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Gentamicin-Based Medium for the Isolation of Group D Streptococci and Application of the Medium to Water Analysis†

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Gentamicin-thallous-carbonate (GTC) medium contained (per liter): 40.0 g of Trypticase soy agar, 5.0 g of KH_2PO_4, 2.0 g of $NaHCO_2$, 1.0 g of glucose, 1.0 g of esculin, 0.5 g of thallous acetate (TA), 0.5 g of ferric citrate, 0.75 ml of Tween 80, and 2.5 mg of gentamicin sulfate. The $NaHCO_3$ (20 ml of a 10% solution that had been heated to boiling) was added after sterilization of the basal medium. The spread plate technique was used to compare GTC agar with Pfizer selective enterococcus, TA, and KF agars by using sewage as well as bovine and swine fecal samples. Significantly greater numbers of group D streptococci were recovered on GTC agar than on Pfizer selective enterococcus or KF agars, within and over all samples. Higher counts also were obtained on GTC than on TA agar, but the differences were not statistically significant. The percentage of false positives was about the same for all four media. Samples of riverwater also were plated on GTC, TA, and KF agars, and significantly higher recoveries were obtained with GTC agar. GTC agar was superior to the other media examined primarily because of increased recoveries of *Streptococcus bovis* and *S. equinus*; other advantages of GTC agar were large colony size and short (24-h) incubation period. The percentage of false positives from riverwater was 13% for GTC agar and 0% for TA and KF agars; therefore, confirmation would be necessary when GTC agar is used with some types of environmental samples.

Numerous media have been proposed for the isolation of group D streptococci from water (12). Nearly all these media contain sodium azide as a selective agent. Azide-containing media possess disadvantages for certain applications, however, because of the instability of azide (12) and the failure of some streptococci, such as *Streptococcus bovis,* to initiate growth on media that contain azide (4, 18, 22). Media containing other selective agents also have been developed (12), but these media were designed primarily for the isolation of enterococci (*S. faecalis* and *S. faecium*). Recovery on non-enterococcal group D streptococci (*S. bovis, S. equinus,* and *S. avium*) and other fecal streptococci (*S. mitis* and *S. salivarius*) has not been given priority. To identify the source of fecal pollution in water by the species of group D streptococcus present, and to more accurately estimate numbers of group D streptococci in water, a medium was needed on which all group D streptococci could grow.

Black and Van Buskirk (3) developed a blood-agar medium containing 5.5 μg of gentamicin per ml for use in isolating beta-hemolytic streptococci; growth of staphylococci and nearly all gram-negative bacilli was inhibited on this medium. Dilworth et al. (7) used a similar medium to detect pneumococci in respiratory secretions. The favorable results obtained when gentamicin was used to select for various cocci (3, 7) indicate that a suitable medium containing gentamicin might be developed for isolation of group D streptococci from natural environments. The purpose of this investigation, therefore, was to devise and evaluate a gentamicin-based medium for the selective isolation of group D streptococci.

MATERIALS AND METHODS

Cultures and samples. Ten cultures were obtained from the culture collection of Paul A. Hartman: *S. faecalis* 4082 and 4083, *S. faecium* R206 and 5403R, *S. faecium* (*durans*) LP5H and PAH105, *S. bovis* Tm/C/101 and 2B, *S. equinus* T2, and *S. uberis* 01155. *S. avium* A and B were obtained from Robert H. Deibel, Department of Bacteriology, University of Wisconsin, Madison. Stock cultures were kept on Trypticase soy agar (TSA) plates stored at 4°C in plastic vegetable crispers. Working cultures were made

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by inoculating tubes of heart infusion broth, which were incubated overnight at 35°C and stored at room temperature for no longer than 1 week.

Three types of fecal samples were obtained: (i) sewage, collected from the influent of the Ames, Iowa, Wastewater Treatment Plant; (ii) swine waste samples, from a confinement lagoon at Iowa State University; and (iii) bovine fecal samples, obtained as solid waste from the Iowa State University cattle barns and suspended in 0.1% peptone water. All samples were processed within 2 h of collection.

Water samples were collected from widely separated rural and urban sites on the Des Moines and Raccoon rivers in west central Iowa. Detailed descriptions of the sampling sites are available (L. S. Donnelly, M.S. thesis, Iowa State University, Ames, 1976). The samples, provided by the Sanitary Engineering Section, Engineering Research Institute, Iowa State University, were placed on ice and processed within 8 h after collection.

