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UNIVERSITY OF MARYLAND AT COLLEGE PARK

DEPARTMENT OF MICROBIOLOGY

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Acting Director, Office of Research
Agency for International Development
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Dear Dr. Daly:

It is a pleasure to forward the final report of my grant, "An Assessment of the Role of Enterotoxin(s) and Other Virulence Associated Features in Aeromonas Infections" (6.333), Grant No. DPE-5542-G-SS-7029-00. Our research with the support of this grant has been very productive and we are very appreciative of this support from the Agency for International Development.

Sincerely yours,

Sam W. Joseph
Professor of Microbiology

cc: BOST ID + EDIE

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Final Report

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"An Assessment of the Role of Enterotoxin(s) and
Virulence Associate Featured in
Aeromonas Infection" (6.333)

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3. Executive Summary

Aeromonas sp., a bacterial genus comprising 14 genospecies (within which are 10 phenospecies) occur naturally in the environment and increasingly over the last 15-20 years have been recognized to cause or be strongly associated with intestinal and extra-intestinal infections of humans worldwide. In Dhaka, Bangladesh, the incidence in intestinal infections of Aeromonas isolation is approximately 20%.

Our intent originally was to collect isolates worldwide, including Bangladesh, and study them to determine which virulence features were primarily associated with strains isolated from clinical sources. We soon discovered that the identification schema and taxonomy for this genus were in a state of flux, so we embarked on what became a lengthy and productive taxonomic alignment of this genus and devised a useful identification schema as well. This was necessary to be able to correctly denote the strains, which we were able to study eventually for virulence assessment. In the process, we designated two new species, A. jandaei and A. trota and contributed valuable new information on a newly described species, A. schubertii. Afterwards, we studied the virulence features of strains representing the majority of the species.

Our findings have been published and have been requested from researchers worldwide. Based on our work, researchers are now able to better identify the Aeromonas isolates to species and all research done on these species can be better assimilated with other research in the future. In the past research reports were a morass because the studies were performed on poorly characterized strains.

We have reported on aerolysin (enterotoxin), elastase and have studied autoagglutination, adhesion and serum susceptibility of Aeromonas sp. These findings have

contributed to the overall knowledge of virulence of this bacterial genus. Such findings will eventually contribute to a means of ~~information~~ intervention against Aeromonas infections in humans.

4. Research Objectives

This project was conducted because it was becoming apparent that Aeromonas was being isolated in high frequencies from patients suffering from intestinal disease worldwide and particularly in tropical countries. The incidence in tropical countries seemed to be much higher in children than in adults.

While this information was accumulating, very little was known about the nature of the virulence of the isolates, thus accounting for their pathogenicity. It was important to understand how disease is caused, since ultimately chemotherapy and other interventions, such as a vaccine, would be based on this type of information.

We became involved in the identification and taxonomy of Aeromonas, because we really wanted our studies to have some validity and to be compared with future studies of a similar nature. We soon found that many of our isolates, collected from various parts of the world, were not correctly identified so we decided to perform a numerical taxonomy study of our isolates. This then led to the development of an identification schema and a copyrighted, computer based identification system (FLOBAN).

This work (see enclosed reprints) fit in with some research performed by Kuijper et al. (1989), Altwegg et al. (1990) and Arduino et al (1988). Each attempted phenotypically to separate the different DNA hybridization groups with limited success. Because these studies did not satisfactorily separate the various genotypes using phenotypic clustering, this study was done. We examined a large set of aeromonads representing as many species as possible,

including clinical, veterinary and environmental isolates and originating from many different geographic sources. We were able to phenotypically delineate all but two of the total recognized genospecies.

We then developed a schema for identifying Aeromonas to the species level (see enclosed reprint). The previous approach (Popoff & Veron, 1976) had become cumbersome and was not useful for some of the new phenospecies identification. With AeroKey II (Carnahan et al., 1991), it is now possible to identify reliably all of the major, important clinical species.

The innovative aspects of the above work were based mainly on the selection of the tests, which were used in this work. Further, research on the identification schema and FLOABN were completely original in their conceptualization and development.

Another major part of the problem was the nature of the virulence of these organisms. We studied the following:

Aerolysin - (see enclosed reprint)

We looked at the aerolysin genes of all of the genospecies and found that A. trota had a unique region which was completely different from all of the other species. This had not been reported for any of the species previously. The only similar work was by Lior et al ( ) who developed a probe for A. hydrophila which could encompass hybridization groups 1, 2, and 3 (motile, mesophilic strains). Jennifer Pryde's work in my lab has shown through PCR and sequencing of the AerC region that the homology for this region in A. trota is >95% (to be published). In the process of doing this work, she found that two strains were atypical in that they were non-hemolytic under the correct temperatures and nutrient availability and when tested against either horse or rabbit erythrocytes (Afsar Ali, to be published).

Elastase - (see enclosed reprint)

We studied 166 aeromonads using a new method developed in my lab. Of the 166, 32% were found to produce elastase. The only species found positive of those tested was A. hydrophila. Two exceptions were one strain of Aeromonas group 501 and one strain of A. schubertii. This reinforced the notion that A. hydrophila was an important pathogen and elastase could be an important contributory virulence feature.

While this feature has been studied sporadically on various strains, this is the first study performed on a large number of well-characterized strains using a reliable assay (Hsu et al., 1981; Renaud et al., 1988).

Autoagglutination, Serum Susceptibility and Adhesion -

Research on autoagglutination, serum, susceptibility and adhesion was performed by Saeed Feize in my lab and is unpublished. These are important virulence features of aeromonads which are part of this research concept. We were interested in determining if there were significant differences between species or between strains within species. This work is described in Section 5 below.

Isozymes -

After studying several isozymes, we concentrated on esterases which demonstrated several bands in many instances. There was substantial difference not only between species, but between strains within species. This finding was important because it can allow us to fingerprint particular strains within species. Since there is a great deal of difference in virulence from strain to strain, esterase patterns provide an ideal biotyping technique.

5. Methods and Results

The methods and results are shown in the eighteen reprints attached to this report. In this section, I have included the summaries of two Master's theses on Aeromonas research which is presently being written up for publication. I would be pleased to supply full copies of both theses if needed.

The following work was performed by an honor's undergraduate student on autogglutination, serum susceptibility and adhesion of aeromonads.

Materials and Methods

Graph of Optical Density vs. Colony Forming Units per ml (CFU/ml).

To obtain the correlation between density of bacterial suspension in PBS and the number of cells per ml in the suspension, the following experiment was performed.

Among the available strains of bacteria, one strain (*A. hydrophila*, AMC#1) was chosen, grown in 50 ml TSB for 4h at 37°C, centrifuged, washed with 20 ml PBS twice and finally resuspended in 20 ml PBS. The optical density of this suspension was measured at 420 nm with the Spectronic 20; then serial 2-fold dilutions of this suspension were made and the optical density of each dilution was measured. To obtain the number of cells in the original suspension, serial 10-fold dilutions of the original suspension were made and 0.1 ml from 10^6, 10^7, 10^8 fold dilution tubes were plated on TSA plates in duplicate, incubated at 37°C overnight and the average number of CFU recorded with the use of an electronic colony counter. Based on this data, the density of the original suspension was determined to be 2.27×10^9

cells/ml. Using this number as the titer of the original suspension and the dilution factors shown in Table 1, the corresponding cell densities (CFU/ml) for the optical densities listed in Table 1 were derived and a graph of cell density vs. optical density was plotted (Figure).

Part I. Serum Survival Assay (Viable Cell Count Method)

A. Bacterial Strains. Fifty strains of *Aeromonas* from various clinical and environmental origins were selected and challenged *in vitro* against normal human serum bactericidal effect. Table A presents the distribution of the clinical and environmental species of *Aeromonas* (*A. hydrophila*, *A. sobria*, *A. caviae*) strains tested.

B. Growth of Bacterial Cells. Each strain was grown on Tryptic Soy Agar (TSA) and incubated at 37°C for 24 hr. Then a heavy inoculum was transferred from the TSA plates to 2 ml small vials containing 1.5 ml broth medium with 12% glycerol (Tryptic Soy Broth + 12% glycerol by volume which was already autoclaved to assure sterility). Vials were placed in a jar containing methanol and immersed in liquid N_2 to quick freeze. Vials were then stored at -70°C.

On the day of each experiment, the selected 1.5 ml vials were partially thawed and a loopful of growth from each vial was streaked on a TSA plate. After 24 hr. of incubation at 37°C, a single colony was transferred to a 250 ml culture flask containing 50 ml Tryptic Soy Broth, incubated in a water bath shaker at 37°C for 3 to 4 hr. at 250 rpm, transferred to 50 ml screw capped

centrifuge tubes and centrifuged for 15 min. at 5000 rpm (4068 g) (4°C). The pellet was washed in 50 ml PBS (pH = 7.4) twice and finally was resuspended in 20 mls of PBS. After appropriate dilutions, a bacterial suspension with an optical density of 0.5 to 0.55 at 420 nm was obtained. By the use of the standard graph of cell density vs. optical density (previously obtained for a single strain of Aeromonas), this suspension of bacterial cells (O.D. = 0.5 to 0.55 at 420 nm) was estimated to contain a cell density on the order of 10^8 cells/ml. After serial 10-fold dilutions, a stock solution of 10^4 cells/ml was used for the serum survival assay following the protocol presented in the method of assay described below. The formulae for all media and PBS used in this assay are listed in the appendix.

Preparation of Serum

Blood was obtained from 5 individuals, allowed to clot at room temperature for 1 hour and refrigerated at 5°C overnight. The next day it was centrifuged at 5000 rpm (4068 g) (4°C) for 5 minutes; the serum was separated from the blood clot using a Pasteur pipette, pooled and stored in 1.5 ml aliquots in Eppendorf tubes at -70°C. On the day of each experiment, one or two tubes of serum were thawed at 0°C and used for the assay.

Method of Assay

The following protocol was used to set up 10%, 30%, 50% and 70% serum suspensions of bacterial cells in 1.5 ml microfuge tubes, to which 75 ul of a bacterial suspension of 10^4 cells/ml was added.

		Serum Suspensions			
	Control	10%	30%	50%	70%
Bacterial suspension	75 ul	75 ul	75 ul	75 ul	75 ul
PBS	175 ul	150 ul	100 ul	50 ul	0
Serum	0	25 ul	75 ul	125 ul	175 ul
TOTAL VOLUME	250 ul	250 ul	250 ul	250 ul	250 ul

Each Eppendorf tube was incubated in the water bath at 37°C immediately after the addition of serum. After 15 and 45 minutes of incubation, 50 ul from each tube was spread on a TSA plate by the use of a sterile glass rod. The plating was done in duplicate. The plates were incubated in a 37°C incubator overnight and the average number of colonies on duplicate plates was recorded.

Part I. Serum Survival Assay (microcolorimetric assay). A variation of the Moll *et al*. method was used (20).

Growth Media. Two kinds of broth media were used: Tryptic Soy Broth (TSB) and Peptone-Glucose Broth (PGB). The formulas for both are listed in the appendix. PGB contained additionally 0.5% of a 1.5% stock solution of Bromthymol Blue in ethanol.

Method of Assay. Frozen aliquots of bacterial strains were thawed at 0°C. A loopful of growth from each aliquot was streaked on a TSA plate and incubated at 37°C overnight. Bacterial colonies from each TSA plate were transferred to corresponding wells of microtiter plates (8x12 wells) containing 0.2 ml of TSB per well by using sterile wooden applicators and incubated overnight at 37°C. A multipoint inocculator

(6x8) was used to inocculate bacteria from overnight cultures to a new microtiter plate containing TSB (.2 ml per well). After incubation of the plate at 37°C for 2 h., the log phase bacteria were transferred to a new microtiter plate containing PGB using a multichannel pipettor (10 ul per well). The plates were incubated at 37°C for 3 hrs. after which the results were read and recorded using the following system:

Yellow	=	0 -------	Resistant
Very light green	=	1 -------	Intermediately Resistant
Light green	=	2 -------	Intermediately Resistant
Blue	=	3 -------	Sensitive

The following illustration shows how a microtiter plate was set up to do the assay.

Content		Microtiter Plate
All across the row		1 2 3 4 5 6 7 8 9 10 11 12
[a]Control (no serum)	A	
[b]30% serum	B	
Control (no serum)	C	
[c]50% serum	D	
Control (no serum)	E	
30% serum	F	
Control (no serum)	G	
50% serum	H	

[a]Control contains 150 ul of PGB + no serum.

[b]30% serum contains 105 ul PGB + 45 ul serum (45ul/150ul=30%)

[c]50% serum contains 75 ul PGB + 75 ul serum (75ul/150ul=50%)

In each column, two different strains (A through D) and (E through H) were challenged against 30% and 50% serum. Therefore in each microtiter plate 24 different strains can be assayed for their serum susceptibility.

Source of Serum in Microcolorimetric Assay. Instead of pooled human serum used in the viable cell count method, my own serum was used in the microcolorimetric assay. The switch to my serum was done for two reasons: firstly, it was more convenient to have my own blood drawn rather than getting the same individuals who donated the blood the first time and drawing their blood. Secondly, a change to my own serum was justified by observing the same results obtained in assays performed on strains AMC #6, and MSH-34 with my own serum. They were both sensitive to my own serum as they were to pooled human serum. The preparation of my own serum was the same as mentioned for pooled human serum.

Part II. Adhesion Assay. A variation of the enzyme-linked assay (Beachey et al., 1986) was used (24).

Reagents. Unless specified otherwise, all reagents and chemicals were purchased from Sigma Chemical Co. The description of each chemical is listed in the appendix.

Bacterial Strains. Fourteen different strains of Aeromonas (as listed in Table C, appendix) were streaked on TSA plates and incubated overnight. A single colony from each plate was transferred to a 250 ml

culture flask containing 30 ml of sterilized TSB and each flask was incubated in a water bath at 37°C for 3 1/2 to 4 hr. at 250 rpm. After this period of incubation, the broth culture was centrifuged, washed twice in 20 ml PBS (pH = 7.2) and finally resuspended in 10 ml bicarbonate buffer (0.1 M sodium bicarbonate, pH = 8.2).

