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Evaluation of Differential and Selective Media for Isolation of *Aeromonas* and *Plesiomonas* spp. from Human Feces

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We studied nine solid and two liquid media for their suitability to select *Aeromonas* and *Plesiomonas* spp. from human stools, using artificially contaminated samples as well as 254 samples from outpatients with and without diarrhea. Media with optimal sensitivity and specificity for *Aeromonas* spp. were alkaline peptone-water, Trypticase soy broth with ampicillin, inositol-brilliant green-bile salts agar, dextrin-fuchsin-sulfite agar, xylose-sodium desoxycholate-citrate agar, and Pril-xylose-ampicillin agar. For *Plesiomonas* sp., alkaline peptone-water and inositol-brilliant green-bile salts agar were optimal. Four strains of *Aeromonas* spp. were detected in patient samples with these media.

Organisms of the primarily waterborne species *Aeromonas hydrophila, A. sobria*, and *Plesiomonas shigelloides* have been isolated from the stools of patients with diarrhea (8, 11, 16, 18), but they have also been isolated from nondiarrheic populations, albeit at a low rate (1, 13, 16–18). The usual enteric media, such as MacConkey agar and eosin methylene blue agar, are not suitable for selection of these organisms since both genera include lactose-negative as well as lactose-positive strains (17). Several media that are selective or differential (or both) have been developed for these bacteria (see Table 1), but only two have thus far been tested for sensitivity and specificity with stool cultures (10, 11). We decided, therefore, to investigate nine agar and two broth media for their suitability in detecting *Aeromonas* and *Plesiomonas* spp. in human stools. The characteristics of two sets of media were published after completion of this study (5; D. Hoban, W. Forsyth, C. Gratton, and T. Williams, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1981, C4, p. 263) and were not included in the experiments.

(Some of the results were presented at the Meeting of the Swiss Society for Microbiology, Chur, Switzerland, and published in abstract form [C. Bucher and A. von Graevenitz, Experientia 37:1217, 1981].)

MATERIALS AND METHODS

Strains. *A. hydrophila* (21 strains), *A. sobria* (12 strains), and *P. shigelloides* (10 strains) were used. Their sources are described elsewhere (A. von Graevenitz and C. Bucher, Infection, in press).

Media. As nonselective media, blood agar (Trypticase soy agar with 5% sheep blood; BBL Microbiology Systems, Cockeysville, Md.) and MacConkey agar (BBL) were used. The other media tested are listed in Table 1 (all abbreviations of medium names are defined in Table 1). With the exceptions of DFS and IBB (*Aeromonas* differential agar and *Plesiomonas* agar, respectively; both from E. Merck AG, Darmstadt, Germany), all were prepared as described in the original publications. Dyes and chemicals used were obtained from E. Merck AG; ampicillin trihydrate was obtained from Beecham AG, Berne, Switzerland.

Identification. Morphologies of *Aeromonas, Plesiomonas*, and coliform colonies on the media tested are listed in Table 2. Colonies suggestive of *Aeromonas* or *Plesiomonas* strains were picked and tested for oxidase on filter paper saturated with tetramethyl-*p*-phenylenediamine dihydrochloride. Colonies showing acid formation were subcultured to blood agar before oxidase testing to avoid false-negative reactions (4). Only IBB was directly flooded with the reagent to differentiate *Plesiomonas* strains from coliforms (14). The final identification was accomplished with the API 20E system (Analytab Products International S.A., Geneva, Switzerland); differentiation between *A. hydrophila* and *A. sobria* was done by the method of Popoff and Véron (7). Salt requirement (3) was tested by growth in Trypticase soy broth (BBL) with 6.5% NaCl.

Growth inhibition by media. Strains were grown overnight in Trypticase soy broth diluted $1:10^3$ and plated in 0.001-ml amounts on the media to be tested. Readings were done after 48 h at 37°C.

