VII. DETECTION OF CHOLERA TOXIN

A. MODE OF ACTION OF CHOLERA TOXIN

The production of cholera toxin (CT) is an essential virulence property of epidemic strains of *Vibrio cholerae* O1. Each CT molecule is composed of five B (binding) subunits and one A (active) subunit. The B subunits bind to G_{M1} ganglioside receptors on epithelial cells of the intestinal mucosa. After attachment, cleavage occurs between the A subunit and the A2 component, facilitating entry of the A1 component into the cell. The A1 component stimulates the production of the enzyme adenylate cyclase, which is responsible for the production of cyclic AMP (cAMP). Increased intracellular levels of cAMP result in a disruption of the active transport of electrolytes across the cell membrane, which hinders fluid absorption and leads to fluid secretion into the small intestine. When the volume of the fluid entering the colon from the small intestine is greater than its reabsorptive capability, diarrhea occurs. CT is very similar to *Escherichia coli* heat-labile enterotoxin (LT), both antigenically and in mechanism of action; therefore, most of the toxin assays for detection of CT are also applicable to LT, and vice-versa.

B. INDICATIONS FOR TESTING FOR CT PRODUCTION

The value of routine CT testing in a diagnostic laboratory varies with the epidemiology of cholera in a specific country or community. During an outbreak of cholera, the isolation of *V. cholerae* possessing the O1 antigen from symptomatic patients correlates well with toxin production and virulence, and there is no need to routinely test isolates for CT. This is also true in most endemic cholera situations with a reasonably high frequency of disease. However, in endemic disease setting where the incidence of cholera is low or in the early stages of an outbreak, most *V. cholerae* O1 strains isolated from diarrheal stool should be tested for toxin. (See Chapter II, “The Role of the Public Health Laboratory,” for a discussion of when it is necessary to test isolates for cholera toxin production.) Since nontoxigenic *V. cholerae* O1 strains are occasionally encountered in environmental specimens (particularly marine and estuarine waters), all food or environmental *V. cholerae* O1 isolates should be tested for cholera toxin production after the identification has been confirmed.

Before testing for toxin, the identity foe isolates as *V. cholerae* O1 should be confirmed. Non-O1 *V. cholerae* strains may produce CT or other toxins such as heat-stable enterotoxin or Shiga-like toxin, but these strains are very rare and have not been associated with epidemic disease. Therefore, there seems to be little public health benefit in testing sporadic isolates of non-O1 *V. cholerae* for CT or other possible toxins.

Although both clinical and public health needs warrant at least some CT assays, those needs are usually most efficiently and economically met at the reference laboratory level. Laboratories should select the most appropriate method for their needs and capabilities.

C. Historical Overview of CT Assay Methods

There are several approaches to assaying for cholera toxin, including tests for toxin activity, toxin antigens, and toxin coding genes. The selection of a specific assay depends on the training, experience, and facilities available to the laboratory. Table VII-1 summarizes important characteristics of some of the more common assays used for detecting cholera toxin.

1. BIOASSAYS

Animal methods

In the early 1950s, investigators discovered that injection of enterotoxin preparations into ligated segments of intestine (ileal loops) of rabbits (and later other animals including pigs, dogs, and calves) caused accumulation of fluid. This discovery resulted in the development of the first cholera enterotoxin assay, the adult rabbit ileal loop, which before the 1970s was the most widely used assay for CT. This model has been used extensively to study the mechanisms of action of CT, *E. coli* LT, and other enterotoxins. After exteriorization and ligation of the rabbit's small intestine, a cell-free supernatant is injected into each ileal loop and the abdomen is closed for 18 hours. The rabbit then euthanized, the intestine removed, and the loops measure and weighed to determine the amount of fluid accumulation stimulated by the toxin. Results are expressed as volume of fluid per length of intestinal loop. This test is not only excessively stressful for the animals but is also time-consuming, cumbersome, and difficult to standardize. The test is relatively expensive in terms of numbers of animals required, since only about 8 to 14 supernatants may be tested per animal, not including positive and negative controls; also, each set of supernatants must be done in duplicate animals and the orientation of supernatants must be reversed from one animal to the next.

The infant rabbit infection model was developed in 1955 and can be used for assay of both *V. cholerae* and *E. coli* enterotoxins. Seven-day-old infant rabbits are infected with the test organism by gastric intubation or by direct intragastric or intralumenal (small intestine) injection. The animals are observed for watery diarrhea and eventual death due to dehydration. Alternatively, after a 7-hour incubation period, the animals are killed; the intestines are removed and the fluid volume is measured per centimeter of intestine. The drawbacks to the infant rabbit method are the variability of results and the expense of using one animal for every isolate to be tested.

The rabbit skin test, or vascular permeability factor assay, has been used to detect either CT or LT activity, with the specificity of the assay determined by the neutralization of activity by a standardized amount of antisera against CT. A cell-free culture supernatant of *V. cholerae* or *E. coli* and dilutions of antisera are injected intradermally into the shaved back of a young adult rabbit. Approximately 30 to 40 supernatants may be tested per rabbit. This is followed 18 hours later by an intravenous injection of Evans blue dye. CT-mediated increased capillary permeability leads to perfusion of the dye in the skin (bluing reaction), with localized induration at the injection sites. The area of "bluing" is measured relative to a negative control. This procedure permits the assay of 30 to 40 cultures per rabbit and is thus more economical in terms of numbers of animals required than other animal systems such as the infant rabbit and ileal loop assays.

TABLE VII-1. Commonly used methods for detection of cholera toxin

Assay	Sensitivity (per ml)	Type of assay	Specific target of assay	Sample tested
Rabbit ilial loop	30 ng	Bioassay	Stimulation of fluid accumulation	Culture supernatant
Infant rabbit assay	250-500 ng	Bioassay	Stimulation of fluid accumulation	Broth culture or supernatant
Rabbit skin test	0.1-3.5 ng	Bioassay	Permeability factor	Culture supernatant
Y1 mouse adrenal cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
Chinese hamster ovary cells	10 pg	Bioassay	Accumulation of cAMP	Culture supernatant
G_{M1}-ELISA	10 pg	Immune	B subunit[b]	Culture supernatant
Coagglutination	50 ng[a]	Immune	B subunit[b]	Culture lysates
Reverse passive latex agglutination	1-2 ng	Immune	B subunit[b]	Culture supernatant
DNA probe	Detects ctx gene	Genetic	ctx gene	DNA (colony blot)
PCR	Detects ctx gene	Genetic	ctx gene	DNA (crude cell lysate)

[a] Sensitivity for detection of cholera toxin using antiserum to *E. coli* heat-labile enterotoxin.

[b] B-subunit of cholera toxin molecule.

Tissue culture methods

Tissue culture methods are very sensitive and reproducible and have been used extensively to assay for toxin production. The action of toxin on cells in culture has allowed successful investigation of the molecular basis of pathogenicity. In addition, these assay systems can be used to detect CT-neutralizing antibody. However, tissue culture methods require skilled workers and special reagents and equipment. These are techniques best suited for use in laboratories with existing tissue culture facilities.

The Y1 mouse adrenal (Y1) and Chinese hamster ovary cell cultures have been the standard tests for detecting CT and LT, although other cell lines, including Vero monkey kidney cells, are also sensitive. The CT or LT, if present in the bacterial culture supernatants added to the cells, stimulates the production of adenylate cyclase, which elevates the intracellular concentration of cAMP. The increased amounts of cAMP result in a morphologic response that can be seen under the microscope (CHO cells elongate and Y1 cells become rounded). Positive reactions may be confirmed by neutralization of the toxic effects in cell culture with antiserum to CT (or LT) or with ganglioside G_{M1}, which is the receptor site for CT and LT in host cell membranes and thus binds either of these toxins. For the toxin assay, a suspension of Y-1 cells is dispensed into 96-well microtiter plates. Usually, one 75-cm^2 flask of Y-1 cells is sufficient to seed up to 25 microtiter plates. Each microtiter plate may be used to test as many as 60 supernatants (30 supernatants if performed in duplicate) if the outside wells of the plate are not used.