From this stock suspension, 0.5 ml was transferred to a clean sterilized cuvette and an adequate amount of bicarbonate buffer was added to adjust the optical density of the suspension to 0.7 at 550 nm. At this point a 1.0 ml aliquot of this suspension was transferred to a 1.5 ml microfuge tube. After addition of 12.5 ul of biotin-n-hydroxy succinamide (10 mg/ml in dimethyl sulfoxide) to 1 ml aliquots of bacterial suspensions, the tubes were incubated for 2 h at ambient temperature. The bacterial suspensions were centrifuged at 5000 rpm for 10 minutes, and pellets washed with PBS three times. After the last centrifugation, the supernatant was discarded and the pellets were stored at -70°C. On the day of each experiment, the cells were thawed, resuspended in PBS and used in the assay.

Preparation of Tissue Culture Cells. Hela cells were provided as a gift from Dr. Ed Oaks at the Walter Reed Army Institute of Research (WRAIR). The Hela cells formed a monolayer after a few days of growth in tissue culture flasks at 37°C and in the presence of 5% CO_2. After formation of a monolayer, (approximately 3 days), the media was poured off and the Hela cells were washed three times with trypsin. After the last wash, the Hela cells were resuspended in fresh media and 0.1 ml was added to all wells of 5 microtiter plates. After incubation in the presence of 5% CO_2 at 37°C overnight, the plates were examined the next

day for formation of a monolayer at the bottom of each well. Then the plates were washed three times with PBS and blot-dried upside down on blotting paper. Then the cells were heat-fixed by incubation of the plates at 70°C for 30 minutes, after which 0.1 ml of a 5% solution of BSA (0.5 g BSA in 10 ml water) was added to each well. The plates were incubated at 37°C for 3 hr and washed 3 times with PBS and blot dried on blotting paper, at which time the Hela cells were ready for the adhesion assay.

A. Determination of the titer of cells in 1 ml aliquots of biotinylated strains. Serial 10-fold dilutions of a 1 ml aliquot of thawed, resuspended biotinylated strain AMC #1 in PBS were made and 0.1 ml from 10^5, 10^6, 10^7 and 10^8 fold dilution tubes was spread on corresponding TSA plates. There were no colonies on 10^8 plates, 2 colonies on 10^7 plates, an average of 50 colonies on 10^6 plates, and more than 300 colonies on 10^5 plates. Therefore, the titer of the suspension was determined to be $5x10^8$ cells/ml.

B. Adhesion Assay. To the corresponding wells of a microtiter plate as illustrated in the following Table, 50 ul of each of 14 biotinylated strains of Aeromonas was added and the plates were incubated at 37°C for 40 min. Then, the wells were washed with PBS 3 times and blot dried upside down on blotting paper for a few minutes. To heat-fix the attached bacteria, the plates were incubated at 70°C for 15 min. After this period of incubation, 100 ul of avidin-peroxidase conjugate (APC) was added to each well and the plates were incubated for 30 min. at 37°C. Then, the plates were washed 3 times with PBS and blot dried as mentioned above, followed by addition of 100 ul of

orthophenylenediamine (OPD) solution to each well. The formulation of OPD is listed in the appendix. The color was allowed to develop for 3 hr., and the absorbance at 450 nm was measured with a microtiter plate reader.

	1	2	3	4 ...12
	H cell + Bacterial Strain	H cell + Bacterial Strain	No H cell + BSA	No H cell + No BSA
A	N6	AMC #1	N6	N6
B	MSH-5	2 AER76	MSH-5	MSH-5
C	AER 218	AMC #2	AER 218	AER 218
D	AER 321	AB3-25	AER 321	AER 321
E	VM94365	AMC #9	VM94365	VM94365
F	VM82255	AB3	VM82255	VM82255
G	N54	PBS	N54	N54
H	AMC #7	PBS	AMC #7	AMC #7

Second Experiment. Since in the first experiment, the strains were not tested in duplicate (two wells for each strain), 8 strains out of 14 strains assayed in Experiment 1 were chosen for a second run of the adhesion assay. These included strains N6, N54, AER218, AB3, AB3-25, AMC #7 and AMC #9 which had the highest absorbances and strain AMC #2 which had the lowest absorbance among 14 strains assayed in the previous experiment.

This assay was performed in two plates in the following manner: In plate #1, the experiment was performed on 8 chosen strains in duplicate (column 1 and 2) exactly as done before. In other columns, different lectins (see Table D in Appendix) were used to study the

inhibition of attachment of the bacteria to Hela cells in the presence of lectins. Thirty-five microliters of each lectin was added to each well containing heat-fixed Hela cells and the plates were incubated at 37°C for 1 hour. Then 50 ul of biotinylated bacteria were added to each well and the plates again were incubated at 37°C for 1 hour. Everything else was done as described in the previous experiment. In this experiment a PBS column on the microtiter plate was intended to be used as a blank in the measurement of absorbance with the microtiter reader (Titertek, Multiscan MC). However, the PBS column did not work (all wells developed color; see explanation provided in discussion), and air was used as the blank.

Third Experiment. Since the PBS control column did not work as described above, the adhesion assay was performed on the 8 chosen strains one more time and with proper precautions to preserve the PBS control column (throughout the entire period of experimentation the plate was kept in the dark). (See discussion for explanation.)

Fourth Experiment. To obtain the correlation between absorbance at 450 nm and the number of bacterial cells present in each well, the following experiment was performed:

The titer of 1 ml aliquot of resuspended biotinylated bacteria in PBS (for strain AMC #1) was determined as explained before ($5x10^8$ cells/ml). Then the resuspended bacteria were centrifuged, the supernatant was discarded and the pellet was resuspended in 1 ml of avidin-peroxidase conjugate. Then the 1 ml suspension was incubated at 37°C for 1 hour and centrifuged and the pellet was washed in 1 ml PBS

three times and finally resuspended in 1 ml PBS. Then serial 10-fold and 2 fold dilutions of this suspension was made. From each dilution made, 50 ul was added to the wells of a microtiter plate, followed by the addition of 100 ul of OPD. The color was allowed to develop for 15 minutes and the absorbance at 450 nm was measured. Then, based on the values of absorbance obtained in this experiment, the graph of the number of bacterial cells per well vs. absorbance at 450 nm was plotted. (See Appendix)

Autoagglutination Experiment

Eighteen different strains of Aeromonas species of clinical and environmental origin (including the 14 strains tested in the adhesion assay) were inoculated into Eppendorf tubes containing 1.5 ml of Brain-Heart Infusion Broth (BHI broth, see Appendix for formulation). After 24 hrs. incubation at 37°C, 10 ul from each Eppendorf tube was then transferred to (16x125 mm) test tubes containing 6 ml BHI broth. A. hydrophila strains were incubated at 37°C, and A. sobria strains were incubated at 29°C. After 18 hours of incubation spontaneous auto-agglutination or pelleting (SP) was detected by absence or presence of a small white button on the bottom of the tubes. Then all tubes were vortexed for 30 seconds to bring all bacteria to suspension and 3 ml of each tube were transferred to a clean test tube. This second set of test tubes was placed in a boiling water bath for 1 hour. The autoagglutination after boiling (PAB) was detected by presence or absence of large aggregates of bacteria at the bottom of the tube.

Results of Virulence Studies

Auto-agglutination -

It was found that strains within species could have differing characteristics for spontaneous pelleting (SP) or pelleting after boiling (PAB). If this phenomenon can be regarded as a virulence factor, these findings suggest that some strains within a species can be more enhanced for virulence because of the auto-agglutination property (Table 1).

Serum susceptibility -

Similar to our auto-agglutination studies, it was observed that serum susceptibility can differ among strains within species suggesting that some are more capable of activating complement than others. Again this property would convey a greater capability to survive, particularly where extra-intestinal infections are involved (Table 1). A large portion of the clinical strains (60%; 12/20) were resistant to serum killing, suggesting the potential pathogencity of a greater number of clinical strains (Table 2).

The frequency of resistant strains was higher with A. hydrophila and A. sobria than with A. caviae. Other studies have suggested that A. caviae is the least pathogenic of the three major species. Thus, serum susceptibility is at least one factor which may account for this observation (Table 3).

Adhesion -

When tested against HeLa cells using biotinylated Aeromonas strains and an avidin peroxidase conjugate (APC0) with orthophylenediamine (OPD) indicator, it was found that all of the strains had some degree of adhesive capability. AB3-25 was the most adhesive strain and AMC-2 was the least adherent (Table 4).

We were able to obtain a saturation curve for the maxium numbers of strain AMC 1

Table 1. Autoagglutination and serum survival analysis of thirty-five strains of Aeromonas sp.

Source and Strain #	Donor #	Organism I.D.	Run #1 Results		Run #2 Results		Serum Survival Assay	
			SP[a]	PAB[b]	SP	PAB	Viable Cell Count Assay	Micro-color-metric
Stool 34	MSH-5	Aeromonas sp	-[c]	+[d]		ND[e]	S	S
10	AB3*	A. trota (N)[f]	-	-	-	-	R	R
11	AB3-5**	A. trota (N)	-	-	-	+[w]	R	R/S
12	AB3-15**	A. trota (N)	-	-	-	-	R	R/S
13	AB3-25**	A. trota (Grp 5A)[g]	-	-	-	-	R	R
Stool 42	UM94303	A. caviae (V) (Grp 5A)	-	-	-	-	ND	R
Stool 44	UM82255	A. caviae (V)	-	-	-	-	ND	R
1	AMC-1	Aeromonas sp	-	+	ND		R	R/S
2	AMC-2	Aeromonas sp	-	+	ND		R	R
6	AMC-7	Aeromonas sp	-	+	ND		R	R
8	AMC-9	Aeromonas sp	-	-	ND		R	S
25	AER-76	Aeromonas sp	-	+	ND		R	R
29	AER-218	Aeromonas sp	-	+	ND		ND	R/S
fecal 28	AER-321	A. hydrophila (H)	+	+	+	+	S	R/S
Env.	NMRI-1	Aeromonas sp (Grp 4)	No Growth		ND		No Growth	
Env. 40	NMRI-2	A. caviae (X)	-	-	-	-	ND	R
Env. 20	NMRI-3	A. hydrophila (H)	-	-	-	+	ND	S
G.I.	NMRI-4	Atyp. A. hydrophila (D)	-	-	-	-	ND	ND
Lung	NMRI-5	A. hydrophila (H)	-	-	-	-	ND	ND

(Cont'd) Table 1. Autoagglutination and Serum Survival Analyses of 35 Strains of Aeromonas sp.

Source & Strain#	Donor #	Organism I.D	Run #1 SP[a]	Run #1 PAB[b]	Run #2 SP	Run #2 PAB	Serum Survival Assay Viable Cell count	Serum Survival Assay micro-colorimetric
Leg wound 23	NMRI-6	Aeromonas sp	-	-	-	-	ND	R/S
Leg wound	NMRI-7	A. hydrophila (H)	-	-	-	+	ND	ND
Env.	NRMI-16	A. hydrophila (H)	-	+	-	+	ND	ND
Env. 24	NMRI-54	A. hydrophila (H)	-	+	-	+	R	R/S
Env. 21	NMRI-55	A. hydrophila (H)	-	-	-	+	ND	S
Env. 22	NMRI-56	A. hydrophila (H)	-	-	-	+	ND	S
Bld.	NMRI-124	Aeromonas sp	-	-	ND		ND	ND
Bld.	NMRI-125	Aeromonas sp	-	-	ND		ND	ND
Foot wound	NMRI-150	Aeromonas sp	-	-	ND		ND	ND
G.I.	NMRI-203	A. hydrophila (H)	+/-	+	+	+	ND	ND
G.I.	NMRI-204	A. hydrophila (H)	-	-	-	+	ND	ND
G.I.	NMRI-206	Aeromonas sp	+	-	-	-	ND	ND
G.I.	NMRI-208	Aeromonas sp (Grp 4)	+	-	-	-	ND	ND
G.I.	NMRI-209	A. caviae (X)	-	-	-	-	ND	ND
G.I.	NMRI-210	Aeromonas sp	+	-	-	ND	ND	ND
Sputum	NMRI-214	Aeromonas sp	-	-	ND		ND	ND

*Nalidixic acid mutant of A. trota AH-2.
**Site directed mutants of AB-3.
[a]SP, Spontaneous pelleting
[b]PAB, Pelleting after boiling
[c]-, negative; [d]+, positive; [e]ND, Not determined
[f]C-J cluster group
[g]DNA hybridization group

Table 2: Experiment I: Adhesion of Aeromonas to Hela cells in Elsa adhsion assay.

Strain#	Donor's #	Absorbance measured at A450		
		Hela cell	No Hela Cell	
		Hela cell+BSA+Aeromonas	CBSA**	SBSA***
23	N6	.169	.191	.272
34	MSH-5	.099	.144	.232
29	AER-218	.177	.148	.259
13	AB3-25	.476	.143	.295
42	VM94303	.103	.146	.241
44	VM82255	.109	.143	.347
24	N54	.152	.310	.240
7	AMC#7	.128	.176	.322
1	AMC#1	.116	_***	_
25	AER-76	.105	_	_
2	AMC#2	.084	_	_
28	AER321	.109	_	_
8	AMC#9	.126	_	_
10	AB3	.148	_	_
	PBS*	.058	_	_

*: PBS was used as control blank.
**: CBSA: Treated with BSA.
***: Not tested.
****: SBSA: Not treated with BSA.

