IV. ISOLATION OF *VIBRIO CHOLERAE* FROM FECAL SPECIMENS

Although *V. cholerae* O1 will grow on a variety of commonly used agar media, isolation from fecal specimens is more easily accomplished with specialized media. Alkaline peptone water (APW) is recommended as an enrichment broth, and thiosulfate citrate bile salts sucrose (TCBS) agar is the selective agar medium of choice for isolating *V. cholerae* O1. In certain instances (for example, when the patient is in very early stages of illness and is passing liquid stool), it may not be necessary to enrich specimens or use selective plating media. However, enrichment broth and a selective plating medium should always be used with convalescent patients, suspected asymptomatic infections, environmental specimens, and whenever high numbers of competing organisms are likely to be present in the specimen.

A. Enrichment in Alkaline Peptone Water

Vibrio spp. grow very rapidly in APW, and at 6 to 8 hours will be present in greater numbers than non-*Vibrio* organisms. Enrichment in APW enhances the isolation of *V. cholerae* O1 when few organisms are present, as in specimens from convalescent patients and asymptomatic carriers.

A number of other broth media have been described for enrichment of *V. cholerae*. These include Monsur's enrichment medium which contains Trypticase, potassium tellurite, and sodium taurocholate (bile salts). A modification of APW, in which potassium tellurite is added in concentrations of 1:100,000 to 1:200,000, is sometimes used. An enrichment medium containing a selective agent may not offer any advantage over APW if it is used with a short incubation time (6 to 8 hours).

B. Selective Plating Media

1. Thiosulfate citrate bile salts sucrose agar

TCBS is the medium of choice for the isolation of *V. cholerae* and is widely used worldwide. TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective (Table IV-1). However, it has a relatively short shelf life once prepared (3 to 5 days) unless plates are carefully protected against drying. TCBS is subject to lot-to-lot and brand-to-brand variations in selectivity, and growth on this medium is not suitable for direct testing with *V. cholerae* O1 antisera.

TCBS agar is green when prepared. Overnight growth (18 to 24 hours) of *V. cholerae* will produce large (2 to 4 mm in diameter), slightly flattened, yellow colonies with opaque centers

Figure IV-1. Overnight colonies of *V. cholerae* on TCBS agar are large (2-4 mm) and yellow because of the fermentation of sucrose. They are characteristically round, smooth, glistening, and slightly flattened.

