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A non-pathogenic vibrio for the routine quality control of TCBS cholera medium

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SUMMARY A non-pathogenic 'indicator' organism to replace *Vibrio cholerae* in the routine quality control of TCBS medium was sought among a large collection of freeze-dried vibrios isolated mostly from environmental sources. One strain, which was consistently more sensitive to inhibition of growth on TCBS medium than strains of *V. cholerae* and *V. parahaemolyticus*, is recommended for this purpose. It has been deposited with, and is available from, the National Collection of Type Cultures as NCTC 11218.

For the isolation of certain bacterial pathogens, especially from faeces, selective media are usually necessary, and it is important that they should be consistently reliable. In the routine quality control of such media it is also important to consider the potential hazards of laboratory-acquired infections from pathogens and ideally to replace them by non-pathogenic 'indicator' organisms. For the isolation of *Vibrio cholerae* in Britain, thiosulphate citrate bile-salt sucrose (TCBS) agar[1] is usually employed now, but different batches and brands of this medium may vary considerably in their inhibitory activity and selectivity.[2 3] For routine quality control purposes, a search was therefore made among a large collection of vibrios for a suitable non-pathogenic strain which would virtually guarantee that TCBS plates would support the growth of minimal numbers of *V. cholerae*, *V. parahaemolyticus*, and other vibrios from clinical and environmental material. We describe the work done and the particular strain recommended for checking the suitability of TCBS medium for the isolation of pathogenic vibrios from such material.

Material and methods

ORGANISMS

Four laboratory strains of *V. cholerae*, including the neotype and the recommended working type, were used as controls throughout the study. After preliminary work, eight initial test strains were selected for comparison with the controls from a large collection of freeze-dried *Vibrio* species derived mostly from cultures submitted for confirmation and serological typing in a survey of the distribution of *V. parahaemolyticus* in British coastal waters.[4] These species were divided into five groups, based on the fermentation of sucrose, salt tolerance, and indole, Voges-Proskauer, and decarboxylase reactions, as follows: (1) *V. parahaemolyticus*; (2) *V. alginolyticus*; (3) non-cholera vibrios; (4) salt-tolerant vibrios; and (5) halophilic vibrios. The initial test strains were chosen at random from these groups; two from each of groups 1, 4, and 5, and one each from the smaller groups 2 and 3. Later, 35 strains from the most promising group—halophilic vibrios—were examined further. The growth of one of these strains, regarded as potentially suitable for the routine quality control of TCBS medium, was then compared with that of three recent isolates of *V. cholerae* and four of *V. parahaemolyticus*. In the later stages of the work, strains of *Escherichia coli*, *Proteus vulgaris*, and *Streptococcus faecalis* isolated from clinical material were used to check the ability of batches of TCBS medium then available to suppress their growth.

MEDIA

A total of 12 batches of TCBS medium from four sources was used: (A) BBL B9DEKV; (B) DIFCO 629969; (C) EIKEN N1208C; (D) OXOID 56 19026; (E) OXOID 323 20453; (F) OXOID 293 22087; (G) OXOID 96 19639; (H) OXOID 207 18860; (I) OXOID 334 14238; (J) OXOID 295 9024; (K) BBL 19DFGY; (L) BBL B9DEKW.

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Each of these was prepared in Petri dishes according to the manufacturers' instructions. Blood agar (Oxoid CM 271 with 2% horse blood) was employed throughout as a non-selective and non-inhibitory control medium.

PROCEDURES

Counts of viable organisms were made by the method of Miles and Misra.[5] Test and control strains of vibrios were inoculated from growth on blood agar plates into tubes of nutrient broth (Oxoid No. 2) containing 2% added NaCl. After incubation overnight at 37°C, serial 10-fold dilutions of the broth cultures were prepared in quarter-strength Ringer solution. With a calibrated Pasteur pipette, 0·02 ml of a range of dilutions, usually from 10^{-3} to 10^{-8}, was inoculated on to each of four identical plates of TCBS medium from each batch. Dilutions from 10^{-4} to 10^{-9} were inoculated similarly on to four blood agar plates. After incubation at 37°C overnight the numbers of colonies formed were counted, and the mean of each set of four plates was calculated. The mean counts were then adjusted so as to represent the same inoculum size for all strains in each experiment.

Acid production from sucrose was determined on TCBS medium and in 1% tryptone water with 2% added NaCl. Survival and growth of vibrios in alkaline peptone water, without extra NaCl, was tested by inoculation of 20 ml with one loopful from nutrient salt broth cultures. After incubation for 4 hours one loopful from the surface was inoculated into a second alkaline peptone water, and a loopful was also plated on TCBS medium. After incubation overnight at 37°C another TCBS plate was inoculated with a loopful from the surface of the second alkaline peptone water. Any inhibitory effects were assessed visually from differences in growth on the plates of TCBS medium.