Table B: Experiment II, Adhesion of Aeromonas to HeLa Cells in ELISA Adhesion

Adhesion Assay

Strain Number	Donor Number	Hela Cells									No Hela Cells	
		slec[a]	L1[b]	L2	L3	L4	L5	L6	L7	PBS[c]	cBSA[d]	sBSA
23	N6	.377	.355	.392	.487	.418	.416	.424	.570	.356	.282	.295
29	AER218	.342	.376	.344	.408	.357	.362	.359	.487	.322	.227	.283
13	AB3-25	.457	.418	.449	.470	.440	.470	.463	.523	.325	.289	.392
24	N54	.385	.325	.449	.396	.363	.380	.372	.480	.358	.240	.430
7	AMC #7	.433	.485	.444	.398	.483	.436	.454	.477	.369	.321	.452
8	AMC #9	.403	.377	.419	.405	.432	.424	.396	.502	.390	.293	.442
10	AB3	.439	.441	.447	.423	.498	.474	.469	.518	.418	.347	.511
2	AMC #2	.388	.430	.380	.354	.457	.446	.460	.598	.351	.325	.427

[a] s lec: The absorbance observed for attached bacteria to Hela cells in absence of lectins. The average of two values obtained for the same organism in two wells (duplicate) was recorded for each strain tested.

[b] The average of two values obtained in the presence of lectins was recorded for each strain. The number affixed to L represents the number of lectin used and it is the same as listed in list of lectins (see List of Lectins, Table D in Appendix).

[c] The PBS control column which was supposed to be used as blank did not work. Air was used as a blank. 50 microliters of PBS, instead of biotinylated bacteria, was added to all wells in this column.

[d] cBSA = Treated with BSA
sBSA = Not treated with BSA

Table 4:Experiment III:Adhesion of *Aeromonas* to HeLa cells in ELSA adhesion assay.

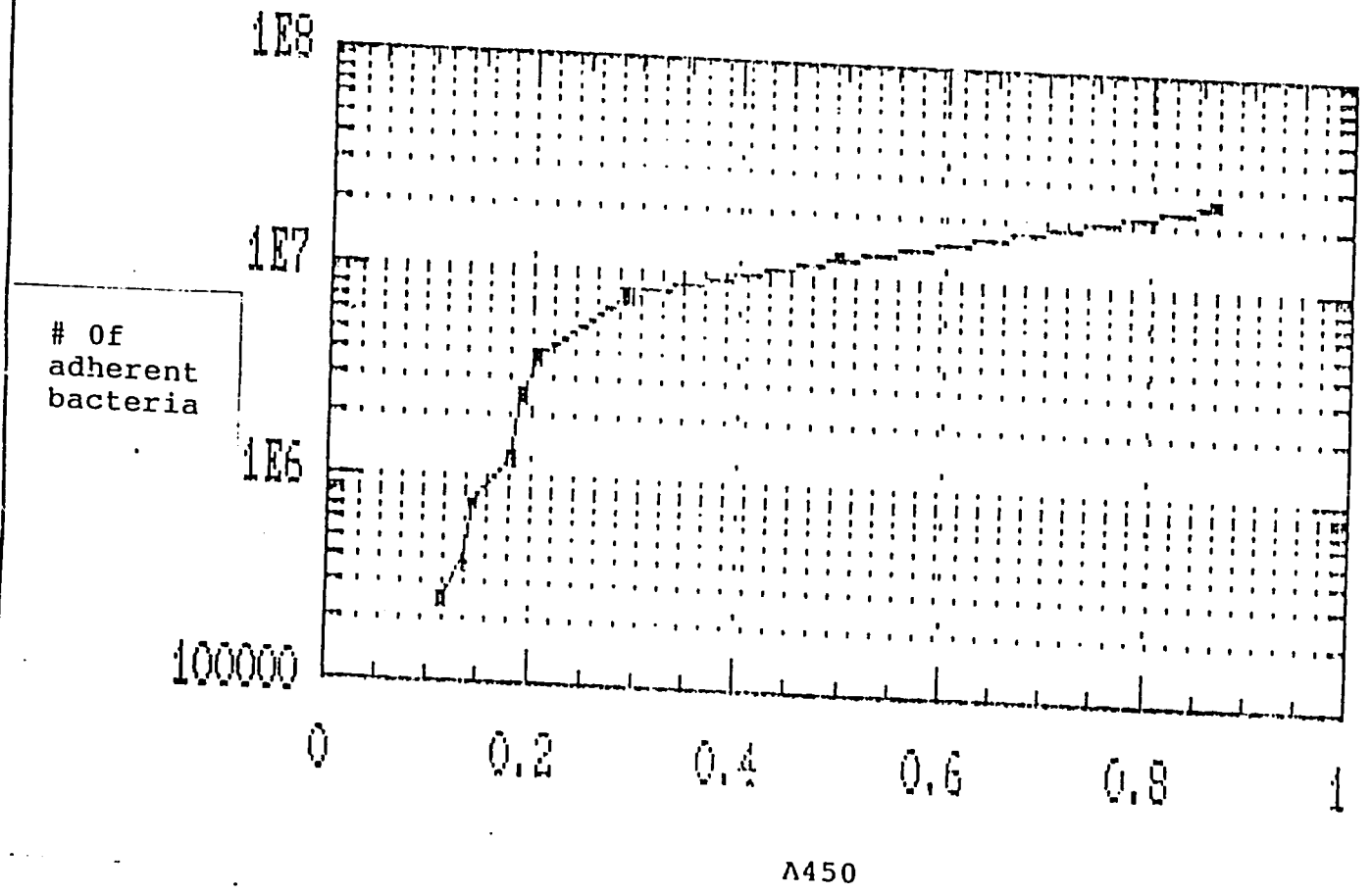
Strain#	Donor's #	A450 Bacteria*	PBS**
23	N6	1.173	.017
29	AER218	1.005	.016
13	AB3-25	1.108	.018
24	N54	1.059	.018
7	AMC#7	1.059	.014
8	AMC#9	1.076	.016
10	AB3	1.045	.015
2	AMC#2	0.924	.021

*The average of two values of A450 was recorded taking PBS control value into conside

** 50 microliters of PBS,instead of biotinylated bacteria,was added to all wells in t column. The column was used as control blank.

FIGURE 6

Plot of number of adherent bacteria VS. absorbance at 450 nm.



which could attach to the HeLa cell monolayer. Using dilutions 1:2 through 1:100 of a heavy suspension of cells and adding 50 μl of biotinylated cells to each well, it was determined that the 1:8 dilution (OD.290) represented the maximum number of binding sites available on the monolayer (Fig. 1).

6. Impact, Relevance and Technology Transfer

The findings of this study will be relevant in Bangladesh and other countries because they will enable scientists and medical technologists to accurately identify their isolates. Our studies on virulence features provide an opportunity for them to study the virulence features of Aeromonas sp. using our techniques and to compare their results with ours. The impact will be on the health of individuals in developing countries. These studies and similar studies will eventually lead to strategies for reducing incidence of diarrheal disease by aeromonads.

Large scale trials are warranted because there is still a need to establish absolute causation of diarrheal disease by Aeronmonas, although strong associations have been shown. A large well-designed epidemiologic study, which addresses the criteria of causation should be performed.

We have trained scientists in Bangladesh and two have been graduate students in my laboratory. These individuals are now independently capable of performing state-of-the-art research on Aeromonas.

As a matter of fact, Dr. Brad Sack at ICDDR,B informs me that they are presently contemplating a study to measure IgA production by antibody secreting cells in response to intestinal infections by various species of Aeromonas.

7. Project Activities/Outputs

- Meetings attended -

American Society for Microbiology Annual Meetings - 1988, 1989, 1990

Interscience Conference on Anti-Microbial Agents & Chemotherapeutics - 1991

International Symposium on Aeromonas and Plesiomonas - 1988, 1990

- Training -

- Trained a technician from ICDDR,B, Afsar Ali, for four months in my lab, 1988-89
- Post-Doc for four months, Volker Husslein, 1991
- Two graduate students from Bangladesh, Afsar Ali and Jafrul Hasan

Graduate students, Post-doctoral associate and technician supported in this work:

1. Volker Husslein, Ph.D. (Post-doctoral associate)
2. Amy Carnahan, M.S.
3. Jafrul Hasan
4. Mohammed Afsar Ali, M.S.
5. Jennifer Pryde, M.S.
6. Paul Macaluso
7. Viola Zaki, guest scientist
8. Saeed Feizi, undergraduate Honor student
9. Marie Head (technician)

In addition, a workshop on Aeromonas identification (see attached copy) was held in which approximately 20 students were trained. This was at no cost to the grant.

Major Contributions:

1. Review on isolation of Aeromonas.
2. Articles on physiologic characteristics.
3. Articles on virulence features including elastases and aerolysin (enterotoxin).
4. Articles on Taxonomy and Identification of Aeromonas.
5. Development of AeroKey II - an identification schema for Aeromonas.
6. Development of FLOABN (For Lack of a Better Name), a computer based system for data storage management and assessment for Aeromonas identification.
7. Workshop on Aeromonas identification held at ICDDR,B, Dhaka, Bangladesh.
8. One technician from ICDDR,B was trained in our lab for four months to be able to analyze and identify Aeromonas.
9. Five M.S. graduate students and 4 others contributed to this work which was supported in part or totally by this AID grant. ~~One part or totally by this AID grant~~. One of these students was from Bangladesh and one other from Bangladesh worked for two years on the project.
10. Seventeen articles, 24 abstracts and one technical article have been published with support of this grant.
11. Designation of two new species Aeromonas jandaei and A. trota and contributed several new strains and information on another new species A. schubertii.
12. Developed a new method for the assay of the enzyme, elastase.

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Patents -

While there are no patents resulting from this research, we do have a copyright and trademark for the FLOABN program.

8. Project productivity -

Overall we probably accomplished much more than we anticipated, because of the additional taxonomic work that was done. I was fortunate that so many people were willing and able to become involved in this work, especially Amy Carnahan who was untiring and outstanding.

9. Future work -

(See my comments in 6 above)

There is additional work to be done on the taxonomy of environmental strains and the molecular genetics of aerolysin.

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WORKSHOP

ON THE

CHARACTERIZATION OF *AEROMONAS*

September 5 - 12, 1989

International Center for Diarrheal Disease Research, Bangladesh (ICDDR,B)
Dhaka, Bangladesh

311

Workshop
on the
Characterization of *Aeromonas*

Organizing Committee

Saul Tzipori	ICDDR,B	Workshop Coordinator
Sam Joseph	University of Maryland	Program Cochairman
Anwarul Huq	ICDDR,B/University of Maryland	Program Cochairman
Jafrul Hasan	University of Maryland	Faculty
Afsar Ali	ICDDR,B	Faculty

Organized & sponsored by:

Department of Microbiology
University of Maryland at College Park, USA,
and
International Center for Diarrheal
Disease Research, Bangladesh (ICDDR,B)

Information provided in this workshop was obtained in part from research accomplished under a research grant from the Agency for International Development (AID), Grant # DPE-5542-G-SS-7029-00.

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III. Biotyping of *Aeromonas* 5

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Program

September 5 10:00 - 12:00 pm 2:00 - 4:00 pm	Jafrul Hasan Afsar Ali	Session 1	The antibiotic susceptibility analysis of *Aeromonas* spp.
September 6 2:00 - 4:00 pm		Session 2	(Session 1 cont'd)
September 7 10:00 - 12:00 pm 2:00 - 4:00 pm	Jafrul Hasan Afsar Ali	Session 3	Electrophoretic isoenzyme typing of *Aeromonas*
September 11 10:00 - 12:00 pm 2:00 - 4:00 pm	Jafrul Hasan	Session 4	Biotyping of *Aeromonas*
September 12 10:00 - 12:00 pm		Session 5	(Session 4 Cont'd)
3:00 - 4:00 pm	Sam Joseph	Session 6	Value of biotyping in the identification of virulent *Aeromonas* species
4:00 - 4:30 pm			Closing Session

Taxonomy, Virulence and Distribution of Aeromonas sp. Recovered from Clinical, Environmental and Other Sources

by

Mohammed Afsar Ali

Thesis submitted to the Faculty of the Graduate School
of The University of Maryland in partial fulfillment
of the requirements for the degree of
Master of Science
1993

Advisory Committee:

Professor Sam W. Joseph, Advisor
Professor Rita R. Colwell
Professor Ronald M. Weiner

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ABSTRACT

Title of Dissertation:	**Taxonomy, Virulence and Distribution of Aeromonas sp. Recovered from Clinical, Environmental and Other Sources**
Name of Degree Candidate:	**Mohammed Afsar Ali**
Degree and Year:	**Master of Science, 1993**
Thesis directed by:	**Sam W. Joseph, Ph.D. Professor Department of Microbiology**

A group of 165 Aeromonas strains of diverse geographical origins and sources were initially phenotyped to the species level (DNA Hybridization Group (HG) level) using a profile of 11 discriminatory tests or AeroKey II. The strains were phenotyped to the species level to enable examination of the distribution of Aeromonas phenospecies in clinical versus environmental samples. The results showed a similar incidence of a particular species found in clinical and water samples in Bangladesh, which corresponded to the spectrum of species in food samples in the U.S.A. The incidence of A. caviae was the highest in both clinical and environmental samples.

Extensive phenotypic characterization and subsequently, genetic analysis, led to the discovery of a new Aeromonas species (A. trota based on 13 strains, which are ampicillin susceptible and are mostly of clinical origin). Another new subspecies, Aeromonas salmonicida subsp bestiarum based on 11 strains (formerly DNA HG2) of mostly environmental and animal origins was designated, also.

Hemolysin activities and specificities for distinct erythrocytes among recently described Aeromonas species, A. trota, and A. jandaei and the long recognized

pathogen, A. hydrophila, were examined. This study revealed that hemolysin activities and specificities not only vary among Aeromonas sp. but also among strains belonging to a particular species. A. trota 5274, which was previously described as non-hemolytic, was in fact beta hemolytic as demonstrated by use of multiple approaches under defined conditions. This finding further proved that Aeromonas hemolysin may be either under the regulation of various environmental controls or that Aeromonas may elaborate similar hemolysins of different specificities.

ANALYSIS OF THE *AerC* AND *AerA* REGIONS OF THE AEROLYSIN GENE OF *AEROMONAS* SP. WITH EMPHASIS ON *AEROMONAS TROTA*

by

Jennifer Newcity Pryde

Thesis submitted to the Faculty of the Graduate School
of The University of Maryland in partial fulfillment
of the requirements for the degree of
Master of Science
1993

Advisory Committee:

Dr. Sam Joseph, Chairman/Advisor
Dr. William Nelson
Dr. Spencer Benson
Dr. Norman Hansen
Dr. Mary Voll

ABSTRACT

Title of Thesis: Analysis of the aerC and aerA regions of the aerolysin gene of Aeromonas sp. with emphasis on Aeromonas trota.