Detection ability of media. Stools with coliform counts between 1×10^7 and 3×10^8/g (as determined by serial dilution on MacConkey agar) were obtained from normal individuals, diluted 1:10, and divided into several portions. We used one portion as a control and added test strains to the others (one received ampicillin-resistant, lysine-negative *A. hydrophila* and one received *P. shigelloides*; both strains had been grown in Trypticase soy broth) in various ratios to the coliform counts. When solid media were tested, these ratios started at 1:10, and 0.001-ml amounts of the mixture were inoculated onto each plate, which was then incubated for 48 h at 37°C. When liquid media were tested, ratios started at $1:10^3$, and 1.0-ml

TABLE 1. Media that are selective or differential (or both) for *Aeromonas* and *Plesiomonas* spp.

Medium (abbreviation)	Inhibitor(s)	Differential substance(s)	Original purpose[a]	Reference
Dextrin-fuchsin-sulfite agar (DFS)	Sodium sulfite, fuchsin	Dextrin	Water (A)	13
DNase-toluidine blue-ampicillin agar (DNTA)	Ampicillin (30 mg/liter)	DNase (toluidine blue)	Stool (A)	18
Inositol-brilliant green-bile salts agar (IBB)	Brillant green, bile salts	Inositol	Stool (P)	14
Peptone-beef extract-glycogen agar (PBG)	Sodium lauryl sulfate	Glycogen	Multipurpose (A)	6
Pril-xylose-ampicillin agar (PXA)	Pril, ampicillin (30 mg/liter)	Xylose	Stool (A)	11
Rimler-Shotts agar (RS)	Citrate, sodium desoxycholate, novobiocin (5 mg/liter)	Lysine, ornithine, maltose	Multipurpose (A)	15
Rippey-Cabelli agar (RC)	Sodium desoxycholate, ethanol, ampicillin (20 mg/liter)	Trehalose	Water (A)	10
Salt-starch-xylose-lysine-sodium desoxycholate agar (SSXLD)	Sodium desoxycholate, citrate, NaCl (1.5%)	Lysine, starch, xylose	Stool (V)	12
Xylose-sodium desoxycholate-citrate agar (XDC)	Citrate, sodium desoxycholate	Xylose	Stool (A)	16
Alkaline peptone-water (APW)	pH 8.6		Stool (A)	16
Trypticase soy-ampicillin broth (TSBA)	Ampicillin (30 mg/liter)		Stool (A)	18

[a] A, *Aeromonas* spp.; P, *Plesiomonas* spp.; V, *Vibrio* spp.

amounts were inoculated into 5-ml portions of each broth, which were incubated for 24 h at 37°C and then subcultured in 0.001 ml to solid media.

Field studies. Stool specimens (254) received in Cary and Blair transport medium (1:5; BBL) from outpatients with and without diarrhea were studied for the presence of *Aeromonas* and *Plesiomonas* spp. by inoculating 0.001-ml suspensions diluted 1:10 onto various selective and enrichment media as described above.

RESULTS

Effects of ampicillin and toluidine blue on *Aeromonas* and *Plesiomonas* spp. Although all strains grew on Mueller-Hinton agar and DNase test agar (BBL), addition of 30 mg of ampicillin per liter inhibited 4 of 12 *A. sobria* strains and 9 of 10 *P. shigelloides* strains. Addition of 0.005% toluidine blue to DNase test agar did not inhibit any of the *Aeromonas* strains. Addition of 0.01% toluidine blue inhibited one *A. sobria* strain. A combination of 30 mg of ampicillin per liter and 0.005% toluidine blue inhibited five *A. sobria* and three *A. hydrophila* strains and was chosen for our experiments.

Elimination of media. The degree to which *P. shigelloides* was inhibited by ampicillin made

TABLE 2. Colony appearances on various media

Medium	*Aeromonas* strains	*Plesiomonas* strains	Coliforms
DFS	Dark red, large, turbid halo	Very small, bright red, light halo	Bright red with light halo or colorless
DNTA	Halo of decolorization	No halo[a]	No halo
IBB	Colorless	Whitish to pinkish	Greenish or pink
PBG	Yellow, typical[b,c]	Yellow, atypical[b,c]	Yellow, atypical[b,c]
PXA	Colorless	Colorless[a]	Yellow
RS	Yellow	Greenish yellow	Greenish yellow
RC	Yellow	Yellow[b]	Yellow
SSXLD	Yellow, halo[c]	Yellow	Yellow
XDC	Colorless	Colorless	Red

[a] Unless inhibited by ampicillin.
[b] See reference 6.
[c] See text.

