APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1979, p. 1023-1026
0099-2240/79/11-1023/04$02.00/0
Vol. 38, No. 5

Medium for the Presumptive Identification of *Aeromonas hydrophila* and *Enterobacteriaceae*

J. KAPER, RAMON J. SEIDLER,† H. LOCKMAN, AND R. R. COLWELL*
Department of Microbiology, University of Maryland, College Park, Maryland 20742

Received for publication 13 August 1979

A medium was devised for the rapid presumptive identification of *Aeromonas hydrophila*. It also offered good differentiation of *Klebsiella*, *Proteus*, and other enteric species. Mannitol fermentation, inositol fermentation, ornithine decarboxylation and deamination, indole production, motility, and H_2S production from sodium thiosulfate and cysteine could be recorded in a single tube of the medium.

Bacterial species comprising microbial communities in aquatic ecosystems often may be of public health significance. Usually, selective and differential media are required for their detection and enumeration. The principles of such media are to eliminate species of secondary interest and to select for specific microorganisms. Unfortunately, most media are not sufficiently selective for individual species, and additional biochemical tests must be applied after selection and purification of isolates before presumptive identification is possible. Biochemical screening on a large scale is laborious and time-consuming. Commercially available miniaturized test kits, i.e., "mini-tests," such as the API (Analytab Products, Inc., Plainview, N.Y.), are too expensive for the routine screening of large numbers of samples, as well as ineffective, in many cases, for environmental isolates.

During studies of bacterial pathogens and pollution indicator bacteria occurring in Chesapeake Bay, an extensive survey of the incidence and distribution of *Aeromonas hydrophila* was undertaken (J. Kaper, H. Lockman, and R. R. Colwell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, N56, p. 188). *A. hydrophila* has received increased attention both as an indicator of pollution (13) and as a pathogen (6, 7, 12, 16–19). The organism has been reported to produce at least three virulence factors, including enterotoxin, cytotoxin, and hemolysin (8), and is recognized to be of public health significance when found in large numbers in the environment. Methods for recovery of *Aeromonas* spp. from environmental samples have been reviewed recently (R. R. Colwell and J. Kaper, *in* B. J. Dutka, ed., *Membrane Filtration: Techniques, Applications and Problems*, in press). Most of the media and procedures presently employed for the isolation and enumeration of *Aeromonas* spp. are not specific and require a number of additional biochemical tests for presumptive and confirmed identification. A specific medium, described by Shotts and Rimler (15), is highly selective and limited in application for natural environments (unpublished data; V. J. Cabelli, personal communication).

Strains of *A. hydrophila* which are novobiocin sensitive and lysine decarboxylase positive will not be detected by this medium. In addition, RS agar cannot be used to distinguish *A. hydrophila* from so-called "group F" or EF6 vibrios (5; I. Huq, B. R. Davis, R. E. Weaver, G. G. Hollis, W. T. Martin, and D. J. Brenner, Abstr. 13th Jt. Conf. Cholera U.S.-Jpn. Coop. Med. Sci. Program, p. 82, 1977) which are widely distributed in the marine and estuarine environments. These vibrios give reactions identical to those of *A. hydrophila* on RS agar and in the API 20 system. Thus, RS agar and the API 20 system can lead to an inaccurate enumeration of *A. hydrophila* in estuarine and marine environments due to interference by group F vibrios. Because of the limitations of the medium, as well as problems encountered with the medium in the enumeration and identification of *Aeromonas* in our laboratory, a multitest medium was developed to screen for *A. hydrophila*. Fortuitously, this medium has also proved useful in the presumptive identification of *Klebsiella* and related *Enterobacteriaceae* occurring in aquatic ecosystems.

