Comparison of Four Plating Media for Isolating *Vibrio cholerae*

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Two brands of thiosulfate citrate bile salts sucrose agar and Monsur taurocholate tellurite gelatin (TTG) agar were compared with two newly developed media, sucrose tellurite teepol agar and *Vibrio parahaemolyticus* agar for isolation and identification of *Vibrio cholerae*. The thiosulfate citrate bile salts sucrose and TTG agars were the best selective media, whereas sucrose tellurite teepol agar was the poorest. Both TTG and sucrose tellurite teepol agars were good for use in follow-up serological tests, whereas only TTG agar could be used for follow-up oxidase tests. In our opinion TTG agar has more advantages for cholera research laboratories routinely culturing large numbers of patients for cholera on a daily basis and where media needs can be accurately predicted. In contrast, in smaller clinical laboratories or in laboratories investigating epidemics, thiosulfate citrate bile salts sucrose agar is best because it is commercially available and easy to prepare and can be used to distinguish colonies of suspect *V. cholerae* from *V. parahaemolyticus*.

Most bacteriology laboratories in the United States, including the Center for Disease Control, use thiosulfate citrate bile salts sucrose (TCBS) agar to isolate *Vibrio cholerae*. Use of TCBS agar was first proposed in 1963 (7), and it is now recommended for diagnosis of cholera by the World Health Organization (16). Sucrose-fermenting organisms grown on TCBS are easily recognizable as large yellow colonies on a blue-green medium. This medium is highly selective and differentiating; few other organisms normally found in diarrheal stools grow on it, and, if they do, they are not likely to be confused with *V. cholerae*. The widespread use of TCBS is due to its availability in commercial dehydrated form, the fact that it does not have to be autoclaved (6), and the ease with which *V. cholerae* is recognized. The disadvantages are its relatively high cost, the difficulty of identifying *V. cholerae* by direct agglutination of colonies from the plates, and variations in the quality of the medium (9).

Another medium used in some laboratories, primarily at the Cholera Research Laboratory in Dacca, Bangladesh, is a medium developed by Monsur, taurocholate tellurite gelatin (TTG) agar (12). This medium, also recommended by the World Health Organization (16), requires autoclaving and is not commercially available. *V. cholerae* colonies grown on TTG are rela-

tively small (1 to 2 mm in diameter after overnight incubation and 3 to 4 mm after 48 h) and are translucent with a cloudy zone surrounding colonies. After 24 h, dark centers develop. In one comparative study, TCBS agar and TTG agar were found to be of equal efficacy for isolating *V. cholerae*, although TTG was found to be better for direct serological agglutination (6).

During 1977, two new plating media were introduced. The first was sucrose tellurite teepol (STT) agar (1), a medium in which teepol is used rather than bile salts and the sucrose concentration is relatively low. It is claimed that *V. cholerae* colonies are easily emulsified, thus permitting slide agglutination and oxidase tests to be performed on colonies directly from the plate. In a comparative study with TCBS agar, STT agar yielded slightly higher isolation rates of *V. cholerae* organisms when fecal specimens were plated directly (1). Like TCBS, this medium is said to be highly selective and does not require sterilization.

The other medium introduced in 1977 was VP agar. It was developed for isolation of *Vibrio parahaemolyticus*, but subsequent studies have shown it to be a reasonably good medium for isolating *V. cholerae* (4). The advantage to VP agar, according to the authors, is that it is inexpensive and easy to prepare.

The purpose of this study was to compare

TCBS agar (two brands), Monsur TTG agar, STT agar, and VP agar to determine the preferred plating media based on total number of positive specimens and the usefulness of colony morphology for the presumptive identification of *V. cholerae* strains. We also evaluated the media for conducting preliminary confirmation tests with colonies directly from the agar plates.