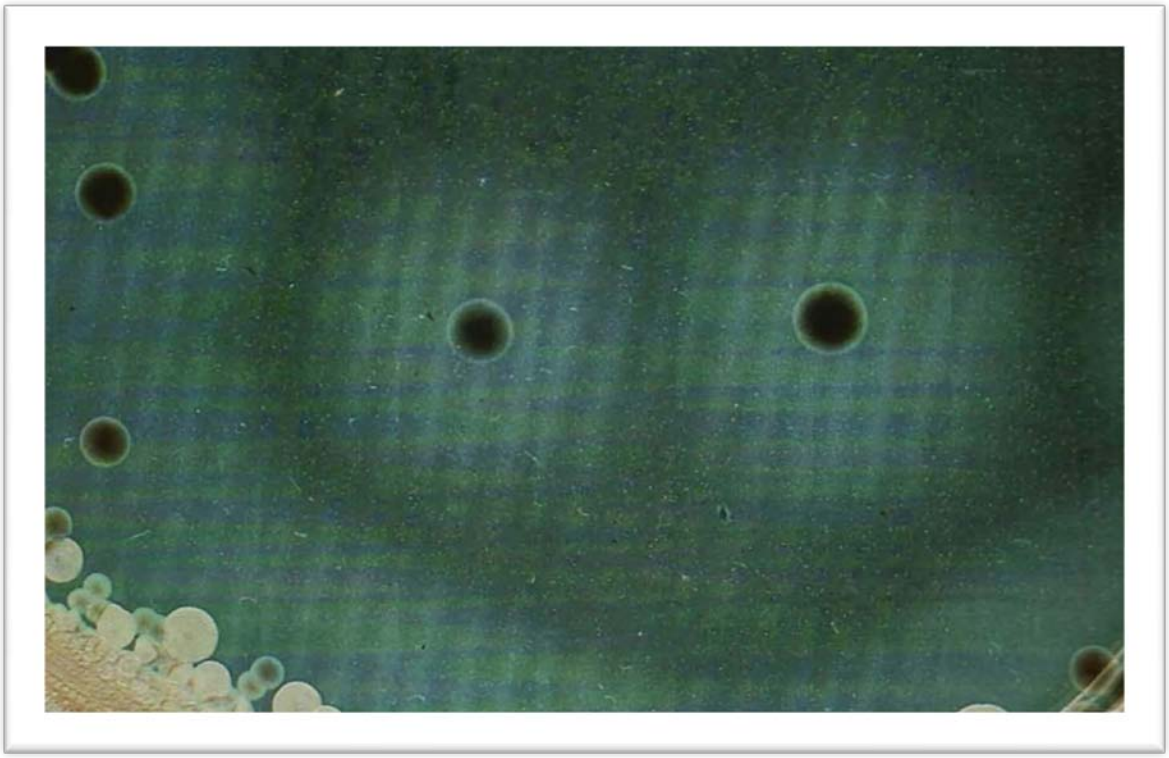

Figure IV-2. On TTGA medium, colonies of *V. cholerae* are grey, flattened, and are surrounded by a cloudy halo formed by the production of gelatinase.

and translucent peripheries (Figure IV-1). The yellow color is caused by the fermentation of sucrose in the medium. Sucrose nonfermenting organisms, such as *V. parahaemolyticus*, produce green to blue-green colonies. Suspicious colonies for further testing should be subcultured to a noninhibitory medium, such as gelatin agar, heart infusion agar (HIA), Kligler's iron agar (KIA), or triple sugar iron agar (TSI).

2. Taurocholate tellurite gelatin agar (TTGA or Monsur's agar)

TTGA is a selective and differential agar specifically designed for the isolation of *V. cholerae*. TTGA has a relatively long shelf life after preparation, and growth directly from the medium may be used for oxidase and agglutination tests (Table IV-1). The disadvantages of this medium are that it is not commercially available, and overnight colonies of *V. cholerae* on TTGA tend to be smaller (1 to 2 mm) than those from the TCBS agar. Potassium tellurite, which is added to the medium to increase selectivity, also varies in its quality, and each lot should be titrated to determine the optimal concentration to use in TTGA medium (see Chapter XI, "Preparation of Media and Reagents).

Overnight growth of *V. cholerae* on TTGA agar appears as small opaque colonies with slightly dark centers (Figure IV-2). After 24 hours, the centers of the colonies become darker, and eventually the entire colony becomes "gunmetal" grey in color. In addition to the dark coloration, which is due to the reduction of tellurite, there is also an opaque zone around colonies which resembles a halo. The halo effect, which is due to the production of the enzyme gelatinase, can be intensified by brief (15- to 30-minute) refrigeration of the plate. Because many members of the genus *Vibrio* have similar characteristics on TTGA, additional tests (antisera and/or biochemicals) are necessary to screen isolates from this medium.

Table IV-1. Selective plating media for *V. cholerae*

Medium	Colony morphology	Colony size	Commercially available	Autoclaved	Direct testing of growth off of plate[a]
TCBS	Yellow, shiny	2-3 mm	Yes	No	No
TTGA	Grey, flattened opaque zone around colony	1-2 mm	No	Yes	Yes
MacConkey[b]	Colorless to light pink	1-3 mm	Yes	Yes	No

Note: TCBS = thiosulfate citrate bile salts sucrose agar; TTGA = taurocholate tellurite gelatin agar.
[a] Direct testing for agglutination in antisera or oxidase reaction.
[b] Not all strains of *V. cholerae* O1 will grow on MacConkey agar.