The *A. hydrophila* medium (AH medium) contains (in grams per liter): proteose peptone (Difco Laboratories, Detroit, Mich.), 5; yeast extract (Difco), 3; tryptone (Difco), 10; L-ornithine hydrochloride, 5; mannitol, 1; inositol, 10; sodium thiosulfate, 0.4; ferric ammonium citrate, 0.5; bromocresol purple, 0.02; agar, 3. To prepare the medium, one must thoroughly mix the in-

† On sabbatical leave from Department of Microbiology, Oregon State University.

gredients in 1 liter of distilled water and adjust the pH to 6.7. The medium is heated to a boil, dispensed in 5-ml quantities in tubes (13 by 100 mm), and autoclaved at 121°C for 12 min. Colonies of *Aeromonas* and enteric bacteria can be picked directly from isolation plates or membrane filters and inoculated into the medium by stabbing to the base of the tube with a straight needle. The inoculated tubes are incubated at 35°C for 18 to 24 h, after which reactions are recorded. For the detection of indole production, 3 to 4 drops of Kovacs reagent are added to each tube.

The reactions of AH medium are based upon the principles of the triple sugar iron agar, lysine iron agar, and motility-indole-ornithine medium (1). Organisms which ferment mannitol and do not decarboxylate ornithine will produce an acid (yellow) butt with a band of purple at the top. Organisms which are ornithine positive and either mannitol positive or negative will give an alkaline (purple) reaction throughout the tube, because the alkaline decarboxylation reaction masks any acid produced via mannitol fermentation. Inositol is present at a concentration of 10 g/liter, compared with mannitol, which is present at a concentration of 1 g/liter, analogous to that of lactose and dextrose in Kligler iron agar or triple sugar iron agar. The small amount of acid that can be produced from 1 g of mannitol permits the top of the medium to revert to a purple color, whereas fermentation of 10 g of inositol will result in the entire tube turning yellow. Because of the reduced concentration of agar included in the medium, i.e., 3 g/liter, motility can be detected with growth extending away from the line of inoculation or throughout the medium only for motile bacteria.

The incorporation of proteose peptone and yeast extract in the medium enhances hydrogen sulfide production, and hydrogen sulfide is detected, as in triple sugar iron agar and Kligler iron agar, by employing sodium thiosulfate and ferric ammonium citrate as the indicator system. The production of H_2S from thiosulfate is noted if the butt of the tube turns black. The production of H_2S from cysteine, but not from thiosulfate, can be important in the classification of *A. hydrophila* (12). In general, H_2S production from cysteine is indicated by blackening at the top of the medium, but not in the butt. Tryptone is included in the medium to test for indole production. The development of a pink to red color, after the addition of Kovacs reagent, indicates that the production of indole has occurred. A yellow color signifies that indole is not present.

Clinical and public health microbiologists, in general, follow the scheme of Ewing and Hugh (4) for the identification of *A. hydrophila,* which, however, differs in several respects from that presented in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (14). Furthermore, the key for *Aeromonas* spp. given in *Bergey's Manual* differs from other proposed schemes (11).

In Table 1 are listed the reactions in AH medium of *A. hydrophila* and selected spp. of *Enterobacteriaceae.* A very distinct, typical combination of reactions was observed for *A. hydrophila,* i.e., a yellow butt with a purple band at the top, since this species fermented mannitol, but not inositol, and did not decarboxylate ornithine. The color of the butt varied from bright yellow to yellowish gray. Certain biotypes of *A. hydrophila* may produce H_2S from cysteine (12), detected by observing a slight blackening only at, or near, the top of the tube. The reaction is enhanced if the inoculated tube of medium is held for 48 h. *A. hydrophila* is described as being

TABLE 1. *Reactions of enteric bacteria in AH medium*

Species	No. of strains tested	Reaction[a]				
		Top	Butt	Motility	H_2S	Indole
A. hydrophila	700	K	A	+	−	+
K. pneumoniae	21	A	A	−	−	−
K. oxytoca	9	A	A	−	−	+
E. coli	10	K	K or A[b]	+ or −	−	+
Salmonella spp.	10	K or A	K or A	+	+	−
Enterobacter spp.	6	K or N	K or N	+	−	−
Proteus spp.	4	R	K or A	+	+ or −	+[b]
Yersinia enterocolitica	2	K or N	K or N	−	−	+ or −
Citrobacter spp.	2	K	A or K	+	+	−
Serratia spp.	2	N or K	N or K	+	−	−

[a] Symbols: K, alkaline reaction; A, acid reaction; R, red; N, bleached neutral color due to the destruction of indicator; +, 90% or more positive; −, 90% or more negative.

