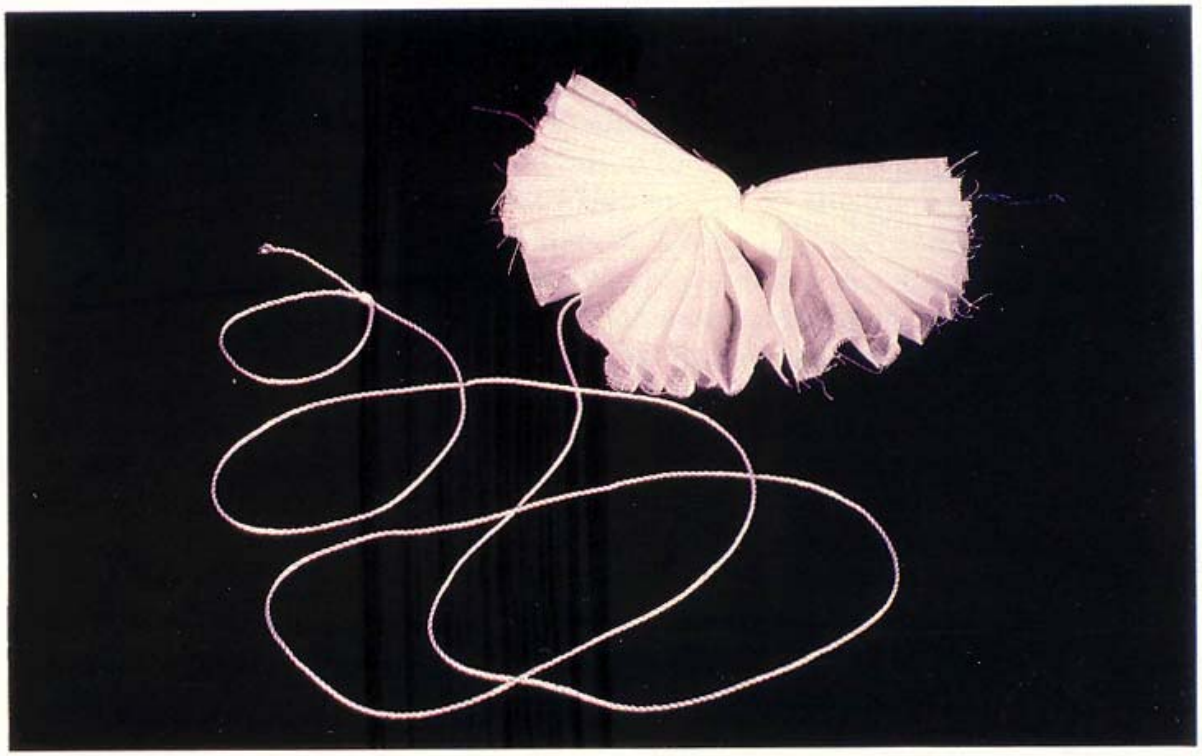

Figure V-2. The Moore swab is a simple device for sampling sewage and contaminated water for the presence of *V. cholerae.*

date. Samples should be transported as quickly as possible to the laboratory in an ice chest with frozen refrigerant packs to prevent possible overheating (see Section A of this chapter for instructions for transport of specimens). If it will be longer than 3 hours between collection of swabs and arrival at the laboratory, the swabs should be placed in APW at the collection site before transport to ensure optimal recovery of *V. cholerae*. APW (300 to 500 ml) should be added to the specimens immediately upon arrival at the laboratory if it was not added at the site. The Moore swab and the associated sample water should make up approximately 10% to 20% of the total volume of the sample with APW added to obtain the optimal ratio of sample to enrichment broth for recovery. Incubate specimens at 35° to 37°C for 6 to 8 hours before plating, as described in Section F of this chapter.