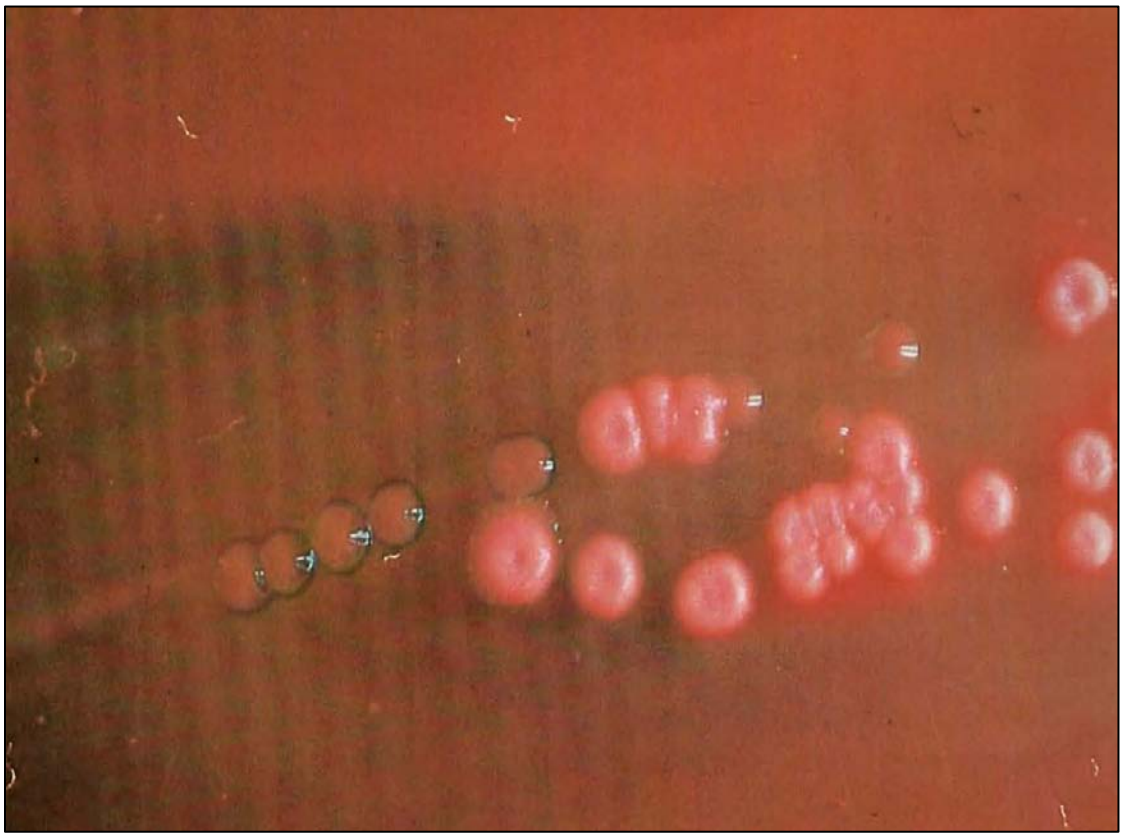

Figure IV-3. Overnight growth of *V. cholerae* on MacConkey agar appears as small (1- to 3 mm), translucent, colorless-to-light pink (lactose-negative) colonies.

3. MacConkey (MAC agar)

MAC is used widely to isolate members of the *Enterobacteriaceae* and will also support the growth of some but not all strains of *V. cholerae*. Overnight colonies of *V. cholerae* on MAC tend to be small to moderately sized (1 to 3 mm) and usually appear as lactose-negative or slightly pink, often resembling colonies of "late" or "slow" lactose-fermenting organisms (Table IV-1;Figure IV-3). Suspicious colonies should be subcultured to noninhibitory media for further testing.