TABLE 3. Inhibition of coliform flora in 23 normal stools by media for *Aeromonas* and *Plesiomonas* spp.

Medium	No. of stools showing inhibition of:[a]			
	<1 log	1–<2 log	2–<3 log	≥3 log
DSF	23	0	0	0
IBB	23	0	0	0
RS	16	3	2	2
RC	14	3	3	3
XDC	5	6	5	7
DNTA	6	1	2	14
PXA	6	1	1	15

[a] As compared with colony counts on MacConkey agar.

the use of PXA, RC, and TSBA impractical as selective media for this species. DNTA and RS were also unsuitable for *P. shigelloides* because of the lack of DNase activity and the presence of decarboxylases for lysine and ornithine in this species (17). In initial experiments, such a substantial amount of normal stool flora indistinguishable from *Aeromonas* and *Plesiomonas* spp. grew on PBG that it was decided to eliminate this medium. Likewise, SSXLD was eliminated because starch hydrolysis by *Aeromonas* strains could not be clearly seen. DNTA was, for the time being, retained for *Aeromonas* strains, however, since inhibition of these strains was only partial and the agar had been used successfully in earlier experiments (18).

Inhibition of coliforms. Results for 23 normal stools are shown in Table 3. As expected, ampicillin-containing media (PXA, DNTA, and RC) were more inhibitory than media that contained only inhibitors of gram-positive bacteria (DFS, IBB, and RS), but XDC was an exception, possibly because of its citrate content. The less-inhibitory quality of RC was probably due to the lower ampicillin concentration.

Inhibition of *Aeromonas* and *Plesiomonas* spp. Compared with MacConkey agar (on which all strains grew), DFS and RC were not inhibitory, whereas RS and DNTA reduced growth of one-third to one-half of the *Aeromonas* strains (Table 4). The growth of one *Plesiomonas* strain was reduced on DFS and XDC.

Recovery of *Aeromonas* and *Plesiomonas* strains from artificially contaminated stools. Sensitivity and specificity of the media tested are listed in Tables 5 to 8. No *Aeromonas* or *Plesiomonas* strains could be detected in the control (unspiked) stools. The sensitivities of *Aeromonas* media decreased significantly from *Aeromonas*-to-coliform ratios of $1:10^2$ on and, for DNTA, RC, and RS, were unsatisfactory, whereas specificity was unsatisfactory only for the latter two media. For *Plesiomonas* media, a similar decrease was observed, with only IBB showing optimal sensitivity and specificity. Enrichment with both APW and TSBA (the latter for *Aeromonas* strains only) was much more effective than direct plating at low *Aeromonas*- and *Plesiomonas*-to-coliform ratios, with no significant difference among the four subculture media except for XDC, which showed a lower sensitivity for *Plesiomonas* strains.

Isolation of *Aeromonas* and *Plesiomonas* spp. from outpatient stool cultures. The above results suggested that the four solid media (IBB, XDC, DFS, and PXA) plus APW be used for all subsequent experiments with *Aeromonas* strains and the same set except PXA be used for

TABLE 4. Inhibition of 23 *Aeromonas* strains and 10 *Plesiomonas* strains on media for *Aeromonas* and *Plesiomonas* spp.

Medium	No. of strains showing inhibition of[a]:					
	Aeromonas spp.			*Plesiomonas* sp.		
	<1 log	1–<2 log	≥2 log	<1 log	1–<2 log	≥2 log
DFS	23	0	0	9	1	0
RC	23	0	0	—[b]	—	—
PXA	21	0	2	—	—	—
XDC	20	2	1	9	1	0
IBB	19	3	1	10	0	0
RS	15	4	4	—	—	—
DNTA	12	7	4	—	—	—

[a] As compared with colony counts on MacConkey agar.
[b] —, Not done.

