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Differential Media for Quantitative Recovery of Waterborne *Aeromonas hydrophila*

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Because of the ubiquity of *Aeromonas* spp., their prevalence in drinking water, and the increasing number of reports on *Aeromonas* sp.-related infections, a standard method for routine and quantitative recovery had to be defined. On the basis of a survey of 10 media for recovery analysis and subsequent differentiation assays in mixed cultures, we conclude that ampicillin-dextrin agar performed the best for the recovery of *Aeromonas* spp. in drinking water and the differentiation by simple criteria of that genus from other common waterborne bacteria.

In recent years, there has been an increasing number of reports on diverse *Aeromonas* sp.-associated infections, including endocarditis, gastroenteritis, hemolytic-uremic syndrome, meningitis, pneumonia, septicemia, urinary tract infections, wound infections, etc. Clinical and epidemiologic evidence indicates that *Aeromonas* spp. are enteropathogenic despite the fact that very few well-documented outbreaks have been reported. Patients with impaired immunity are particularly at risk for *Aeromonas* infections (1, 13, 14).

Aeromonas spp. can survive standard chlorination and thus recolonize the water distribution networks after the chlorination process (5, 6, 25, 26). In routine evaluations of the bacteriological quality of drinking water, the identification of lactose-negative colonies on m-Endo agar revealed a frequency of distribution of *Aeromonas* spp. ranging from 17% (6) to 25% (unpublished data) of the total indicator bacteria recovered. Environmental *Aeromonas* strains have been shown to produce a wide variety of extracellular toxins (4, 10, 18). Thus, drinking water may represent an important source of pathogenic *Aeromonas* spp. To our knowledge, only The Netherlands (26) and the Province of Ontario in Canada (3) have legislated to control the presence of *Aeromonas* spp. in drinking water.

A high rate of isolation of *Aeromonas* spp. in drinking water and time-consuming identification protocols justified the identification of a simple, standardized, and reliable method for *Aeromonas* screening. Commercially available miniaturized test kits or new molecular technologies are still too expensive for the routine screening of large numbers of samples. The purpose of this study was to compare the potentials of 10 solid media described in the literature for recovering and differentiating *Aeromonas hydrophila*. The conditions and methods used in this study were those already used in routine laboratories for determination of microbiological water quality.

Bacterial strains and culture media. One hundred *A. hydrophila* isolates were recovered from chlorinated and unchlorinated water supplies by the standard procedure for coliform screening in drinking water (2). Lactose-negative colonies on m-Endo agar were tested for oxidase production and starch hydrolysis (19). Confirmation was obtained with an API 20E system (Analytab Products Inc.) and by the following additional biochemical tests: production of gas from glucose, L-arabinose, and salicin fermentation; growth at 42°C; esculin hydrolysis; and utilization of L-arginine and L-ornithine. Tryptic soy agar was chosen as a nonselective medium. The selective and differential media used are listed in Table 1. Two modified media were also included: (i) brilliant green bile-salts-starch (BGBSS) agar, a modified medium consisting of brilliant green bile agar base, recommended for coliform screening (8), supplemented with starch (10 g/liter), and (ii) Ecosan-xylose-ampicillin (EXA) agar, a modification of the Pril-xylose-ampicillin agar (23), consisting of nutrient agar (15 g/liter), xylose (10 g/liter), phenol red (25 mg/liter) (as a pH

TABLE 1. Selective and differential media for *A. hydrophila*

Medium	Selective compound(s) (concn [mg/ml])	Differential agent(s)	Reference
AD	Ampicillin (10)	Dextrin	Havelaar et al. (11)
mA	Ampicillin (10), sodium deoxycholate, ethanol	Trehalose	Rippey and Cabelli (22)
MIX	Ampicillin (20), bile salts, citrate	Xylose, *meso*-inositol	Cunliffe and Adcock (7)
PBG	Sodium lauryl sulfate	Glycogen	McCoy and Pilcher (16)
RS	Novobiocin (5), citrate, sodium deoxycholate	Lysine, ornithine, maltose	Shotts and Rimler (24)
SA	Ampicillin (10)	Starch	Palumbo et al. (19)
m-Endo	Bile salts	Lactose	American Public Health Association (2)
TCBS	Citrate, oxgall	Saccharose	Difco Laboratories (8)
BGBSS	Bile salts, oxgall	Starch, lactose	Modified from the method of Difco Laboratories (8)
EXA	Ampicillin (30), Ecosan-2	Xylose	Modified from the method of Rogol et al. (23)