Results

After preliminary work the collection of vibrios was divided into five groups. Strains of *V. parahaemolyticus*, *V. alginolyticus*, and non-cholera vibrios were first identified, and the remaining strains were then regarded as either salt-tolerant vibrios or as halophilic vibrios according to their degree of tolerance to salt. The initial eight test strains from these groups were compared on nine different kinds of TCBS medium. The limits of their growth, after adjustment to represent equal inocula, are shown in Figure 1. The strains of salt-tolerant vibrios and non-cholera vibrios varied in their sensitivity to inhibition compared with the four control strains of *V. cholerae*, while those of *V. parahaemolyticus* and

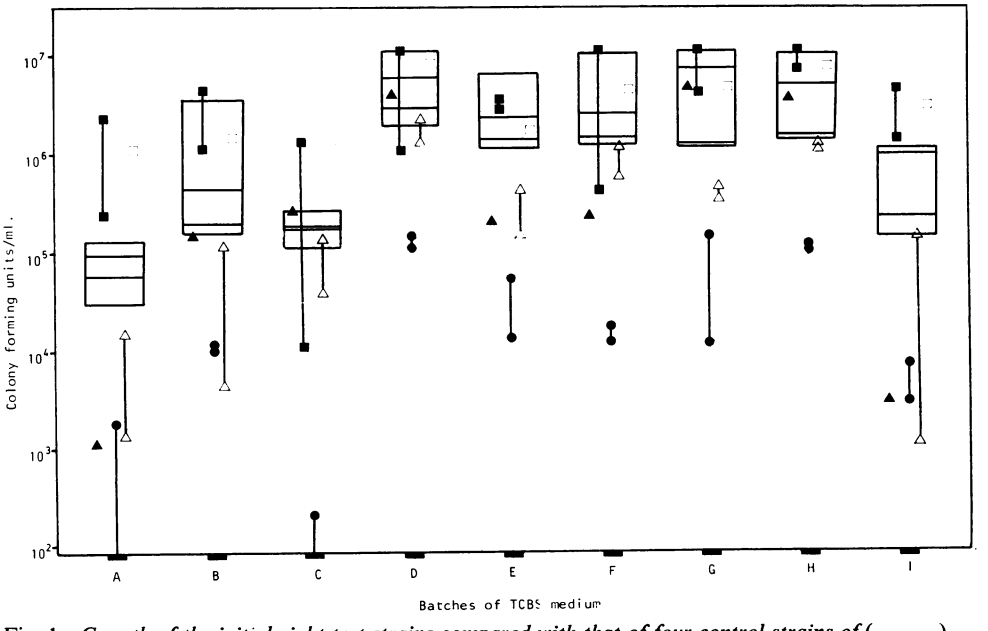

Fig. 1 *Growth of the initial eight test strains compared with that of four control strains of* (———) V. cholerae *on TCBS medium,* (▲) *non-cholera vibrio,* (■) V. parahaemolyticus, (●) *halophilic vibrio,* (△) *salt-tolerant vibrio,* (□) V. alginolyticus. *Inoculum for each strain*: $1{\cdot}6 \times 10^7$ *cfu/ml.*

V. alginolyticus were consistently less sensitive. In contrast, both the halophilic vibrio strains were consistently more sensitive to inhibition than the control strains of *V. cholerae*. All the strains in this group were therefore examined further. Thirty-five strains were identified which produced acid from sucrose and, of these, 15 survived passage through alkaline peptone water. Accordingly, the growth of these 15 halophilic strains was compared with that of the control strains of *V. cholerae* on the nine

