Enhancement by Sodium Chloride of the Selectivity of Thiosulfate Citrate Bile Salts Sucrose Agar for Isolating *Vibrio cholerae* Biotype El Tor

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In this study we utilized the salt-tolerant characteristics of vibrios to develop a more selective medium by addition of NaCl to thiosulfate citrate bile salts sucrose (TCBS) agar. The effect of adding salt to TCBS agar varied greatly among brands of TCBS agar and between lots of the same brand. The addition of salt at concentrations as high as 1.5% (2.5% total NaCl) caused the inhibition of growth of three species of commonly encountered normal bowel flora and one strain of classical *Vibrio cholerae* but did not compromise the use of TCBS agar for isolation of *V. cholerae* biotype El Tor.

Cholera is a major international public health problem. The disease, caused by *Vibrio cholerae*, during the past decade has spread from Indonesia to Asia, the Middle East, Europe, and Africa (2). During the past few years there have been major epidemics of cholera in Italy (1, 5) and in Portugal (4), with six cases reported in Guam (3) and one case in Texas—the first case of cholera in the United States, with the exception of laboratory-acquired illness, in over 50 years (8). The disease is most frequently a problem in developing countries lacking modern sanitary facilities. However, it is becoming clear that there will be imported cases in developed countries from time to time (9); thus, all diagnostic bacteriology laboratories must be able to isolate and identify the organism quickly and accurately.

An excellent selective agar medium which allows good recovery of *Vibrio* organisms and inhibits most normal fecal flora has been developed; this is thiosulfate citrate bile salts sucrose (TCBS) agar. This medium has been produced commercially in Japan, since its development in 1963 (Eiken Chemical Co.), and since 1966 has been produced commercially by two companies in the United States. (Difco Laboratories and the BBL Division of Becton-Dickinson & Co.). We have previously evaluated the three brands of this medium and found striking variation in the ability of these media to inhibit normal fecal flora, not only among brands but

also among various lots of the same brand (6). Optimal selectivity of isolation media is very important in the investigation of cholera. This is due to the urgency for rapid diagnosis and to the fact that cholera usually occurs in areas with minimal laboratory facilities.

The objective of this study was to use NaCl to improve the selectivity of TCBS agar. This paper describes our studies on elevation of the salt content of TCBS agar and its effect on the growth of pathogenic vibrios and on normal fecal bacteria.

MATERIALS AND METHODS

Dehydrated TCBS agar was purchased from Eiken Chemical Co., Becton-Dickinson & Co. (BBL), and Difco Laboratories and was rehydrated by suspending 86 g (BBL and Eiken) and 89 g (Difco) in 1 liter of water. The Center for Disease Control TCBS agar was prepared by suspending 5 g of yeast extract, 10 g of proteose peptone no. 3, 10 g of sodium citrate, 10 g of sodium thiosulfate, 8 g of oxgall, 20 g of sucrose, 10 g of sodium chloride, 1 g of ferric citrate, 0.04 g of bromothymol blue, 0.04 g of thymol blue, and 15 g of agar in 1 liter of distilled water. All the TCBS agars alone or with added amounts of NaCl were heated to boiling until completely dissolved and were then poured into petri dishes without further sterilization.

All cultures used in this study were isolated from fecal specimens cultured during laboratory investigations of diarrheal diseases. The bacterial species referred to as normal bowel flora were strains of *Klebsiella*, *Proteus*, and enterococci representative of frequently occurring non-vibrios isolated from TCBS agar during an earlier investigation (6). They were held in agar medium for 2 to 6 months before use in this study. The *Vibrio parahaemolyticus*

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strains were isolated in an outbreak of foodborne disease in the United States (7). They were received soon after the epidemic and lyophilized for 1 year. The classical strains of *V. cholerae* were isolated from patients in India and Pakistan and held for 7 to 21 years. The *V. cholerae* El Tor strains were isolated from patients in Indonesia, Viet Nam, and Iran and held for 1 to 8 years. The *V. cholerae* strains (both classical and El Tor) were held on agar medium until 1969, when they were lyophilized. The non-cholera vibrio strain was a stock culture of unknown source.

For use in this study, cultures were inoculated into heart infusion broth and incubated overnight at 37°C. Overnight broth cultures were quantitated by preparing serial 10-fold dilutions in physiological saline and inoculating 0.1 ml of each dilution onto heart infusion agar. The inoculum was spread evenly on the agar surface with a bent glass rod and incubated overnight at 37°C. The colonies were counted, and the total number of organisms in the culture was calculated.