D. Isolation of *V. cholerae* from Water Specimens

All water specimens should be collected in sterile containers and transported to the laboratory under refrigeration (see Section A of this chapter) to prevent overheating. Generally the larger the water sample, the greater the chance of isolating *V. cholerae*. Collecting and processing multiple samples is another way to enhance the chances of isolation. Selection of the isolation method should depend on the type of water sample to be cultured, and salinity of the water source should be the determining factor. For example, ship ballast water, a documented source of *V. cholerae*, should be cultured by the same method as seawater.

1. Direct culture technique

Add 450 ml of water to 50 ml of 10x concentrated APW. An alternate method is to make a 10^{-1} dilution of the water sample in 1x APW (for example, 10 ml of water in 90 ml of APW). The latter method is particularly useful for heavily contaminated samples. If estuarine or sea water samples are cultured, two additional tenfold dilutions should be made in APW to give 10^{-2} and 10^{-3} dilutions. Additional dilutions to 10^{-6} may be done if enumeration is desired (see the MPN technique below). Incubate all dilutions at 35° to 37°C, and plate at 6 to 8 hours as described in Section F of this chapter. If laboratory resources permit, a duplicate set of dilutions may be prepared to be incubated at 42°C. When culturing fresh water samples, further dilutions and incubation at 42°C may not be necessary to enhance isolation since the numbers of competing organisms (particularly *Vibrio* spp.) may be considerably fewer than in marine or estuarine waters.

2. Membrane filter technique

The membrane filter technique is most appropriate for clear water that does not contain debris, mud, or silt. If it is used for cloudy water, a clarifying agent, filter aid, or prefilter to remove suspended materials may be necessary. Filter 100 to 300 ml of sample water (or a larger

amount if possible) through a 0.22- to 0.45-µm membrane (Millipore) filter. Place the filter in 100 ml of APW in a flask. Incubate at 35° to 37°C for 6 to 8 hours and plate to TCBS. If laboratory resources permit, a duplicate specimen may be prepared to be incubated at 42°C. Alternatively, the filter may be placed directly on the surface of a nonselective agar plate (e.g., T_1N_1, heart infusion agar), incubated for 3 hours at 35° to 37°C, then transferred to a TCBS agar plate and incubated for 18 to 24 hours at 35° to 37°C. If estuarine or marine samples are to be cultured, smaller water samples should be filtered, or filters should be placed in larger volumes of APW to dilute the samples adequately.

3. Moore swab

The Moore swab can be used for sampling water as well as sewage, but it is useful only for rivers and flowing water sources and offers no particular advantage over other sampling methods for stationary water sources. As with sewage, the Moore swab should be left at the sampling site for 24 to 48 hours. (Refer to Section C of this chapter for assembly, collection, and examination of Moore swabs.)

4. Spira swab

The Spira swab procedure is a sampling method in which water is filtered through cotton gauze. The gauze is placed in a large plastic bottle (for example, a 500-g media bottle or other equivalent container) with a 2-cm hole cut in the bottom (Figure V-3). The size of the hole in the bottom

Figure V-3. The Spira swab is used for filtering large volumes of water through cotton gauze to recover *V. cholerae*.

of the bottle is critical; if it is too large, the gauze is pulled out by the water as it flows through, and if it is too small the water passes through very slowly. Gauze 30 cm (12 in) wide is packed into the bottle in a layered fashion so that when water is poured into the top of the bottle it passes through the gauze and out of the bottle through the 2-cm hole in the bottom. The gauze should be properly layered to prevent the water from being channeled around the layers of gauze instead of being filtered. Enough gauze (approximately 4 to 6 ft or 120 to 180 cm) should be used to form a firm but still compressible pack and should fill about two-thirds of the bottle's volume. Sterilization of Spira swabs is optional. If swabs are to be sterilized, wrap each bottle in foil or brown paper and autoclave. Pour water to be sampled into the top and allow to drain out the bottom. Aseptically remove the gauze swab and place in flask or jar containing 100 ml of 10x APW. Add sufficient source water to final volume of 1 liter. Incubate at 35° to 37°C for 6 to 8 hours and plate to TCBS.

