XI. Preparation of Media and Reagents

A. Storage of Media

A number of different media are used in cholera studies, some of which are available as dehydrated preparations from commercial sources. Commercially prepared media usually have an expiration date; however, the actual shelf-life of prepared and dehydrated media is difficult to predict. The laboratory environment has a marked effect on even the most stable medium, especially if extremes of heat, cold, humidity, or dryness prevail for prolonged periods. Generally, dehydrated media remain satisfactory for longer periods than prepared media. Dry media must be kept tightly closed and stored in a cool, dry place, ideally with the temperature not exceeding 25° to 28°C. Dehydrated medium is generally considered usable if it does not show caking, discoloration, or other signs of deterioration.

B. Quality Control

Each new batch or lot of medium should be examined before routine use to ensure sterility and proper growth characteristics of control strains. Out-of-date media that are still in use should also be examined periodically. Sterility of the medium should be determined by incubating it for 18 to 24 hours at 35° to 37°C. Reference strains with known characteristics should be inoculated to the medium and incubated for the correct length of time. After incubation, the medium should be examined for growth (or absence of growth, if appropriate), colony characteristics, and differential characteristics unique to the medium. Written records should be kept of results.

Quality Control of Selective Agar Media

The following is an example of a procedure that may be used for evaluating the quality of a large lot of selective plating media. Other methods may also be used successfully. Laboratory personnel should establish a method and should use that method to evaluate each batch or lot of media. Whatever method or modification is used, it should give accurate and reproducible results and it should be used regularly with different lots or batches of media. Written records should be kept. As long as these criteria are met, the quality control procedures themselves may vary from laboratory to laboratory.

An example of a quality control test report for thiosulfate citrate bile salts sucrose (TCBS) agar is given in Table XI-1. A broth culture of each organism was diluted and plated in duplicate on each lot of TCBS medium to be tested. The organisms were also plated onto heart infusion agar (HIA), a noninhibitory medium, to assess the number of colony-forming units in each dilution. Quality control records should be kept for reference.

Media and reagents

- Heart infusion agar and heart infusion broth (HIB)

[Other nonselective, nondifferential media such as Trypticase soy agar or blood agar base may be substituted. If testing a selective medium for *V. cholerae,* do not use nutrient agar or nutrient broth since they do not contain NaCl and *V. cholerae* grows best on an NaCl-containing medium.]

- Sterile saline (0.85%)
- New lot of selective medium to be tested
- Tested lot of same medium

Strains

- Two or more isolates of the appropriate etiologic agent(s)

[For example, when using TCBS agar, select *Vibrio cholerae* and *V. parahaemolyticus*, which are sucrose positive and negative, respectively.]

- One or more isolates of anticipated competing organisms

[For example, when using TCBS agar, competitors could be *Escherichia coli, Proteus, Aeromonas,* and *V. alginolyticus.*]

Method

Day 1

Obtain sterile, quality controlled HIA agar slants or plates. If plates are used, make sure the agar surface is dry before use. Streak test organisms onto the HIA slants or plates. Incubate overnight.

Day 2

Lightly inoculate HIB with strains grown on HIA from day 1. Incubate overnight.

Day 3

1) Make 10-fold dilutions (10^{-1} through 10^{-7}) of the overnight HIB cultures in saline.

2) Inoculate each plating medium to be tested with 0.1 ml of tube dilutions. Perform this on duplicate plates. Spread the inoculum on the plates with a sterile glass spreading rod until it is completely absorbed into the medium. Incubate plates under conditions appropriate for each medium.

Day 4

1) Record the plate counts. Calculate the colony-forming units.

2) Measure the diameter of 3 to 5 colonies per plate, and calculate the average colony diameter for each medium.