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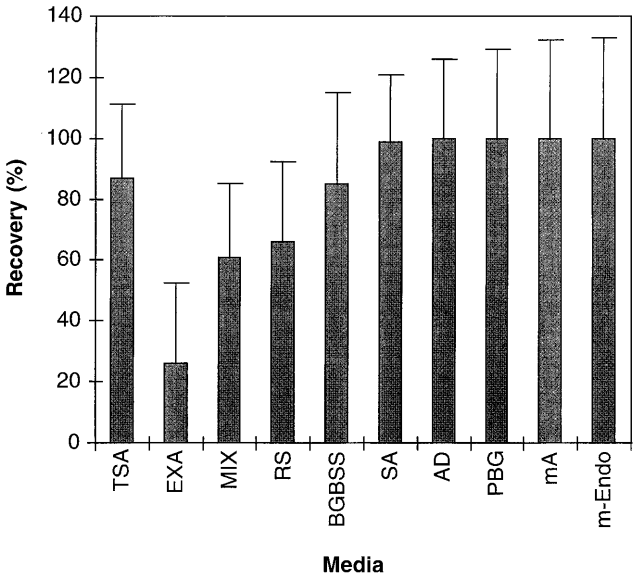

FIG. 1. Recovery of *A. hydrophila* on selected media at 35°C. Error bars indicate standard deviations. MIX, *meso*-inositol xylose agar; RS, Rimler-Shotts agar; TSA, tryptic soy agar.

indicator), ampicillin (30 mg/liter), and Ecosan-2 (20 μl/liter; Ecochimie Ltée), a quaternary ammonium detergent.

Recovery analysis. The first part of this study dealt with the recovery potential of diverse culture media for the isolation of waterborne *A. hydrophila*. The rates of recovery of 100 different water isolates, along with *A. hydrophila* ATCC 7966, in pure culture were compared for nine media. Every isolate was precultured in tryptic soy broth overnight at 35°C. Decimal serial dilutions were then made in order to obtain an inoculum of 100 ml containing 30 to 50 CFU per membrane; microorganisms were recovered on each medium after 24 h at 35°C. Tryptic soy agar was used as the control for the nonselective recovery medium. Even though it was reported that optimal temperature culture conditions for mesophilic aeromonads range between 22 and 28°C (20), cultivation under aerobic conditions at 35°C was used since laboratories already follow these standards for coliform screening in drinking water. Cell counts and colony characteristics (color and size) on each medium were noted. Thiosulfate-citrate-bile-sucrose (TCBS) agar, recommended for screening of vibrios in water, was also assessed, but it was eliminated after preliminary assays because of its low recovery potential. Results obtained are depicted in Fig. 1. Recovery percentages of the nine tested media were compared by using the Duncan analysis of variance for multiple comparisons. This analysis revealed that the percentages of recovery of *A. hydrophila* for six of the nine media were equal to (BGBSS agar) or significantly greater than (starch-ampicillin [SA] agar, ampicillin-dextrin [AD] agar, peptone-beef extract-glycogen [PBG] agar, ampicillin-trehalose [mA] agar, and m-Endo agar) that with tryptic soy agar ($P < 0.001$). Five of these media were subsequently chosen for the differentiation assay.

Differentiation assay. Since the purpose of this work was to select the optimal medium for routine laboratory tests, the criteria for the presumptive identification of *A. hydrophila* needed to be as simple as possible, and thus colony size and color were chosen. These criteria were investigated with 20 isolates of *A. hydrophila* in pure cultures or in cultures mixed with equal proportions of the following bacteria frequently

TABLE 2. Differential scheme of *A. hydrophila* in mixed cultures on selected media at 35°C

Species	Colony color (size [mm]) on medium:					
	AD	BGBSS	mA	PBG	SA
A. hydrophila (ATCC 7966)	Yellow with dark center (1–1.5)	Purple with dark center (1–1.5)	Yellow (1.5–2)	Green with dark center (1–1.5)	Yellowish (1–1.5)
A. calcoaceticus subsp. *anitratus* (ATCC 19606)	Light green (1)	Pink (1–2)	Yellowish green (1)	Green (1)	Colorless (1)
C. freundii (MUL-B-605)[a]	Light green (0.5)	Pink (1)	Yellow (1)	Light green (1)	Colorless (0.75)
E. cloacae (MUL-B-P99)[a]	Light green (1–1.25)	Pink to purple (1)	Yellow (1)	Light green (0.75–1)	Yellowish (1)
E. coli (ATCC 17958)	Light green (2–3)	Light blue (3)	Light green (2)	Light green (1–2)	Colorless (2)
K. pneumoniae (ATCC 10031)	Light green (1)	Pink to purple (0.5)	Yellow (1)	—[b]	Yellowish (1)
E. faecalis (ATCC 23241)	Yellow (0.1)	—	—	Colorless (0.2)	—

[a] Collection of the Département de Microbiologie, Faculté de Médecine, Université Laval.
[b] —, no growth.