5. MPN Technique

The most probable number (MPN) method uses a multiple dilution-to-extinction approach for estimating microbial population. It is useful in situations where extremely low densities of organisms are encountered and where potential competitors complicate other enumeration methods. This method may be used to locate the source of contamination by establishing a gradient of concentrations of *V. cholerae* O1. Estimates of *V. cholerae* populations in water may be determined by an MPN procedure consisting of a 3-dilution, 3- or 5-tube replication series that uses enrichment in APW followed by plating to TCBS. However, if the water to be sampled is heavily contaminated with sewage, dilutions out to 10^{-6} may be necessary to reach an endpoint. If laboratory resources permit, a duplicate set of dilutions may be prepared to be incubated at 42°C. Methods for the MPN procedure are described in *Standard Methods for the Examination of Water and Wastewater*, published by the American Public Health Association.

E. Isolation of *V. cholerae* from Food, Sediment, and Other Environmental Samples

In addition to water, contaminated food can serve as a vehicle for the transmission of cholera. Foods commonly associated with cholera transmission have included fish (particularly shellfish harvested from contaminated waters), milk, cooked rice, lentils, potatoes, kidney beans, eggs, chicken, and vegetables. Freshly harvested oysters and fish are frequently cultured as sentinel specimens for surveillance purposes. The intestines, and to a lesser extent, the skin of freshly caught fish are more likely to harbor *V. cholerae* organisms than is the muscle tissue, but for fish in the market that have been scaled and cleaned, the flesh may be culture positive because of cross-contamination during cleaning and storage on ice.

Sediment, aquatic plants, plankton, and other environmental specimens may be sampled to monitor the incidence and ecology of *V. cholerae* O1 in various ecosystems and the importance of these as reservoirs in the transmission of cholera.

1. Preparation of food, sediment, and other environmental samples

Samples should be kept refrigerated (4°C) until cultured (see Section A of this chapter). Aseptically weigh a 25-g food sample into a sterile blender jar or stomacher bag (see Figure V-4). Cut large samples into smaller pieces before blending. Add a small amount of APW to the jar and blend thoroughly. After blending, add additional APW to bring the total amount added to 225 ml (10^{-1} dilution). For sediment and other environmental samples that do not require blending, weigh a 25-g sample and place into 225 ml of APW (10^{-1} dilution). If resources permit, prepare duplicate samples (for incubation at both 35° to 37°C and 42°C). Prepare two tenfold dilutions (10^{-2} and 10^{-3}) of the blended food samples or plankton/sediment samples in APW. If duplicate samples are prepared, both should be diluted. Dilutions may be made to 10^{-6} if enumeration of *V. cholerae* is desired. Refer to the *Bacteriological Analytical Manual* (U.S. Food and Drug Administration) for more information.

2. Preparation of oyster samples

To culture oysters, remove and combine the meat from 10 to 12 animals; include the shell liquor. Blend to mix. Blend 25 g of this composite with 225 ml of APW (10^{-1} dilution). Prepare two tenfold dilutions (10^{-2} and 10^{-3}) of the oysters in APW. If duplicate samples are prepared, both should be diluted. Because certain components of oyster meat are toxic to *V. cholerae* O1, process only a few oysters at a time and dilute the samples as quickly as possible. This process will dilute out competing microorganisms, as well as the bactericidal effects of the oyster meat on *V. cholerae*. A duplicate set of dilutions incubated at 42°C greatly enhances the chances of isolating *V. cholerae* and is strongly recommended when culturing oysters, even more than for other types of food specimens.