Table XI-1. Example of quality control test report for TCBS agar

		TCBS Brand A		TCBS Brand B		HIA LOT 790278	
Organism	Tube dil	1	2	1	2	1	2
V. cholerae O1	10-5	53	67	67	47	211	198
Inaba - strain #1	10-6	3	2	10	8	31	26
	10-7	2	1	0	0	3	4
V. cholerae O1	10-5	424	368	TNTC	TNTC	TNTC	TNTC
Ogawa - strain #2	10-6	59	53	89	115	85	114
	10-7	4	4	7	4	22	22
V. cholerae O1	10-5	47	30	232	256	TNTC	TNTC
Ogawa - strain #3	10-6	3	7	32	30	74	65
	10-7	1	0	1	0	6	8
V. para-	10-5	79	83	116	104	244	284
haemolyticus	10-6	14	16	11	10	38	32
	10-7	1	1	1	0	3	4
V. alginolyticus	UNDIL	TNTC	TNTC	TNTC	TNTC		
	10-1	TNTC	TNTC	TNTC	TNTC		
	10-2	TNTC	TNTC	TNTC	TNTC		
	10-3	60	70	138	124		
	10-4	10	13	11	17	TNTC	TNTC
	10-5	1	0	1	1	89	63
	10-6	1	0	0	0	3	6
	10-7	0	0	0	0	1	0
P. mirabilis	UNDIL	TNTC	TNTC	0	0		
	10-1	40	48	0	0		
	10-2	1	2	0	0		
	10-3	0	0	0	0		
	10-4	0	0	0	0		
	10-5	0	0	0	0	TNTC	TNTC
	10-6	0	0	0	0	146	136
	10-7	0	0	0	0	17	14
A. hydrophila	UNDIL	0	0	0	0		
	10-1	0	0	0	0		
	10-2	0	0	0	0		
	10-3	0	0	0	0		
	10-4	0	0	0	0		
	10-5	0	0	0	0	TNTC	TNTC
	10-6	0	0	0	0	79	53
	10-7	0	0	0	0	4	4
E. coli	UNDIL	0	0	0	0		
	10-1	0	0	0	0		
	10-2	0	0	0	0		
	10-3	0	0	0	0		
	10-4	0	0	0	0	TNTC	TNTC
	10-5	0	0	0	0	184	156
	10-6	0	0	0	0	36	41
	10-7	0	0	0	0	4	0

Note: Tube dil = tube dilution; TNTC = too numerous to count; UNDIL = undiluted.

3) Record the results and determine the quality of the medium tested compared with results with previously examined acceptable lots of the same medium.

C. Media Formulas

AKI medium for in vitro production of cholera toxin

NaCl	0.5 g
$NaHCO_3$	0.3 g
Yeast extract	0.4 g
Bacto-peptone	1.5 g
Distilled water	100.0 ml

Preparation: Dissolve the ingredients except $NaHCO_3$ in a half volume of distilled water, and autoclave. $NaHCO_3$ (0.6%) should be sterilized by filtration. Gently mix equal volumes (5 ml each) of both sterilized ingredients in a test tube.

Alkaline peptone water (APW)

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

Peptone	10.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Preparation: Add ingredients to the water and adjust to pH 8.5 with 3N NaOH solution. Distribute and autoclave at 121°C for 15 minutes. Store the APW in bottles or test tubes with tightly closed caps to prevent a drop in pH or evaporation.

Arginine glucose slant

[Note: Formula from *Bacterial Analytical Manual*, U.S. Food and Drug Administration.]

Peptone	5.0 g
Yeast extract	3.0 g
Tryptone	10.0 g
NaCl	20.0 g
Glucose	1.0 g
L-arginine-(hydrochloride)	5.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.3 g
Bromcresol purple	0.03 g
Agar	13.5 g
Distilled water	1000.0 ml

Preparation: Suspend ingredients in distilled water, boil to dissolve, and dispense in 5-ml amounts to 13 x 100-mm tubes. Final pH should be 6.8 to 7.0. Autoclave 10 to 12 minutes at 121°C. After sterilization, incline tubes to solidify as slants.

Procedure: Inoculate individual colonies by stabbing the butt and streaking the slant of the agar surface. Incubate for 18 to 24 hours at 35° to 37°C with caps or stoppers loose. Organisms that produce arginine dihydrolase cause an alkaline reaction (purple color) throughout the medium. Organisms without this enzyme typically produce an alkaline slant (purple) and an acid butt (yellow). Read tube as with Kligler's iron agar or triple sugar iron agar, noting the reactions in the butt and on the slant, as well as gas and H_2S formation. *V. cholerae* gives a K/A reaction (arginine negative) in this medium, with no gas or H_2S.