Name of degree candidate: Jennifer Newcity Pryde

Degree and Year: Master of Science, 1993

Thesis directed by: Sam W. Joseph, Ph.D.
Professor
Department of Microbiology

The genus Aeromonas has been implicated as a possible pathogen in gastrointestinal disease. A possible virulence mechanism widely studied is the beta hemolysin aerolysin. DNA hybridization studies, using probes from Aeromonas trota AH2, have revealed that although the aerolysin structural gene denoted as aerA could be detected in all Aeromonas definition strains tested, a proposed regulation region upstream of aerA, designated as aerC hybridized only to strains belonging to A. trota. The aerC and aerA regions of the known A. trota strains were sequenced and the resultant nucleotide and protein sequences were compared for homologies. This was accomplished in a three step procedure, consisting of the Polymerase Chain Reaction (PCR), automated DNA sequencing, and subsequent computer analysis.

The homology of the nucleotide sequences for the aerC and aerA regions was above 90%, while the protein sequences for the structural gene (aerA) was found to average 98% or

above. Only two strains, 5274 and 5439 were relatively less homologous (<95%) in both the nucleotide and protein sequences. Their nucleotide sequences contained significant differences in the aerA region compared to the other sequenced strains of A. trota which also resulted in amino acid changes. Two of the amino acid changes could be responsible for the non-hemolytic phenotype displayed by these two strains. These results indicate that the aerC and aerA regions are highly conserved within the species A. trota. In addition, attempts to amplify other Aeromonas species in the aerC region had very limited success. Although earlier studies suggested a high degree of homology in the aerA region among Aeromonas DNA hybridization groups (HG), some PCR primers in the aerA region were found to be highly specific for particular HG's.

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SYSTEMATIC AND APPLIED MICROBIOLOGY

SEMPER BONIS ARTIBUS

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Systematic Assessment of Geographically and Clinically Diverse Aeromonads

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Summary

Numerical taxonomy was used to analyze data obtained on 167 *Aeromonas* strains collected from the USA, N.E. Africa, Bangladesh, India, Indonesia, and Puerto Rico. The strains included clinical, environmental, and veterinary isolates. Each strain was tested at 36 °C ± 1 °C for 80 unit characters that included morphological, biochemical, antimicrobial resistance, and virulence-associated markers. The data for 50 of these characters were analyzed by the SAS/TAXAN* program using Simple Matching and Jaccard coefficients. At a similarity coefficient of 85%, the 167 strains clustered into 12 phenons plus 13 single unclustered strains. All clusters but one contained only one DNA group definition strain. The major clusters resembled the three clinical species *Aeromonas hydrophila*, *Aeromonas veronii* biovar sobria, and *Aeromonas caviae*, with smaller clusters for the species *Aeromonas veronii* biovar veronii and *Aeromonas schubertii*. Additional clusters delineated were: an "arabinose-negative" biovar of *A. hydrophila* and clusters for two newly validated species, *A. jandaei* and *A. trota*. This systematic assessment provides a comprehensive taxonomic study of a geographically and clinically diverse group of aeromonads and delineates, for the first time, essentially all of the currently recognized genospecies using phenotypic clustering.

Key words: *Aeromonas* – Taxonomy – Clinical Isolates – Systematics – Phenospecies – Genospecies

Introduction

Aeromonads are ubiquitous, oxidase-positive, facultatively anaerobic, glucose-fermenting, gram-negative microorganisms that are autochthonous to the aquatic environment. They have been associated with a myriad of disease processes among both cold- and warm-blooded animals (*Janda*, 1991). Parallel to the recognition of *Aeromonas* as a human pathogen, the taxonomy of the genus *Aeromonas* has evolved amidst much confusion. Although the genus currently resides in the family Vibrionaceae, phylogenetic studies (*Colwell* et al., 1986) have resulted in the proposal of a new family Aeromonadaceae. Once considered a single mesophilic species, *A. hydrophila*, this family now includes at least 8 phenospecies among at least 14 DNA hybridization groups. A comprehensive review of *Aeromonas* taxonomy is that of *Farmer* et al. (1991).

Popoff and *Veron* (1976) first used numerical taxonomy (NT) to analyze 68 mesophilic aeromonads, primarily from environmental sources, based on 203 morphological, biochemical, and physiological characteristics tested at 30 °C. Using the coefficient of Jaccard-Sneath, they found that the 68 strains could be divided into two well-segregated groups on the basis of 59 variable characters, of which seven were of diagnostic value. These two groups were *A. hydrophila* (biovar X_1) and a new species *Aeromonas sobria* (biovar Y). *Popoff* et al. (1981) followed this study with a genetic analysis of 55 motile *Aeromonas* strains using DNA/DNA hybridization by the S1 nuclease method. The results led to the proposal of a third mesophilic species, *Aeromonas caviae*, formerly an "anaerogenic" biovar (X_2) of *A. hydrophila*. However,

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these DNA studies revealed also that within each of the three biochemically distinct species (phenospecies), there were at least 2–3 distinct DNA hybridization groups (genospecies) that could not be distinguished by phenotypic traits.

Fanning et al. (1985) reported the results of an extensive DNA/DNA hybridization study conducted with a large number of reference, environmental and clinical strains. They confirmed the 7–8 genospecies of *Popoff* et al. (1981) and extended the number of hybridization groups to at least 10. Over the next six years, five more phenotypic species were proposed: *A. veronii (Hickman-Brenner* et al. 1987), *A. schubertii (Hickman-Brenner* et al., 1988), *A. eucrenophila (Schubert* and *Hegazi*, 1988), *A. jandaei* (*Carnahan* et al. 1991a) and *A. trota* (*Carnahan* et al., 1991c). Thus the number of DNA hybridization groups of *Aeromonas* was extended to 14 (Table 1).

Table 1. Current taxonomic status of mesophilic *Aeromonas* species

DNA Hybridation Groups	Phenospecies
1	*A. hydrophila* (ATCC 7966[T])
2	Unnamed
3*	Unnamed*
4	*A. caviae* (ATCC 15468[T])
5a**	Unnamed**
6	*A. eucrenophila* (ATCC 23309[T])
7	*A. sobria* (CIP 7433[T])
8X/10	*A. veronii* biovar sobria (CDC 0437-84; Popoff C224)
8Y/10	*A. veronii* biovar sobria (ATCC 9071)
9	*A. jandaei* (ATCC 49568[T])
10/8	*A. veronii* biovar veronii (ATCC 35624[T])
11	*A. spp. ornithine positive*
12	*A. schubertii* (ATCC 43700[T])
13	*Aeromonas Group 501*
14	*A. trota* (ATCC 49657[T])

* Also includes the psychrophilic species, *A. salmonicida*.
** DNA Group 5b includes the psychrophilic species, *A. media*.

Concurrently, there were several taxonomic studies conducted with large groups of aeromonads, usually mostly fecal isolates from a single geographic source (*Arduino* et al., 1988; *Kuijper* et al., 1989; and *Altwegg* et al., 1990). Each attempted to phenotypically separate the different DNA hybridization groups with limited success. Because these studies did not satisfactorily separate the various genotypes using phenotypic clustering, this study was initiated. We sought to examine a large set of aeromonads representing as many species as possible, including clinical, veterinary and environmental isolates, and originating from many different geographic sources. We used numerical taxonomy to measure the phenotypic similarities among these strains with the 14 DNA group definition and other mesophilic ATCC reference strains for the genus *Aeromonas* as control strains. All but two of the currently recognized genospecies were phenotypically delineated for the first time using cluster analysis, and information on the proportionate distribution of all genospecies was updated and expanded.

Materials and Methods

Bacterial strains. One hundred-fifty-two strains, received between 1985–1989 as *Aeromonas* spp., were from several countries, including the United States (n = 78), N.E. Africa (Somalia, the Sudan, and Egypt) (n = 50), Bangladesh (n = 19), India (n = 1), Indonesia (n = 3), and Puerto Rico (n = 1). The majority of the strains came from a variety of clinical sources (n = 131), but also included veterinary (n = 8), and environmental strains (n = 13). The CDC definition strains for the DNA hybridization groups 1, 2, 3, 4, 5a, 5b, 6, 7, 8X, 9, 10, 11, 12 and ATCC reference strains for *Aeromonas sobria* group 8Y and *Aeromonas* Group 501 were included as reference strains (Table 1). The strains were stored at −70°C in Trypticase soy broth with 10% Glycerol (Remel, Lenexa, KS.) and subsequently subcultured to Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, MD.) and maintained at ambient temperature. Unless stated otherwise, all tests were done at 36°C ± 1°.

Phenotype analysis. Strains were presumptively identified as *Aeromonas* and examined for 80 phenotypic traits that represented a range of morphological,biochemical, physiological and tolerance tests as previously described (*Carnahan* et al., 1991b). Excluding the API–20E (Analytab products, Plainview, NY), all tests were performed by conventional methods (*Carnahan* et al., 1991b). The 50 differential tests used in the NT analysis are listed in Table 3.

Antibiotic resistance studies. Minimum Inhibitory Concentrations (MICs) were determined for seventeen antimicrobial agents plus a "growth" or "no growth" response to a single dilution of four additional antimicrobials in a 96-well microdilution plate according to NCCLS standards as previously described (MicroScan Gram Negative Panel 7, Baxter Healthcare Corp., MicroScan Division, West Sacramento, CA) (*Carnahan* et al., 1991b). The results included in the NT analysis are listed in Table 3.

Coding of data and computer analyses. Analysis of the frequency (per cent positive) results revealed those tests out of the original 80 which gave variable results among the strains. Consequently, fifty of the 80 tests were encoded for use in SAS/TAXAN[T] (Table 3). Nine tests were excluded from the analysis with positive results at a percentage frequency >95%: gram stain reaction (gram-negative rods), positive oxidase activity, fermentation of glucose, gelatin hydrolysis, ONPG production, resistance to cetrimide, resistance to penicillin, kanamycin and nitrofurantoin. Nine tests were excluded with positive results at a positive percentage frequency <5%: presence of diffusible brown pigment, production of tryptophan deaminase and urease, fermentation of amygdalin, inositol, sorbitol, rhamnose, and melibiose, and resistance to ciprofloxacin. The results for arginine dihydrolase and citrate utilization were not used because these results were only available from the API–20E strip and not done conventionally. The results of bile esculin agar and nine additional tests (based on the API–20E) were eliminated because they duplicated tests performed by conventional methods: lysine decarboxylase, ornithine decarboxylase, H_2S production, indole production, Voges-Proskauer, and acid production from arabinose, sucrose and mannitol. The results for growth in KCN were

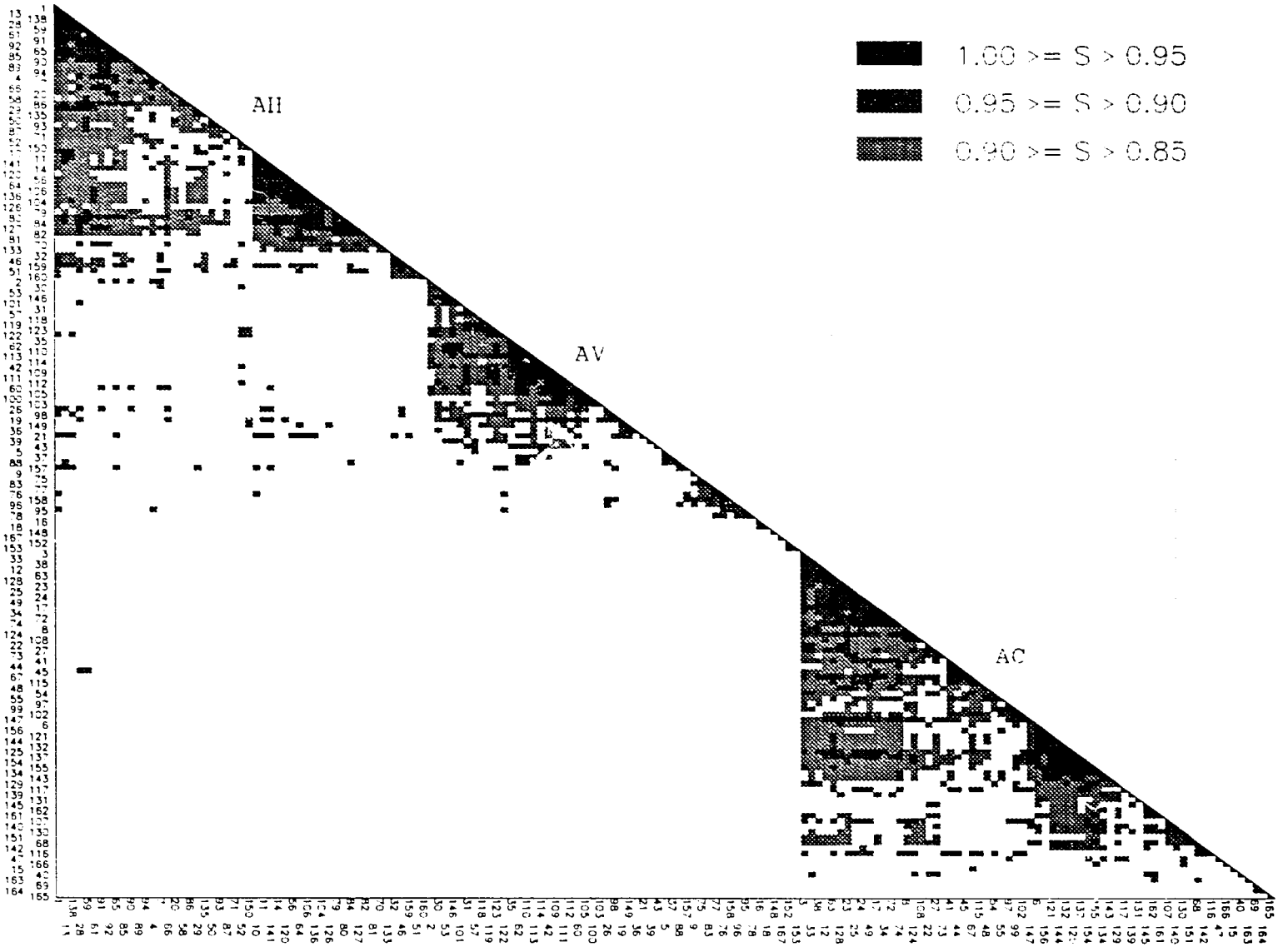

Fig. 1. Similarity matrix triangle of the 167 strains examined using the Simple Matching (S_{SM}) coefficient at a similarity level of S = 85%. AH denotes *A. hydrophila* (DNA Group 1), AV denotes *A. veronii* biovars sobria and veronii (DNA Groups 8/10), and AC denotes *A. caviae* (DNA Group 4).

not used even though this test gave results consistent with those of *Popoff* et al. (1976) because it was considered a highly subjective test to read and a hazardous medium to prepare.