2. Immunoassays

ELISA

CT is highly immunogenic for humans and laboratory animals. As a result, many immunologic techniques have been developed for detecting CT. The discovery the G_{M1} ganglioside is the natural receptor for CT and LT and its subsequent purification led to the development of ganglioside-capture enzyme-linked immunosorbent assay (G_{M1}-ELISA, culture supernatants are added to microtiter plate wells coated with G_M1 ganglioside. Toxin bound to the G_{M1} receptors is then detected by adding antiserum to CT, followed by enzyme-conjugated antiglobulin antibody. Instead of using G_{M1} to coat the plate, a second antibody may be used to bind CT. If 96-well microtiter plates are used for the toxin assay, 30 supernatants may be tested in duplicate per microtiter plate (the outside wells are not usually used to test supernatants).

Coagglutination

Because many strains of *Staphylococcus aureus* have an outer coat of protein A that can directly combine with IgG, serologic reagents have been prepared in which a specific IgG antibody is absorbed onto the surface of staphylococcal cells for use in an agglutination reaction. The test reagent is relatively inexpensive to prepare but requires a specific anti-CT (or anti-LT) antibody. Coagglutination for *E. coli* LT is rapid and simple to perform and requires little specialized laboratory equipment or training of laboratory personnel; however, this test has never been used to detect CT from culture supernatants or lysates of *V. cholerae* O1.

Latex agglutination

The latex agglutination test uses specific anti-CT or anti-LT bound to latex particles. The latex agglutination technique requires high quality antiserum or purified antibodies, a suitable latex preparation, and readily available laboratory supplies. A commercial version of this test is available in kit form and is described later in this chapter.

3. DNA-based Assays

DNA Probes

Molecular tests that identify pathogenic microorganisms based on DNA sequences unique to the pathogen have many applications in diagnostic and public health microbiology. Specific DNA sequences within the cholera toxin gene(s) have been used as probes to detect homologous DNA sequences in toxigenic *V. cholerae* isolates. In practice, a variety of DNA molecules can be used as probes, including cloned DNA, restriction fragments of cloned DNA, polymerase chain reaction-generated DNA amplicons, and oligonucleotides. The probe is first labeled with an easily detectable molecule, such as a radioisotope, an enzyme, or a ligand, and is then hybridized to DNA from the test organism. The probe hybridizes only to those organisms containing homologous sequences. Nonhybridized probe is washed away, and the remaining hybridized probe is detected in a specific assay.

Polymerase chain reaction (PCR)

A second approach to molecular diagnostic tests that are based on specific DNA sequences is PCR. In PCR, the enzyme DNA polymerase is used to synthesize or amplify multiple copies of a specific DNA sequence (amplicon), which can then be detected on an agarose gel or with DNA probes. The DNA amplicon is defined by the location of two short, specific DNA oligonucleotides that bracket the sequence of interest and are used as primers by the DNA polymerase. Like DNA probes, the target for a PCR test is a virulence gene or DNA sequence that is unique to a pathogen. The toxigenicity of a *V. cholerae* isolate can be tested using PCR and primers that specifically amplify only CT genes. PCR has the advantages of being a very rapid technique that does not require pure cultures or even viable organisms. Some PCR tests can amplify DNA segments directly from stool, food, or environmental specimens; thus, the presence of an organism in a specimen can be determined without culturing the organism. Also, PCR has applications in other molecular techniques. It can be used to rapidly produce labeled DNA probes for restriction fragment length polymorphism (RFLP) analysis and colony blots.

The sensitivity and specificity of DNA-based assays provide an advantage over conventional methods. The use of DNA probes or PCR avoids the difficulties encountered with *V. cholerae* strains that do not express CT at detectable levels but possess *ctx* genes. The use of nonradioactive DNA labels, such as biotin and digoxigenin, has eliminated the technical problems associated with radioisotopic labels. While DNA-based diagnostic tests require specific training in molecular methods and more expensive reagents, the advantages of simplicity, sensitivity, safety, and stability have made DNA-based techniques invaluable to research and clinical laboratories.

D. Production of CT for Laboratory Assays

Optimal growth conditions for *V. cholerae* do not always correspond with optimal CT production in vitro. The optimal conditions for CT production vary according to the medium used. For this reason, specialized media have been developed for production of CT. In general, an incubation temperature of 30˚C has been found to be superior to 37˚C for toxin production with *V. cholerae* of either biotype. With El Tor strains, using Craig's medium, the best combination of time and

temperature is 30˚C for 48 hours without aeration. For classical biotype strains, 30˚C with vigorous shaking for 48 hours provides the best yield of toxin. If 37˚C incubation temperatures must be used, AKI medium is recommended. Although AKI allows the production of CT at 37˚C, it has the disadvantage of having a short shelf life and must be prepared weekly. *V. cholerae* El Tor grown in AKI medium should be incubated at 37˚C for 20 hours without shaking. AKI medium has not been evaluated for toxin production by classical biotype strains.

CT is actively exported by *V. cholerae* into the culture medium, unlike *E. coli* LT, which is usually found in the periplasmic space. Antimicrobial agents such as polymyxin B or lincomycin, which enhance LT accumulation in media, have no effect on the release of CT. It is therefore unnecessary to use either drug in media for production of CT.

Most cholera toxin assays, both immunologic methods and bioassays, test for CT in culture supernatants. Assays for toxin activity require intact CT, whereas some antigenic assays only detect the B subunit of CT and do not require complete toxin. The growth conditions for CT production described below are recommended for optimal expression of complete (active) CT, as required by rabbit ileal loop, rabbit skin test, and tissue culture assays.

Production of cholera toxin

1) Streak cultures to be tested on a nonselective slant (such as heart infusion agar). Incubate overnight at 35˚C to 37˚C. Include four control strains (two positive and two negative).
2) Inoculate strains into 5 ml of Craig's medium in 16 X 125-mm screw cap tubes. Keeping caps loose, incubate at 30˚C for 48 hours without shaking. [Note: cultures may be tested after only 24 hours' incubation, but 48 hours is optimal for CT production.]
3) Centrifuge to sediment the bacterial cells and draw off supernatant with a Pasteur pipette. Store supernatant at 4˚C until ready to test. Freeze at -70˚C if the supernatant is to be stored for longer than 7 days.

E. Y-1 Assay for CT

The Y-1 clone of mouse adrenal tumor cells is sensitive to concentrations of CT as low as 10 picograms per milliliter. However, *Vibrio* and other bacteria genera may produce extracellular heat-labile products that cause nonspecific rounding, which may be misinterpreted as being caused by CT. It is preferable to neutralize positive or doubtful reactions with specific antisera to the toxin or other specific binding substances such as G_{M1} ganglioside, the natural receptor for the toxin in cell membranes.

Materials

- CO_2 incubator set at 37˚C, 5.0% CO_2
- Laminar flow hood
- Inverted phase-contrast microscope
- Sterile conical centrifuge tubes
- Centrifuge
- Sterile tissue culture flasks (75 cm^2 growth area)
- Sterile, flat-bottom, 96-well microtiter plates for tissue culture

- Ham's F-10 Nutrient Mixture (GIBCO Laboratories, Grand Island, N.Y.) with 15% horse serum, 2.5% fetal calf serum, and gentamicin (10 µ/ml)
- 0.2% trypsin

Weekly procedures for tissue culture assay and maintenance

Y-1 adrenal cells are maintained in mono layer culture in Ham's F-10 nutrient mixture. Routinely, tissue culture flasks with a 75-cm^2 growth area are filled with 25 ml of medium and incubated at 37C in a humidified 5% CO_2 atmosphere. All cell manipulations are done in a laminar flow hood, and cells are examined by using an inverted phase-contrast microscope.

1) Pour off the F-10 medium from the confluent monolayer (1 week of growth is usually required). Wash the cell monolayer with 5 ml sterile phosphate-buffered saline (PBS) and pour off.

2) Add 1.5 ml of 0.2% trypsin to the flask, and leave the trypsin-covered monolayer at room temperature until cells begin to loosen from the plastic surface (5 to 10 minutes).

3) Add 5 ml of F-10 medium to the flask to neutralize the trypsin. (The Ham's F-10 is stored at 4˚C, and should be brought to 37˚C in a water bath before all medium changes and cell subcultures). If any monolayer remains, scrape it from the flask surface with a sterile rubber scraper.

4) Transfer the suspended cells (approximately 6.5 ml) to a sterile conical centrifuge tube and centrifuge at 500 to 1000 *x g* for 5 minutes.