[b] The response of individual strains of the species listed may vary as indicated, consistent with biochemical patterns compiled by Edwards and Ewing (2).

motile (14) and will produce turbidity throughout the medium after inoculation and incubation. However, nonmotile strains may be encountered (11) and should be considered. Indole production is also usually positive for *A. hydrophila,* with the reported incidence of indole production among strains of *A. hydrophila* varying with the classification scheme employed (4). The combination of reactions produced by *A. hydrophila* was unique among the facultatively anaerobic bacteria that were examined. An occasional strain of *Escherichia coli* or *Enterobacter agglomerans* produced the combination of characteristics for identifying *Aeromonas* spp., but the oxidase test readily separated them from the aeromonads. Alkalinity at the surface permitted the oxidase test to be done by picking from growth at the surface of the tube since an acid reaction on a primary isolation medium will interfere with the oxidase test (10; A. H. Havelaar, C. J. Hoogendorp, A. J. Wesdorp, and W. A. Scheffers, Abstr. 12th Int. Congr. Microbiol., p. 159, 1978). The AH medium is also useful in distinguishing *Aeromonas* from some of the oxidase-positive *Vibrio* spp. that occur with high frequency in aquatic environments since, without added sodium chloride, the growth of *V. parahaemolyticus* and related halophilic vibrios will not occur.

AH medium has proved useful in the presumptive identification of *Klebsiella* spp. from both clinical and nonclinical sources. *Klebsiella* spp. produce a unique pattern of reactions due to the production of acid from inositol throughout the tube. Even though a few strains of *Klebsiella ozaenae* may prove to be exceptions to a generalization, klebsiellae ferment inositol but do not decarboxylate ornithine. The pattern of characteristics for *Klebsiella* spp., together with the lack of motility and results in the indole test, permits the identification of the two *Klebsiella* species, *Klebsiella pneumoniae* and *Klebsiella oxytoca,* most frequently encountered both in the hospital environment and in polluted waters.

AH medium can be used also to separate the major groups of coliforms, i.e., *Klebsiella, Enterobacter,* and *E. coli,* chiefly on the basis of motility and indole production (Table 1). Other enteric species have been found to be readily differentiated in AH medium. Selected results are given in Table 1. Reactions of *Enterobacter* spp. differ from those of other enterics since a purple-gray or "bleached" purple color may be produced throughout the medium, a result of the combination of inositol fermentation and ornithine decarboxylation, yielding neither a totally alkaline nor a totally acid reaction, but, rather, an intermediate coloration due to the alteration of the bromocresol purple indicator (9).

Species of *Proteus* and *Providencia* produce a distinct appearance, i.e., a red surface over a black, alkaline or acid butt, arising from oxidative deamination of the ornithine. As occurs in lysine iron agar, an orange color is produced when the bromocresol purple indicator is omitted; the red coloration arises from the combination of the orange color and the purple indicator (3).

Variations in the basic formula of the AH medium are possible. For example, agar can be added to a final concentration of 15 g/liter, and the medium can be prepared as a slant, rendering gas production obvious by the splitting of the agar. Lactose may be substituted for inositol, if lactose-negative organisms are sought.

This investigation was supported by Public Health Service grant 1R22AI14242-01 from the National Institute of Allergy and Infectious Diseases and grant DEB-77-14646 from the National Science Foundation.