Blood agar

Blood agar may be prepared by using a dehydrated basal medium, such as blood agar base or Trypticase soy agar, to which 5% sterile defibrinated sheep blood is added after autoclaving and cooling to 50°C. Do not use human blood, as it may contain antimicrobial agents, traces of other pharmaceutical agents, antibodies, other immune factors, or bloodborne pathogens. Mix well after adding blood, and pour 15- to 20-ml volumes into petri dishes. Allow surfaces of plates to dry before inoculation.

Carbohydrate test broth

Peptone	10.0 g
NaCl	5.0 g
Distilled water	1000.0 ml
Andrade's indicator (see below)	10.0 ml
Carbohydrate to be tested	10.0 g

Preparation: Mix ingredients with gentle heating. Adjust the pH to 7.4 to 7.5. Dispense in 3- to 5-ml amounts in tubes. Sterilize by autoclaving. A positive reaction (fermentation of the carbohydrate) is recorded when the indicator changes from amber to pink.

Andrade's indicator:

Acid fuchsin	0.5 g
NaOH, 1 N	16.0 ml
Distilled water	100.0 ml

Dissolve the acid fuchsin in the distilled water and add the sodium hydroxide. The fuchsin should be slightly orange after sitting overnight. If it is not, add an additional small amount of NaOH (up to 1 ml) to decolorize.

Cary-Blair transport medium

Sodium thioglycolate	1.5 g
Na_2HPO_4	1.1 g
NaCl	5.0 g
Agar	5.0 g
Distilled water	991.0 ml

Preparation: Dissolve the ingredients in the water while heating in a boiling water bath until the solution is clear (do not allow to boil). Cool to 50°C, add 9 ml of freshly prepared 1% calcium chloride solution, and

adjust to pH 8.4 with 10 N NaOH. Dispense 5- to 7-ml amounts into sterile 13 x 100-mm screw cap tubes (caps loosened). Sterilize by steaming (do not autoclave) at 100°C in a boiling water bath for 15 minutes. Tighten the caps after sterilization. Cary-Blair is quite stable if stored in a cool, dark place and not allowed to dry out. It may be used as long as there is no loss of volume, contamination, or color change.

Note: dehydrated Cary-Blair medium is available commercially.

Craig's medium for cholera toxin production

Casamino acids	30.0 g
Yeast extract	4.0 g
K_2HPO_4	0.5 g
Distilled water	1000.0 ml

Preparation: Dissolve casamino acids, yeast extract, and K_2HPO_4 in distilled water. Final pH should be adjusted to 7.0 with 3 M NaOH. After autoclaving, add filter-sterilized glucose (10 ml/liter of a 20% solution) to achieve a final glucose concentration of 0.2%. Dispense 5 ml into sterile 16 x 125-mm screw cap tubes or 10 ml into sterile 50-ml screw cap flasks. Refrigerate until ready to use.

Decarboxylase/dihydrolase broths, (Möeller formulation)

NaCl	10.0 g
Peptone	5.0 g
Beef extract	5.0 g
Bromcresol purple	0.10 g
Cresol red	0.005 g
Glucose	0.5 g
Pyridoxal	0.005 g
Distilled water	1000.0 ml

Preparation: Mix ingredients with gentle heating until dissolved. Adjust pH to 6.0. Aliquot into four flasks. Add one of the following to one of the flasks: 1% L-arginine monohydrochloride, L-lysine dihydrochloride, or L-ornithine dihydrochloride (if pure L-form amino acids are not available, 2% DL amino acids may be used). Reserve one portion for use as a control with no amino acids added. Readjust the pH of the ornithine portion to 6.0. Dispense in 3- to 4-ml amounts in screw cap test tubes and autoclave for 10 minutes at 121°C.