5) Suction off the supernatant leaving a sediment of Y-1 adrenal cells in the centrifuge tube. Resuspend the cells in 5 ml of fresh F-10 medium with a Pasteur pipette.

6) Using a Pasteur pipette, dispense 6 drops of the cell suspension into each flask, to which has been added 25 ml fresh F-10 medium. As a general rule, duplicate flasks are seeded and carried.

7) For the toxin assay, which is run in flat-bottom, 96-well microtiter plates, make a 1:50 to 1:100 dilution of the cell suspension. Dispense the suspension, approximately 0.15 ml per well. Therefore, one flask usually can seed 12 to 25 plates. Stack the plates and cover the top plate to prevent contamination and evaporation.

8) Place flasks (with loose caps) and/or microtiter plates in the CO_2 incubator.

Day 2

Check flasks and/or wells under the microscope for cell growth

Day 3 (late afternoon)

Using a Pasteur Pipette inoculate each well of the microtiter plate containing a monolayer of Y-1 adrenal cells, with 2 drops (50 µl) of the toxin supernatants (including positive and negative controls). See Section D for methods for growing *V. cholerae* cultures for CT production.) Be sure the microtiter plates and appropriate record sheets are coded before the actual transfer. Restack microtiter plates, and incubate in CO_2 at 37˚C overnight.

Day 4 (morning)

Read assay results. Examine wells at 100x or 200x magnification, using an inverted stage phase microscope. Compare test wells with positive control wells. CT and LT cause rounding of Y-1 adrenal cells (Figure VII-1). For the CT or LT assay, a positive well contains more than 10% rounded Y-1 cells. Occasionally, cytotoxic activity in the supernatant will result in dead, lysed, or detached cells, which may mask the rounding effect of CT. If this occurs, dilution of the supernatant (in an attempt to dilute out the cytotoxin) may allow the rounded cells to be visualized.

Method for neutralization of cholera enterotoxin

Refer to Section D in this chapter for methods for growing *V. cholerae* cultures for CT production. Dilute test supernatants 1:4 by using PBS (pH 7.2) containing 0.1% gelatin (PBS/G). If available, pure cholera toxin preparation should be used as a positive

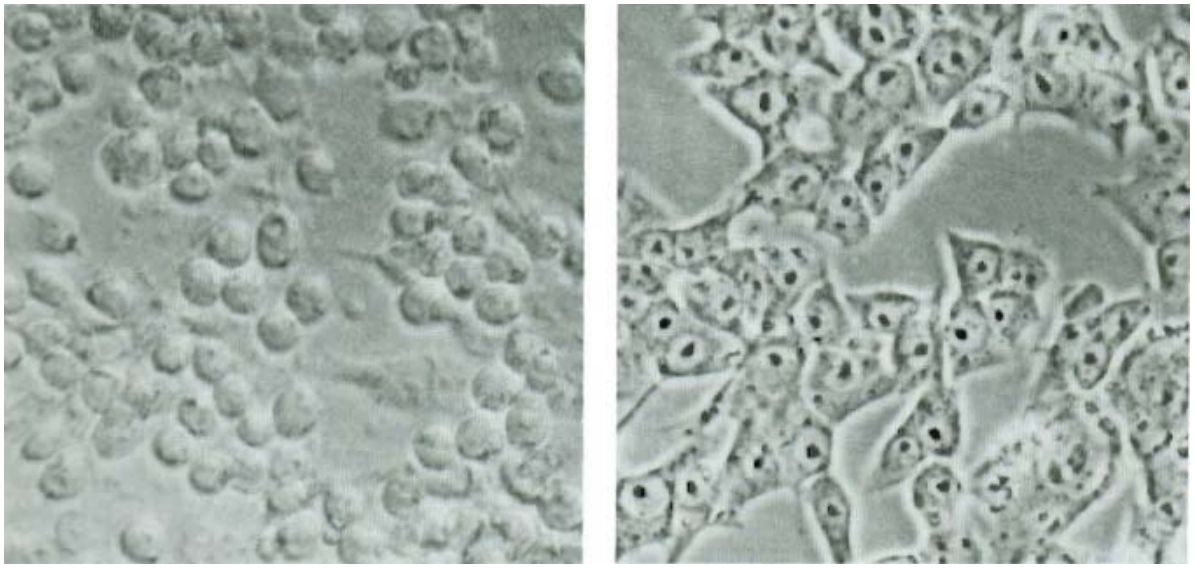

Figure VII-1. The panel on the left shows typical rounding of Y-1 mouse adrenal cells caused by the presence of cholera toxin. Normal Y-1 cells are shown in the panel on the right.

control. Use high-titer antiserum to cholera toxin in this assay (at CDC the anti-CT antiserum used for the G_{M1}-ELISA is also used for neutralization in Y1 cells). Dilute the antiserum in PBS/G. Prepare twofold dilutions of the anti-CT antiserum in PBS/G starting at 1:10 and ending at 1:10, 240. Mix equal volumes of the antiserum dilutions with undiluted supernatant and the 1:4 dilution of the supernatant. Repeat the procedure for the rest of the supernatants and purified CT. Incubate the supernatant with antiserum mixtures in a water bath at 37˚C. After 1 hour, transfer 50 µl of each supernatant with antiserum mixture in duplicate to the Y1 monolayer in the microtiter plate. Add non-neutralized supernatant for each test culture to one well for use as a control. Incubate cells overnight, and read to determine the highest dilution of anti-CT that neutralizes the rounding effect of the CT.

F. G_{M1}-ELISA FOR CT

The G_{M1}-ELISA is a sensitive immunoassay for the detection of CT. It uses an enzyme-labeled immunoglobulin, which is quantified by causing its activity on a specific substrate. Microtiter wells are coated with ganglioside G_{M1}, the natural receptor for CT (Figure VII-2). Alternatively, microtiter wells may be coated with antibody to CT, but the coating antibody must be prepared in a different animal species than the second anti-CT antibody used in the assay. Culture supernatants are then added to the coated wells. CT molecules in the supernatant bind to the G_{M1} in the well and react with the anti-CT antibody. Antibody to the specific animal species'

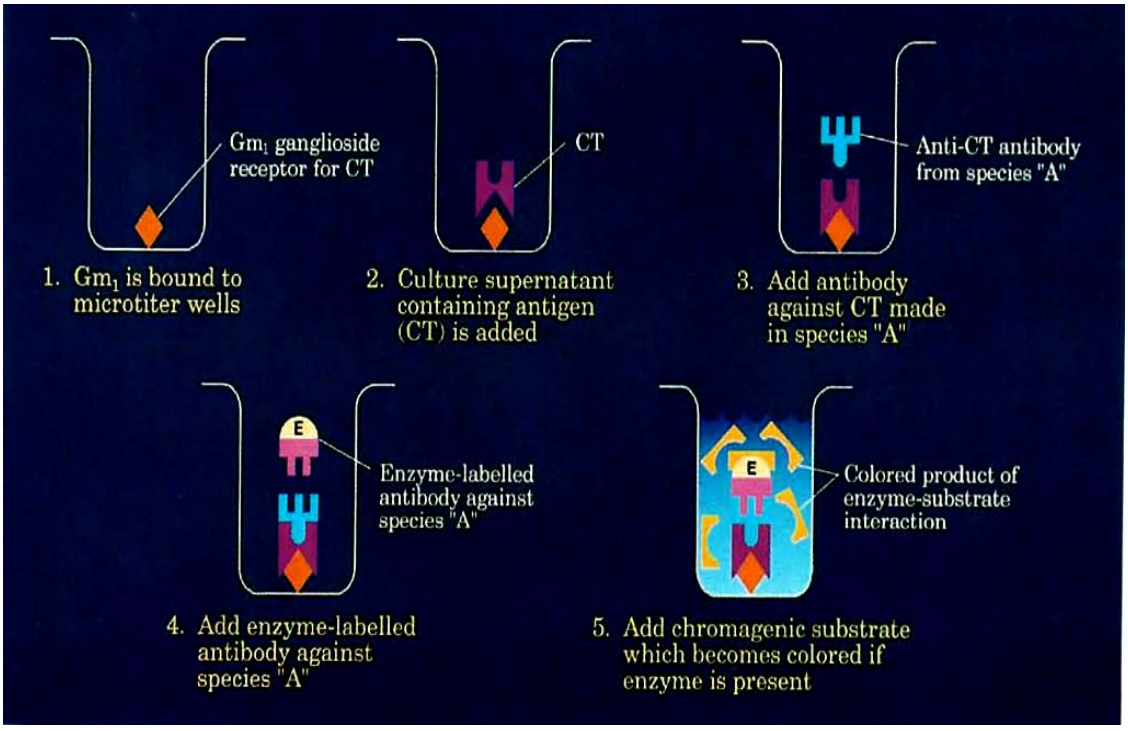

Figure VII-2. Schematic diagram of the G_{M1}-ELISA for the detection of cholera toxin.