Cultures were inoculated onto TCBS agar by streaking 0.1 ml of heart infusion broth culture on the agar surface. After overnight incubation at 37°C, the presence or absence of growth was recorded. Quantitative studies with TCBS agar were conducted by plating 0.1 ml of appropriate 10-fold dilutions in physiological saline. The TCBS agar plates were incubated at 37°C for 24 h, and the colony counts were multiplied by the dilution factor.

RESULTS

The effect of adding 0, 0.5, 2.0, 3.5, and 5.0% NaCl to TCBS agar on the growth of *V. cholerae* and two common fecal contaminants, *Klebsiella* and *Proteus*, is shown in Table 1. All strains were inhibited when the TCBS agar contained 2% or more of added NaCl. A subsequent study indicated that TCBS agar with 1.5% added NaCl did not inhibit *Vibrio* strains (Table 2).

The results found when undiluted broths of classical *V. cholerae* (four strains), *V. cholerae* biotype El Tor (four strains), *V. parahaemolyticus* (three strains), *Proteus* (three strains),

TABLE 1. Effects of added NaCl to TCBS agar (Difco 554984) on growth of *V. cholerae* and on common fecal contaminants *Klebsiella* and *Proteus*

Organism	Inoculum	Added NaCl (%)				
		0.0	0.5	2.0	3.5	5.0
<i>Proteus</i>	7×10^7	+	+	—	—	—
<i>Klebsiella</i>	9×10^7	+	+	—	—	—
<i>V. cholerae</i>	1×10^6	+	+	—	—	—
Pool ^b		+	+	—	—	—

^a Sign indicates presence (+) or absence (—) of growth.

^b Pooled inoculum contained 7×10^4 *Proteus*, 9×10^4 *Klebsiella*, and 1×10^3 *V. cholerae*.

TABLE 2. Effects of added NaCl to TCBS agar (Difco 554984) on growth of *V. cholerae* and common fecal contaminants

Culture	Inoculum	Added NaCl (%)				
		0.0	0.5	1.0	1.5	3.5
<i>V. cholerae</i>	2×10^3	+	+	+	+	—
	2×10^7	+	+	+	+	—
Non-cholera Vibrio	6×10^3	+	+	+	+	—
	6×10^7	+	+	+	+	—
<i>Proteus</i>	3×10^4	+	+	—	—	—
	3×10^8	+	+	—	—	—
<i>Klebsiella</i>	8×10^3	+	—	—	—	—
	8×10^7	+	+	—	—	—

^a Sign indicates presence (+) or absence (—) of growth.

Klebsiella (three strains), and enterococci (three strains) were plated on multiple lots of three brands of TCBS agar and onto TCBS agar prepared at the Center for Disease Control and the same media to which 1.5% of NaCl had been added (2.5% total NaCl) are shown in Table 3. Some normal fecal bacteria were suppressed on every brand by the added salt. However, one strain of classical *V. cholerae* was inhibited when salt concentrations of one lot each of Difco and BBL TCBS were increased.

Results of quantitative studies to determine the effect of added salt on growth of *V. cholerae*, *V. cholerae* biotype El Tor, *V. parahaemolyticus*, *Klebsiella*, *Proteus*, and enterococci are given in Table 4. Three commercial brands of TCBS agar and TCBS agar prepared from ingredients at the Center for Disease Control were used with and without 1.5% added NaCl. In the case of Eiken TCBS, *Vibrio* species were suppressed by less than 1 log. Normal fecal flora was inhibited both with or without added salt. With Difco TCBS agar, added salt completely suppressed the growth of *Klebsiella* and *Proteus* but also caused the suppression of growth of classical *V. cholerae* (by 3 to 5 logs), although this suppression was not always consistent from experiment to experiment, even with the same lots of media. Very little effect was noted on *V. cholerae* biotype El Tor, *V. parahaemolyticus*, and enterococci. With BBL TCBS agar, added salt suppressed *Proteus*, but some suppression was also noted in all three *Vibrio* species. With the media prepared from basic ingredients, the effect of added salt caused only slight suppression of vibrios. *Klebsiella* strains were inhibited, but *Proteus* strains and enterococci were not.