C. Nonselective Plating Media

1. Gelatin agar (GA)

GA is a good nonselective growth medium for *V. cholerae*. Gelatinase production, a characteristic of vibrios in general, may be determined on GA and is indicated by the production of an opaque zone around colonies which resembles a halo. The halo effect can be intensified by brief (15- to 30- minute) refrigeration of the plate. Colonies of *V. cholerae* on GA are smooth, opaque, white, and 2-4 mm in diameter after overnight incubation at 35˚ to 37˚C. When viewed with obliquely transmitted light with 10X to 20X magnification, colonies may appear finely

granular and iridescent with a greenish-bronze sheen. Colonies from this medium may be tested directly for agglutination with antisera as well as oxidase and string test reagents. Salt-free GA may be used as a screening medium to rule out halophilic (salt-requiring) marine vibrios resembling *V. cholerae*, which are frequently isolated from seafood and environmental specimens. See Chapter XI, "Preparation of Media and Reagents," for special instructions for preparation of gelatin agar.

2. Meat extract agar (MEA, or alkaline nutrient agar)

MEA is similar to GA in its ability to support the growth of *V. cholerae*. However, unlike GA, MEA does not produce colonies with differential characteristics. Colonies of *V. cholerae* on NEA are 2 to 4 mm in diameter after overnight incubation and are smooth, opaque, and cream colored. When viewed with oblique light with 10X to 20X magnification, colonies may appear finely granular and iridescent with a greenish bronze sheen. The oxidase test, string test and screening with antisera may be performed directly with suspicious growth on MEA plates.

D. Isolation and Presumptive Identification

1. Direct inoculation of selective plating media from fecal specimens

Inoculate highly selective media (TCBS, TTGA) with a heavy inoculum from liquid stool, fecal suspension, or a rectal swab (Figure IV-4). With media of low selectivity (GA, MEA, blood agar), use a light inoculum. The inoculum should be streaked with a wire loop to give a large number of isolated colonies. It is not necessary to flame the loop between streaking different quadrants on the plate. Media of high selectivity require more cross streaking into previous quadrants than media with lower selectivity. After inoculation, incubate plates for 18 to 24 hours at 35˚ to 37˚C.

2. Inoculation of APW from fecal specimens

APW can be inoculated with liquid stool, fecal suspension, or a rectal swab (Figure IV-4). The stool inoculums should not exceed 10% of the volume of the broth. Incubate the tube with the cap loosened at 35˚ to 37˚C for 6 to 8 hours. After 6 to 8 hours' incubation, subcultures to TCS should be made with one to two loopfuls of APW from the surface and topmost portion of the broth, since vibrios preferentially grow in this area. Do not shake or mix the tube before subculturing. If the broth cannot be subcultured after 6 to 8 hours of incubation, subculture at 18 hours to a fresh tube of APW. This second tube should be subcultured to a solid medium after 6 to 8 hours incubation.

3. Isolation of suspicious colonies from plating media

Select several suspect colonies from the TCBS plate and use these to inoculate HIA slants or another nonselective medium. Do not use nutrient agar because it has no added salt and does not allow optimal growth of *V. cholerae*. Incubate at 35˚ to 37˚C.