During an investigation of cholera cases on Guam in 1974, environmental strains of *V. cholerae* were discovered that appeared as sucrose-negative (green) colonies on TCBS agar, but later studies with sucrose fermentation broth revealed that these strains did ferment sucrose slowly (11). A review of the literature revealed only one previous report of sucrose-negative strains, and this was from the Cholera Research Laboratory (14). These strains are probably missed by workers who routinely use only TCBS agar and follow the procedure of selecting only sucrose-positive (yellow) colonies for identification as suspect *V. cholerae*. Because TTG agar does not use sucrose fermentation reactions to detect *V. cholerae*, these slow sucrose-fermenting strains would not be missed by workers using this medium, but they might not be recognized as slow sucrose fermenters. Another objective of this study was to determine the frequency of non-sucrose-fermenting or slow sucrose-fermenting *V. cholerae* by testing the sucrose-negative colonies from TCBS agar, STT agar, and VP agar.

MATERIALS AND METHODS

The study was conducted at the Cholera Research Laboratory during September through November 1977. The clinical specimens used were rectal swabs obtained each day from patients who visited the Cholera Research Laboratory hospital and had a history of acute watery diarrhea requiring rehydration therapy.

Plating media. TCBS agars were obtained from two commercial sources: Eiken TCBS agar lot NK504L from Tanabe Seiyaku Co., Ltd., Osaka, Japan and TCBS agar lot J6DCJT from Baltimore Biological Laboratory (BBL), Cockeysville, Md. They were prepared, boiled, and poured according to the instructions of the manufacturers.

The composition of the STT agar (1) was the corrected formula containing 1% sucrose and 0.5% NaCl (2) and was prepared as follows. A 1-g amount of beef extract (Difco Laboratories, Detroit, Mich.), 1 g of peptone (Difco), 1 g of sucrose, 0.5 g of sodium chloride, 0.2 ml of teepol (British Drug House, Bombay, India), 1 ml of a freshly made 0.05% solution of potassium tellurite, 2.5 ml of a freshly prepared 0.2% aqueous solution of bromothymol blue, and 2 g of agar (Difco) were added to 100 ml of distilled water. The medium was adjusted to pH 8.0, heated to boiling, and poured into petri plates.

The VP agar (4) was prepared as follows. A 1-g amount of peptone (Difco), 0.5 g of yeast extract

(Difco), 0.5 g of sodium taurocholate, 1.0 g of sodium thiosulfate, 2.0 g of sodium chloride, 0.02 g of sodium lauryl sulfate, 1.0 g of sodium citrate, 2.0 g of sucrose, 0.004 g of bromothymol blue, 0.004 g of thymol blue, and 2.0 g of agar were added to 100 ml of distilled water. The medium was adjusted to pH 8.6, heated to boiling, and poured into petri plates.

The TTG agar (12) was prepared as follows. A 1.0-g amount of Trypticase (BBL), 1.0 g of sodium chloride, 0.5 g of sodium taurocholate, 0.1 g of sodium carbonate, 3.0 g of gelatin, and 1.5 g of agar were added to 100 ml of distilled water. The medium was sterilized by autoclaving at 121°C for 15 min and cooled to 50°C, and potassium tellurite was added to a final concentration of 1:200,000 before the plates were poured.

Inoculation of plating media. Three rectal swabs were collected from each patient. Within 30 min the plates were inoculated by swabbing the agar surface in a circular fashion over an area about 1.5 inches (ca. 3.75 cm) in diameter. After inoculating the first plate, the swab was then rotated to expose a fresh side of the swab, and a second plate was inoculated. The second swab was used in a similar fashion to inoculate a third and fourth plate, whereas the third swab was used to inoculate the fifth plate. The sequence of inoculating the plates was rotated so that each plating medium was equally represented in being inoculated by swab 1, swab 2, or swab 3. Finally, the inoculated plates were streaked with a wire loop in order to get isolated colonies.

Picking the colonies. The plates were read and recorded after overnight incubation at 37°C, usually 18 h. Three suspect *V. cholerae* colonies, if present, were subcultured from each plate to Kligler iron agar (KIA) slants. From TCBS, STT, and VP agars, three sucrose-negative (blue-green) colonies were also subcultured. The KIA tubes were incubated overnight, and those slants showing reactions typical (alkaline slant, acid butt, no H₂S, no gas) of *V. cholerae* were confirmed by follow-up tests.