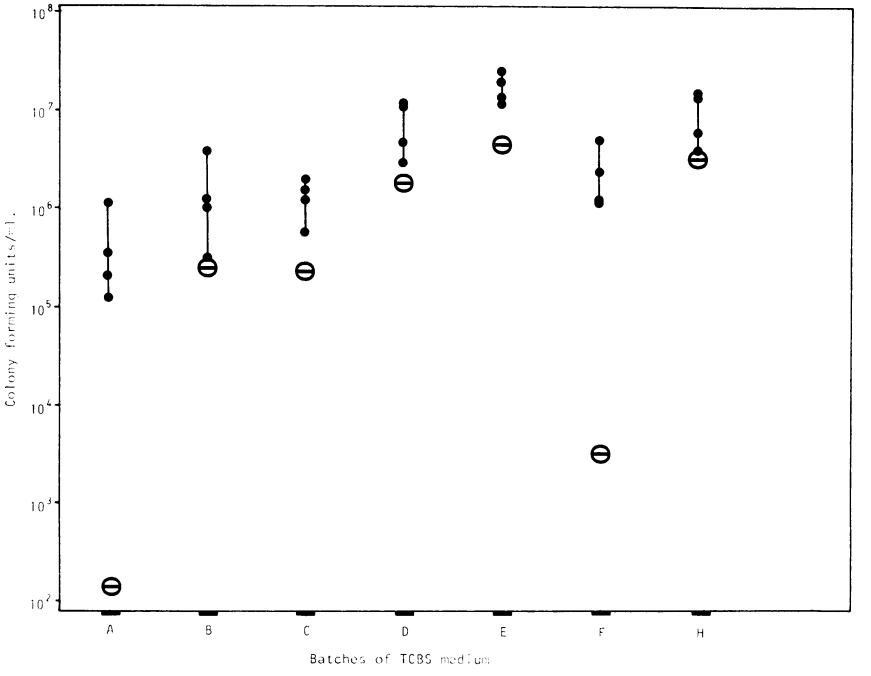

Fig. 2 *Growth of* (⊖) *vibrio strain 2510/74 compared with that of four control strains of* (●) V. cholerae *on TCBS medium. Inoculum for each strain: 9·5 × 10⁷ cfu/ml.*

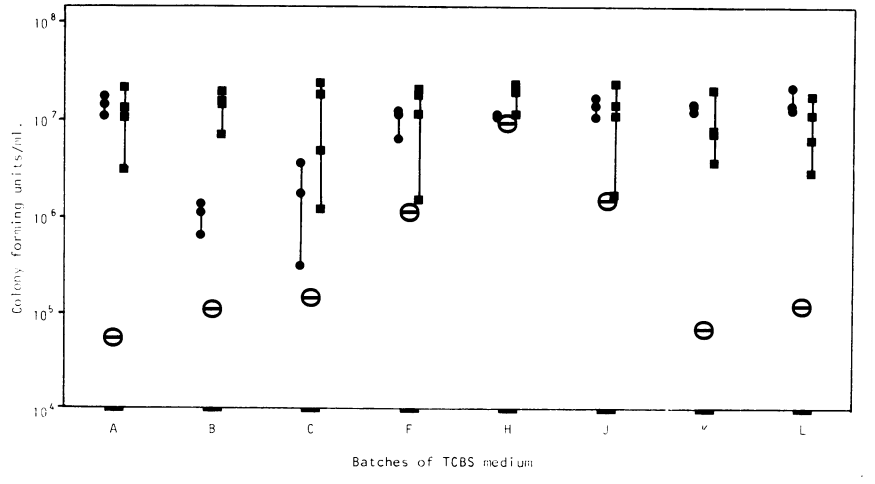

Fig. 3 *Growth of* (⊖) *vibrio strain 2510/74 recommended for quality control of TCBS medium compared with that of three recent isolates of* (●) V. cholerae *and four of* (■) V. parahaemolyticus. *Inoculum for each strain: 4·5 × 10⁷ cfu/ml.*

different batches of TCBS medium. Two of these test strains (2510/74 and 4446/75) emerged as consistently more sensitive to inhibition of growth than the control strains. The remaining strains varied considerably in their sensitivity, and they were therefore rejected as unsuitable for the quality control of TCBS media. Strain 2510/74 was the more useful of the two selected halophilic vibrios as it followed closely the pattern of sensitivity to inhibition of the control strains of *V. cholerae* (Fig. 2). The growth of strain 2510/74 was then further compared with those of three recent isolates of *V. cholerae* and four of *V. parahaemolyticus*—two clinical Kanagawa-positive and two environmental Kanagawa-negative strains (Fig. 3). In repeated experiments, this test strain consistently showed less growth than all of these recent isolates, thus confirming its suitability for quality control purposes. It was subsequently characterised (Table) as a member of '*Vibrio* sp. group F', biotype 2.

Discussion

Inhibitory activity of the media was measured by inoculation of TCBS and blood agar plates with 10-fold dilutions of broth cultures of both the test and control strains and a comparison of the resulting viable colony counts. As the two initial test strains of halophilic vibrios, unlike those from the other four groups (Fig. 1), both grew less well on all the batches of TCBS medium than the four control strains of *V. cholerae* used as controls, this seemed the most promising group for further investigation. All the halophilic vibrios in the collection were therefore

Biochemical characteristics of strain 2510/74 (Vibrio *sp. group F*)