TABLE 3. Recovery of *A. hydrophila* in mixed cultures on selected media[a]

Species in mixed culture	% Recovery on medium:				
	AD	BGBSS	PBG	mA	SA
A. calcoaceticus subsp. *anitratus*	80	76	—[b]	69	61
C. freundii	87	87	79	—	92
E. cloacae	94	91	105	—	—
E. coli	87	65	83	78	74
K. pneumoniae	72	81	91	—	—
E. faecalis	92	59	79	73	73
Mean (SE)	85 (3)	77 (5)	87 (5)	73 (3)	75 (6)

[a] Percentage recovery is expressed as the number of *Aeromonas* colonies recovered in mixed culture divided by the number of *Aeromonas* colonies in pure culture on the same medium.

[b] —, not evaluated.

found in drinking water: *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterococcus faecalis*, and *Acinetobacter calcoaceticus* subsp. *anitratus*. All bacterial strains were precultured in tryptic soy broth overnight at 35°C. Decimal dilutions were then made in order to obtain a total inoculum (100 ml) of 50 to 100 CFU per membrane, and the microorganisms were recovered on each medium after 24 h at 35°C. Table 2 presents the differential pattern of *A. hydrophila* on the selected media. Although these media gave satisfactory results in recovery tests with bacteria in pure cultures (Fig. 1), only AD medium showed sufficient selectivity for colonial morphology differentiation in mixed cultures. These results are consistent with recent data from Holmes and Sartory (12). AD medium shows yellow colonies for the genus *Aeromonas* and light green colonies for all other genera, except for the genus *Enterococcus*, whose colonies were also yellow, but enterococci could not be mistaken for members of the genus *Aeromonas* because the former have pinpoint-sized colonies compared with *Aeromonas* colonies (which have diameters of 1 to 1.5 mm). As expected, the recovery percentages found in mixed cultures were lower than those in control pure cultures. However, the AD and PBG media showed optimal rates of recovery (Table 3). AD agar is a modification of the mA medium (22). Combined with the nutritive quality of the mA agar for *Aeromonas* recovery (Fig. 1), AD agar makes use of the high specificity of dextrin fermentation for the detection of aeromonads in environmental samples. Until now, no dextrin-negative *Aeromonas* strains have been encountered (11). Ampicillin was chosen for adequate suppression of the background microflora as it is a selective agent with a broad spectrum. It has been shown by Havelaar et al. (11) that the effect of ampicillin on *Aeromonas* spp. is generally negligible. Nevertheless, it has occasionally been reported that a certain proportion of *Aeromonas* strains can be sensitive to ampicillin (21, 27), but strains sensitive to 10-mg/ml concentrations of ampicillin normally occur at a frequency of ≤1% (9, 15, 17).

PBG and BGBSS media are the best alternatives to AD medium for *Aeromonas* screening in drinking water, as determined on the basis of recovery percentages and ease of differentiating *Aeromonas* spp. in pure and mixed cultures. However, PBG medium remains ambiguous for differentiating members of the genera *Aeromonas* and *Acinetobacter* while BGBSS medium remains ambiguous for differentiating members of the genera *Enterobacter* and *Klebsiella.*

Drinking water bacterial contamination is usually ascribed to one dominant genus, but rarely to two or more simultaneously. It was demonstrated that the use of AD medium with a standard membrane filtration procedure was efficient for both recovery and differentiation in mixed cultures in which relatively large inoculum sizes of the most frequently encountered water bacterial contaminants were used. It is expected that this technique will be applicable to standard determinations of microbiological drinking water quality in routine control laboratories.

We thus recommend the AD medium for routine *Aeromonas* screening and propose that this medium could be used to conduct a broad survey of the prevalence of *Aeromonas* spp. in drinking water. This could help to define a standard for water quality in order to control the presence of *Aeromonas* spp. in drinking water supplies.

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