Plating media and procedures. Gentamicin-thallous-citrate (GTC) medium was developed by first testing stock cultures on TSA that contained different levels of gentamicin and then examining a number of combinations of other chemicals that would impart additional selectivity to the medium. Details of this extensive series of tests (Donnelly, M.S. thesis) will not be reported here. The final formula of GTC medium was (per liter): 40.0 g of TSA (Baltimore Biological Laboratory, Cockeysville, Md.), 5.0 g of KH_2PO_4, 2.0 g of $NaHCO_3$, 1.0 g of glucose, 1.0 g of esculin, 0.5 g of thallous acetate (TA), 0.5 g of ferric citrate, 0.75 ml of Tween 80 (polyoxyethylene sorbitan monooleate), and 2.5 mg of gentamicin sulfate (Schering Corp., Kenilworth, N.J.). A gentamicin stock solution was prepared in sterile water to contain 1.0 mg/ml and stored at 4°C for up to 3 months (3); 2.5 ml was used per liter when media were prepared. All the constituents except the $NaHCO_3$ were sterilized together at 121°C for 15 min. After the medium was cooled to 55°C, 20 ml of 10% $NaHCO_3$ in water (made fresh and pasteurized by boiling) was added to each liter and mixed before plates were poured.

Gentamicin and TA were the major selective agents in GTC agar. Addition of $NaHCO_3$, Tween 80, and KH_2PO_4 resulted in stimulation of group D streptococci as reported by others (8, 13). Without these agents, laboratory cultures of *S. bovis, S. equinus,* and *S. avium* were inhibited by levels of gentamicin and TA necessary for appropriate selectivity. The concentration of KH_2PO_4 was increased from the 0.2% level used by Lachica and Hartman (14) to maintain a pH (measured after plates were dried overnight) of 6.8 to 7.0. Esculin was included in GTC agar because all group D streptococci reportedly hydrolyze esculin (5); in the presence of ferric citrate, dark halos of iron salts surround esculin-hydrolyzing colonies. Colony size and numbers also were increased when esculin and ferric citrate were added to the medium; these effects were accentuated when 0.1% glucose was added.

Selective media used as controls were: Pfizer Selective Enterococcus (PSE) medium (Pfizer, Inc., New York, N.Y.), KF agar (BBL), and Barnes (2) TA agar. A 0.1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; ICN Pharmaceuticals, Inc., Cleveland, Ohio) was sterilized by filtration through a 0.22-μm membrane filter and stored at 4°C in the dark for use in the media. In some experiments, however, TTC was omitted.

In most experiments, dilutions of the samples were prepared in 0.1% peptone water, and inocula were surface plated in triplicate. After each inoculum was spread with a sterile, bent glass rod, plates were incubated at 35°C for 18 to 24 h unless otherwise noted. Colonies were counted according to standard procedures (1).

Identification media and procedures. To determine whether the bacteria that formed colonies on the experimental and control media were fecal streptococci, 10 colonies (or as many colonies as were available) were randomly picked from each medium and streaked on TSA containing 0.01% TTC and the same proportions of KH_2PO_4, $NaHCO_3$, glucose, and Tween 80 as described for GTC agar. After incubation for 18 to 24 h at 35°C, several isolated colonies of similar color and morphology were suspended in 1 ml of modified Minitek (BBL) inoculum broth. The formulation of Minitek inoculum broth, as given by the manufacturer (7.5 g of peptone, 0.25 g of L-tryptophan, and 0.59 g of HEPES [*N*-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid] buffer per liter), was modified by the addition of 0.1% yeast extract; the yeast extract was necessary to prevent the occurrence of false-negative tests when isolates were examined. A 96% correlation with conventional biochemical tests was obtained when the modified inoculum broth was used.

The modified Minitek inoculum broth was used to inoculate tests for esculin hydrolysis and fermentation of arabinose, glycerol, lactose, mannitol, raffinose, sorbitol, and sucrose, which were performed by using the BBL Minitek system. Tryptose phosphate broth (5 ml; Difco) also was inoculated. The broth was incubated for 18 to 24 h at 35°C and used to inoculate other test media. The bile-esculin test was made on Swan (21) bile-esculin agar, except that horse serum was excluded from the formulation. Starch agar contained 0.2% soluble starch (17) and 0.1% glucose (9). Bile agar (40%; without blood), 6.5% NaCl broth, 5% sucrose agar, and media for fermentation of inulin and sorbose were prepared as described by Facklam (9, 10). Pyruvate fermentation and arginine dihydrolase tests were those of Gross et al. (11). A wet mount also was made of each culture and was examined with a phase-contrast microscope.