Thus, the final data set contained test results for 50 different unit characters (Table 3) for each of the 167 strains, designated as OTUs (operational taxonomic units). Most characters existed in one of two mutually exclusive states and were scored as positive (1) or negative (0). Quantitative multistate characters, such as "Intermediate" instead of "resistant" or "susceptible" for antimicrobial susceptibility or "alpha or gamma hemolysis" in lieu of beta-hemolysis were coded by the additive method of *Sneath* and *Sokal* (1973). Those tests that measured quantitation of growth or motility had values of 4+, 3+, or 2+ for growth or motility coded as (1) and values of 1+, +/− or no growth coded as (0). The data were examined with the SAS/TAXAN® clustering program (Maryland Sea Grant College, College Park, MD) *Jacobs*, 1988) on an IBM 4381 Mainframe computer using the Simple Matching (S_{SM}), and Jaccard (S_J) coefficients (*Austin* and *Priest*, 1988; *Sneath* and *Sokal*, 1973). Hierarchical clustering of the 167 strains was by UPGMA (unweighted pair method with arithmetic averages). This resulted in the generation of a similarity matrix (Fig. 1), a dendrogram (Fig. 2), and two PCA (Principal Component Analysis) plots (Fig. 3 and 4) as means of visualizing the cluster separation. Emphasis was placed on results obtained using the S_{SM} coefficient since the more compact clusters and aggregate clusters are generally produced with this coefficient (*Austin* and *Priest*, 1988). Finally, the frequency of positive test results for each cluster and test used in the SAS/TAXAN® analysis are displayed as a "per cent positive" frequency matrix (Table 3).

Test and performance reproducibility. Both positive and negative control strains of *Aeromonas* (i.e., an ATCC[1] or DNA definition group strain) were included for each battery of tests. Additionally, 18 of the strains were randomly selected by computer and tested in duplicate (Table 2). Comparison of these duplicate results for each test was then used to determine an estimate of individual test variance (S_i^2). The average test variance was calculated to obtain a pooled variance (S^2), which in turn was used to estimate the average probability (p) of an erroneous test result (*Austin* and *Priest*, 1988; *Sneath* and *Sokal*, 1973).

Results

Test reproducibility

The average probability (p) of an erroneous result, calculated from the pooled variance (S^2 = 0.3825) of all the unit characters scored for the 18 duplicate strains was 3.8%. Values of S_i^2 were zero for most of the tests, with a range of .028–.222. Most discrepancies were in the hemolysis reactions on Sheep Blood Agar (.083) and the antimicrobials, cefazolin (.111), gentamicin (.194), and imipenem (.222).

Correlation

Cophenetic correlation, i.e., the correlation between the respective values in the similarity matrix and its corresponding dendrogram, was calculated for each coefficient using the correlation coefficient "r" (*Austin* and *Priest*, 1988; *Sneath* and *Sokal* 1973). These values were 0.992 for the S_{SM} and 0.994 for the S_J coefficients, well within acceptable limits for studies utilizing UPGMA.

Clustering of strains by SAS/TAXAN®

With the initial analysis of the S_{SM} matrix at 85% similarity (Fig. 1), three distinct smaller triangles were readily discernible within the single large similarity triangle. These three phenons represented the three major recognized species of motile, mesophilic aeromonads as described by *Popoff* and *Veron* (1976): *A. hydrophila*, *A. sobria*, and *A. caviae* and included 153 (92%) of the total strains studied. Examination of the S_J coefficient similarity matrix (not shown), revealed the same three phena but at a lower level of similarity (S = 70%), because of the nature of this coefficient.

The S_{SM} data were also analyzed using SAS/TAXAN® to construct a dendrogram (Fig. 2). Of the 167 strains analyzed, 165 (99%) joined at 68% similarity or higher using the S_{SM} coefficient with only two atypical strains (Cluster A) not joining at this level. These two strains were later confirmed as atypical *Vibrio cholerae* (non–01) resistant to 0/129 (Vibriostatic agent). At 85% similarity, a generally accepted level for species designation (*Austin* and *Priest*, 1988) there were 12 aeromonad clusters ranging in size from (n = 2) to (n = 33) and containing 152 (91%) of the total strains. Most of the remaining 13 single strains were considered "highly atypical", i.e., they differed from their nearest cluster by at least eight phenotypic characters. All of the clusters and single strains were labeled as Clusters A–Z and are listed in Table 2. The phenotypic description of each cluster and single strain are in Table 3. Construction of a dendrogram with the S_J coefficient at a level of S = 70% (not shown) gave similar results to the S_{SM} dendrogram just described. The Jaccard dendrogram revealed 14 aeromonad clusters ranging in size from (n = 2) to (n = 46) and representing 143 (86%) of the total strains within the study.

In the dendrogram constructed using the S_{SM} coefficient at S = 85% (Fig. 2), clusters and single strains A–P constituted approximately the upper half of the S_{SM} dendrogram. This demarcation included 100/167 or 60% of the total strains of this study and represented the DNA groups 8Y, 9, and 10 which phenotypically resemble *A. veronii* biovar sobria, DNA group 9 *A. sobria* (now validated as *A. jandaei*), and *A. veronii* biovar veronii. This upper half also included the DNA groups 1, 2, and 3 of the species *A. hydrophila*, DNA group 12 of *A. schubertii*, a single *Aeromonas* Group 501 strain, seven ampicillin-susceptible *A. sobria-like* strains (now validated as *A. trota*), four single atypical aeromonads and the aforementioned two atypical *Vibrio cholerae* (non–01) strains. Clusters and single strains Q–Z comprised approximately the lower half of the S_{SM} dendrogram and included 65/167 or 40% of the total strains in the study. They represented the DNA groups 4, 5a, 5b, 6, and 11 which phenotypically resemble *A. caviae*, *A. eucrenophila* and *Aeromona* spp. "ornithine positive". Also residing in this region were the DNA groups 7 and 8X of *A. sobria* and *A. veronii* biovar sobria.

SAS/TAXAN® was then utilized to generate PCA plots which used four principal components (P1, P2, P3 and P4) to construct a three-dimensional view of the strains (Figs. 3 and 4). These plots allow the viewing of relationships

between OTUs in terms of "phenetic (taxonomic) hyperspace as a continuum" and are actually a more accurate representation of the distribution of the bacterial species within a genus as they occur in nature (*Austin* and *Priest*, 1988). The first PCA plot (Fig. 3) displays only the 17 type strains, reference strains and DNA definition strains examined in this study, while the second PCA plot displays the entire collection of 167 strains. This latter PCA plot confirmed the results of the previous analyses (similarity matrix and dendrogram) in that the majority of the strains fell into a few large clusters representing the major species, with smaller clusters for less commonly found species.

Discussion

While *Aeromonas* was emerging as a clinically significant pathogen, several major studies were conducted in the latter part of the eighties to reconcile the 5–6 proposed phenotypic species with the 10–12 established genospecies. Arduino et al. (1988) analyzed 132 previously hybridized (DNA/DNA hybridization) aeromonad strains from the Centers for Disease Control (CDC) collection that represented all of the 12 DNA group definition strains. The strains were tested for 165 phenotypic traits at 25 °C and the majority were in DNA groups 1, 4 and 8 with most in DNA group 4 (phenotype *A. caviae*). DNA groups 4, 5 and 6 were the most difficult to separate. That same year, *Renaud* et al. (1988) published work which had phenotypically characterized 66 lake water and 34 various, clinical isolates using a combination of conventional tests and rapid assimiliation strips. When the results were analyzed by NT using the S_J coefficient, all but 4 strains were found among only 4 clusters which represented *A. sobria*, *A. hydrophila*, *A. hydrophila* (arabinose-negative) and *A. caviae*.

A major clinical study to relate genotype to phenotype involved 189 fecal isolates collected in the Netherlands between 1982–1986 by *Kuijper* et al. (1989). The strains were analyzed for 30 conventional phenotypic traits at 37 °C, and most phenotypically resembled *A. caviae*, *A. hydrophila*, and *A. veronii* biovar sobria. When a subset of 142 strains was subjected to DNA hybridization using the DNA definition strains for DNA groups 1–11, a majority of the strains were in DNA groups 1, 4 and 8, but there were increased frequencies of the other rarer genospecies 2, 3 and 5a. However, they were unable to reliably identify all of the strains using their phenotyping methods.

The most recent study to attempt to reconcile genotype with phenotype was by *Altwegg* et al. (1990). They examined 138 clinical (mostly fecal) isolates plus a few environmental isolates, all collected in Switzerland and included the CDC definition strains for DNA groups 1–9. All were tested for 100 phenotypic characters, but only 63 different characters were actually evaluated because of redundant tests. They used only ten conventional tests plus fifty three biochemical, carbohydrate, and enzyme tests from three rapid identification strips (API–20E, API–50E, and API ATB 32GN). Their computer analysis utilized a similarity coefficient similar to Jaccard with UPGMA clustering. Cluster analysis revealed only three phenons at S = 88%. These resembled *A. hydrophila*, *A. caviae* and *A. sobria* (now *A. veronii* bv. sobria), but each phenon contained more than one genospecies. A subset of 126 strains was subjected to DNA-DNA hybridization and confirmed that these phenons corresponded to DNA groups 1, 4, and 8 with slightly larger numbers in groups 2, 3, and 5 than previously reported. Like the studies of *Kuijper* et al. (1989) and *Arduino* et al. (1988), no DNA group 6, group 7 (type strain for *A. sobria*), group 11, or group 12 strains were recovered.

After assessing the results of these studies, it was evident that no single study thus far had thoroughly examined a large group of aeromonands that were diverse in both geographic and clinical source of isolation and included all 12 known DNA Group definition strains. Because the aforementioned studies had failed to phenotypically separate all the known genotypes correctly, our objective was to establish whether a systematic phenotypic correlation to genotype was possible using standardized and conventional methods and analyzing the data with the most capable NT computer programs available.

Excluding the two *V. cholerae* (non–01) strains, at a S_{SM} level of S = 85% there were 12 clusters and each included only one DNA Group definition strain, except for Cluster J which contained the definition strains for both DNA Groups 2 and 3. These two genospecies appear to be rare among clinical species, since the only strains in this cluster, except for one, were from veterinary sources. *Renaud* et al. (1988) and *Altwegg* et al. (1990) have proposed that the tests for growth on DL-lactate and acid production from sorbitol may be useful in separating these groups from each other and from DNA Group 1 *A. hydrophila*.

Likewise, the majority of our strains were in clusters that contained the DNA definition strains for groups 1, 4, and 8 (Figs. 1 and 2), and phenotypically resembled *A. hydrophila*, *A. veronii* sobria, and *A. caviae*. However, we also found clusters for *A. hydrophila* (arabinose negative), *A. veronii* biovar veronii (DNA Group 8/10), DNA Group 9 (now validated as *A. jandaei*), DNA Group 12 *A. schubertii*, and a group of ampicillin-susceptible aeromonads, now validated as *A. trota*. Although the majority of the *A. caviae* strains resided with the definition strain for DNA Group 4, we found increased numbers of strains similar to the definition strains for DNA Groups 5a and 5b, with the latter cluster (5b) exhibiting susceptility to ampicillin. Interspersed among these clusters were 8 single strains, of which 5 were either DNA definition strains or ATCC type strains for rare genospecies, i.e., DNA Group 501, *A. eucrenophila*, *Aeromonas* "ornithine positive", the type strain for *A. sobria* and the definition strain for *A. veronii* bv sobria (Group 8X). Some or all of these single strains may represent either "bridge" organisms between the more predominant genospecies found in clinical specimens or the nucleus for similar NT studies of large groups of environmental and veterinary isolates.