immunoglobulin conjugated to an enzyme (alkaline phosphatase or horseradish peroxidase) is added and reacts with the well-bound anti-CT antibody. Finally, the enzyme substrate is added, and the bound enzyme degrades the substrate, forming a colored product that indicates the presence of CT, LT, or other immunologically related molecules. These reactions can be read spectrophotometrically or visually. A summary of the ELISA method is presented in Table VII-2. All materials commercial sources, including antiserum to CT are individually available from commercial sources, including antiserum to CT.

Equipment

- Polystyrene or polyvinyl microtiter plates, flat-bottom or round-bottom
- Micropipettes and multichannel pipettes
- ELISA plate reader

Reagents

(See Chapter XI, "Preparation of Media and Reagents," for instructions for preparing the following reagents.)

- G_{M1} ganglioside (Sigma Chemical Co., St. Louis, Mo.)
- PBS with and without 0.05% Tween 20 and bovine serum albumin
- Goat antibody to CT_b (Calbiochem Corp., La Jolla, Calif.) [Note: As an alternative to goat anti-CT antibody, antibody produced in another animal species or monoclonal anti-CT antibody can be used.]
- Anti-goat (or whatever species was used to produce the anti-CT antibody) globulin labeled with alkaline phosphatase or horseradish peroxidase (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.).
- *p*-nitrophenyl phosphate (Sigma Chemical Co.) dissolved in diethanolamine buffer [Note: Several substrates are available for horseradish peroxidase, each with specific requirements for preparation.]

The optimal dilution of each specific reagent preparation should be determined by titration. A sample titration procedure is described in this section.

Table VII-2. ELISA for detection of cholera toxin

Step	Reagent	Diluent	Concentration	Volume/well	Incubation time
Coating	GM1	PBS	2µg/ml[a]	100µl	Overnight
Blocking	BSA	PBS	1%	150µl	30 minutes
Sample	Culture supernatant	None	Undiluted	100µl	60 minutes
Blocking	BSA	PBS	1%	150µl	30 minutes
Antibody	Antibody to CT prepared in goat	PBS with 0.1% BSA	1:2000[a]	100µl	60 minutes
Conjugate	Alkaline phosphatase-labeled anti-goat	PBS with 0.1% BSA	1:500[a]	100µl	60 minutes
Substrate	*p*-nitrophenyl phosphate	Diethanolamine buffer	1 mg/ml	100µl	10-20 minutes
Stop reaction	NaOH	H_2O	3 M	50µl	None

[a] The optimal dilution of each reagent should be determined by titration.

Controls

At least two known *V. cholerae* positive control and two known negative control supernatants should be included on each microtiter plate.

Performance of the test

1) Add 100 µl of G_{M1} diluted appropriately in PBS to the inner 60 wells of the microtiter plate. Fill empty wells around the perimeter of the plate with PBS/Tween. Cover with a plate sealer or place the plate in a moist chamber and let it stand at room temperature overnight or at 35˚ to 37˚C for 4 hours.
2) Wash the plate three times with PBS/Tween as follows: Invert the plate and gently tap out the contents onto an absorbent towel. With a wash bottle or another suitable dispensing device, fill each well with PBS/Tween (approximately 200 µl). Let the plate stand for 3 minutes. Remove the PBS/Tween and repeat twice. To store the plate for later use, leave the third wash in the plates and refrigerate (4˚C). The plate may be stored for 4 to 6 weeks.
3) Block the remaining binding sites by filling each well with 150 µl of PBS with 1% BSA. Incubate the plate at room temperature for 30 minutes. Remove the contents and wash the wells three times with PBS/Tween as described in step 2.
4) With a micropipette, add 100 µl of each supernatant to duplicate wells in the plate. Leave a single row of wells around the perimeter of the plate empty. This will allow 30 tests per plate. Fill empty well around the perimeter of the plate with PBS/Tween. Place the plate in a moist chamber or seal it and incubate it at 35˚ to 37˚C for 1 hour. Wash plates three times as described in step 2.
5) Add 150 µl of PBS with 1% BSA to each of the wells, and incubate at room temperature for 30 minutes. Wash three times (step 2).
6) Add 100 µl of goat anti-CT_b serum (diluted in PBS containing 0.1% BSA) to each of the test wells. Place the plate in a moist chamber or seal it and incubate it at 35˚C for 1 hour. Wash plate three times.
7) Add to each test well 100 µl of alkaline phosphatase-labeled rabbit anti-goat globulin diluted appropriately in PBS containing 0.1% BSA. Place the plate in a moist chamber or seal it and incubate it at 35˚ to 37C˚ for 1 hour. Wash plate three times.
8) Add 100 µl of the enzyme substrate solution (*p*-nitrophenyl phosphate in diethanolamine buffer) to all of the wells. Incubate the plate at room temperature until color development in the positive control wells reaches a suitable intensity but not so long that excessive color develops in the negative wells (approximately 10 to 20 minutes, but no longer than 30 minutes).
9) Stop the reaction by adding 50 µl of 3 M NaOH to each well. Mix well.

Reading the test results

1) Compare the positive control wells with the negative control supernatants. There should be little or no color in the negative control wells. In the positive control wells, a distinct yellow color should be visible (Figure VII-3).
2) Compare the amount of color in the test wells with the color in the negative control wells. Specimens that clearly develop a stronger color than the negative controls are considered positive.

3) If a microtiter plate reader is used, the wavelength should be set to the appropriate setting for the substrate used. For the *p*-nitrophenyl phosphate, the wavelength should be set to 405 nm. A positive to negative (P/N) ratio is calculated by dividing the optical density (OD) of the unknown sample by the mean OD of the wells containing the negative controls. Samples with a P/N ratio of 2.0 or greater are considered positive.

Titration of reagents for G_{M1}-ELISA for CT

1) Dilute GM1 in PBS to concentrations listed below:

0.5 µg/ml
1.0 µg/ml
2.0 µg/ml
5.0 µg/ml

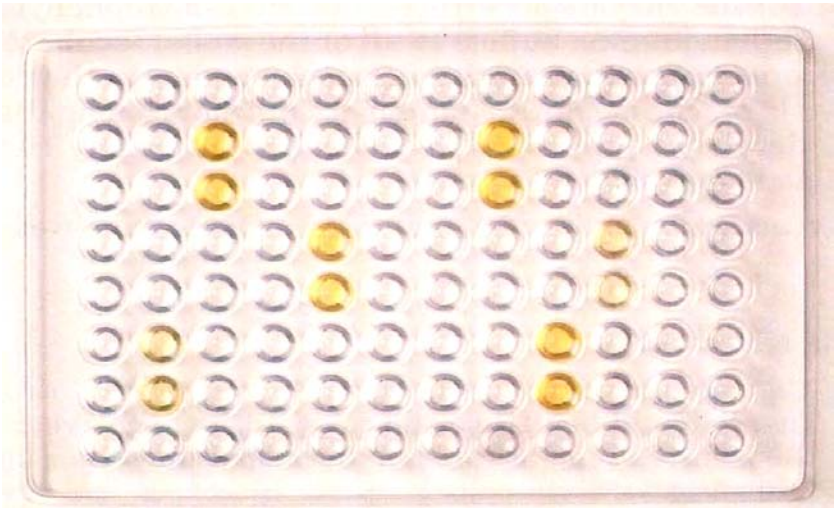

Figure VII-3. In the G_{M1}-ELISA for CT, a positive reaction is indicated by the development of a distinct yellow color in the

2) Add 100 µl of G_{M1} ganglioside (Sigma Chemical Co.) to each of the inner 60 wells of polyvinyl round-bottom microtiter plates or polystyrene, flat-bottom plates containing the following G_{M1} dilutions:

Plate 1	0.5 µg/ml
Plate 2	1.0 µg/ml
Plate 3	2.0 µg/ml
Plate 4	5.0 µg/m

Cover with a plate sealer or place the plates in a moist chamber and let them stand at room temperature overnight. Wash plates three times with 200 µl of PBS/Tween.