TABLE 5. Recovery of *Aeromonas* strains in 23 artificially contaminated stools—direct plating

Medium	Control[a]	No. of stools positive at an *Aeromonas*/coliform ratio of:			Sensitivity (%)[b]	Specificity (%)
		$1:10^1$–$<10^2$	$1:10^2$–$<10^3$	$1:10^3$–$<10^4$		
IBB	0	23	21	5	100	100
DFS	1	21	18	6	92	96
PXA	0	21	16	11	92	100
XDC	0	20	19	5	96	100
RC	18	17	15	8	74	22
DNTA	1	17	14	9	74	96
RS	16	14	6	2	51	30

[a] Number of normal stools with false-positive colonies.
[b] At a ratio of $1:10^1$ to 10^2.

Plesiomonas strains. Of 254 stool specimens, four *Aeromonas* strains were isolated (all from individuals with diarrhea), whereas no *Plesiomonas* strains were detected (Table 9). One *Aeromonas* strain was detected only after enrichment.

Only one *A. hydrophila* strain was beta-hemolytic and did not ferment lactose on MacConkey agar. One *A. sobria* strain was lysine positive. All four *Aeromonas* strains isolated were resistant to 30 mg of ampicillin per liter and failed to grow in 6.5% NaCl broth.

DISCUSSION

Our results clearly indicate that some of the media originally designed for isolation of *Aeromonas* strains are not optimal for this purpose as far as stool specimens are concerned. Ampicillin is unsuitable as a selective agent for *Plesiomonas* strains since, contrary to earlier statements (17), up to 55% of these strains may be inhibited by ≤16 mg of the drug per liter (8). Likewise, the minimal inhibitory concentrations for 4 of 103 strains of *A. hydrophila* subspp. *hydrophila* and *punctata* and 6 of 15 strains of *A. hydrophila* subsp. *caviae* were recently found to be ≤25 mg/liter (9). Whether ampicillin alone accounted for the relatively poor performance of DNTA and RC is unclear in view of the good performance of PXA and TSBA. Sensitivity and specificity of RC are higher when pure cultures are used (10). Both DNTA and RC contain other inhibitors, i.e., toluidine blue and ethanol. A toluidine blue concentration lower than 0.005% in DNTA was not chosen since visualization of DNA depolymerization was only accomplished at 0.005%.

RS inhibited gram-negative stool flora less than did DNTA, presumably owing to the lack of inhibitors of gram-negative species and perhaps also to the presence of novobiocin-sensitive *Aeromonas* strains (10). Furthermore, RS failed to recognize lysine-positive *Aeromonas* strains. These have been recognized more frequently as of late and are more often cytotoxic than are lysine-negative strains (5). Finally, halophilic non-*Aeromonas* gram-negative species with low G+C ratios, which were not encountered in our study, have morphologies similar to those of *Aeromonas* spp. on RS and RC (3). The specificity of RC was also found to be insufficient, owing to the lack of clear characteristics to distinguish *Aeromonas* colonies from *Enterobacteriaceae* colonies (trehalose being fermented by most members of the *Enterobacteriaceae*). The fermentable-carbohydrate content in the media (0.5 to 1.5%) was such that fermenting colonies could be clearly distinguished from nonfermenting ones. Likewise, the lack of peptones did not seem to be a limiting factor for growth since RC, which contains only 5.0 g of tryptose per liter plus the inhibitors listed in Table 1, was less inhibitory than IBB,

TABLE 6. Recovery of *Plesiomonas* strains in 10 artificially contaminated stools—direct plating

Medium	Control[a]	No. of stools positive at a *Plesiomonas*/coliform ratio of:			Sensitivity (%)[b]	Specificity (%)
		$1:10^1$–$<10^2$	$1:10^2$–$<10^3$	$1:10^3$–$<10^4$		
IBB	0	9	5	2	90	100
XDC	0	7	5	2	70	100
DFS	3	6	2	0	60	70

[a] Number of normal stools with false-positive colonies.
[b] At a ratio of $1:10^1$ to 10^2.

