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Evaluation of Different Assay Systems for Identification of Environmental *Aeromonas* Strains

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Important biochemical reactions in conventional tests were compared with counterpart reactions in two multiple test systems, API-20E (Analytab Products, Plainview, N.Y.) and *Aeromonas hydrophila* medium, to evaluate their accuracy for the identification of motile *Aeromonas* spp. isolated from fish. In a total of 49 *Aeromonas* spp. isolates and 10 *A. hydrophila* reference strains, false-negative or -positive reactions were detected in the Voges-Proskauer test, indole production, gelatinase activity, production of gas, fermentation of arabinose, and lysine decarboxylase reaction. A good correlation was found, among the three identification systems, for the fermentation of mannitol and inositol as well as for the arginine dihydrolase and ornithine decarboxylase tests. The failure of *A. hydrophila* medium in the detection of gas indicates that this medium is not entirely suitable for defining aerogenic or anaerogenic strains. From the results of the present study, we consider that of the identification method and taxonomic scheme to be adopted for environmental *Aeromonas* spp. must be standardized.

Motile *Aeromonas* species, which are widely distributed in nature, are autochthonous inhabitants of aquatic environments (11, 13, 18). *Aeromonas hydrophila* is one of the main opportunistic pathogens for fish (3, 17, 23, 29) and other poiquilotherm animals (25) and more recently has been recognized to be a causative agent of human disease (8, 12). *Aeromonas* spp. may possess virulence factors such as proteases, enterotoxins, cytotoxins, and hemolysins, but their role in disease has not been clearly identified (1, 2, 16).

Few attempts have been made to correlate the biochemical characteristics of *Aeromonas* species with toxigenicity (4, 5, 30). The taxonomy of *Aeromonas* spp. is confused, and it has been demonstrated that a great variation in virulence exists within the motile *Aeromonas* spp. (9, 19, 20, 30). At present it is not clear whether *A. hydrophila* or *Aeromonas sobria* has more virulent characteristics.

Most of the *Aeromonas* species isolated from either clinical or environmental sources are being classified with multitest systems such as API-20E (Analytab Products, Plainview, N.Y.). However, it was reported that the commercial miniaturized systems are not always adequate for the identification of environmental strains (6, 10, 15, 24). Kaper et al. (14) devised a multitest medium for the rapid presumptive identification of *A. hydrophila* (AH medium). The present study was undertaken to compare two multiple-test systems (API-20E and AH medium) with conventional biochemical methods in strains of motile *Aeromonas* isolated from fish to allow a more adequate classification of these organisms in relation to their virulence characteristics.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We used 49 motile *Aeromonas* strains isolated from gills, liver, kidney, intestine, and external lesions of diseased and healthy rainbow trout (*Salmo gairdneri*) as well as from water tanks.

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In all of the identification tests we have included 10 *A. hydrophila* reference strains from the American Type Culture Collection and particular donors (Table 1). The strains were routinely cultured on tryptic soy agar or broth (Difco Laboratories, Detroit, Mich.) at 25°C for 24 to 48 h and were stored on tryptic soy agar slants at 4°C under mineral oil and frozen at −70°C with 15% glycerol.

Comparison of identification systems. Two multiple-test systems, API-20E (La Balme, Les Grottes, France) and AH medium (14), were evaluated to test several important reactions for the presumptive identification of motile *Aeromonas* spp. in comparison with the conventional tube or plate tests.

Cultures (24 h) on tryptic soy broth or agar of the strains to be tested were used as inocula for the different assays.

The commercial miniaturized API-20E system was utilized by following the manufacturer's instructions.

AH medium contains the following (in grams per liter): proteose peptone, 5; yeast extract, 3; tryptone, 10; L-ornithine hydrochloride, 5; mannitol, 1; inositol, 10; sodium thiosulfate, 0.4; ferric ammonium citrate, 0.5; bromocresol purple, 0.02; and agar, 3. This medium was adjusted to pH 6.7, dispensed in tubes, and autoclaved. Tubes were inoculated into the medium by stabbing from colonies grown on tryptic soy agar.