F. Incubation of APW

Incubate the APW (with caps loosened on all jars and dilution tubes) at 35° to 37°C for 6 to 8 hours. If duplicate dilutions are prepared, incubate the second set of jars and dilutions at 42°C for 6 to 8 hours. After incubation, streak to TCBS using one large or two smaller loopfuls from the surface and topmost portion of the broth, since vibrios preferentially migrate to this area. Do not shake or mix the tube before subculturing. If the APW cannot be subcultured after 6 to 8 hours of incubation, at 18 hours subculture to a fresh tube of APW. This second APW tube should be subcultured to TCBS following 6 to 8 hours of incubation. Incubate TCBS plates for 18 to 24 hours at 35° to 37°C.

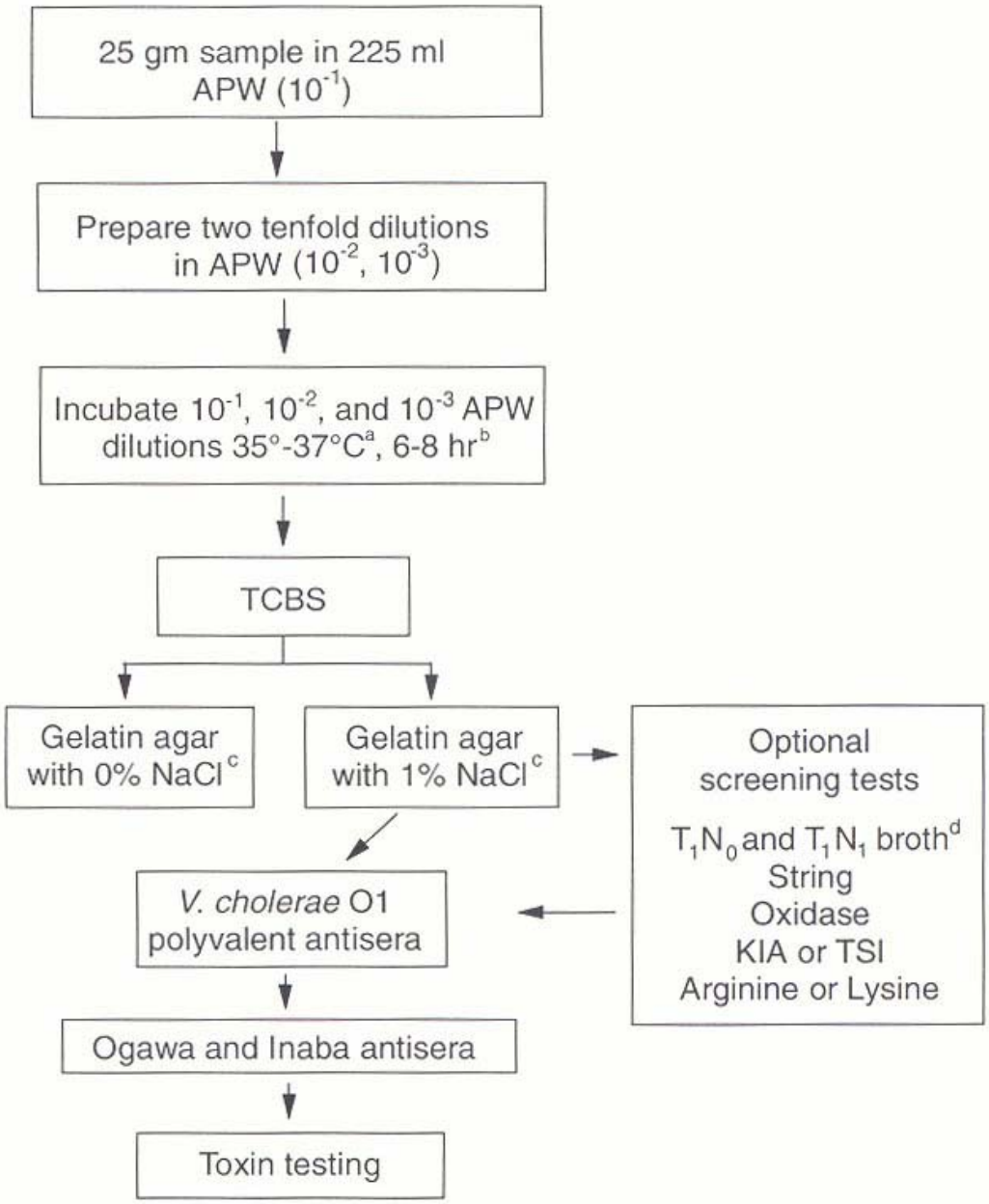

Figure V-4. Procedure for recovering *Vibrio cholerae* O1 from food or environmental specimens