Confirmation of isolates. Isolates were confirmed as *V. cholerae* by three tests: (i) agglutination in *V. cholerae* 01 antiserum (Biological Products Division, Center for Disease Control), (ii) oxidase test by the method of Kovac (8), which consisted of a smear of fresh agar-grown culture on reagent-impregnated filter paper, read within 10 s, and (iii) string test which involves emulsifying growth from a culture in 0.5% sodium deoxycholate (15). The isolates were then biotyped into classical or El Tor biotypes by polymyxin B sensitivity (5) and sensitivity to classical phage IV (13).

Analysis of data. The comparison of the plating media was statistically analyzed by the Cochran Q test (3, 10), an extension of the McNemar test for more than two related samples. This test is designed for use when a number of treatments applied to the same group of subjects are compared.

RESULTS

The results of plating 157 fecal specimens on the five media are shown in Tables 1 and 2. A total of 88 were positive for *V. cholerae* on one or more media. The differences among the five

TABLE 1. *Evaluation of five plating media for isolation of V. cholerae from 157 specimens*

No. of specimens	Medium ^a				
	Eiken TCBS	BBL TCBS	STT	VP	TTG
68	+	+	+	+	+
3	+	+	+	-	+
8	+	+	-	+	+
5	+	+	-	-	+
2	+	-	-	+	+
1	+	-	+	-	-
1	-	-	-	-	+
69	-	-	-	-	-

^a STT agar was significantly different from TTG ($P < 0.01$), Eiken TCBS ($P < 0.01$), and BBL TCBS ($P < 0.05$), but not VP agar ($P > 0.9$). VP agar was not significantly different from TTG ($P > 0.05$), Eiken TCBS ($P > 0.05$), and BBL TCBS ($P > 0.9$). Probability multiplied by 10 to correct for multiple comparisons (Bonferroni's inequality correction).

^b Symbols indicate whether fecal specimen was positive or negative on indicated medium.

TABLE 2. *Summary of media comparisons with 88 positive specimens*

Agar medium	No. positive in each medium	% Of total
TTG	87	99
Eiken TCBS	87	99
BBL TCBS	84	95
VP	78	89
STT	72	81

media were statistically significant ($P < 0.001$). Monsur TTG agar and Eiken TCBS agar yielded the greatest percentage of positive specimens, although the differences between them and the BBL TCBS and VP agars were not statistically significant. The STT agar yielded the poorest results. The poor results of STT agar appeared to result from the poor selectivity of the medium, resulting in abundant overgrowth by competing non-*Vibrio* bacteria.

The media were also evaluated for use in serological confirmation with *V. cholerae* 01 antiserum by testing colonies directly from the agar plates (Table 3). Both STT agar and TTG agar were excellent for this purpose. The TCBS agars (both Eiken and BBL) and VP agar were unsatisfactory in that colonies of positive cultures reacted poorly on the slide agglutination tests because of difficulty in removing colonies from the plates and emulsifying the colonies on the slide. To determine whether the poor emulsification was due to the higher sucrose content (20 g/liter) in TCBS and VP agars, we tested the agglutinability of nine cultures on STT agar modified to contain 20 g of sucrose per liter. The

results were similar to those on STT agar plates containing 10 g.

The media were also evaluated for conducting the oxidase tests directly from colonies on the agar plates. Colonies from all of the TTG agar plates gave strong positive results in the oxidase tests; the other four media gave poor results (Table 4).

Examination of stools resulted in the picking and evaluation of 132 sucrose-negative colonies from 54 patients. None of the isolates was *Vibrio* species.

DISCUSSION

The TCBS and TTG agars were preferred over STT and VP agars for primary isolation of *V. cholerae* from fecal specimens collected during this study in Bangladesh. The characteristics of the four types of media are compared in Table 5. Both TCBS agar and TTG agar had excellent selectivity, whereas STT agar was definitely inferior in selectivity. With TTG agar and STT agar, colonies could be confirmed in the serolog-

TABLE 3. *Slide agglutination reaction on individual colonies directly from primary plating media with Vibrio cholerae 01 antiserum*

Agar medium	Reaction ^a					2+ or stronger reaction	
	Negative	1+	2+	3+	4+	No.	%
Eiken TCBS	20	31	36	8	1	45/96	47
BBL TCBS	15	24	41	13	2	56/96	58
STT	2	1	2	30	54	86/89	97
VP	12	15	43	15	1	59/86	69
TTG	1	5	7	28	55	90/96	94

^a Reactions: negative, no detectable agglutination; 1+, poor emulsification, questionable agglutination, or agglutination delayed more than 1 min; 2+, poor emulsification but definite agglutination; 3+, homogenous suspension, almost complete or slightly delayed agglutination; 4+, homogenous suspension, rapid complete agglutination.