The reactions of AH medium are based upon the principles of the triple sugar iron agar. (i) Organisms which ferment mannitol and do not decarboxylate ornithine will produce an acid (yellow) butt with a band of purple at the top. (ii) Organisms which are ornithine positive and either mannitol positive or negative will give an alkaline reaction throughout the tube. Tryptone is included in the medium to test indol production which is conducted by the addition of Kovacs reagent. In general, it is considered that in the AH medium, mannitol and inositol fermentation, ornithine decarboxylation, indole production, motility, H_2S production from sodium thiosulfate and cysteine, and gas production could be recorded in a single tube.

The biochemical characteristics evaluated by using the

TABLE 1. Reference strains of *A. hydrophila* used in this study

Strain	Origin and location	Source[a]
ATCC 7966	"Red-leg" frog, United States	ATCC
ATCC 15467 (subsp. *anaerogenes*)	Used oil emulsions, United States	ATCC
1.25 (subsp. *anaerogenes*)	Human, United States	D. P. Anderson
1.54	*Salmo trutta*, United States	D. P. Anderson
Y-62	*Anguilla japonica*, Japan	T. Aoki
67-P-24	*Plecoglossus altivelis*, Japan	T. Aoki
81–83	*Lepomis macrochirus*, United States	A. E. Toranzo and F. M. Hetrick
80-A1	*Salmo gairdneri*, Spain	A. E. Toranzo and J. L. Barja
80-A2	*Salmo gairdneri*, Spain	A. E. Toranzo and J. L. Barja
80-A3	*Salmo gairdneri*, Spain	A. E. Toranzo and J. L. Barja

[a] ATCC, American Type Culture Collection, Rockville, Md.; D. P. Anderson, National Fisheries Health Laboratory, Leetown, W.V.; T. Aoki, Faculty of Agriculture, Miyazaki University, Japan; F. M. Hetrick, Department of Microbiology, University of Maryland, College Park, Md.

conventional tube or plate assays are listed in Table 2. These tests were basically conducted as previously described (19). Acid production from carbohydrates was determined in O/F basal medium (Difco) containing 1% (wt/vol) sugar substrate. The test for acid and gas production from glucose was conducted on phenol red basal medium with glucose (1%, wt/vol) added and containing inverted Durham tubes.

Indole production was determined with Kovac reagent on cultures grown in 1% tryptone broth (Difco), and the Voges-Proskauer (VP) reaction was done in MR-VP medium (Difco). Production of nitrite from nitrate was tested by using nitrate broth (Difco).

Arginine dihydrolase, lysine, and ornithine decarboxylase tests were carried out at pH 6.0 under mineral oil in Moeller decarboxylase base (Difco) supplemented with 1% (wt/vol) of the corresponding amino acid.

Gelatinase activity was examined in agar plates by using a basal medium (0.4% neopeptone, 0.1% yeast extract, and 1.5% agar) supplemented with 1.5% (wt/vol) of gelatin.

A battery of additional standard physiological and biochemical tube and plate tests was also conducted for a better characterization of our *Aeromonas* isolates to the species level.

The incubation temperature used along this study was 25°C, and the readings of the tests in all the identification systems were performed at 48 h. Only the standard tube reactions were examined for 7 days before being discarded as negative.

RESULTS

On the basis of morphological, physiological, and biochemical conventional tube and plate tests, we identified the *Aeromonas* strains isolated from fish and water tanks to the species level, following basically the classification of Popoff and Véron (22). From a total of 49 motile *Aeromonas* isolates, 31 were identified as *A. hydrophila* biovar *hydrophila*, 1 was identified as *A. hydrophila* biovar *anaerogens*, and 15 were assigned to the species *A. sobria*. All of these strains were positive for β-galactosidase, fermented glucose and mannitol, produced arginine dihydrolase, were nitrate positive, and produced indole, whereas none of the isolates produced ornithine decarboxylase, fermented inositol, produced urease, or produced hydrogen sulfide from thiosulfate or triptophane deaminase. Only two strains could not be adequately identified, remaining as *Aeromonas* spp.