Growth at 5°	−	Single carbon sources:	
25°	+	Alanine	+
30°	+	Arginine	+
37°	+	Asparaginine	+
42°	+	Aspartic acid	+
		Glutamic acid	+
Growth in tryptone plus salt 0%	−	Glutamine	+
0·5%–8%	+	Glycine	−
10%	−	Histidine	+
Growth aerobically	+	Leucine	−
anaerobically	+	Lysine	−
Growth on CLED	+	Proline	+
MacConkey	+	Serine	+
Catalase	+	Threonine	+
Oxidase	+	Adonitol	−
Swarming	−	Arabinose	+
Motility	+	Cellobiose	−
Nitrate reduction	+	Dulcitol	−
Nitrite reduction	−	Galactose	+
Simmons' citrate	−	Glucose	+
Luminescence	−	Glycerol	+
Urease	−	Inositol	−
Gelatinase	+	Lactose	−
Chitinase	+	Maltose	+
Amylase	−	Mannitol	+
Lipase	+	Mannose	+
Alginase	−	Raffinose	−
Lecithinase	+	Rhamnose	−
Malonate	−	Salicin	+
Phenylalinine	−	Sorbitol	−
ONPG	+	Starch	+
Arginine dihydrolase	+	Sucrose	+
Lysine decarboxylase	−	Trehalose	+
Ornithine decarboxylase	−	Xylose	−
Methyl red	+	Formate	−
Voges-Proskauer	−	Acetate	+
Gluconate	−	Pyruvate	+
Indole	−	Succinate	+
Glucose acid	+	α-Ketoglutarate	+
gas	+	Butyrate	−
Glycerol acid	+	Gluconate	+
gas	+	Glucuronate	−
Sucrose acid	+	Propionate	−
Sensitivity:		Ethanol	+
0/129* 150 μg S		Propanol	−
10 μg R		Putrescine	+
1% methylene blue S		p-Hydroxybenzoate	−
		Caproate	−

*2,4-diamino-6,7-diisopropyl-pteridine phosphate.

tested for acid production from sucrose and survival in alkaline peptone water so that the strain finally selected would yield yellow colonies on TCBS medium like *V. cholerae*, and could also be used to demonstrate cholera enrichment and isolation techniques. Although the two halophilic vibrio test strains were eliminated at this stage because they did not survive passage through alkaline peptone water, 15 other halophilic strains satisfied both these criteria. Their growth was compared with that of the control strains of *V. cholerae*, and the response was encouraging: two strains were consistently more sensitive to inhibition of growth on TCBS medium, and these were re-examined against the four control strains of *V. cholerae*. This confirmed the observation that one of them (strain 2510/74) was clearly more suitable (Fig. 2). It followed the pattern of sensitivity of the *V. cholerae* strains much more closely than the other which was considerably more sensitive to inhibition, and would therefore lead to the unnecessary rejection of some batches of TCBS medium as unsuitable for routine use.

At this stage comparisons had been made only against laboratory reference strains of *V. cholerae* and *V. parahaemolyticus*. As there is always a possibility that some of their characteristics have altered during storage, it was important to compare the selected test strain 2510/74 with some recent isolates of *V. cholerae* and of *V. parahaemolyticus*. These comparisons (Fig. 3) confirmed the previous results that this halophilic vibrio was likely to be suitable for routine TCBS quality control purposes. It had already been in storage for a considerable time and had undergone several subcultures; it was therefore more likely to have undergone adaptive changes to laboratory conditions. It has been deposited with and is available from the National Collection of Type Cultures as NCTC 11218. Characterisation of this strain showed that it belonged to biotype 2 of a group of vibrios recently designated 'group F'.[6 7] This group of vibrios is becoming recognised as a separate phenon.[8] The aerogenic biotype 2 is regarded as an environmental organism with no known pathogenic role.

For satisfactory quality control of selective media such as TCBS it is important that a non-selective medium such as blood or nutrient agar should be inoculated similarly for comparison of subsequent growth. For this purpose, the most accurate though rather laborious technique is viable colony counts from serial dilutions. A less accurate but more rapid method is to subculture standard loopfuls of broth cultures on to both control and test plates. A practicable alternative tried successfully in this laboratory is the use of combined half-plates. These are prepared by cutting diametrically across blood-agar plates with a sterile blade, removing half the agar, and then filling with the TCBS agar under test. A loopful of the test culture is then inoculated across the join; after overnight incubation the degree of inhibition is readily apparent on the TCBS side of the plate.

In this study the pattern of sensitivity of the vibrios tested could not be related to the degree of suppression of faecal flora on the different TCBS media, as judged either by heavy inocula of faeces or with clinical isolates of *E. coli*, enterococci, and *Proteus* species. In general, certain makes of TCBS were better at suppressing growth of non-vibrio organisms, and others were less inhibitory to recent isolates of *V. cholerae* and *V. parahaemolyticus* or to laboratory-adapted strains (Figs 1 to 3). Ideally, maximum recovery of vibrios from clinical material should be combined with maximum selectivity, and the routine use of the non-pathogenic strain described in this paper for the quality control of TCBS media may also help to achieve this objective.

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