LITERATURE CITED

1. **Ederer, G. M., and M. Clark.** 1970. Motility-indole-ornithine medium. Appl. Microbiol. **20:**849–850.
2. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of *Enterobacteriaceae,* 3rd ed. Burgess Publishing Co., Minneapolis, Minn.
3. **Edwards, P. R., and M. A. Fife.** 1961. Lysine-iron agar in the detection of *Arizona* cultures. Appl. Microbiol. **9:**478–480.
4. **Ewing, W. H., and R. Hugh.** 1974. *Aeromonas,* p. 230–237. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology. American Society for Microbiology, Washington, D.C.
5. **Furniss, A. L., J. V. Lee, and T. J. Donovan.** 1977. Group F. A new vibrio? Lancet **ii:**565–566.
6. **Hanson, P. G., J. Standridge, F. Jarrett, and D. G. Maki.** 1977. Freshwater wound infection due to *Aeromonas hydrophila.* J. Am. Med. Assoc. **238:**1053–1054.
7. **Ljungh, A., M. Popoff, and T. Wadström.** 1977. *Aeromonas hydrophila* in acute diarrheal disease: detection of enterotoxin and biotyping of strains. J. Clin. Microbiol. **6:**96–100.
8. **Ljungh, A., B. Wretlind, and T. Wadström.** 1978. Evidence for enterotoxin and two cytolytic toxins in human isolates of *Aeromonas hydrophila,* p. 947–960. *In* P. Rosenberg (ed.), Toxins: animal, plant and microbial. Proceedings of the 5th International Symposium. Pergamon Press, Inc., Elmsford, N.Y.
9. **MacFaddin, J. F.** 1976. Biochemical tests for identification of medical bacteria. The Williams & Wilkins Co., Baltimore.
10. **Neilson, A. H.** 1978. The occurrence of aeromonads in activated sludge: isolation of *Aeromonas sobria* and its possible confusion with *Escherichia coli.* J. Appl. Bacteriol. **44:**259–264.
11. **Popoff, M., and M. Véron.** 1976. A taxonomic study of the *Aeromonas hydrophila-Aeromonas punctata* group. J. Gen. Microbiol. **94:**11–22.
12. **Qadri, S. M. H., L. P. Gordon, R. D. Wende, and R. P. Williams.** 1976. Meningitis due to *Aeromonas hydrophila.* J. Clin. Microbiol. **3:**102–104.
13. **Schubert, R.** 1976. The detection of aeromonads of the

"hydrophila-punctata-groups" within the hygienic control of drinking water. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B **161**:482–497.

14. **Schubert, R. H. W.** 1974. Genus *Aeromonas* Kluyver and van Niel, p. 345–349. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.
15. **Shotts, E. B., and R. Rimler.** 1973. Medium for the isolation of *Aeromonas hydrophila*. Appl. Microbiol. **26**:550–553.
16. **Tapper, M. L., L. R. McCarthy, J. B. Mayo, and D. Armstrong.** 1975. Recurrent *Aeromonas* sepsis in a patient with leukemia. Am. J. Clin. Pathol. **64**:525–530.
17. **von Graevenitz, A., and A. H. Mensch.** 1968. The genus *Aeromonas* in human bacteriology. N. Engl. J. Med. **278**:245–249.
18. **Washington, J. A.** 1972. *Aeromonas hydrophila* in clinical bacteriologic specimens. Ann. Intern. Med. **76**:611–614.
19. **Zajc-Satler, J.** 1972. Morphological and biochemical studies of 27 strains belonging to the genus *Aeromonas* isolated from clinical sources. J. Med. Microbiol. **5**:263–265.

U.S. POSTAL SERVICE

STATEMENT OF OWNERSHIP, MANAGEMENT AND CIRCULATION

(Required by 39 U.S.C. 3685)

1. TITLE OF PUBLICATION: Applied and Environmental Microbiology
A. PUBLICATION NO.:
2. DATE OF FILING: 10/1/79
3. FREQUENCY OF ISSUE: Monthly
A. NO. OF ISSUES PUBLISHED ANNUALLY: 12
B. ANNUAL SUBSCRIPTION PRICE: mem$17;nonmem.$70
4. LOCATION OF KNOWN OFFICE OF PUBLICATION *(Street, City, County, State and ZIP Code) (Not printers)*: 1913 I St., N.W., Washington, D.C. 20006
5. LOCATION OF THE HEADQUARTERS OR GENERAL BUSINESS OFFICES OF THE PUBLISHERS *(Not printers)*: 1913 I St., N.W., Washington, D.C. 20006
6. NAMES AND COMPLETE ADDRESSES OF PUBLISHER, EDITOR, AND MANAGING EDITOR