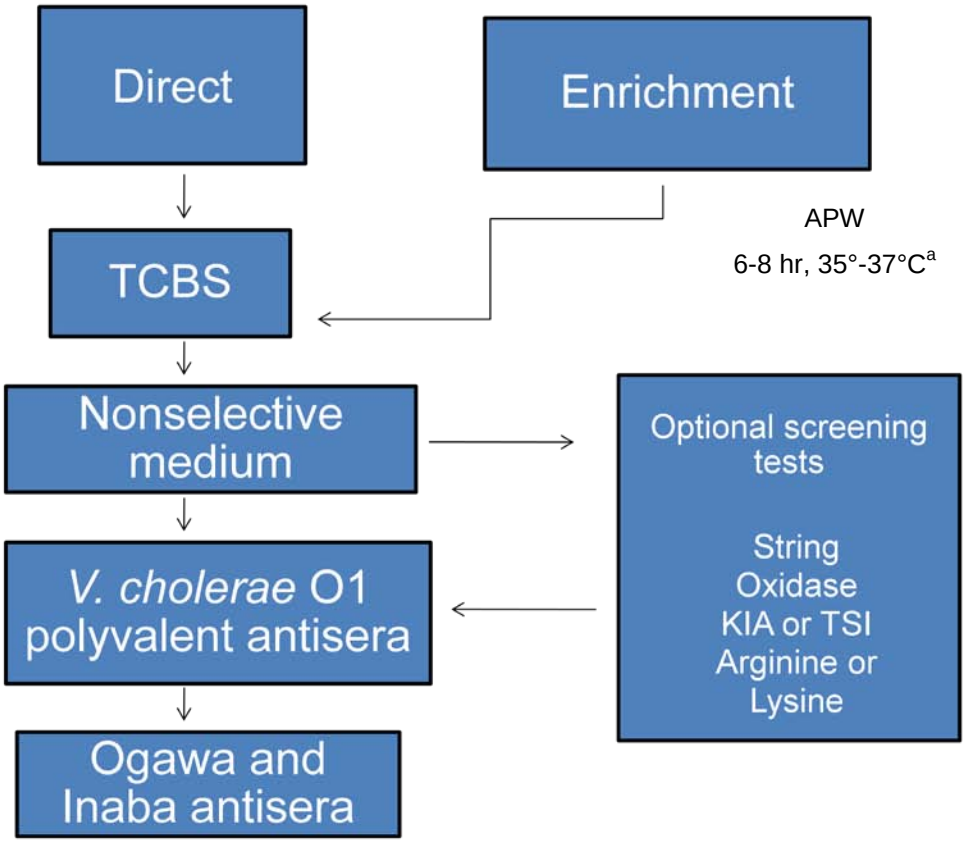

Figure IV-4. Procedure for recovery of *Vibrio cholerae* O1 from fecal specimens

[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.

4. Slide agglutination

Fresh growth of suspect *V. cholerae* on a nonselective agar medium may be tested in *V. cholerae* O1 polyvalent antiserum. Usually after 5 to 6 hours of incubation, growth on the surface of the slant is sufficient to perform slide serology with *V. cholera* O1 polyvalent antisera; if not, re-incubate overnight. Isolates that agglutinate in polyvalent antiserum to the O1 serogroup are presumptively identified as *V. cholerae* O1 (see Chapter VI, "Laboratory Identification of *V. cholerae*," for description of the slide agglutination method). Presumptive *V. cholerae* O1 may be confirmed with agglutination in either monovalent Ogawa or Inaba antisera, but confirmation may not be necessary for all isolates, particularly when the supply of antisera is limited. The minimum identification of *V. cholerae* O1 requires only serologic confirmation of the presence of O1 serotype antigens with suspect isolates. However, a more complete characterization of the organism is sometimes necessary and may include toxin or hemolysis testing, as well as determination of antimicrobial sensitivity, biochemical identification, biotype, or molecular subtype. These types of tests should be performed only on selected isolates, such as those recovered early in an outbreak or during surveillance in areas threatened by epidemic cholera. Only isolates that are serologically confirmed to be *V. cholerae* O1 should be further

characterized. (See Chapter II for discussion of when further characterization of isolates is necessary, and Chapters VI, VII, IX for a description of these tests.)

5. Biochemical screening tests

Generally for suspect *V. cholerae* isolates from fecal specimens, screening with biochemical tests is not necessary since slide serology with polyvalent O1 antisera is sufficient for a presumptive identification. However, if the supply of antisera is limited, the string and oxidase tests or various other biochemical tests may be useful for additional screening of isolates before testing with antisera. No single screening procedure for *V. cholerae* O1 is ideal for all laboratories and every sample. The laboratorian should select a screening procedure on the basis of available resources (for example, the availability of antisera), the types and numbers of competing organisms likely to be present in the specimens being cultured, and the ability of the selective plating medium to inhibit the growth of those competing organisms.