3) To block the remaining binding sites of the wells, fill each well with 150 µl of PBS with 1% BSA and incubate the plate at room temperature for 30 minutes. After incubation, remove the contents and wash the wells three times with 200 µl of PBS/Tween.

4) Rehydrate pure CT (Calbiochem Corp.) according to manufacturer's instructions. Dilute in PBS with 0.1% BSA as follows (at least 4 ml of each dilution is needed for titration on four plates):

Dilution	**CT concentration**
10^{-3}	1.0 µg/ml
10^{-4}	100.0 ng/ml
10^{-5}	10.0 ng/ml
10^{-6}	1.0 ng/ml
10^{-7}	100.0 pg/ml
10^{-8}	10.0 pg/ml

Add to each plate 100 µl of each different dilution of CT so that each dilution takes up one row on the plate (rows B, C, D, E, F, G). Fill empty wells with PBS/Tween. Incubate at 35˚ to 37˚C for 1 hour. Wash plates three times with 200 µl of PBS/ Tween.

5) Block with 150 µl of 1% BSA. Incubate at room temperature for 30 minutes. Wash the plate three times.
6) Prepare five dilutions of anti-CT in PBS with 0.1% BSA:

   1:500

   1:1000

   1:2000

   1:5000

   1:10000

   Note: monoclonal antibody should be tested at dilutions 1:100, 1:200, 1:500, 1:1000.

   Add 100 µl of diluted anti-CT to each plate so that each dilution takes up one column of the pate (columns 2-6). Repeat for columns 7-11. Fill empty wells with PBS/Tween. Incubate at 35˚ to 37˚C for 1 hour. Wash the plate three times.
7) Dilute alkaline phosphatase-labeled conjugate in PBS with 0.1% BSA as follows:

   1:500

   1:1000

Add 100 µl of each conjugate dilution to each plate so that each dilution takes up half the plate (columns 2-6 for one dilution and columns 7-11 for the other dilution). Fill empty wells with PBS/Tween. Incubate at 35˚C to 37˚C for 1 hour. Wash the plate three times.

8) Add 100 µl of *p*-nitrophenyl phosphate substrate. Incubate for 30 minutes at room temperature.
9) Add 50 µl of 3 M NaOH to stop the reaction, and read the results spectrophotometrically or visually. Note the highest dilution of each reagent which provides suitable color intensity. The reagent that is the most difficult to obtain or the most expensive should be used in the highest dilution possible.

G. Latex Agglutination Assay for CT

The VET-RPLA kit (Oxoid Limited, Hampshire, England) is designed for the detection of CT or LT in culture supernatant fluids. This test procedure is known as reversed passive latex agglutination (RPLA). Polystyrene latex particles are sensitized with purified antiserum produced in rabbits immunized with purified *V. cholerae* enterotoxin. These latex particles will agglutinate in the presence of CT or LT. A control reagent, which consists of latex particles coated or "sensitized" with nonimmune rabbit globulins, is provided.

The test is performed in V- or U-bottom microtiter plates (V-bottom plates are preferred; flat-bottom plates are not suitable for this procedure). Dilutions of culture supernatant to be tested are made in two columns of wells. [Note: test supernatants should be prepared according to instructions in Section D of this chapter; do not prepare supernatants by the method described in the instructions included with the kit because *V. cholerae* does not usually produce CT in sufficient quantities in alkaline peptone water (APW).] A suspension of antibody-coated latex particles is added to the first column, and unsensitized control latex is added to the second column. If sufficient amounts of either CT or LT are present in the supernatant, agglutination will occur. Agglutination results from the formation of cross-linkages among the latex particles bound to CT molecules. This lattice-like structure will settle and form a diffuse layer at the bottom of the well. If enterotoxin is absent or at a concentration below the detection level, no lattice structure will be formed, and a "button" of unagglutinated latex particles will form on settling.

Materials Required

- 96-well plates (V-bottom)
- Fixed or adjustable micropipette and tips (25 µl)
- Sensitized latex (latex suspension sensitized with specific antibodies [rabbit IgG] against cholera toxin, provided with kit).
- Control latex (latex suspension coated with nonimmune rabbit globulins, provided with kit).
- Control cholera toxin (dehydrated, provided with kit).
- Diluent: PBS containing BSA (provided with kit).

Performance of the test

1) Each sample requires two columns (eight wells per column) on the plate. With a pipette or dropper, dispense 25 µl of diluents in each well except the first well in each column.
2) Add 25 µl of test sample to the first and second well of each set of two columns.
3) Using a pipette or diluter and starting at the second well of each column, remove 25 µl and perform twofold dilutions. Stop at the seventh well in the column so that the eighth well contains diluent only. Discard the extra 25 µl from the seventh well.

4) Add 25 µl of sensitized latex to each well of the first column for each test and control supernatant. Add 25 µl of unsensitized control latex to each well of the second column for each test and control supernatant.

5) Mix the contents of each well by agitating the microtiter plate gently by hand.

6) To avoid evaporation, cover the plate with a lid and place in a plastic storage container along with a wet paper towel. Leave the plate undisturbed on a vibration-free surface at room temperature for 20 to 24 hours.

Reading the results

1) Examine each well in each column against a black background for agglutination, demonstrated by the formation of a lattice structure, which upon settling forms a diffuse layer on the bottom of the well (Figure VII-4). If the enterotoxins are absent or at a

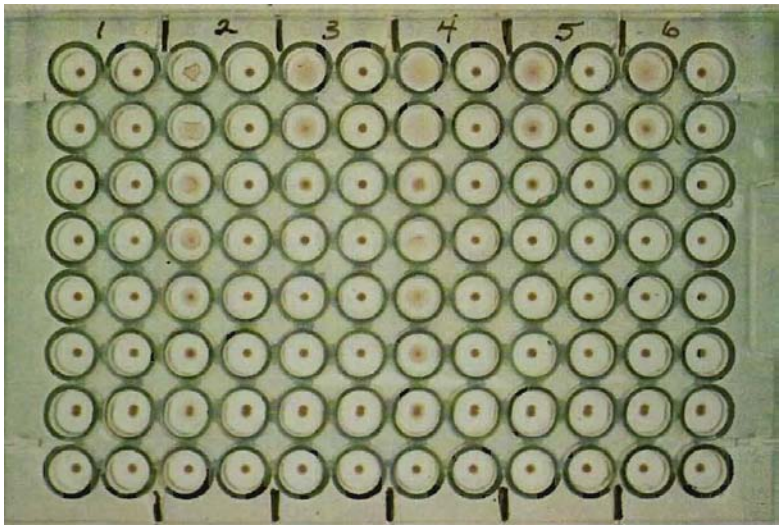

Figure VII-4. Microtiter plate with results of VET-RPLA test. Sample 1 is negative; samples 2,3,4,5, and 6 are positive.

concentration below the assay detection level, no such lattice structure can be formed; therefore, upon settling a tight button will be observed.

2) Results in the column of wells containing control latex should be negative. The last well in all columns should be negative. If positive patterns are observed in some of these wells, the reaction should be regarded as invalid.

3) When excess CT is present, a prozone effect may be observed; i.e., a negative pattern is obtained in wells containing test sample and sensitized latex. However, as a result of the doubling dilutions, the concentration of CT in each well along the column is progressively reduced, negating the prozone effect due to excess amounts of CT. A positive pattern of agglutination will be seen after negative patterns in the first few wells of the column. With such results, the test sample should be classified as positive.

4) The sensitivity of this test kit in detecting CT is 1 to 2 ng/ml. Enterotoxin present at concentrations lower than this will, therefore, give negative results. The method is

slightly less sensitive than the G_{M1} ELISA, and rarely, a toxigenic strain will give negative results.