Procedure: Inoculate test and control tubes with a light inoculum from a young culture. Cover with 4 to 5 mm of sterile mineral oil. Incubate at 35° to 37°C and read at 24 and 48 hours or for up to 7 days if negative. Positive reactions are indicated by an alkaline (purple) reaction (pH 6.2 or above) when accompanied with an acid (yellow) reaction in the control tube. It is normal for the medium to first turn yellow because of fermentation of the glucose. However, if the color remains yellow, the reaction is negative.

Gelatin agar (0% and 1% NaCl)

Peptone	4.0 g
Yeast extract	1.0 g
NaCl	0 or 10.0 g
Gelatin	15.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Preparation: For salt-free gelatin do not add any NaCl; otherwise add 10 grams of NaCl per liter. Dissolve ingredients with heating and bring to a boil with constant stirring. Adjust pH to 7.2 to 7.4. Autoclave at 121°C for 15 minutes. Cool to 50°C. Pour into petri dishes and allow to solidify.

Quality control: Gelatin agar prepared with 0% and 1% NaCl is recommended for use as a screening medium to differentiate *V. cholerae* from halophilic organisms (see Chapter V, "Examination of Food and Environmental Specimens"). When gelatin agar is used for this purpose the peptone used in the medium must be chosen carefully. Different peptones contain varying amounts of NaCl, which may affect the growth of organisms sensitive to NaCl concentration. Each peptone source must be quality controlled using a standard strain of *V. cholerae* and a halophilic organism.

Meat extract agar (MEA; also called alkaline nutrient agar)

Peptone	10.0 g
NaCl	10.0 g
Meat extract (concentrated)	3.0 g
Agar	20.0 g
Distilled water	1000.0 ml

Preparation: Add ingredients to the water and heat to boiling while stirring to melt the agar. Adjust the pH to 8.4. Autoclave at 121°C for 15 minutes. Pour plates aseptically (20 ml per plate). Allow to cool slowly and store in inverted position under refrigeration (4°C).

Polyvinylpyrrolidone (PVP) medium for lyophilization

Polyvinylpyrrolidone	5.0 g
Sucrose	5.0 g
Sodium glutamate	1.0 g
Distilled water	100.0 ml

Preparation: Dissolve the above ingredients in 90 ml of water and adjust the pH to 7.4 with NaOH. Bring volume up to 100 ml with water. Sterilize by filtration or autoclave 15 minutes.

Salt test broth

Nutrient broth (Difco Laboratories)	8.0 g
Distilled water	1000.0 ml

Preparation: Combine ingredients with mixing and gentle heating. Adjust the pH to 7.4 to 7.5. Divide into equal portions, the number of portions depending on the number of different NaCl concentrations to be prepared. Do not add salt to one portion (0% NaCl); to the other portions add salt to equal the percentage desired. Dispense in screw cap test tubes and sterilize by autoclaving at 121°C for 10 minutes.

Quality control: *V. cholerae* will grow in nutrient broth (Difco Laboratories, Detroit, Mich.) with no added salt, but halophilic organisms will grow only in nutrient broth to which 1% NaCl has been added (see Chapters IV and VI). These reactions are based on using Bacto nutrient broth (Difco Laboratories), and any other basal medium must be thoroughly evaluated before use in this test. Different peptones contain varying amounts of NaCl, which may affect the growth of organisms sensitive to NaCl concentration. Each peptone source or basal medium must be quality controlled using a standard strain of *V. cholerae* and a halophilic organism.

Skim milk freezing medium

Add 20 g powdered skim milk to 100 ml distilled water; dissolve, autoclave at 116°C for 20 minutes. Avoid overheating, because it will caramelize the milk.

Taurocholate tellurite gelatin agar (TTGA, or Monsur's medium)

Trypticase	10.0 g
NaCl	10.0 g
Sodium taurocholate	5.0 g
$NaHCO_3$	1.0 g
Gelatin	30.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Potassium tellurite (1%) (see below for titration procedure)

Preparation: Dissolve the ingredients, with heating, in the water. Adjust pH to 8.5. Sterilize at 121°C for 15 minutes. Cool to 50°C, add predetermined amount of filter-sterilized 1% aqueous potassium tellurite solution (see below). Mix well and pour plates. If only a few plates are needed at a time, the media may be dispensed in measured amounts into screw cap bottles. After autoclaving, the medium can be stored refrigerated until needed. Before use, melt and cool the medium to 50° to 55°C, add the appropriate amount of potassium tellurite, mix, and pour plates.