DISCUSSION

In a prior study (6), we described brand-to-

TABLE 3. Comparison of growth of pathogenic vibrios versus normal fecal flora on TCBS agar with and without added NaCl

Media source	Lot no.	NaCl added (%)	<i>V. cholerae</i> (classical) (4) ^a	<i>V. cholerae</i> (El Tor) (4)	<i>V. parahaemolyticus</i> (3)	<i>Proteus</i> (3)	<i>Klebsiella</i> (3)	<i>Enterococcus</i> (3)
CDC	—	0	4 ^b	4	3	3	3	3
		1.5	4	4	3	3	0	3
Eiken	TC102E	0	4	4	3	0	0	0
		1.5	4	4	3	0	0	0
Eiken	M002P	0	4	4	3	3	0	3
		1.5	4	4	3	0	0	2
Difco	496296	0	4	4	3	3	0	3
		1.5	3	4	3	0	0	2
BBL	701638	0	4	4	3	3	0	2
		1.5	4	4	3	0	0	1
BBL	106601	0	4	4	3	0	0	2
		1.5	3	4	3	0	0	0

^a Number of strains tested.^b Number of strains that grew on TCBS agar plates when plated with 0.1 ml of an overnight broth culture.TABLE 4. Comparison of total plate counts of *Vibrio* organisms and normal fecal flora on TCBS agar containing normal and elevated NaCl concentrations

Culture	Strain no.	Heart infusion agar	TCBS agar									
			Eiken TC102E		Difco 554984		BBL 701638		BBL 106601		CDC	
			No NaCl added ^a	1.5% NaCl added	No NaCl added	1.5% NaCl added	No NaCl added	1.5% NaCl added	No NaCl added	1.5% NaCl added	No NaCl added ^b	1.5% NaCl added
<i>V. cholerae</i>	65	8 × 10 ⁷	7 × 10 ⁵	1 × 10 ⁵	1 × 10 ⁵	0 ^c	4 × 10 ⁵	0	1 × 10 ⁵	0	2 × 10 ⁷	7 × 10 ⁶
<i>V. cholerae</i>	236	2 × 10 ⁸	1 × 10 ⁸	1 × 10 ⁷	5 × 10 ⁷	1 × 10 ⁴	6 × 10 ⁷	8 × 10 ⁶	2 × 10 ⁷	4 × 10 ⁶	1 × 10 ⁸	6 × 10 ⁷
<i>V. cholerae</i>	27	8 × 10 ⁷	1 × 10 ⁷	6 × 10 ⁶	3 × 10 ⁷	1 × 10 ³	4 × 10 ⁷	4 × 10 ⁶	3 × 10 ⁷	1 × 10 ⁸	9 × 10 ⁷	1 × 10 ⁸
<i>V. cholerae</i>	569B	8 × 10 ⁷	1 × 10 ⁷	3 × 10 ⁶	3 × 10 ⁶	4 × 10 ³	3 × 10 ⁶	1 × 10 ⁵	2 × 10 ⁶	1 × 10 ²	2 × 10 ⁷	3 × 10 ⁶
<i>V. cholerae</i> El Tor	15	7 × 10 ⁸	6 × 10 ⁸	2 × 10 ⁸	6 × 10 ⁸	1 × 10 ⁸	7 × 10 ⁸	5 × 10 ⁸	6 × 10 ⁸	1 × 10 ⁷	7 × 10 ⁸	3 × 10 ⁸
<i>V. cholerae</i> El Tor	464	3 × 10 ⁸	3 × 10 ⁸	1 × 10 ⁸	2 × 10 ⁸	1 × 10 ⁷	4 × 10 ⁸	8 × 10 ⁷	1 × 10 ⁸	8 × 10 ⁶	4 × 10 ⁸	3 × 10 ⁸
<i>V. cholerae</i> El Tor	4418	5 × 10 ⁸	6 × 10 ⁸	3 × 10 ⁸	5 × 10 ⁸	4 × 10 ⁸	6 × 10 ⁸	2 × 10 ⁸	6 × 10 ⁸	3 × 10 ⁸	6 × 10 ⁸	4 × 10 ⁸
<i>V. cholerae</i> El Tor	116	4 × 10 ⁸	3 × 10 ⁸	3 × 10 ⁸	3 × 10 ⁸	1 × 10 ⁸	3 × 10 ⁸	7 × 10 ⁷	3 × 10 ⁸	1 × 10 ⁷	5 × 10 ⁸	3 × 10 ⁸
<i>V. parahaemolyticus</i>	MD7	4 × 10 ⁷	1 × 10 ⁶	5 × 10 ⁶	1 × 10 ⁷	8 × 10 ⁶	1 × 10 ⁷	5 × 10 ⁶	2 × 10 ⁷	1 × 10 ⁶	2 × 10 ⁷	2 × 10 ⁷
<i>V. parahaemolyticus</i>	MD14	6 × 10 ⁸	1 × 10 ⁸	2 × 10 ⁷	2 × 10 ⁸	5 × 10 ⁷	3 × 10 ⁸	4 × 10 ⁷	2 × 10 ⁸	1 × 10 ⁷	3 × 10 ⁸	3 × 10 ⁸
<i>Klebsiella</i>	E45	4 × 10 ⁸	0	0	1 × 10 ³	0	0	0	0	0	2 × 10 ²	0
<i>Klebsiella</i>	E46	4 × 10 ⁸	0	0	1 × 10 ⁸	0	0	0	0	0	1 × 10 ⁵	0
<i>Klebsiella</i>	E47	4 × 10 ⁸	0	0	2 × 10 ⁷	0	0	0	0	0	1 × 10 ³	0
<i>Proteus</i>	E39	7 × 10 ⁸	0	0	8 × 10 ⁷	0	1 × 10 ³	0	0	0	1 × 10 ⁸	1 × 10 ⁷
<i>Proteus</i>	E40	5 × 10 ⁸	0	0	3 × 10 ⁷	0	1 × 10 ⁴	0	0	0	1 × 10 ⁸	9 × 10 ⁶
<i>Proteus</i>	E41	5 × 10 ⁸	0	0	1 × 10 ⁸	0	0	0	0	0	1 × 10 ⁸	5 × 10 ⁶
<i>Streptococcus</i>	28314	3 × 10 ⁸	0	0	1 × 10 ³	1 × 10 ³	1 × 10 ⁵	1 × 10 ³	1 × 10 ³	1 × 10 ³	4 × 10 ⁸	3 × 10 ⁸
<i>Streptococcus</i>	30714	1 × 10 ⁸	0	0	1 × 10 ³	1 × 10 ³	1 × 10 ³	1 × 10 ³	1 × 10 ³	0	3 × 10 ⁷	4 × 10 ⁷
<i>Streptococcus</i>	32630	5 × 10 ⁸	0	0	1 × 10 ³	1 × 10 ³	1 × 10 ⁵	1 × 10 ³	1 × 10 ³	1 × 10 ³	3 × 10 ⁸	2 × 10 ⁸