All tests were incubated at 35°C. Starch and 5% sucrose agar plates, and results of the arginine dihydrolase tests, were read at 24 h. A catalase test also was done at this time by adding 3% H_2O_2 to growth on 5% sucrose agar. Bile-esculin and bile agar plates, inulin, pyruvate, and sorbose fermentation tests, and growth in the presence of 6.5% $NaCl_2$, were examined presumptively at 24 h and finally at 72 h (unless otherwise noted). Results of the Minitek tests were recorded presumptively at 24 h and finally at 48 h.

An identification schema was developed (Fig. 1), following the reports of Facklam (9) and Gross et al. (11). Tests included in the schema were based on usefulness, convenience of medium preparation and test performance, and ease of interpretation. Since many isolates were examined, the number of tests necessary for presumptive species identification was

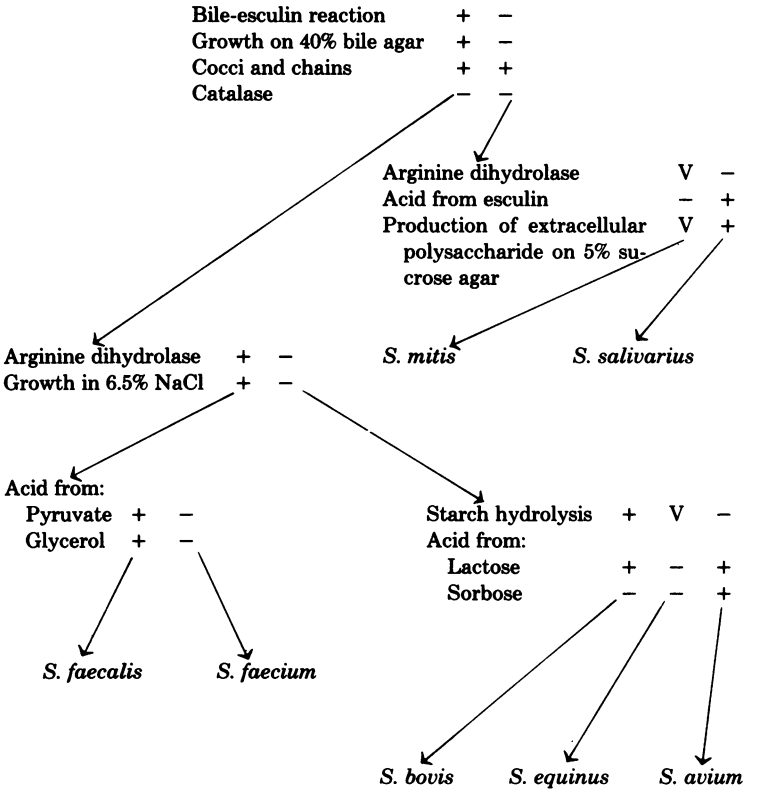

FIG. 1. *Diagram demonstrating the use of key tests for the identification of fecal streptococci.*

minimized. Furthermore, dependence was placed on a spectrum of characteristics (6) rather than on complete adherence to the schema, because both control cultures and isolates from environmental samples often varied in one or more specific tests.

RESULTS

Comparative growth of stock cultures. To compare the ability of selective media to recover specific species, four laboratory cultures were plated on five media. The results of a typical experiment are shown in Table 1. Except for the inability of *S. bovis* and *S. equinus* strains to grow on the azide-containing media (KF and PSE agars), approximately the same numbers of colonies were recovered on each medium.

Fecal sample tests. Sewage, swine, and bovine samples were plated on GTC, TA, KF, and PSE agars; the TA and KF media did not contain TTC. The highest counts were obtained on GTC agar (Table 2); an analysis of variance (19) revealed that there were significant differences ($P = 0.05$) between GTC versus KF and GTC versus PSE agars, both within samples and over all samples. Differences existed between counts on GTC and TA agars, but the differences were not statistically significant.