Regarding the major clusters produced by the SAS/TAXAN® analysis, several points deserve further attention. First, the NT analysis showed that Clusters B and C, which contained DNA definition strains for Groups 8Y

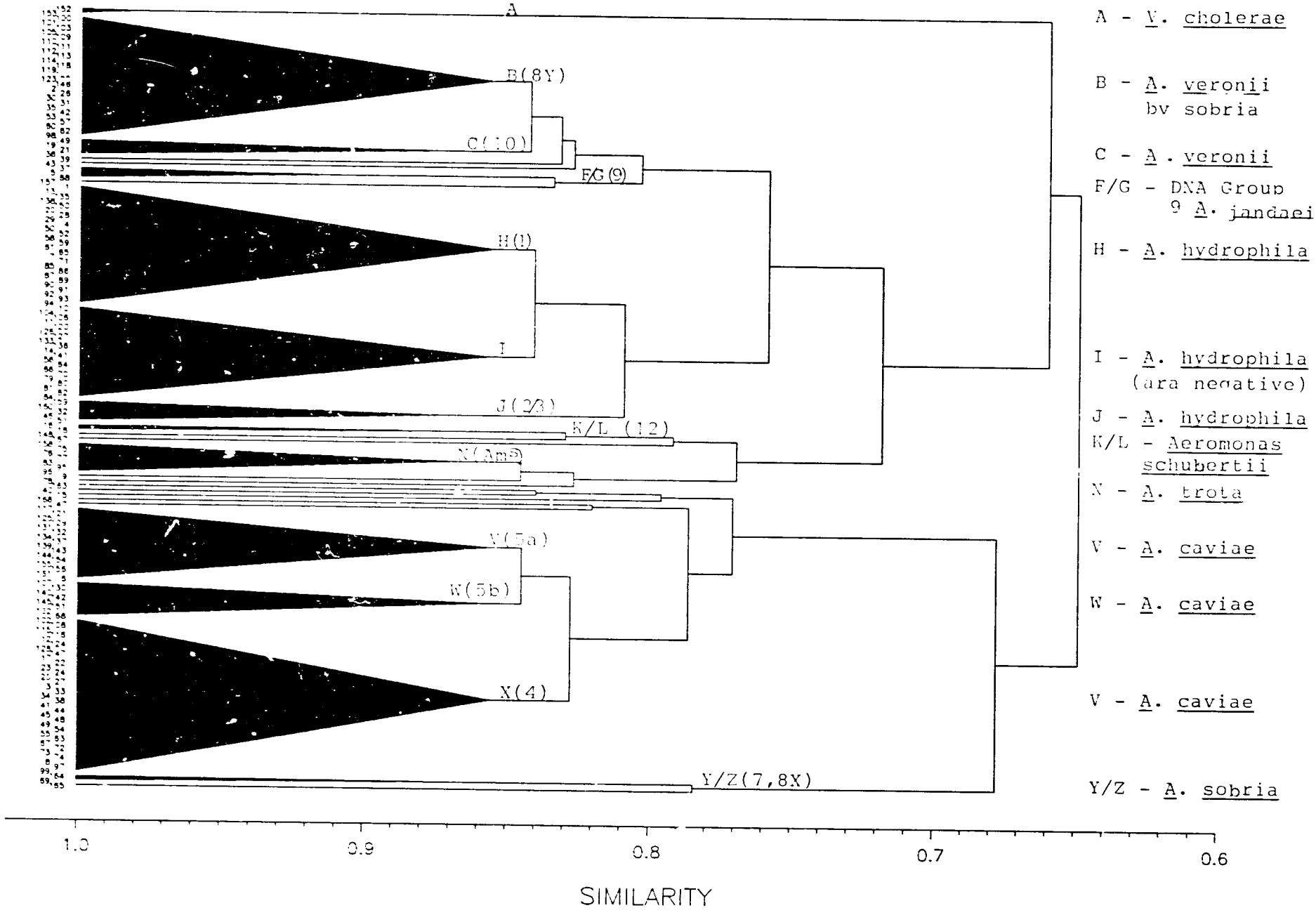

Fig. 2. Simple matching dendrogram at S = 85% using UPGMA. Cluster designations A–Z correlate with Table 2.

Table 2. Bacterial strains assigned to clusters in the S_{SM} UPGMA analysis

Cluster or single stream	Lab reference numbers	Donor designations	Geographic Source	Source of Isolation
A (n = 2)	152; 153	VM 17408; 17810	Bangladesh	fecal
B (n = 26)	2	ATCC 9071 (DNA Group 8Y *A. veronii* by sobria	ATCC	frog/leg
	26; 30; 31; 35	AMC 4130-E; 8581-E; 15918-E; 3110-E	USA/MD	fecal
	42	AMC 1123-W	USA/MD	finger
	53; 57	AMC 6192-E; 7612-E	USA/MD	fecal
	60	AMC 4995-V	USA/MD	duck
	62	AMC 6573-W	USA/MD	foot
	98; 100; 103; 105; 109; 110; 111; 112; 113; 114	L063; L142; L164; L205; L346; L386; L400; L424; L426; L472	Sudan	fecal
	122; 123; 146	WR154; WR511; BR539	Somalia	fecal
	101	L147	Sudan	well water
	118; 119[a]	BR525; B0147	Somalia	fecal
C (n = 4)	149[a]	ATCC 35624[T] (DNA Group 10) *A. veronii* by veronii	ATCC/USA/MI	sputum
	19; 21	AMC 12628-E; 2052-E	USA/MD	fecal
	36	WRI/4659	USA/DC	eye
D (n = 1)	39	NMRI-4	USA/MD	fecal
E (n = 1)	43	AMC 2930-c	USA/MD	fecal
F (n = 3)	37	WRII/4658	USA/DC	eye
	5	AS-14	USA/NY	blood
	88	NMRI-6	USA/MD	leg
G (n = 1)	157	ATCC 49568[T] (DNA Group 9) *A. jandaei*	USA/CDC	fecal
H (n = 26)	1[a]	ATCC 7966[T] (DNA Group 1) *A. hydrophila*	ATCC	canned milk
	13[a]	AMC 12723-W	USA/MD	wound
	135[a]	EGY 51857	Egypt/Sinai	water tank
	138[a]	MM241	Egypt/Alex[b]	duck/fecal
	150[a]	AER-321	USA/CA	fecal
	20	AMC 7056-W	USA/MD	wound
	28; 29	AMC 10774-E; 3387-E	USA/MD	fecal
	4	AS-4	USA/NY	leg
	50	AMC 7059-W	USA/MD	wound
	52	AMC 4474-W	USA/MD	toe
	58	AMC 305-W	USA/MD	wound
	59	AMC 8174-W	USA/MD	hand
	61	AMC 10570-V	USA/MD	bird/eye
	65	AMC/Kilbe	USA/MD	knee
	7	NMRI-16	USA/MD	river water
	71	AMC/SWJ	USA/MD	goldfish/gill
	85	AMC 2123-W	USA/MD	wound
	86	NMRI-3	USA/MD	river water
	87	NMRI-5	USA/MD	lung
	89[a]	NMRI-7	USA/MD	leg
	90; 91[a]; 92	NMRI-54; 55; 56	USA/MD	river water
	93	NMRI 203	USA/NY	fecal
	94	NMRI 204	USA/CT	fecal
I (n = 20)	10	AMC 6437-W	USA/MD	foot
	104[a]; 106	L172; L206	Sudan	fecal
	11	AMC 12148-E	USA/MD	fecal
	120; 126; 127	W107; WY520; WY561	Somalia	fecal
	133	EGY 48536	Egypt/Cairo	fecal
	136	EGY 51855	Egypt/Sinai	water tank
	14	AMC 3276-W	USA/MD	wound
	141	TERRY/NIH	Puerto Rico	leg
	56	AMC 3469-I	USA/MD	fecal
	64	AMC/RICE	USA/MD	knee
	66	AMC/CAMARE	USA/MD	heel
	70	AMC/GALLBLADDER	USA/MD	gallbladder
	79; 80; 81; 82; 84	MOB/A12; A/13; /A14; /A15; /A17	Bangladesh	fecal
J (n = 5)	159	CDC 9533-76 (DNA Group 2)	CDC	fish
	160	CDC 0434-84 (DNA Group 3)	CDC	fresh water
	32	AMC 15228-V	USA/MD	condor/choana
	46	AMC 14228-V	USA/MD	duck/neck
	51	AMC 3294-R	USA/MD	respiratory

Table 2. Continued

Cluster or single strain	Lab reference numbers	Donor designations	Geographic Source	Source of Isolation
K (n = 2)	16; 18	AMC 1108-W; 4396-W	USA/MD	leg
L (n = 1)	148	ATCC 43700[1] (DNA Group 12) *A. schubertii*	CDC/TX	forehead abscess
M (n = 1)	167	ATCC 43946[1] (DNA Group 13) *Aeromonas* Group 501	CDC/LA	leg
N (n = 7)	158	ATCC 49657[1] (DNA Group 14) *A. trota*	India	fecal
	75; 76; 77[a]; 83	MOB/A7; /A8; /A9; /A16	Bangladesh	fecal
	95; 96	NMRI-206; -208	Jakarta	fecal
O (n = 1)	9	AMC 5439-E	USA/MD	fecal
P (n = 1)	78	MOB/A11	Bangladesh	fecal
Q (n = 1)	63	ATCC 23309[1] (DNA Group 6) *A. eucrenophila*	CDC	fresh water
R (n = 1)	40	AMC Leg-W	USA/MD	leg
S (n = 1)	15	AMC 5761-R	USA/MD	throat
T (n = 1)	166	ATCC 35924 (DNA Group 11)	CDC/NewZealand	leg
U (n = 1)	47	AMC 1051-W	USA/MD	ear
V (n = 16)	117; 121[a]; 125; 129; 131	BN14; WR100; WY505; Y175; MO114	Somalia	fecal
	132	EGY 44678	Sudan	fecal
	134[a]	EGY 48872	Egypt/Cairo	water tank
	137	EGY AH340	Egypt/Cairo	fecal
	139	EGY W26A	Egypt/Alex	salt lake
	143; 144	BE505; WR508	Somalia	fecal
	154; 155; 156	VM 82255; 94303; 98145	Bangladesh	fecal
	161	CDC 0862-83 (DNA Group 5a)	CDC	fish
	6	AS-34	USA/NY	blood
W (n = 8)	107	L321	Sudan	fecal
	130	YD64	Somalia	fecal
	140	L226	Sudan	well water
	142; 145	BR541; 536	Somalia	fecal
	151	VM 4947-48	Bangladesh	fecal
	162	CDC 0435-84 (DNA Group 5b)	CDC	fish
	68	AMC/Leftwich	USA/MD	fecal
X (n = 33)	102	L149	Sudan	well water
	108	L327	Sudan	fecal
	115; 116	B101; BR545	Somalia	fecal
	12	AMC 12338-W	USA/MD	rectal wound
	124; 128; 147	WR537; WY568; W102	Somalia	rectal
	17	AMC 3204-W	USA/MD	foot
	22; 23	AMC 1685-E; AMC 5142-E	USA/MD	fecal
	24; 25	AMC 514-W; 511-W	USA/MD	leg
	27	AMC 7980-R	USA/MD	throat
	3	ATCC 15468[1] (DNA Group 4) *A. caviae*	ATCC	guinea pig
	33	AMC 5217-V	USA/MD	duck/respiratory
	34	AMC 11953-E	USA/MD	fecal
	38[a]	NMRI-2	USA/MD	fecal
	41	AMC 1554-E	USA/MD	fecal
	44[a]; 45	AMC 23B (opaque); AMC 23B (clear)	USA/MD	blood
	48; 49	AMC 18786 SM; 1936-E	USA/MD	fecal
	54[a]	AMC 13814-V	USA/MD	horse/foot
	55	AMC 8205-E	USA/MD	fecal
	63	AMC/SINGER	USA/DC	fecal
	67	AMC/KAISER	USA/MD	fecal
	72[a]; 73; 74	MOB/A1; /A2; /A5	Bangladesh	fecal
	8	NMRI-209	Jakarta	fecal
	97; 99	L023; L077	Sudan	fecal
Y (n = 2)	164	CIP 7433[1] (DNA Group 7) *A. sobria*	CDC	fish
	69	AMC/MULDOON	USA/MD	fecal
Z (n = 1)	165[a]	CDC 0437-84 (DNA Group 8X) *A. veronii* by sobria	CDC	fish

[a] Strains examined in duplicate to assess possibility of test error. [b] Alexandria. [c] Centers for Disease Control, Atlanta, GA.

Table 3. Frequency matrix for SAS/TAXAN[a] clusters as percentage of positive reactions

Unit Characters	A (n = 2)	B (n = 26)	C (n = 4)	D (n = 1)	E (n = 1)	F (n = 3)	G (n = 1)	H (n = 26)	I (n = 20)	J (n = 5)	Cluster and K (n = 2)
0/129 Resistance	100	100	100	100	100	67	100	100	100	100	100
string test	100	0	0	0	0	0	0	8	10	20	0
indole	100	96	100	100	100	100	100	100	100	100	0
β stab SBA[a]	100	100	100	100	100	100	100	100	100	100	100
β stab HBA[b]	100	100	100	100	100	100	100	100	100	100	100
β on SBA	100	100	100	100	100	100	100	100	100	100	100
α on SBA	0	0	0	0	0	0	0	0	0	0	0
γ on SBA	0	0	0	0	0	0	0	0	0	0	0
β HBA	100	100	100	100	100	100	100	100	100	100	100
α on HBA	0	0	0	0	0	0	0	0	0	0	0
γ on HBA	0	0	0	0	0	0	0	0	0	0	0
esculin hydrolysis	0	0	100	0	0	0	0	100	100	80	0
gas/glucose	0	96	100	0	100	100	100	92	100	80	0
Voges-Proskauer	100	96	100	100	100	100	100	92	85	100	0
lysine decarboxylase	100	96	100	0	100	100	100	100	100	0	100
ornithine decarboxylase	100	0	50	0	0	0	0	0	0	0	0
arabinose[c]	100	4	0	0	100	0	0	100	0	80	0
salicin[c]	50	4	75	0	0	0	0	54	85	20	0
mannitol[c]	100	92	100	100	100	100	100	100	100	100	0
sucrose[c]	100	100	100	100	100	0	0	88	100	100	0
motility	100	88	75	100	100	100	100	100	100	80	50
H_2S/GCF	0	100	100	100	100	100	100	100	100	100	0
colistin[d]	100	0	25	0	0	100	100	50	75	40	0
cephalothin[d]	100	8	0	0	0	100	100	92	100	100	0
self-pelleting	0	0	0	0	100	0	100	15	0	20	0
PAB[b,e]	100	23	0	0	0	33	0	50	45	0	50
arbutin hydrolysis	0	8	75	0	0	0	0	100	100	100	0
42° growth	100	100	100	100	100	100	100	81	100	0	100
Camp (aerobic)	100	96	100	100	0	33	100	73	95	100	0
Camp (anaerobic)	100	62	100	0	0	0	100	100	100	100	0
acriflavine agglutination	100	31	75	100	0	0	100	23	40	100	0
pyrazinamidase	100	4	0	0	0	33	100	92	100	20	0
amikacin[d]	0	8	0	0	0	0	0	4	0	0	50
ampicillin[d]	100	100	100	100	100	100	100	100	100	100	50
cefazolin[d]	100	8	0	0	0	100	0	65	95	80	50
ceftazidime[d]	0	0	0	0	0	0	0	0	5	0	0
cefuroxime[d]	0	0	0	0	0	0	0	0	15	0	0
gentamicin[d]	100	0	0	0	0	0	0	0	0	0	0
gm intermediate[d]	0	4	0	0	0	0	0	0	0	0	0
imipenem[d]	0	62	100	0	100	33	0	0	60	20	0
piperacillin[d]	100	65	25	0	100	100	0	8	5	0	0
tetracycline[d]	100	8	0	100	0	0	0	23	5	0	0
te intermediate[d]	0	4	0	0	0	0	0	0	0	0	0
ticarcillin clavulanate[d]	0	12	25	0	100	0	0	4	15	0	0
ticarcillin[d]	100	65	75	100	100	67	0	19	30	0	0
tobramycin[d]	100	19	0	100	100	0	0	0	0	20	50
tobramycin intermediate[d]	0	12	25	0	0	0	0	15	5	20	50
SXT[d,f]	100	0	0	0	0	0	0	4	5	0	0
cefoxitin[d]	0	0	0	0	0	0	0	0	95	0	0
ceftriaxone[d]	0	0	0	0	0	0	0	0	5	0	0