[a] Duplicate APW dilutions may be prepared and incubated at 42°C.
[b] If the APW cannot be streaked after 6-8 hours of incubation, subculture at 18 hours to a fresh tube of APW; incubate for 6-8 hours, and then streak to TCBS.
[c] T_1N_0 and T_1N_1 agar may be used as an alternative to gelatin agar with 0% and 1% NaCl.
[d] Testing for growth in 0% and 1% NaCl may be done by subculturing from TCBS to a nonselective medium (e.g., gelatin agar with 1% NaCl, T_1N_1 agar, HIA) and then to T_1N_0 and T_1N_1 broth.

In some circumstances, it may be advantageous to incubate APW for 18 hours. For frozen food and oysters, incubate the homogenate in APW for 6 to 8 hours, then subculture an inoculum to TCBS. Reincubate the original homogenates for total incubation time of 18 hours, and replate the specimens to TCBS.

G. Isolation and Presumptive Identification

When culturing samples from an estuarine or marine environment, special methods must often be used to screen out the numerous other *Vibrio* species that grow well in APW and on TCBS agar. No single screening procedure for *V. cholerae* O1 is ideal for each laboratory and sample. The laboratorian should select a screening procedure based on available resources (for example, the availability of antisera), the competing organisms present in the environment being sampled, and the ability of the selective plating medium to inhibit the growth of those competing organisms. An effective procedure using a salt-free medium followed by biochemical screening and serologic tests is described below.

1. Salt-free media

Most of the *Vibrio* species other than *V. cholerae* that are frequently encountered in marine or estuarine specimens are halophilic or at least require a minimum amount of salt in culture media. For this reason, the ability of *V. cholerae* to grow on culture media with no added salt can be a useful selective characteristic.

Three or more colonies suspicious of *V. cholerae* may be subcultured from TCBS to two gelatin agar plates, one with 0% NaCl and the other with 1% NaCl (Figure V-4). See Chapter XI, "Preparation of Media and Reagents," for special instructions for preparation of gelatin agar. If the gelatinase reaction does not offer any advantage in the screening process, T_1N_0 (1% tryptone and 0% NaCl) agar and T_1N_1 (1% tryptone and 1% NaCl) agar may be used. Plates may be inoculated with as many as eight colonies by dividing them into eight sections. Growth should be inoculated in a straight line in the middle of each sector. Growth should be added to the gelatin plate without salt first since *V. cholerae* does not grow as well without salt and a larger inoculum may be required for that medium.

Since halophilic *Vibrio* spp. grow only on the medium with 1% NaCl, select isolates that grow on both the medium with 0% NaCl and medium with 1% NaCl. The gelatinase reaction (*V. cholerae* is positive) may be observed by holding the plate above a black surface or up to a light to observe the halo effect around the growth of gelatinase-positive organisms. Refrigeration of the gelatin agar plate for 15 to 30 minutes enhances the halo and makes it easier to observe this effect (see Chapter IV, "Isolation of *V. cholerae* from Fecal Specimens," for a description of gelatin agar). Growth from the agar medium with 1% NaCl may be tested directly in

V. cholerae O1 polyvalent antiserum or screened with the oxidase and string test before further biochemical testing.

An alternate procedure is to inoculate colonies suspicious of *V. cholerae* from TCBS to a nonselective medium (e.g., gelatin agar with 1% NaCl, T_1N_1 agar, HIA). Plates or tubed media may be used. Growth from this medium may then be inoculated into T_1N_0 and T_1N_1 broth to test for growth in the absence of salt before proceeding with further testing.