H. PCR for CT Genes

The polymerase chain reaction is a technique that employs two short, specific DNA oligonucleotides (primers) and the enzyme DNA polymerase to synthesize multiple copies of DNA in the portion of the bacterial gnome that is flanked by the two primers. In the PCR for cholera toxin describe here, the specific primers detect only the gene encoding the A subunit of cholera toxin (*ctxA*; Figure VII-5). The amplified DNA from *ctxA* is detected as a 564-bp band in an agarose gel. The PCR amplicon can be further characterized by restriction digests or hybridization with a specific internal probe to ensure that the amplicon band in the gel is from the *ctxA* gene. This PCR test is a colony confirmation test, designed for distinguishing toxigenic from nontoxigenic *V. cholerae* O1 strains taken from pure

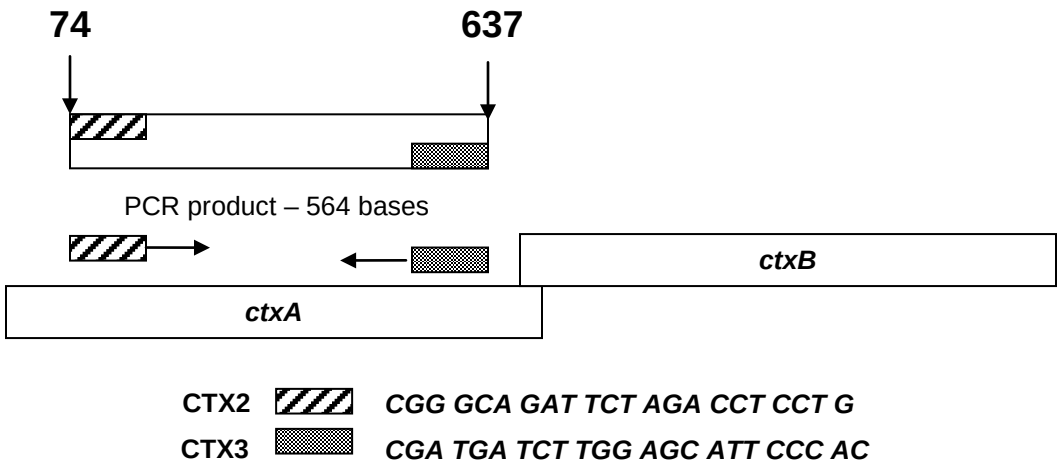

Figure VII-5. Location of the primers and the size of the amplified DNA fragment in the PCR assay for detection of *ctxA*.

cultures. Others have described PCR assays that can detect toxigenic *V. cholerae* directly in stool or in food after enrichment in APW. Direct detection of *V. cholerae* by PCR in stool or food may be hampered by substances present in these types of samples which inhibit DNA polymerase.

Equipment and supplies

- Boiling water bath
- Thermocycler (Perkin-Elmer, Norwalk, Conn.; or M.J. Research Inc., Watertown, Mass.)
- Vortex
- Electrophoretic apparatus
- Power supply
- Camera and UV-transilluminator
- Micropipettes: 0.5-20 µl, 20-50 µl, and 100-1000 µl
- Sterile, disposable micropipette tips
- Microfuge tubes: 1.5 and 0.5 ml
- Gloves
- Dust/particle mask

Reagents

- Sterile water (make aliquots of 10-20 ml, open fresh aliquot for each assay)
- 10X PCR buffer (100 mM Tris-HCl, 500 mM KCI, 15 mM MgCl, 0.1% [wt/vol] gelatin)
- 12.5X dNTP (2.5 mM each, dATP, dCTP, dGTP, dTTP in sterile distilled water)
- Primer CTX2: 5' CGG GCA GAT TCT AGA CCT CCT G 3'; 100 µM in distilled water
- Primer CTX3: 5' CGA TGA TCT TGG AGC ATT CCC AC 3'; 100 µm in distilled water
- AmpliTaq DNA polymerase (5 units/µl, Perkin-Elmer)
- Mineral oil
- Agarose (FMC BioProducts, Rockland, Maine)
- 10x Tris borate EDTA (TBE) buffer (108 g of Tris base, 55 g of boric acid, 40 ml of 0.5 M EDTA, and distilled water to 1,000 ml; adjust pH to 8.0)
- 10x gel loading buffer (0.5% sodium dodecyl sulfate, 10 mM EDTA, 50% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol)
- Ethidium bromide (stock solution is 10 mg/ml water)
- Quick spin columns (G-50, Boehringer Mannheim, Indianapolis, Ind.)
- Restriction endonucleases *Alu*I and *Rsa*I (Gibco BRL, Gaithersburg, Md.)
- 10x restriction buffer 1 (Gibco BRL)

- DNA molecular size marker: 1 kb ladder (Gibco BRL)
- TEMED (Sigma Chemical Co.)
- Ammonium persulfate (10% in water, made fresh daily, Gibco BRL)
- 30% acrylamide (29 parts acrylamide: 1 part bisacrylamide in water), store in dark at 4˚C.
- Note: Acrylamide is neurotoxic; wear a face mask when preparing the stock solution.

Contol Strains

- *V. cholerae* ATCC 14035 (Classical, Ogawa, toxin-positive)
- *V. cholerae* ATCC 14033 (El Tor, Inaba, toxin-negative)

Performance of the test

SPECIAL NOTE: Only a very small amount of template DNA (DNA from which the PCR amplicon is generated) is required for PCR. Small amounts of contaminating DNA, especially the products of previous PCR assays, may result in false-positive results. It is important to use negative as well as positive controls in every experiment. Water in

place of template DNA and a known negative strain are appropriate negative controls. Positive controls should be a known positive strain that is prepared along with the test strains and a known positive template preparation.

To reduce the possibility of contaminating the PCR test with the PCR products of previous reactions, the reactions should be prepared in one room and the amplification and electrophoresis should be performed in another room. The PCR product should never be taken into the PCR set-up room. A separate set of micropipettes, preferably positive displacement pipettes, should be reserved only for setting up PCR reactions.

1) To prepare a template DNA, suspend a 1-µl loopful of the control or the test strains in 0.5 ml of water to obtain a concentration of 10^5 to 10^6 organisms/ml. Boil the sample for 20 minutes to release DNA. One to 10 ng of DNA is sufficient; too much DNA can inhibit the amplification reaction. It is important to use water rather than PBS or any other phosphate-containing buffer because phosphate inhibits PCR. Heme-groups from blood agar plates can also inhibit PCR.

2) For each PCR test, a 50 µl reaction mix consists of 38.75 µl of sterile water, 5 µl of 10X PCR buffer, 4µl of 12.5x dNTPs, 0.5 µl of each primer (1 µl total volume), 0.25 µl of AmpliTaq DNA polymerase, and 1 µl sample template. Prepare a “master mix” of

all reagents except the sample DNA. This reduces pipetting errors and produces more consistent concentrations of reagents. Mix in one tube enough PCR buffer, dNTPs, *Taq* polymerase, primers, and water for all the tests being performed, and aliquot 49 µl of the master mix into each 0.5-ml microfuge tube. Then add 1 µl of the sample template.

3) Overlay the reaction mix with 1 drop of sterile mineral oil, and close the tubes. Program the thermocycler for a preincubation step at 95°C for 5 minutes, then 30 cycles of 1 minute at 95°C, 1 minute at 60°C, 1 minute at 72°C, and a final incubation at 72°C for 10 minutes. A final step of holding the tubes at 4°C can be added to refrigerate the samples until they are loaded on the gel. Place tubes in thermocycler and start.

4) Prepare a 0.8% agarose gel with TBE buffer. Mix 10 µl of the PCR mix and 1 to 2 µl of the 10x gel loading buffer, and load the wells. When removing the 10 µl of PCR reaction, make sure the pipette tip is beneath the oil layer. Use appropriate positive and negative controls with each set of PCR reactions, including a molecular size standard on each gel.

5) Run the gel until the bromphenol blue (purple color) has migrated about two-thirds of the way down the gel. Actual voltage and time will vary with the gel apparatus used (generally 2 to 3 hours at 60-80 V is sufficient). The 564-bp *ctx*A Amplicon should migrate close to the bromphenol blue. Stain the gel with ethidium bromide (1 drop of stock solution in 500 ml of water) for 20 minutes, then destain in water for 10 to 20 minutes. [Note: ethidium bromide is mutagenic; wear gloves at all times.] Place the gel on the UV-transilluminator, and take photographs for documentation.