TABLE 1. *Mean counts ($\times 10^6$) of control organisms per milliliter recovered on four selective media and TSA*

Medium	Control organisms			
	S. faecalis 4082	*S. faecium* R206	*S. bovis* Tm/C/ 101	*S. equinus* T2
TSA	2,100[a]	970	1,100	66
GTC	2,400	1,100	980	36
TA	2,200	1,000	1,000	51
KF	2,100	1,000	—[b]	—
PSE	1,900	840	—	—

[a] Mean counts were calculated from the average number of colonies on three plates of the appropriate dilution.

[b] —, Counts were less than 10^6/ml, and colonies were too small to count.

Table 2 also shows the percentages of each species and/or biotype recovered on the four media. No *S. faecalis* strains were isolated on GTC agar, but this was because of the vast preponderance of other biotypes in the samples and not the inability of GTC agar to support the growth of *S. faecalis*. On the other hand, PSE agar was very selective; thus, a substantial por-

TABLE 2. *Mean counts and percentages of species, unidentifiable strains, and false-positive isolates recovered from sewage, swine, and bovine samples on four selective media*[a]

Parameter measured	Sewage count in medium:				Swine count in medium:				Bovine count in medium:			
	GTC	TA	KF	PSE	GTC	TA	KF	PSE	GTC	TA	KF	PSE
Mean count ($\times 10^4$)	22	12	3	6	1,300	850	140	51	1,800	1,300	1,200	900
S. faecalis	0	3	3	24	0	4	0	3	0	0	0	0
S. faecium	54	66	63	59	33	46	60	59	0	3	14	34
Starch$^+$ *S. bovis*	18	3	7	10	30	8	3	0	76	62	61	62
Starch$^-$ *S. bovis*	0	24	20	3	0	0	0	3	0	0	0	0
S. equinus	0	0	0	0	27	21	13	3	21	17	11	0
S. avium	0	0	0	0	0	8	0	0	0	0	0	0
S. mitis	21	0	0	3	0	4	0	3	0	0	4	0
Unidentifiable[b]	7	3	7	0	10	8	23	28	3	17	11	3
Percent false positives	7	0	0	3	0	17	0	3	3	3	7	3

[a] The experiment was repeated three times; three plates were counted per replication. Mean counts are averages of the three replications. Counts are per milliliter for sewage and swine samples and per gram (wet weight) for bovine samples. Ten colonies were examined from each medium for each of the three replications; 30 colonies were examined from each medium for each sample type.

[b] These possessed properties of fecal streptococci but did not conform closely to any described (5) species.

tion of colonies from the sewage sample were *S. faecalis*. Similarly, with the sewage sample, a large proportion of colonies that grew on PSE agar were *S. faecalis;* none was observed on GTC agar, because most strains in the bovine sample were *S. bovis* and *S. equinus* biotypes that grew on GTC but not on PSE agar. GTC agar most effectively recovered *S. bovis, S. equinus,* and *S. mitis* strains. The few *S. avium* strains that were recovered grew on TA agar. No *S. salivarius* was isolated. All media were more than 93% selective for fecal streptococci except when swine samples were plated on TA agar. False positives recovered on GTC agar were catalase-positive, motile rods; false positives recovered on control media were catalase-positive cocci.

Water sample tests. Water samples were plated on GTC, TA, and KF agars. The water samples were collected at eight urban and rural sites on the Des Moines River that would be representative of different point-sources of pollution (sewage outflows, hog feedlots, and cattle feedlots). The goals were to determine the relative efficiencies of the media in affecting recovery of fecal streptococci and in ability to differentiate between point-sources of surface-water pollution. PSE agar was not used because of the very low recoveries obtained when swine samples were plated (Table 2).

The highest average counts for the eight sampling sites were obtained on GTC agar (200 colony-forming units [CFU]/ml), which was significantly ($P = 0.0001$) more efficient than TA (48 CFU/ml) or KF (5 CFU/ml) agars.

Isolates obtained from sample locations 2, 3, and 6 were identified to species. Two locations were rural; location 2 was above Des Moines on the Des Moines River and location 6 was on the Raccoon River. Samples obtained at location 3, below the city, should have contained a flora indicative of human wastes. Species distributions are shown in Table 3. We expected that the largest percentages of *S. faecalis* would be isolated from location 3 because effluent from the Des Moines Water Pollution Control Plant entered the river 2 miles upstream from sampling point 3, and the contributions of streptococci from a meat processing plant in Des Moines should have been relatively minor. Likewise, samples from location 3 should have contained the lowest percentages of starch$^-$ *S. bovis* and *S. equinus*. This did not occur; however, significantly higher recoveries were demonstrated by using GTC agar.