The string test, using fresh growth from non selective agar, is useful for ruling out non-*Vibrio* spp., particularly *Aeromonas* spp. (Table IV-2). Oxidase can also be used to screen out non-*Vibrio* spp. such *Enterobacteriaceae*. Kligler's iron agar (KIA) or triple sugar iron (TSI) agar rules out *Pseudomonas* and certain *Enterobacteriaceae* spp. Arginine is generally more helpful than lysine for screening out *Aeromonas* and certain *Vibrio* spp., but either medium may be used. There is no need to use two biochemicals that rule out the same organism. For example, if arginine is used, it is usually not advantageous to also screen with lysine since they generally rule out the same species. 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 reaction of *V. cholerae* may not be exhibited. (See Chapter VI, "Laboratory Identification of *V. cholerae*," for description of these biochemical tests.)

Table IV-2. Differential characteristics of selected members of *Vibrionaceae* and *Enterobacteriaceae*.

TEST	ORGANISM						
	Vibrio cholerae	*Vibrio mimicus*	*Halophilic vibrios*	*Aeromonas hydrophila*	*Aeromonas veronii*	*Plesiomonas shigelloides*	*Enterobacteriaceae*
KIA	K/A	K/A	V	V	K/AG	K/A	V
TSI	A/A	K/A	V	V	A/AG	K/A	V
String	+	+	+[a]	-	-	-	-
Oxidase	+	+	+	+	+	+	-
Gas from glucose	-	-	-[b]	+	+	-	V
Sucrose	+	-	V	V	+	-	V
Lysine	+	+	V	V	+	+	V
Arginine	-	-	V	+	-	+	V
Ornithine	+	+	V	-	+	+	V
VP	V	-	V	V	+	-	V
Growth in 0% NaCl[c]	+	+	-	+	+	+	+
Growth in 1% NaCl[c]	+	+	+	+	+	+	+

Note: V=variable reaction
[a] *V. parahaemolyticus*, *V. cincinnatiensis*, and *V. damsel* give variable reactions.
[b] *V. furnissii* and *V. damsel* are variable for gas from glucose.
[c] Nutrient broth base (Difco Laboratories, Detroit, MI)

E. Rapid Diagnostic Methods

Prompt laboratory diagnosis of cholera is often advantageous for monitoring the spread of the disease and rapidly instituting control measures. Several rapid methods have been developed and used to detect *V*. *cholerae* O1 directly from stools of acutely ill patients or from enrichment broth. In certain situations, these rapid methods may be practical, but they provide only a preliminary diagnosis. Despite their advantages of speed and (sometimes) simpler requirements for reagents and equipment, for the most part, rapid diagnostic methods cannot entirely replace traditional culture methods. Traditional techniques must be relied on when an isolate is required for further tests, such as assays for cholera toxin production, antimicrobial sensitivity, hemolysis, biotype, or molecular subtyping. Rapid methods may be most useful in field situations where beside diagnosis is required to monitor the course of an outbreak.

1. Dark-field and phase-contrast methods

Dark field and phase-contrast microscopy have been used for screening fecal specimens for the presence of *V. cholerae*. With these techniques, liquid stools are microscopically examined for the presence of organisms with typical darting ("shooting star") motility. The observation of characteristic motility can only be considered a screening test, and the diagnostic accuracy is not high when compared with standard culture techniques.

The motility detection method is improved with the use of specific *V. cholerae* O1 antisera. In this method, stools or enrichment broths are examine with and without antisera added. If the addition of polyvalent antisera against *V. cholerae* O1 results in the cessation of motility, as observed by either dark-field or phase-contrast microscopy, the test is considered positive.