^a TCBS agar normally contains 1.0% NaCl.^b Medium was prepared at the Center for Disease Control (CDC) (see Materials and Methods). The indication of no added NaCl indicates that this medium contained the normal concentration of NaCl (1.0%).^c Zero indicates that there was no growth when 0.1 ml of undiluted broth was inoculated on media.

brand and lot-to-lot variation in the ability of TCBS agar to inhibit organisms which may be found in the normal fecal flora, especially *Klebsiella*, *Proteus*, and enterococci. This prompted us to conduct this study in which we have attempted to utilize the salt-tolerant characteristics of vibrios to develop a more selective medium by the addition of salt to TCBS agar.

This study indicates that when available supplies of commercial TCBS are found not to be adequately inhibitory for non-vibrio organisms, salt at concentrations as high as 1.5% (2.5% total NaCl) may be added to the medium to suppress normal bowel flora and facilitate isolation and identification of *V. cholerae* El Tor. As in our previous study, we again found that, in general, TCBS agar made by Eiken Chemical Co. was more effective than Difco TCBS agar and BBL TCBS agar in inhibiting organisms other than *Vibrio*. The effect of adding salt to TCBS agar varied greatly among brands of TCBS agar and between lots of the same brand. Very striking suppression of organisms other than *Vibrio* was noted in some cases. However, all four classical strains of *V. cholerae* were inhibited to some extent on Difco and BBL TCBS agar with added salt, and one classical strain of *V. cholerae* did not grow at all on Difco and BBL TCBS agar containing 1.5% added salt. The addition of salt did not compromise the use of TCBS for the isolation of *V. cholerae* biotype El Tor or *V. parahaemolyticus*. Since *V. cholerae* biotype El Tor is responsible for much of the cholera in the world today, the suppression of the classical *V. cholerae* strains by TCBS with added salt does not appear to be a problem.

An important consideration in the interpretation of these data, however, is the fact that this study was conducted with laboratory strains, some of which had been held for many years. The ultimate test of the usefulness of adding NaCl to TCBS agar requires corrobora-

tion of these data in a clinical setting.

The addition of NaCl to TCBS agar should only be carried out in laboratories capable of conducting appropriate quality control on the modified medium. The ultimate responsibility of modifying TCBS agar is that of the commercial manufacturers of this medium. The clinical laboratory microbiologist should not have to modify this medium to improve the selectivity for pathogenic vibrios.

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