Interpretation of the results

The PCR test generates a 654-bp amplicon from the *ctxA* gene. This amplicon migrates just above the 0.5-kb band of th 1-kb DNA ladder size standard. The primers may be visible as a faint smear migrating just below the 200-bp size standard (Figure VII-6). The primers, but not the 564-bp amplicon band, will be visible for negative samples. Bands that are the wrong size should be considered negative. Faint bands that appear to be the correct size should be interpreted cautiously. When the results are in doubt, the amplified DNA can be verified as from the *ctxA* gene by digestion with restriction enzymes or hybridization with an internal probe after Southern blotting. For routine application, restriction analysis is simpler and faster than probing.

Verification of the PCR amplicon

1) Remove the mineral oil for the sample by placing the total volume of the PCR reaction on an angled parafilm strip. Mineral oil sticks to the parafilm as the aqueous drop slides downward. Purify the DNA fragment on a Quick Spin G-50 column. To prepare the column for use, centrifuge it at 600 x *g* for 5 minutes. Add the sample (10 to 50 µl) and repeat the centrifugation. The purified DNA will pass through the column in the same volume as was added to the column.
2) Transfer 15 µl of the PCR solution to a microcentrifuge tube; add 2 µl of water, 2 µl of restriction buffer 1 (BRL), and 1 µl of *Rsa*I or *Alu*I (or both). Incubate for 2 hours at 37˚C.
3) Separate the restricted fragments by electrophoresis in an acrylamide gel. Acrylamide gels produce better resolution of small DNA fragments. For a minigel apparatus, prepare an 8% gel with 6.6 ml of 30% acrylamide (29:1 acrylamide: bisacrylamide in water), 15.6 ml of water, 2.5 ml of 10x TE, 0.2 ml of 10% ammonium persulfate, and 20 µl of TEMED. Pour the gel and let it polymerize for 1 hour before loading the sample (10 µl of the PCR reaction and 1 µl of 10x loading buffer). Let the bromphenol blue migrate half way down the gel before staining DNA, as for an agarose gel. Restricting the 564-bp *ctxA* amplicon with *Rsa*I generates three fragments of 480, 70, and 14 bp; the 14-bp fragment may not be visible. *Alu*I produces two fragments of 499 and 65 bp. Combination of the two enzymes generate four fragments: 415, 70, 65, and 10 bp.

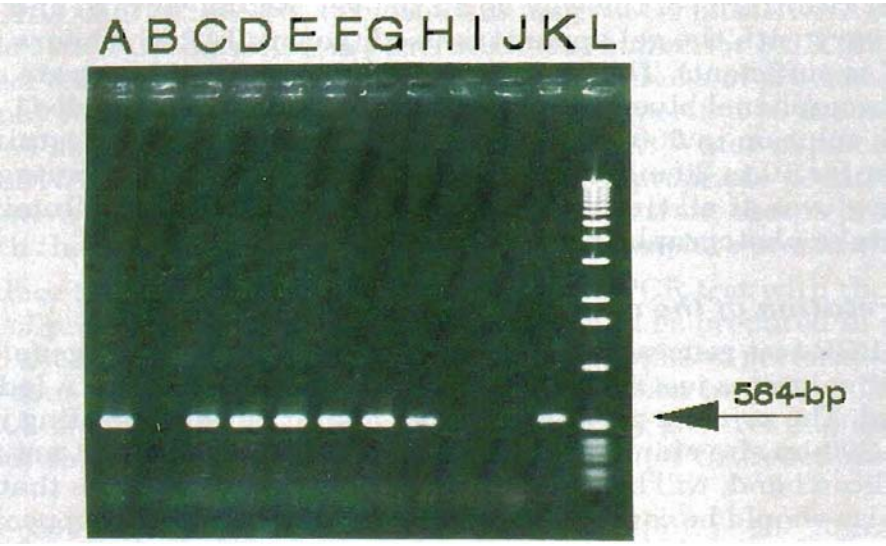

***Figure VII-6. Agarose gel with results of a typical cholera toxin PCR test. Lanes A, C, D, E, F, positive test strains; lanes B, J, negative test strains; lanes G, K, positive controls; lanes H, I,* negative control strains; lane L, I-kb DNA ladder.**

I. DNA Probes for CT Genes

In colony blots, isolated colonies to be tested for cholera toxin genes are inoculated in a grid-fashion on a nonselective agar plate and incubated until the patches of growth reach the desired size. The patches are then transferred to a nylon filter, where they are treated by a series of steps that lyse the cells and fix the denatured or single-stranded DNA to the filter. The filter is then hybridized with a specific DNA probe that corresponds to the *ctx* gene. Although cloned DNA and oligonucleotides have been tested, a PCR-generated amplicon that contains digoxigenin-labeled bases has proved to be sensitive, stable, and safe.

1. Generation of digoxigenin-labeled PCR amplicons

The procedure for generating digoxigenin-labeled PCR probes is similar to that for detecting *ctx* genes with PCR (Section H of this chapter), except that a well-characterized CT-positive strain of *V. cholerae* O1 is used to produce the template DNA and a second amplification is used to incorporate the digoxigenin-labeled base.

Equipment and supplies

See Section VII-H (PCR for CT genes)

Reagents

- See Section VII-H (PCR for CT genes)
- 10x "-digoxigenin" dNTP mix (1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP)
- Digoxigenin-11-dUTP (Boehringer Mannheim; concentration is 1 mM)

Procedure

1) Generate the PCR amplicon that will be used as the template in a labeling PCR by following the procedure described in Section VII-H using a known CT-positive strain (ATCC 14035 or another well-characterized, CT-positive strain) as the source of template DNA. Incorporation of digoxigenin-labeled dUTP into a DNA molecule is less efficient than incorporation of standard dNTPs; therefore, the PCR amplicon is used as template DNA rather than total genomic DNA in the labeling PCR to increase the amount of labeled DNA (probe) produced.
2) Run 5 to 10 µl of the PCR mix on a 0.8% agarose gel to confirm production of the appropriate size and concentration of the DNA amplicon. Stain and photograph for documentation.
3) Make a dilution of the PCR mix so that about 50 ng of the PCR fragment is used as the template in the labeling PCR (usually, 1 to 3 µl of a 1:10 dilution).

4) Set up the labeling PCR in a 0.5-ml microcentrifuge tube: 1 to 3 µl of the diluted PCR-generated template DNA, 1 µl of each primer (2 µl total volume), 10 µl of 10x PCR buffer, 10 µl of 10x "-digoxigenin" dNTP mix, 7 µl of digoxigenin-11-dUTP (1mM), 0.5 µl of *Taq* polymerase, 67.5 to 69.5 µl of sterile distilled water. Overlay the mixture with 2 drops of mineral oil.
5) Program the thermocycler for 40 cycles of 30 seconds at 95˚C, 1.5 minutes at 60˚C, 3 minutes at 72˚C; then a 10 -minute incubation at 72˚C; hold at 4˚C. Place tubes in thermocycler and start.
6) Remove unincorporated dNTPs and primers by purifying the DNA over a Quick Spin G-50 column.
7) Run 2 to 3 µl of the PCR mix on a 0.8% agarose gel. Stain and photograph. The digoxigenin-labeled amplicon will migrate more slowly (i.e., at a higher apparent molecular weight) than the nonlabeled amplicon because of the presence of digoxigenin in the molecule.