Titration of potassium tellurite: The optimum concentration of potassium tellurite for TTGA may vary according to the quality of each lot of potassium tellurite. Before regular use, each lot (or bottle) of potassium tellurite should be titrated to determine the proper working dilution to use in TTGA. See Table XI-2 for amounts of 1% potassium tellurite to use in preparing media for evaluation.

On each test batch of TTGA containing the different dilutions of potassium tellurite, streak one or more well-characterized strains of *V. cholerae* for isolated colonies . Incubate plates at 35° to 37°C for 18 to 24 hours. After incubation, examine each plate for the amount of growth, the size and color of the colonies, and the production of gelatinase. Record the results and re-incubate the plates for an additional 18 to 24 hours, after which plates will be re-examined. Typical *V. cholerae* colonies will, after 18 to 24 hours, be small (1 to 2 mm) and opaque with a slight darkening of the center of the colony. Growth may or may not exhibit gelatinase activity at this time. After 36 to 48 hours of incubation, colonies should be 2 to 4 mm in diameter, have a distinct cloudy "halo" caused by the formation of gelatinase, and should be gunmetal grey in color. Most lots of potassium tellurite are satisfactory at a final dilution of 1:100,000 or 1:200,000.

Table XI-2. Potassium tellurite (K_2TeO_3) concentration per liter

Amount of 1% potassium tellurite solution per liter	Final concentration of potassium tellurite in TTGA
0.25 ml	1:400,000
0.5 ml	1:200,000
1.0 ml	1:100,000
2.0 ml	1:50,000
4.0 ml	1:25,000

Tryptone salt broths or agars (T_1N_0 and T_1N_1)
[Note: Formula from *Bacteriological Analytical Manual*, U.S. Food and Drug Administration.]

Trypticase or tryptone	10.0 g
NaCl	0 or 10.0 g
Agar	0 or 20.0 g
Distilled water	1000.0 ml

Preparation: Add agar if solid medium is to be made, omit for broth formulation. For the salt-free T_1N_0, do not add any NaCl. For the 1% NaCl formulation (T_1N_1), add 10 g NaCl. Dissolve ingredients in distilled water. Autoclave for 15 minutes at 121°C, and dispense into appropriate containers (tubes for broth, plates or tubes for agar). If agar formulations are made, cool to 50°C before pouring.

Quality control: Different peptones contain varying amounts of NaCl, which may affect the growth of organisms sensitive to NaCl concentration. Each peptone source must be quality controlled using a standard strain of *V. cholerae* and a halophilic organism.

Voges-Proskauer broth with 1% NaCl for vibrios

Peptone	7.0 g
K_2HPO_4	5.0 g
Glucose	5.0 g
NaCl	10.0 g
Distilled water	1000.0 ml

Preparation: Combine ingredients; adjust pH to 6.9. Dispense in 2-ml volumes in screw cap test tubes. Sterilize by autoclaving at 121°C for 10 minutes.

VP reagents A + B:

VP reagent A

Absolute ethanol	100.0 ml
Alpha-naphthol	5.0 g

VP reagent B

Potassium hydroxide (KOH)	40.0 g
Creatine	0.3 g
Distilled water to a volume of	100.0 ml

Separately prepare VP reagents A and B, stirring ingredients well to ensure complete mixing.

Procedure: After 48 hours of incubation of the test culture in MR-VP broth, add approximately 0.6 ml of MR-VP reagent A and 0.2 ml of MR-VP reagent B. Cap the tube, shake well, and let stand for up to 5 minutes. The production of a dark pink to cherry red color within 5 minutes is a positive test. No color development or yellow to orange color development within this time is a negative test.