GTC agar was 87% selective overall (mean of data obtained from all three locations), compared with 10% for TA and KF agars (Table 3). Most of the false positives on GTC agar came from location 2; all were catalase-positive, motile rods.

DISCUSSION

Gentamicin has a broad spectrum of activity, is stable over wide ranges of pH and temperature, and can withstand sterilization in an autoclave. These characteristics make it an ideal agent for use in selective media such as GTC agar. Enterococci (15), and presumably other streptococci (5, 6), present a permeability barrier to aminoglycosides. Thus, low levels of gentamicin are not toxic to streptococci unless another antibiotic is present that facilitates deterioration of the permeability barrier. The mechanism of action of TA, a second selective agent of GTC agar, is not known. Pavlova et al. (16) stated,

TABLE 3. *Mean counts and percentages of species, unidentifiable strains, and false-positive isolates recovered from water samples obtained from locations 2, 3, and 6 and plated on three selective media*[a]

Parameter measured	Location 2 count in medium:			Location 3 count in medium:			Location 6 count in medium:		
	GTC	TA	KF	GTC	TA	KF	GTX	TA	KF
Mean count per milliliter	110	14	1	800	74	54	420	150	14
S. faecalis	5	0	0	7	10	10	3	4	13
Atypical *S. faecalis*	0	0	0	0	0	3	0	4	4
S. faecium	38	95	100	48	57	73	33	93	61
Starch$^+$ *S. bovis*	10	0	0	30	13	10	13	0	9
Starch$^-$ *S. bovis*	14	5	0	0	0	0	1	0	0
S. equinus	5	0	0	11	7	0	23	0	0
S. avium	0	0	0	0	3	0	0	0	0
Unidentifiable[b]	29	0	0	11	0	3	23	0	13
Percent false positives	30	0	0	10	0	0	0	0	0

[a] The mean counts reported are averages of three replications, three plates per replication. Ten colonies were examined from each medium for each of the three replications of the experiment. A total of 30 colonies were examined for each medium from each sample. The locations are described in the text.

[b] These possessed properties of fecal streptococci but did not conform to any described (5) species.

however, that catalase-negative bacteria are more resistant than catalase-positive bacteria to TA. Hence, GTC agar selects for gentamicin-resistant, catalase-negative bacteria.

GTC agar was superior to TA, KF, and PSE agars for the enumeration of fecal streptococci in fecal and surface-water samples (Tables 2 and 3). This superiority was caused by increased recoveries of *S. bovis* and *S. equinus* biotypes (Tables 1 to 3). Switzer and Evans (22) previously observed that azide-containing media were inhibitory to *S. bovis*. The major contribution of our work, therefore, was the development of a selective, direct-plating medium upon which most biotypes of fecal streptococci present in environmental samples would initiate growth and form colonies. Furthermore, the use of GTC agar revealed that the streptococcal flora of fecal samples and surface waters are much more diverse than other investigators had reported or suspected. The short incubation time (18 to 24 h) and large size of colonies produced on GTC agar were further advantages of the medium. These features were especially desirable when using miniaturized differentiation or confirmation tests that rely upon substantial inocula to perform the tests.

It was hoped that more adequate differential tests might be incorporated into GTC agar so that the number of false positives could be decreased. The esculin hydrolysis reaction partly fulfilled this requirement, but additional ways of distinguishing species would be desirable. TTC reduction has been used traditionally for this purpose and seems to yield qualitative indications of species present. The incorporation of TTC into GTC agar, however, was undesirable because the TTC was inhibitory to some fecal streptococci (Donnelly, M.S. thesis). Solberg and Proctor (20) flooded plates with 0.1% TTC after primary incubation and, in most instances, reduction of TTC occurred within 1 h. A preliminary examination of this technique with GTC agar indicated that it had merit; flooding the plates with TTC, however, eluted colonies and precluded picking colonies for further testing. To avoid these problems, 0.1% TTC was sprayed on GTC-agar plates. This technique was effective, and TTC reduction occurred within 15 min.

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