TABLE 4. *Oxidase reaction in 13 isolates on individual colonies directly from primary plating media*

Agar medium	No. with the following reaction: ^a			
	Strong positive	Weak	Very weak	Negative
Eiken TCBS	0	1	0	12
BBL TCBS	0	0	1	12
STT	0	1	4	8
VP	0	0	0	13
TTG	13	0	0	0

^a Strong, weak, very weak, and negative indicate the intensity of color reaction.

TABLE 5. Comparison of the characteristics of TCBS, TTG, STT, and VP agars

Character of medium	Agar			
	TCBS	TTG	VP	STT
Selectivity of <i>Vibrio</i> sp.	Excellent	Excellent	Good	Fair
Use for follow-up serological tests	Fair	Excellent	Fair	Excellent
Use for follow-up oxidase tests	Poor	Excellent	Poor	Poor
Indicator of sucrose fermentation	Yes	No	Yes	Yes
Gelatinase zone around colony	No	Yes	No	No
Commercially available	Yes	No	No	No
Requires autoclaving	No	Yes	No	No

ical test by using growth directly from the agar plates, but the oxidase test could be done only from the TTG agar. The TCBS agars and VP agar were unsuitable for the oxidase test and only fair for the serological test. The reason for the negative serological results with colonies from TCBS agar and the VP agar plates was poor emulsification of the colonies. When the colonies were emulsified, they usually agglutinated (although this was frequently delayed). This confirms previous reports that colonies grown on TCBS agar may agglutinate slowly or weakly (16), as well as previous observations in our own laboratories. Another advantage of TCBS agar as well as STT and VP agars is the indicator for sucrose fermentation. It allows a microbiologist to know, when the plates are read, whether the colonies are suspect *V. cholerae* (yellow colony, sucrose fermenter) or suspect *V. parahaemolyticus* (green colony, non-sucrose fermenter). TTG agar contains gelatin, and the zone of gelatinase activity around the colony is advantageous in picking colonies, especially from crowded areas. The disadvantages of TTG, VP, and STT agars are that they are not commercially available and must be prepared from individual ingredients, which might lead to variation in quality between laboratories. An autoclave is required for sterilization of TTG agar. Either TCBS or TTG agar is preferred over VP and STT agars. In our opinion TTG agar has more advantages for the cholera research laboratory routinely culturing large numbers of patients for cholera on a daily basis. In this situation setting up for media preparation presents no problem because large numbers of plates are used daily, and media needs can be accurately predicted. On the other hand, in smaller clinical laboratories or in laboratories investigating epidemics, TCBS is advantageous because of its commercial availability, simplicity and rapidity of preparation, and ability to distinguish suspect

V. cholerae and *V. parahaemolyticus* by colony appearance.

STT agar was a poor medium for isolating *V. cholerae* because of its poor selectivity, which is in contrast to the report by Chatterjee et al. (1), who found that it yielded almost pure growth of *Vibrio*. It was equivalent to TTG agar for the serological confirmation of colonies from plates but, unlike Chatterjee et al., we found it unsuitable for the confirmatory oxidase test.

VP agar, although not designed specifically for isolating *V. cholerae*, gave indications of being a better selective medium than STT (although the difference was not statistically significant). This work tends to support the results of De et al. (4), who found VP agar slightly inferior to TCBS agar for isolating *V. cholerae*. VP agar is identical to TCBS except for the composition of bile salts, the amount of NaCl, and the presence of sodium lauryl sulfate in VP agar. It might be improved for isolating *V. cholerae* without compromising its usefulness for *V. parahaemolyticus* by lowering the NaCl content to 1 or 1.5%.

The occurrence of sucrose-negative *V. cholerae* does not appear to be common in Bangladesh; none was found in this study. Previous studies in which sucrose-negative strains were found (11, 14) may represent unusual occurrences.

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