2. Colony hybridization for cholera toxin genes

Equipment and supplies

- 8 cm diameter circular nylon filters (Micron Separations, Inc., Westboro, Mass.)
- Whatman 3MM chromatography paper
- Drying oven
- Platform shaker
- Plastic, heat-sealable bags
- Heat-sealing apparatus
- Petri dishes
- Luria broth (LB) plates or other rich medium (do not use MacConkey plates because they seem to cause more background staining on the blots)

Reagents

- Digoxigenin-labeled probe (amplicon prepared as in section I.1.)
- 10 N NaOH
- 1 M Tris, pH 8.0
- 5 M NaCl

- 20% SDS
- 20x SSC (175.3 g NaCl, 88.2 g sodium citrate in 1 liter water, pH 7.0)
- Prehybridization solution: 5x SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 0.02% SDS (can be prepared in large quantities in advance, aliquoted, and stored at -20˚C) [Note: Prehybridization solution is used for both prehybridizations and hybridizations.]
- Buffer A (100 mM Tris, pH 7.5, 150 mM NaCl)
- Buffer C (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$)
- Nonfat dry milk
- Anti-digoxigenin Fab fragment/alkaline phosphatase conjugate (Boehringer Mannheim)
- Nitroblue tetrazolium (NBT) (75 mg/ml in water)
- 5-bromo-4-chloro-3-indolylphosphate toluidinum (BCIP) 175 g/ml in water)

Preparation of colony blots

1) Transfer isolated colonies of each test strain to a dry LB plate using a grid to organize the colonies. Incubate the plate for several hours or overnight at 37˚C until all the colonies are clearly visible on the plate.
2) Mark an 8-cm circular nylon filter (50 cm^2 total area) with an arrow to orient the position of the colonies and a number or date to identify the filter. Place the filter (labeled side down) over the inoculated LB plate with the arrow at the top of the plate. Remove any trapped air bubbles. Incubate the LB plate for 0.5 to 2 hours at 37˚C. [Note: nitrocellulose filters can also be used, but they tear more easily than nylon filters. Glass filters, such as Whatman 541, cannot be used in this assay because digoxigenin sticks nonspecifically to these filters.
3) Saturate two sheets of 3MM chromatography paper with 0.5 M NaOH in a glass pan. Lift the nylon filter off the plate and place on the 3MM paper, colony side up. Visible patches of each colony should be stuck to the filter. Allow the cells to lyse for 15 minutes.
4) Saturate two sheets of 3MM chromatography paper with 1.0 M Tris pH 8.0, in a glass pan. Transfer the filter from the paper saturated with NaOH to the paper saturated with Tris, again colony side up. Allow neutralization to occur for 10 minutes.
5) Saturate two sheets of 3MM chromatography paper with 0.7 M Tris 1.5 M NaCl, pH 8.0, in a glass pan. Transfer the filter from the paper saturated with 1.0 M Tris to the paper saturated with Tris/NaCl colony side up. Allow neutralization to occur for 10 ten minutes.

6) Rinse the filter briefly in 2x SSC. Blot the filter on dry 3MM chromatography paper and air dry at room temperature or 37˚C. Bake the filter at 80˚C in an oven for 0.5 to 2 hours. The filter is now ready for hybridization but it can be stored in an airtight container at room temperature until needed.

Hybridization of colony blots

1) Place the filter in a plastic, heat-sealable bag. Add 10 ml (0.2 ml/cm2) of prehybridization solution. Remove air bubbles by rolling a pipette across the bag and squeezing the bubbles to the top. Do not squeeze the fluid out of the bag. Seal the bag with a heat sealing apparatus. Incubate the filter in the bag at 65˚C for 1 hour in a shaking water bath.
2) Prepare the probe 15 to 20 minutes before the end of the prehybridization time (see section I.1. for instructions for probe preparation). Dilute the probe in a total volume of 100 µl in a microfuge tube; use 1 to 5 µl of probe, depending on the concentration. [Note: The optimal concentration of probe may need to be determined empirically by testing several concentrations on replicate filters.] Denature the probe by incubating in a boiling water bath for 10 minutes. Quickly cool the probe by placing it on ice for a few minutes. Centrifuge the probe in a microfuge for a few seconds, and hold on ice until needed.
3) Remove the filter from the water bath. Cut one corner of the bag and remove all the prehybridization solution from the bag. Add 2.5 ml (0.05 ml/cm^2) of prehybridization solution and the probe to the bag. Remove the air bubbles and seal the bag so that it conforms to the shape of the filter as closely as possible to maximize contact between the filter and the probe.
4) Hybridize the filter at 65˚C overnight in a shaking water bath.
5) Prepare about 500 ml of wash solution (1x SSC, 0.1% SDS), and preheat to 65˚C. Remove the filter from the plastic bag and place in a glass pan or dish. Add 100 ml of wash solution to the glass pan and rinse the filter briefly. Pour off the used wash solution.
6) Add 200 ml of wash solution to the glass pan and wash the filter at 65˚C in a shaking water bath for 15 minutes. Pour off the used wash solution. Add the remaining wash solution and incubate again at 65˚C for 15 minutes. Air dry the filter and hold for later development, or proceed directly to the developing steps.

Detection of digoxigenin-labeled probes

1) Rinse the filter with 40 ml of buffer A in a petri dish on a platform shaker for 1 minute.
2) Remove the filter from buffer A, allowing excess solution to drip off for a few seconds.

Place the filter in another petri dish containing 40 ml of buffer A + 5% nonfat dry milk (blocking solution). Incubate with gentle shaking for 1 hour.

3) Pour off the blocking solution and replace with the anti-digoxigenin/alkaline phosphatase conjugate diluted 1:5000 in 10 ml of buffer A + 5% nonfat dry milk. Incubate for 30 minutes with gentle shaking.
4) Transfer the filter to a petri dish containing 40 ml of buffer A. Incubate with gentle shaking for 15 minutes. Repeat the wash step once.
5) Rinse the filter in a petri dish containing 40 ml of buffer C on a shaker tray for 2 minutes.

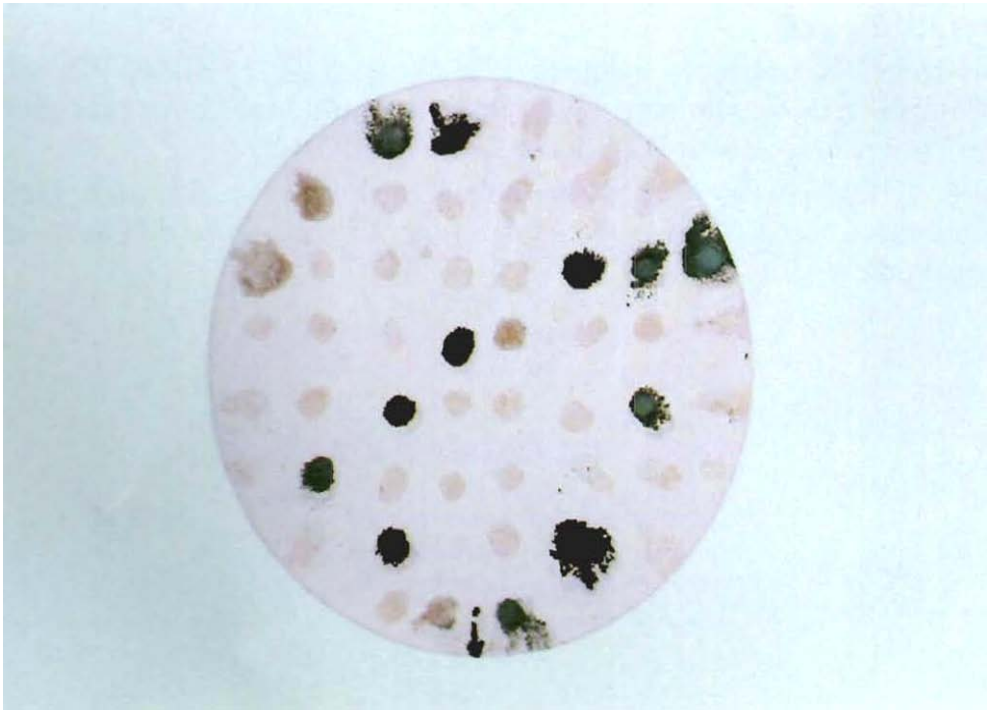

Figure VII-7. Results of hybridization with a digoxigenin-labeled probe for *ctxA*; dark purple color indicates a positive reaction.

6) Remove the filter from buffer C, allowing excess solution to drip off for a few seconds. Place the filter in a petri dish containing 10 ml of buffer C with 45 µl of NBT (75 mg/mL) and 35 µl of BCIP (175 µl/ml). Seal the petri dish with parafilm. Place the petri dish in a dark place and check periodically. Results may be observed within a few hours, although overnight exposure may be required for complete development.

Interpretation of results

ctxA-positive isolates will appear as dark purple/brown patches on the nylon filters (Figure VII-7). Negative controls and negative isolates may be visible as very faintly stained patches.

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