TABLE 7. Recovery of *Aeromonas* strains in 16 artificially contaminated stools—enrichment

Medium	No. of stools positive at an *Aeromonas*/coliform ratio of:			
	$1:10^3$–$<10^4$		$1:\geq10^4$	
	APW	TSBA	APW	TSBA
IBB	14	14	13	13
DFS	13	13	13	12
PXA	14	11	13	11
XDC	13	12	13	11

(Table 8) which contains 10 g of proteose peptone plus 5 g of Lab Lemco meat extract (Oxoid Ltd., Basingstoke, England).

On the basis of our results, we recommend APW as *Aeromonas* enrichment medium, IBB, DFS, XDC, and PXA as *Aeromonas* plate media, and APW and IBB as *Plesiomonas* media. The use of traditional media will certainly lead to underreporting (2), since lactose-fermenting and nonhemolytic *Aeromonas* strains are not uncommon (17) and TCBS is also inhibitory (2). In Germany, Schubert (13) was unable to detect any *Aeromonas* strain in 300 stool specimens from healthy individuals with the use of traditional lactose broth and Endo agar but was able to find one strain in 11 specimens with DFS. In a study in the United States, DNTA plus TSBA enabled the detection of six *Aeromonas* strains in 188 stool samples from hospitalized patients without diarrhea (18); three strains were prompt lactose fermenters and two were nonhemolytic; thus, these would not have been detected by the use of traditional enteric media and blood agar, respectively. In England, Shread et al. (16), using XDC and APW, isolated 5 *Aeromonas* strains from 402 feces of healthy individuals, 14 strains from 106 diarrheal specimens containing a "known bacterial enteropathogen," and 24 strains from 339 diarrheal specimens not containing such a pathogen. Only 16 strains (1 from a healthy individual) were isolated by direct plating. Rogol et al. (11) found seven *Aeromonas* strains (six as the only bacterial pathogen) in 100 diarrheal specimens with PXA. Our strains, exclusively isolated from patients with diarrhea, were the first enteric strains reported from Switzerland. At least three of them would not have been detected on enteric media.

TABLE 8. Recovery of *Plesiomonas* strains in 10 artificially contaminated stools—enrichment

Medium	No. of stools positive at a *Plesiomonas*/coliform ratio of:	
	$1:10^3$–$<10^4$ (APW)	$1:\geq10^4$ (APW)
IBB	8	7
DFS	8	6
XDC	5	3

TABLE 9. Isolation of *Aeromonas* and *Plesiomonas* spp. from 254 outpatient stool specimens

Medium	No. of strains isolated			
	Aeromonas		*Plesiomonas*	
	Direct	APW	Direct	APW
IBB	3[a]	4[b]	0	0
XDC	3[a]	3[a]	0	0
DFS	2[c]	—[d]	0	—
PXA	3[a]	—	—	—

[a] Two *A. hydrophila* strains and one *A. sobria* strain were isolated.

[b] Two *A. sobria* and two *A. hydrophila* strains were isolated.

[c] Two *A. hydrophila* strains were isolated.

[d] —, Not done.

It is possible that geographical and seasonal variations account for the differences in rates of isolation from healthy as well as diarrheic individuals. The DNTA study, for instance, was conducted in a coastal area during the summer and fall, when the density of *Aeromonas* strains in water bodies (5) and the incidence of human infections (17) is highest.

It is not surprising that we isolated no *Plesiomonas* spp. from our samples. *Aeromonas* diarrhea is ubiquitous, but *Plesiomonas* diarrhea and carriers have mostly been reported from subtropical and tropical areas and Japan (17). Determined with traditional media, *Plesiomonas* carrier rates, even in healthy citizens of Tokyo, were quite low (3 of 38,454 individuals [1]).

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