Important biochemical reactions obtained in the conventional tests were compared with counterpart reactions in the API-20E system or AH medium or both to evaluate the accuracy of these multitest systems for the identification of motile *Aeromonas* spp. (Table 2). Variable characteristics included the VP reaction, indole production, gelatinase activity, production of gas, fermentation of arabinose, and the lysine decarboxylase (LDC) test. The false-positive and false-negative reactions encountered in the use of multitest systems in our *Aeromonas* isolates as well as in 10 *A. hydrophila* reference strains are based upon the results of standard tube and plate tests.

The results shown on Table 2 can be divided into two categories: (i) reactions which can be recorded in the three assay systems compared, and (ii) reactions which can be only evaluated in the API-20E system and in conventional tests.

Production of gas, fermentation of mannitol and inositol, indole production, arginine dihydrolase, and ornithine decarboxylase are reactions falling in the first category. At 2 days, we found a high correlation among the three systems in the fermentation of both alcohols as well as in the arginine dihydrolase and ornithine decarboxylase tests either for the reference strains or for our fish isolates. However, we detected false-negative reactions in the production of gas only in the AH medium, finding that the use of 1.5% agar in its formulation did not improve the detection of gas. In addition, although the indole test performed in the AH medium revealed concordance with the API-20E and standard tube systems in the reference strains, a low number (eight strains) of our *Aeromonas* isolates exhibited false-negative reactions.

Within the second category, variable results between API-20E system and conventional tests were detected in gelatinase activity, VP reaction, fermentation of arabinose, and the LDC test, whereas the nitrate reaction gave excellent correlation in both systems. The false-negative reactions recorded in the API-20E system for the gelatinase test in four *A. hydrophila* reference strains as well as in a number of our isolates are noteworthy, since a key characteristic of this species is to be gelatinase positive.

The VP test displayed either false-positive or false-negative reactions in the API-20E system. However, some strains that were negative in the conventional assay at 2 days became positive after 4 days of incubation. In addition, the majority of the false-negative reactions for the arabinose fermentation in the API-20E system occurred in strains having delayed positive conventional tube tests. When we extended the incubation time to more than 48 h in the API-20E system before adding the reagents, to avoid their interference with other tests, most of the sugars reactions showed reversion, causing a very confused reading of the results.

TABLE 2. Comparison of different assay systems to test important biochemical characteristics for the identification of motile *Aeromonas* spp.

Biochemical test	Assay systems compared[a]		No./total[b]	
			A. hydrophila reference strains	*Aeromonas* spp. isolates
VP	API-20E	+ in both systems	6/10	33/49
	Tube	− in both systems	2/10	10/49
		+ only in API-20	2/10	3/49
		+ only in tube	0/10	3/49
Indole	API-20E	+ in all systems	10/10	38/49
	Tube	− in all systems	0/10	3/49
	AH medium	− only in AH medium	0/10	8/49
Nitrate	API-20E	+ in both systems	10/10	49/49
	Tube			
Gelatinase	API-20E	+ in both systems	6/10	42/49
	Plate	− in both systems	0/10	1/49
		+ only in plate	4/10	6/49
Gas production	API-20E	+ in all systems	7/10	29/49
	Tube	− in all systems	2/10	1/49
	AH medium	+ only in tube	1/10	19/49
Acid from mannitol	API-20E	+ in all systems	9/10	47/49
	Tube	− in all systems	0/10	2/49
	AH medium	(+) only in tube	1/10	0/49
Acid from inositol	API-20E	− in all systems	10/10	49/49
	Tube			
	AH medium			
Acid from arabinose	API-20E	+ in both systems	10/10	21/49
	Tube	− in both systems	0/10	11/49
		+ only in tube	0/10	17/49
Arginine dihydrolase	API-20E	+ in both systems	10/10	47/49
	Tube	− in both systems	0/10	2/49
LDC	API-20E	+ in both systems	0/10	0/49
	Tube	(±) in both systems	4/10	23/49
		− in both systems	2/10	1/49
		(±) only in tube	4/10	25/49
Ornithine decarboxylase	API-20E	+ in all systems	0/10	2/49
	Tube	− in all systems	10/10	47/49
	AH medium			

[a] (+), weak reaction; (±), doubtful or difficult to interpret reaction.
[b] Number of strains giving positive or negative reactions/number of strains tested.