[a]Tryptic Soy Agar with 5% Sheep Blood, [b]Tryptic Soy Agar with 5% Horse Blood, [c]Acid production, [d]Resistance, [e]PAB = Pelleting after

Single Strains L (n = 1)	M (n = 1)	N (n = 7)	O (n = 1)	P (n = 1)	Q (n = 1)	R (n = 1)	S (n = 1)	T (n = 1)	U (n = 1)	V (n = 16)	W (n = 8)	X (n = 33)	Y (n = 2)	Z (n = 1)
100	100	100	100	100	100	100	100	0	100	100	100	100	50	0
0	0	14	0	100	100	0	100	0	0	6	13	0	50	0
0	100	100	100	100	100	100	0	100	100	100	50	97	100	100
100	100	100	100	100	0	0	0	0	100	31	38	61	0	0
100	100	100	100	100	100	100	100	0	0	19	13	97	100	100
0	100	100	0	100	0	0	100	0	100	6	0	39	0	0
0	0	0	100	0	100	100	0	0	0	94	100	67	100	100
100	0	0	0	0	0	0	0	100	0	0	0	0	0	0
100	100	100	100	100	0	100	100	0	0	0	13	88	0	0
0	0	0	0	0	0	0	0	0	0	0	13	12	100	0
0	0	0	0	0	100	0	0	100	100	100	75	0	0	100
0	0	0	0	0	0	100	100	0	100	100	100	100	0	0
0	0	86	0	0	0	0	0	0	0	0	0	0	50	100
0	100	14	0	0	0	0	0	0	0	0	0	6	0	0
100	0	100	100	100	0	0	0	0	0	0	0	0	100	100
0	0	0	0	0	0	0	0	100	0	0	0	0	0	0
0	0	14	0	0	100	100	100	100	100	100	88	100	0	0
0	0	0	0	0	100	0	100	100	100	94	75	94	0	0
0	0	71	100	100	100	100	100	100	100	100	100	100	100	100
0	0	14	0	0	0	0	100	100	100	100	100	100	100	100
100	100	100	0	100	0	100	100	100	0	81	75	76	100	0
0	0	100	100	100	100	100	100	0	0	0	0	0	100	100
0	0	0	0	0	0	0	0	0	0	0	0	6	0	0
0	100	100	100	100	0	0	100	100	100	100	50	91	0	100
100	0	0	0	0	100	0	0	0	0	0	38	6	50	0
0	100	0	0	0	0	0	0	0	100	13	0	3	0	100
0	0	57	0	0	100	100	100	100	100	100	100	97	0	0
100	100	100	100	100	0	0	0	0	0	69	75	85	50	100
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	100	57	0	100	0	0	0	0	0	0	0	0	0	0
100	0	71	100	0	100	100	100	100	100	69	100	70	100	0
0	0	43	0	100	100	0	0	100	100	94	63	97	0	100
0	0	14	0	100	0	0	0	0	0	0	0	3	0	0
100	100	0	0	0	0	0	0	0	0	100	13	85	50	100
0	100	43	100	0	0	0	100	0	0	75	0	61	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	100	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	13	9	50	100
0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
0	100	14	0	100	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	31	0	9	0	0
0	100	14	0	0	0	0	0	0	100	25	0	24	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

boiling, [1]SXT = Trimethoprim-sulfamethoxazole.

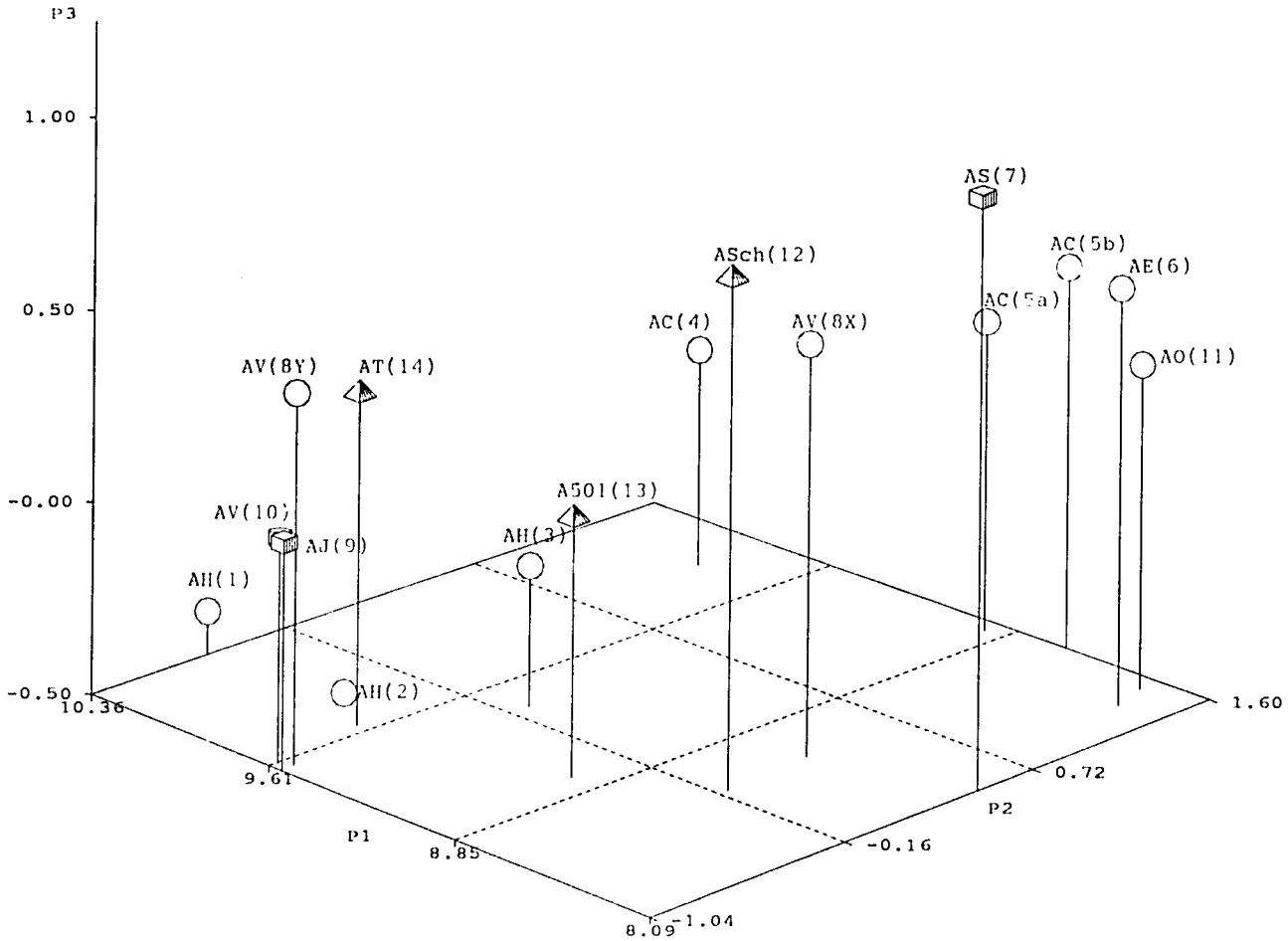

Fig. 3. Simple Matching PCA plot for all of the DNA Definition Group strains and ATCC type and reference strains. The DNA hybridization Group Number is in parantheses. The 4 principal components are labeled P1, P2, P3, and P4 where P3 denotes height and P4 denotes shape. AH denotes *A. hydrophila*, AV denotes *A. veronii*, AC denotes *A. caviae*, AJ denotes *A. jandaei*, ASch denotes *A. schubertii*, AT denotes *A. trota*, A501 denotes *Aeromonas* Group 501, AE denotes *A. eucrenophila*, AO denotes *Aeromonas* spp. "ornithine positive", and AS denotes *A. sobria*.

and 10, were extremely similar, merging to one cluster at S = 84% thereby confirming that they are indeed two biochemically distinct biovars of the same species, *A. veronii (Hickman-Brenner* et al., 1987). Next, the small Cluster F of "sucrose-negative" strains merged to one cluster of four with single strain G at S = 84% and resulted in the proposal and validation of *A. jandaei (Carnahan* et al., 1991). Clusters H and I both resembled *A. hydrophila* and merged to a single large cluster at S = 84%, but at the initial level of S = 85% appear to hint at the existence of an "arabinose-negative" biovar, first described by *Renaud* et al. (1988), within the DNA group 1 *A. hydrophila*. It's interesting to note that 88% of the "arabinose-positive" strains were from the USA, while the "arabinose-negative" strains were found in the USA, NE Africa, Bangladesh, and Puerto Rico. Cluster K merged into a cluster of 3 with single strain L (DNA Group 12 definition strain) at S = 83% and all three strains had been confirmed by DNA/DNA hybridization as *A. schubertii (Carnahan* et al., 1989). However, the single strain M, which represents *Aeromonas Group* 501, did not join this *A. schubertii* cluster until S = 79%, thereby phenotypically confirming that it is divergent enough to constitute its own DNA group (*Hickman-Brenner* et al., 1988). Cluster N did not contain a DNA definition or ATCC type strains; subsequent DNA homology studies resulted in its proposal and validation as *A. trota* (*Carnahan* et al., 1991c). Single strains Q and T which represent DNA Group 6 *A. eucrenophila (Schubert* and *Hegazi*, 1988) and *Aeromonas ornithine positive (Hickman-Brenner* et al., 1987) indeed appear to be rare genospecies, at least among clinical strains.

Finally, Cluster Y which contained the type strain for *A. sobria* (DNA Group 7) was confirmed as highly "atypical" of clinical strains formerly described as *A. sobria*, but now proposed as *A. veronii* biovar sobria (*Carnahan* et al., 1991a). This small cluster Y (n = 2) was found far below the Clusters B and C described above and more closely resembled *A. caviae*. To date, no clinical *A. sobria*-like strains have hydribized to DNA group 7, and the fecal

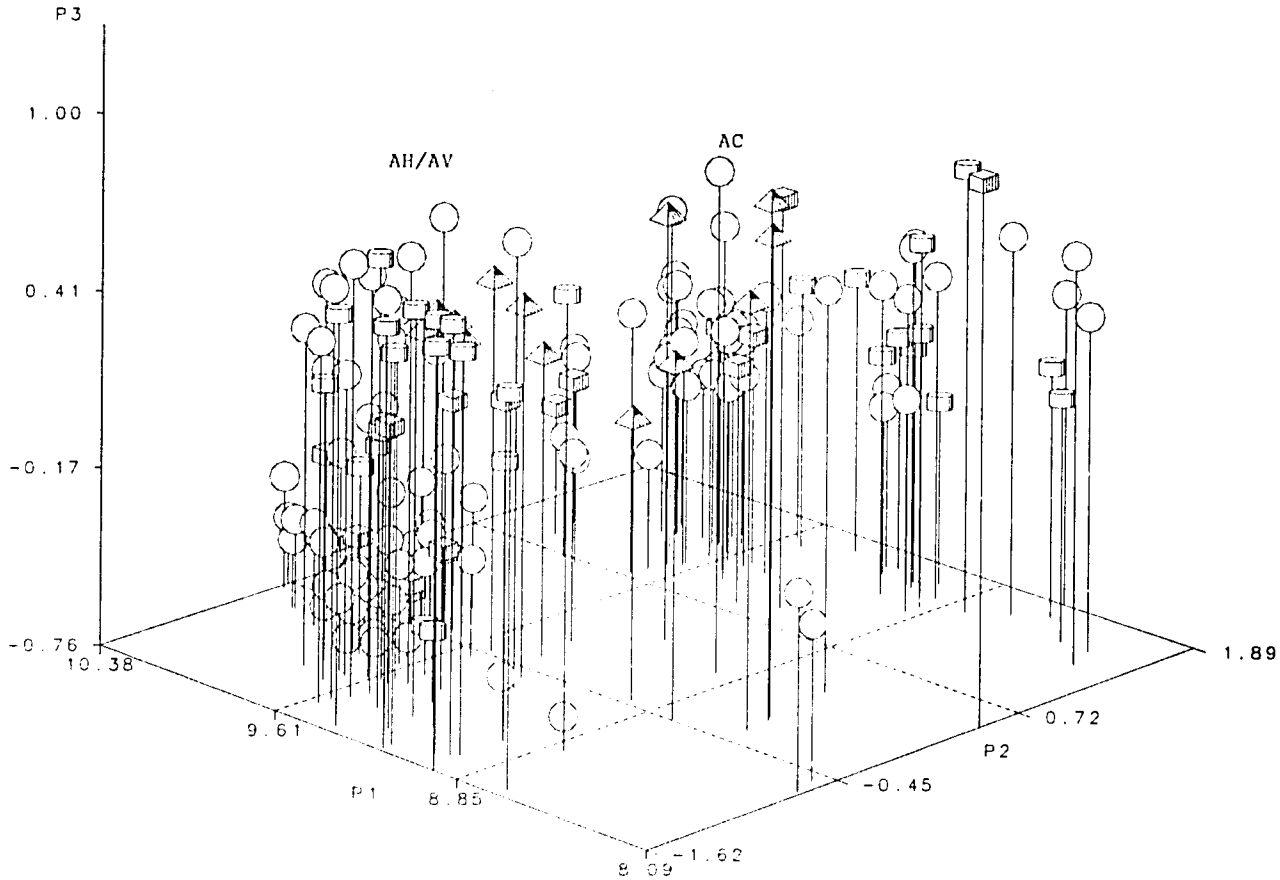

Fig. 4. Simple Matching PCA plot of all 167 strains examined with the three major clinical clusters: AH for *A. hydrophila*, AV for *A. veronii* and AC for *A. caviae*.

isolate "Muldoon" from this collection may indeed represent the first clinical *A. sobria*, pending confirmatory DNA/DNA hybridization. The last strain of the NT analysis, single strain Z, was the definition strain for DNA Group 8 and resides in DNA Group 8X. Although, genetically similar to DNA Group 8Y (Cluster B), there were significant phenotypic differences, particularly among markers previously reported to be indicative of virulent biotypes, (beta-hemolysis and positive Voges-Proskauer reaction). Thus, SAS/TAXAN® found this DNA definition strain more similar to the "atypical" *A. sobria* type strain of DNA Group 7 than to the clinical *A. veronii* biovar sobria of DNA Group 8Y (Cluster B). This is additional evidence that indeed there may exist among a single genospecies, virulent subsets of aeromonads more capable of causing human disease, i.e. DNA Group 8Y may be a virulent biovar of DNA Group 8.