D. Preparation of Reagents

Carbonate buffer, pH 9.6 (coating buffer for anti-CT ELISA)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

Bring volume to 1 liter with distilled water. Store at 4°C for up to 2 weeks.

Diethanolamine buffer (10%) for alkaline phosphatase substrate

Diethanolamine	97.0 ml
Sodium azide (NaN_3)	0.2 g
$MgCl_2 \cdot 6H_2O$	100.0 mg
Distilled water	800.0 ml

Preparation of diethanolamine buffer: Adjust the pH to 9.8 with 1 M HCl, and adjust volume to 1.0 liter. Store buffer in the dark at 4°C.

Safety note: In addition to being highly toxic, sodium azide combines with the salts of metals commonly used in plumbing (copper and lead) to form highly explosive shock-sensitive compounds. If azide-containing materials are regularly discarded in drains or sewers, metallic pipes must be periodically treated to remove the buildup of these compounds.

Preparation of alkaline phosphatase substrate: Remove an aliquot of the diethanolamine buffer (described above) and warm to room temperature before use. Dissolve 1 phosphatase substrate tablet (*p*-nitrophenyl phosphate, disodium, 5 mg tablet; Sigma Chemical Co., St. Louis, Mo.) in 5 ml diethanolamine buffer.

McFarland turbidity standards

Prepare a 1.75% solution (wt/vol) of barium chloride dihydrate ([$BaCl_2 \cdot 2H_2O$]; if using anhydrous barium chloride [$BaCl_2$], prepare a 1% aqueous solution). Prepare a 1% (by volume) solution of chemically pure sulfuric acid and mix the two solutions according to the volumes given in Table XI-3. Seal the tubes with wax or parafilm or by some other means to prevent evaporation. Refrigerate or store at room temperature in the dark for up to 6 months. Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube. The turbidity of a well-mixed tube will approximate the turbidity of the corresponding bacterial suspensions given in Table XI-3.

Oxidase reagent

Tetramethyl-*p*-phenylenediamine	1.0 g
Distilled water	100.0 ml

Preparation: Dissolve the reagent in distilled water (do not heat to dissolve). Alternatively, a 1% solution of dimethyl-*p*-phenylenediamine may also be used for the oxidase test. The reagent should be colorless when prepared and should be refrigerated in a glass-stoppered bottle, protected from light. To protect from light, the reagent may be stored in dark brown bottles, or if a clear glass bottle is used, it should be wrapped in aluminum foil or dark paper. Alternatively, the reagent may be dispensed in small amounts (2-5 ml) in screw cap vials and frozen until needed. Vials should not be refrozen after being thawed. With time, the reagent will

Table XI-3. Composition and corresponding bacterial concentrations of McFarland turbidity standards

Tube no.	Contents (ml)	
	Barium chloride (1.75%)	Sulfuric acid (1%)
0.5	0.5	99.5
1	0.1	9.9
2	0.2	9.8
3	0.3	9.7
4	0.4	9.6
5	0.5	9.5
6	0.6	9.4
7	0.7	9.3
8	0.8	9.2
9	0.9	9.1
10	1.0	9.0

slowly oxidize and turn blue. A blue tint to the reagent is acceptable; however, when the reagent alone gives a blue color on filter paper, it must be discarded.

Phosphate-buffered saline (PBS), 0.01 M, pH 7.2

Concentrated stock solution (50x):

Na_2HPO_4, anhydrous	54.8 g
$NaH_2PO_4 \cdot H_2O$	15.75 g
Distilled water to final volume of	1000.0 ml

Working (diluted) solution:

Stock solution (50x, above)	20.0 ml
NaCl	8.5 g
Distilled water to a final volume of	1000.0 ml

(Final pH should be 7.2 to 7.3.)
For PBS-Tween, add 0.5 ml Tween 20 to 1000 ml of PBS.

Sodium deoxycholate reagent (0.5%) for string test

Sodium deoxycholate	0.5 g
Sterile distilled water	100.0 ml

Preparation: Add sterile distilled water to sodium deoxycholate and mix well. Store at room temperature.

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