A reaction that was doubtful or difficult to interpret in both systems was the LDC test. This confused interpretation was more frequent in the conventional Moeller medium where the color changes often were difficult to distinguish. The only clear negative LDC strains in both identification systems corresponded to anaerogenic *A. hydrophila* strains.

DISCUSSION

Until now, few studies have been conducted to associate the biochemical characteristic of *Aeromonas* species with virulence factors, mainly in environmental strains (5, 7, 13, 30). A major problem encountered in carrying out this type of study is that the taxonomy of the genus *Aeromonas* has undergone significant change in recent years. Schubert (26) describes two species, *A. hydrophila* and *A. punctata*, with several subspecies. Popoff and Véron (22) combined both species to form a single species, *A. hydrophila* (including biovar *hydrophila* and biovar *anaerogenes*), and in addition described a new species, *A. sobria*. Recently, Popoff (21) recognized the *A. hydrophila* biovar *anaerogenes* as the new species *A. caviae*. In the present work we have followed basically the taxonomic scheme of Popoff and Véron (22), using the following as the main differential traits: esculin hydrolysis, fermentation of arabinose and salicin, and production of gas.

Biochemical reactions such as the VP test, production of gas, fermentation of arabinose, gelatinase activity, and the LDC test have been considered correlated with virulence in motile *Aeromonas* species isolated from different sources (4, 5, 7, 13, 30). Interestingly, our results (Table 2) indicated a high variation in these important characteristics among the multitest systems and conventional methods. In general, the failure of miniaturized systems in some of those tests was also demonstrated in bacterial isolates from marine fish and

shellfish (6, 24), in pathogenic *Vibrio* and *Pasteurella* species (10, 15), and in *Yersinia ruckeri* isolates (27).

Although De Figueiredo and Plumb (9) reported false-negative indole reactions with the API-20E system in *A. hydrophila* isolated from fish, we have obtained good correlation between this system and the conventional tube test. However, the AH medium exhibited failure in this reaction and, in addition, did not detect all of the aerogenic *Aeromonas* strains utilized. Thus, the AH medium seems to be useful for a presumptive and fast identification of motile *Aeromonas* spp., but is not entirely suitable for defining aerogenic or anaerogenic strains.

We have found that a large number of isolates are not listed in the API-20E Profile Index (i.e, strains with anomalous LDC, gelatinase, or arabinose fermentation reactions were not codified); some strains were misidentified. Another problem with the API-20E Index is that the results are clear and simple to score after the same time of incubation. From the results of this study, we consider that the API-20E strips can provide a tool for examining environmental motile *Aeromonas* strains only if these recommendations are followed: (i) the API 20E Profile Index must not be used; (ii) important biochemical reactions such as gelatinase, fermentation of arabinose, VP, and citrate must be backed up by standardized testing; (iii) the conventional esculin hydrolysis and fermentation of salicin tests should be conducted simultaneously with the API-20E system to classify the *Aeromonas* isolates to the species level.

The overall false reactions found in the multiple test systems, designed for use at 37°C, indicate that these tests are not sufficiently reliable to identify bacterial isolates from fish or other environmental sources. Therefore, precautions must be taken, especially when some of the reactions included in these systems are used to look for a correlation with virulence (28). In conclusion, since biotypic designations are susceptible to slight variation in methodology, we believe that future work should focus on standardization of the identification system (including medium, temperature, and time of incubation) and taxonomic criteria.

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