2. Biochemical screening tests

Slide serology is sufficient for a preliminary identification of *V. cholerae* O1 without further testing. However, it may be advantageous to screen with biochemical tests before testing with *V. cholerae* O1 antiserum if the TCBS medium used for isolation is not sufficiently selective to inhibit competitors such as *Aeromonas, Pseudomonas,* and *Enterobacteriaceae*; if those competitors are especially numerous in the environment sampled; or if the supply of antiserum is limited. Tests found to be useful for eliminating non-*V. cholerae* organisms are arginine (or arginine glucose slant), lysine (or lysine iron agar), string, oxidase, and Kligler iron agar (KIA) or triple sugar iron agar (TSI) (see Table IV-2 in Chapter IV, "Isolation of *V. cholerae* from Fecal Specimens"). Biochemical tests should be selected according to their ability to rule out the greatest number of competitors and will vary with different types of specimens. There is no need to use two biochemicals that rule out the same organism. For example, if arginine is used, it is not advantageous to also screen with lysine since they generally rule out the same organisms.

The string and oxidase tests may be performed on fresh growth from the gelatin with 1% NaCl agar plate (T_1N_1 or other nonselective medium), offering information immediately. The string test is useful for ruling out non-*Vibrio* spp., particularly *Aeromonas*. Arginine and lysine can be used to rule out *Aeromonas* and certain *Vibrio* spp. Arginine is generally more helpful than lysine, but lysine may be used if arginine is not available. KIA or TSI will rule out *Pseudomonas* and certain *Enterobacteriaceae* spp. Oxidase can be used to eliminate non-*Vibrio* spp., particularly the *Enterobacteriaceae*. (For descriptions of these tests see Chapter VI, "Laboratory Identification of *V. cholerae*")

Caps on all tubes of biochemicals should be loosened before incubation. This is particularly important for KIA or TSI slants since, if the caps are too tight and anaerobic conditions exist, the characteristic reactions of *V. cholerae* may not be exhibited.

3. Slide agglutination

Suspect *V. cholerae* isolates should be tested by slide agglutination with polyvalent *V. cholerae* O1 antiserum. Isolates that agglutinate in polyvalent antiserum may be reported as presumptive *V. cholerae* O1 but should be confirmed with agglutination in monovalent Ogawa and Inaba

antisera. Since nontoxigenic *V. cholerae* O1 is frequently encountered in environmental specimens (particularly marine and estuarine), all *V. cholerae* O1 isolates from environmental or food specimens should be tested for cholera toxin production after their identification has been confirmed. Isolates that are serologically confirmed to be *V. cholerae* O1 may be further characterized for hemolysis, biochemical identification, antimicrobial sensitivities, biotype, or molecular subtype. (Refer to Chapter VI, "Laboratory Identification of *V. cholerae,*" for a description of methods for serologic, biochemical, biotype, hemolysis, and sensitivity tests. Chapter VII describes methods for detection of cholera toxin production.)

References

1. Barrett TJ, Blake PA, Morris GK, Puhr ND, Bradford HB, Wells JG. Use of Moore swabs for isolating *V. cholerae* from sewage. J Clin Microbiol 1980;11:385-8.
2. Spira WM, Ahmed QS. Gauze filtration and enrichment procedures for recovery of *Vibrio cholerae* from contaminated waters. Appl Environ Microbiol 1981;42:730-3.
3. U.S. Food and Drug Administration. Bacteriological Analytical Manual, 7th ed. Arlington, Virginia: Association of Official Analytical Chemists, 1992.
4. American Public Health Association, American Water Works Association, and the Water Pollution Control Federation. Standard Methods for the Examination of Water and Wastewater, 17th ed. Washington, D.C.: American Public Health Association, 1989.