The diluents for the antisera and stool must be selected carefully to avoid nonspecific inhibition of motility (for example certain preservatives such as sodium azide or merthiolate used in antisera). Distilled water is not a suitable diluents for stool specimens because It can extinguish the motility of *V. cholerae*. Other disadvantages include the requirements for a dark-field or phase-contrast microscope and a technician experienced in this technique. Despite these disadvantages, this technique has been widely used.

Dark-field procedure

Freshly collected liquid ("rice-water") stool is the specimen of choice for the dark-field procedure. If stool is liquid but not rice-water in nature, it should be dilute 1:1 with saline before direct testing. If freshly collected stool is negative, perform a 6- to 8-hour APW enrichment and retest the enrichment broth. Antisera may be saved by first determining that the stool or enriched specimen is motile before preparing the anti-serum-containing specimen for examination. A known motile strain of *V. cholerae* O1 should be used as a positive control.

The following materials are needed:

- Microscope with dark-field condenser (if an oil immersion lens is used [100X], the objective must have an iris diaphragm)
- Polyvalent *V. cholerae* O1 antiserum

- Clean microscope slides (standard size) and cover slips
- Sterile physiological saline or phosphate-buffered saline, pH 7.0 to 8.0

To perform the test, place a drop of polyvalent *V. cholerae* O1 antiserum near the end of a clean microscope slide. Next, mix a drop of freshly collected rice-water stool or a drop of a 6- to 8-hour APW enrichment broth culture with the antiserum, and place another drop of stool or APW at the opposite end of the slide. Place cover slips over the drops at each end of the slide. Using dark-field, examine the drop containing the stool or enrichment broth for "shooting star" motility by using the 40X objective. If this type of motility is detected, examine the antiserum-containing mixture. If the motile organism is *V. cholerae* O1, the motility will be completely extinguished. If the organisms under both cover slips are non-motile or if there is no difference between the motility of either mixture, the organism is not *V. cholerae* O1.

2. Immunofluorescence

Immunofluorescence techniques using antisera conjugated to fluorescein isothiocyanate have been used to visualize *V. cholerae* O1 cells in a variety of specimen types. Despite the utility of immunofluorescence methods, they are not widely used as primary diagnostic tools because the requirements for expensive equipment, high quality immunologic reagents, and trained technicians.

3. Latex agglutination

A commercially manufactured slide agglutination kit (Denka Seiken, Tokyo, Japan), developed for serotyping *V. cholerae* O1 isolates, has been used to detect the organism directly in fecal specimens. The kit uses latex particles coated with monoclonal antibodies directed against the A, B, and C antigens of *V. cholerae* O1. During an investigation of an epidemic of cholera, the kit was evaluated for its ability to confirm the diagnosis of cholera at bedside using rectal swabs. The latex agglutination test detected 63% of culture-positive patients from rectal swabs, but gave false positive results for 12% of culture-negative patients. The sensitivity and specificity of this test with liquid stool specimens have not been determined.

4. Coagglutination

In the coagglutination method, antibodies against *V. cholerae* O1 are bound to the surface of *Staphylococcus aureus* (Cowan 1) cells while retaining their binding capacity and specificity. In a *positive* reaction, staphylococcal cells are bound together in a lattice-like arrangement caused by the formation of linkages between them created by the binding of the antibody on their surface to the *V. cholerae* bacterial cells.

Problems using this technique have been attributed to substances in stool which nonspecifically inhibit agglutination and lattice formation of staphylococcal cells. Recently, a commercially prepared monoclonal anti-body-based coagglutination test (CholeraScreen, New Horizons Diagnostics, Columbia, MD) has been marketed and appears to have overcome these

obstacles. Reports of evaluations of the product with culture collections in the United States and with clinical specimens in Guatemala and Bangladesh have been encouraging.

5. Other rapid isolation/identification techniques

Other techniques for the rapid diagnosis of *V. cholerae* O1 include methods based on the multiplication of *bacteriophage*, addition of antisera to growth medium to precipitate *V. cholerae* cells in broth, and the use of antibody-coated magnetic beads to physically aggregate *V. cholerae* bacterial cells.

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