PUBLISHER *(Name and Address)*: American Society for Microbiology, 1913 I St., N.W.,Washington, D.C. 20006

EDITOR *(Name and Address)*: Marvin P. Bryant, University of Illinois, Urbana, Illinois

MANAGING EDITOR *(Name and Address)*: Robert A. Day, 1913 I St., NW, Washington, D.C. 20006

7. OWNER *(If owned by a corporation, its name and address must be stated and also immediately thereunder the names and addresses of stockholders owning or holding 1 percent or more of total amount of stock. If not owned by a corporation, the names and addresses of the individual owners must be given. If owned by a partnership or other unincorporated firm, its name and address, as well as that of each individual must be giving. If the publication is published by a nonprofit organization, its name and address must be stated.)*

NAME	ADDRESS
American Society for Microbiology	1913 I St., N.W., Washington, D.C. 20006

8. KNOWN BONDHOLDERS, MORTGAGEES, AND OTHER SECURITY HOLDERS OWNING OR HOLDING 1 PERCENT OR MORE OF TOTAL AMOUNT OF BONDS, MORTGAGES OR OTHER SECURITIES *(If there are none, so state)*

NAME	ADDRESS
None	

9. FOR COMPLETION BY NONPROFIT ORGANIZATIONS AUTHORIZED TO MAIL AT SPECIAL RATES *(Section 132.122, PSM)*
The purpose, function, and nonprofit status of this organization and the exempt status for Federal income tax purposes *(Check one)*

[X] HAVE NOT CHANGED DURING PRECEDING 12 MONTHS [ ] HAVE CHANGED DURING PRECEDING 12 MONTHS *(If changed, publisher must submit explanation of change with this statement.)*

10. EXTENT AND NATURE OF CIRCULATION	AVERAGE NO. COPIES EACH ISSUE DURING PRECEDING 12 MONTHS	ACTUAL NO. COPIES OF SINGLE ISSUE PUBLISHED NEAREST TO FILING DATE
A. TOTAL NO. COPIES PRINTED *(Net Press Run)*	10,581	10,447
B. PAID CIRCULATION 1. SALES THROUGH DEALERS AND CARRIERS, STREET VENDORS AND COUNTER SALES	None	None
2. MAIL SUBSCRIPTIONS	9,211	9,529
C. TOTAL PAID CIRCULATION *(Sum of 10B1 and 10B2)*	9,211	9,529
D. FREE DISTRIBUTION BY MAIL, CARRIER OR OTHER MEANS SAMPLES, COMPLIMENTARY, AND OTHER FREE COPIES	80	80
E. TOTAL DISTRIBUTION *(Sum of C and D)*	9,291	9,609
F. COPIES NOT DISTRIBUTED 1. OFFICE USE, LEFT OVER, UNACCOUNTED, SPOILED AFTER PRINTING	1,290	838
2. RETURNS FROM NEWS AGENTS	None	None
G. TOTAL *(Sum of E, F1 and 2—should equal net press run shown in A)*	10,581	10,447

11. I certify that the statements made by me above are correct and complete.
SIGNATURE AND TITLE OF EDITOR, PUBLISHER, BUSINESS MANAGER, OR OWNER: Robert A. Day, Managing Editor

12. FOR COMPLETION BY PUBLISHERS MAILING AT THE REGULAR RATES *(Section 132.121, Postal Service Manual)*

39 U. S. C. 3626 provides in pertinent part: "No person who would have been entitled to mail matter under former section 4359 of this title shall mail such matter at the rates provided under this subsection unless he files annually with the Postal Service a written request for permission to mail matter at such rates."

In accordance with the provisions of this statute, I hereby request permission to mail the publication named in Item 1 at the phased postage rates presently authorized by 39 U. S. C. 3626.

SIGNATURE AND TITLE OF EDITOR, PUBLISHER, BUSINESS MANAGER, OR OWNER: Robert A. Day, Managing Editor

PS Form 3526 *(Page 1)* Aug 1978 (See instructions on reverse)