Janda and *Kokka* (1991) recently reported on the pathogenicity of *Aeromonas* genospecies and phenospecies in Swiss-Webster mice based on LD_{50} values. They found that *A. hydrophila* and *A. veronii* biovar sobria were inherently more pathogenic for mice than *A. caviae*, but most intersting was the wide range of pathogenicity among DNA Group 8 *A. veronii* biovar sobria strains. The least virulent strain studied was the just described single strain of Cluster Z (definition strain for DNA Group 8X), while the DNA Group 8Y strain of Cluster B (ATCC 9071) was one of the most virulent strains studied.

In conclusion, this study of a geographically and clinically diverse group of aeromonads, including a small number of environmental and veterinary strains, expanded the geographic predominance of the three major phenospecies, *A. hydrophila*, *A. veronii* biovar sobria and *A. caviae*, as well as the species *A. veronii* biovar veronii and *A. schubertii*. The SAS/TAXAN® NT analysis also phenotypically delineated clusters for the newly validated species *A. jandaei* and *A. trota*, and confirmed the existence among clinical strains of an "arabinose-negative" biovar of *A. hydrophila*. Most importantly, this systematic study has demonstrated that essentially all of the currently recognized DNA hybridization groups of *Aeromonas* can be correctly separated by phenotypic analysis.

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ISOLATION, ENUMERATION AND IDENTIFICATION OF *AEROMONAS* SP.

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ABSTRACT

Methodologies for the isolation of Aeromonas sp. *from various sources, utilizing a broad range of media, are presented. Recommendations are made for the identification of* Aeromonas *isolates to the species level. The current nomenclature of* Aeromonas sp. *is discussed with reference to previously used taxonomic systems.*

INTRODUCTION

Since its original recognition in 1890 by Zimmerman and by Sanarelli in 1891, *Aeromonas* has been isolated from water, soil, humans, and other animals. Its role in human infections has become better defined in recent years causing us to delve into the human epidemiology of this disease on a world-wide basis. *Aeromonas* is increasingly being recognized as a primary pathogen in humans both in normal and immunocompromised humans.

Some microorganisms occurring in the aquatic environment can be classified as having a primary animal or terrestrial habitat while existing only incidentally in aquatic, estuarine or marine circumstances. However, organisms, such as *Aeromonas*, appear to be autochthonous to the aquatic environment and are incidentally found in animal and terrestrial situations. *Aeromonas* is generally regarded as occurring widely in fresh waters. While it is not regarded as a marine organism, Hazen *et al.* 1978, isolated *Aeromonas* in higher density from saline habitats of varying concentrations.

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Leclerc and Buttiaux (1962) found that motile aeromonads cause an important error in the total coliform count in drinking water, noting that they were present in 30% of 9,036 water samples which were presumptive coliform positive. Neilson (1978) isolated motile aeromonads in high number from activated sludge samples, and noted difficulty in differentiating them from *E. coli*. Schubert (1976), while studying distribution of aeromonads in surface water, showed that the aeromonads in waste water were not of fecal origin. Since large numbers of aeromonads were found in mud from siphons of sinks and in the first portion of waste water drainage system, these were considered the source of the organisms.

Hazen *et al.* (1978) reported a wide range of prevalence and distribution of *A. hydrophila* in 147 natural waters in 30 states in the U.S. and Puerto Rico and concluded that abundance of *A. hydrophila* is so many different systems would seem to indicate an important role for the aeromonads in natural aquatic processes.

Vibrios in the environment clearly show a capacity for attachment to and degradation of chitin, chondroitin and collagen; probably as a part of the survival process. Aeromonads, which are also widely distributed, probably provide similar contributions in their ecological spheres. Clearly, it is an important resident of the total polymicrobial flora of the aquatic environment, while being pathogenic for some of the animal residents.

Motile aeromonads are the causative agent of "red leg" disease in frogs, and have been reported as etiologic agents for a number of diseases in fish, reptiles, guinea pigs, snails, cattle and humans.

The nonmotile aeromonad, *Aeromonas salmonicida*, has a wide geographic distribution in freshwater fishes and produces furunculosis and bacteremia, particularly in salmon and trout. It is probably the most important cause of disease in "farmed" salmon.

General Characteristics

Members of the genus *Aeromonas* are straight-celled, gram negative rods, possess a single polar flagellum, and utilize carbohydrates. Most species are motile, some are nonmotile. Metabolism is both respiratory and fermentative. They are oxidase positive and resistant to the vibriostatic agent 2, 4, diamino-6,7-diisopropylpteridine (0/129). The mol % G+C of the DNA is 57–63 (Bd, T_m).

Identifying and Differential Characteristics

The common characteristics which define the motile aeromonads are possession of motility, morphology, growth in nutrient broth at 37 °C, indole production, fermentation of sucrose and mannitol, breakdown of inositol, and *oxidase activity* (Table 1). In contrast, the nonmotile aeromonads grow best at 22–25 °C

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with a maximum of 35 °C. Speciation of the motile aeromonads is based on esculin hydrolysis, growth in KCN broth, L-histidine and L-arginine utilization, L-arabinose utilization, fermentation of salicin, gas from glucose, and H_2S from cysteine (Table 2).

TABLE 1.
IDENTIFYING CHARACTERISTICS FOR MOTILE AEROMONADS

Catalase	+
Oxidase	+
Motility	+
Morphology	Rods in singles and pairs
Growth in nutrient broth at 37 °C	+
Arginine dihydrolase	+
Ornithine decarboxylase[1]	−
Indole production	+
Fermentation:	
sucrose and mannitol	+
dulcitol, rhamnose, xylose,	−
raffinose, inositol, and adonitol	−
Breakdown of inositol	−
NO_3 reduction to NO_2	+
Growth in peptone H_2O without NaCl	+
0/129 resistance	+
Starch, gelatin, ONPG, RNA and DNA hydrolysis	+
Tween 80 esterase	+

−*Aeromonas veronii* is ODC +.

Taxonomy

The name *Aeromonas* was first proposed by Kluyver and Van Niel and the genus was represented in the Seventh Edition of Bergey's manual. *Aeromonas* is a member of the family Vibrionaceae along with the genera *Vibrio* and *Photobacterium*. The earlier designations by Schubert (1969) identified the species *A. hydrophila*, and *A. punctata*, with three subspecies (*hydrophila*, *anaerogenes* and *proteolytica*) under *A. hydrophila* and two (*punctata* and *caviae*) under *A. punctata*. More recently, Popoff and Veron (1976) have proposed species designations of *hydrophila*, *sobria* and *caviae* for the motile, and *salmonicida* for the nonmotile, aeromonads. Other proposed species include *Aeromonas media*, isolated from an aquaculture pond, and *Aeromonas veronii*, an ornithine decarbodylase positive organism. (Allen *et al.* 1983; Hickman-Brenner *et al.* 1987).

TABLE 2.
DIFFERENTIATING BIOCHEMICAL REACTIONS FOR MOTILE AEROMONADS

Test	*A. hydrophila*	*A. sobria*	*A. caviae*
Esculin hydrolysis[1]	+	−	+
Acid from salicin[1]	+	−	+
Acid from arabinose (API)[1]	+	−	+
Lysine decarboxylase (API[1] and Meoller's)	+	+	−
Voges-Proskauer (API)[1]	+	+	−
Gas from glucose[1] (Durham Gas vial)	+	+	−
Beta hemolysis[1]	+	+	−
Gluconate[1]	+	+	−
Elastase	+	V	−
Growth in KCN broth[1]	+	−	+
mannose	+	+	−
alpha-methylglucopyranoside	+	+	−
H_2S from Glucose Cysteine[1] Fermentation medium	+	+	−
L-Histidine utilization[1]	+	−	+
L-Arginine utilization[1]	+	−	+

[1]Minimal characteristics required to differentiate motile *Aeromonas* sp.

DNA Analyses

DNA hybridization studies have verified the distinction of the three species, *A. hydrophila*, *A. sobria*, and *A. caviae*. Within these species there are at least seven DNA hybridization groups, three in *A. hydrophila*, two in *A. caviae*, and at least two in *A. sobria*. Thus far these DNA-related new groups have not been distinguished phenotypically one from the other (Popoff *et al.* 1981). Similarly, Farmer *et al.* (1986) using DNA hybridization (hydroxyapatite, ^{32}P, 60C), found only 5 of 60 Aeromonas strains to be highly related to the type strain of *A. hydrophila* (70% or greater). The type strain of *A. sobria* was highly related to only two other strains, suggesting that many strains of the motile, mesophilic group of *Aeromonas* belong to species other than *A. hydrophila*, *A. sobria*, and *A. caviae*. There appear to be nine to twelve different DNA hybridization groups.

5S rRNA Sequence Analysis

Results of 5S rRNA sequence analysis showed that *Aeromonas* spp. should be included in a family, Aeromonadaceae, separate from the Enterobacteriaceae and the Vibrionaceae (Colwell *et al.* 1986). Molecular genetic information compiled to date, including results of 16S ribosomal ribonucleic acid cataloging and

5S ribosomal ribonucelic acid sequence analyses, suggest that *Aeromonas* demonstrate an evolutionary divergence which is significantly at variance with that of other members of the *Vibrionaceae* (MacDonell *et al.* 1986). This is based on a method of ribonucleotide sequencing of 5S rRNA which appears to be useful in resolving questionable relationships (at least at the genus level) where extensive homology with the 5S rRNA is extant. This approach appears to be another dimension of the polyphasic method of bacterial taxonomy, where phylogenetic analysis of phenotypic characteristics embedded in the evolutionary history of these organisms can be correlated with fundamental properties of the species.

Isolation and Enumeration

Aeromonas can be isolated from food, environmental, and clinical sources on numerous types of media with varying success (Table 3). It is generally not a difficult organism to culture, but separation from other organisms in mixed populations and eventual identification is more complex and difficult.

Several factors should be considered in choosing a suitable culture medium. Sensitivity and selectivity are, of course, important. Achieving an increase in one many result in a reduction of the other.

Environment. *Aeromonas* sp. have been isolated from estuarine and nonsaline waters and sediment in high numbers approaching and sometimes surpassing the total coliform counts. Studies performed in the Anacostia River near Washington, D.C. (Joseph *et al.* 1979; Seidler *et al.* 1980) and in the Chesapeake Bay (Kaper *et al.* 1981) indicated that this observation was due probably to a high degree of pollution, and not fecal contamination. Such findings probably account for the high proportion of this resident organism, in relation to other organisms in the environment. The study in the Anacostia Riber was done after a diver suffered an infection of a puncture wound from which *Aeromonas sobria* and *A. hydrophila* were isolated (Joseph *et al.* 1979). In the Chesapeake Bay study, incidence of *A. hydrophila* was determined using a three-tube replication, MPN series. Presence of the organism was determined by inoculating appropriate volumes of water or sediment into an enrichment broth (Kaper *et al.* 1981). After 24 h at 35 °C, aliquots were plated either on RS media or MacConkey's agar with trehalose substituted for lactose. Isolated colonies were presumptively identified as *Aeromonas* using the screening medium of Kaper *et al.* (1979).

In the Anacostia study (Seidler *et al.* 1980), a somewhat different approach was used for detection and enumeration. Water samples were filtered through 0.45 uM filters which were placed in tubes of lactose broth. Presumptive *Aeromonas*-containing tubes were pour plated on PBG agar and enumerated as presumptively positive *Aeromonas* sp. These colonies were then analyzed by the

TABLE 3.
AEROMONAS ISOLATION MEDIA

MEDIUM	APPLICATION	INHIBITORY AGENT	DIFFERENTIAL AGENT	INCUBATION TEMP.	REFERENCE
Phenol red broth base starch Ampicillin Agar (SA)	Food	Ampicillin	Starch, Phenol red	25 °C	Palumbo, *et al.* 1985
Pectin Agar (PA)	Food	--	Pectin	25 °C	Myers, B.R. *et al.* 1982
Sorbitol Bile Broth (SBB)	Food	Bile	Sorbitol	5 °C	Myers, B.R. *et al.* 1982
Rimler-Shotts Agar (RS)[1]	Environmental	Citrate, novobiocin, desoxycholate	Lysine, ornithine maltose	35 °C	Shotts & Rimler 1973
MacConkey (trehalose substituted for lactose) MAC[1]	Environmental	Crystal violet, bile salts	Trehalose	35 °C	Kaper, *et al.* 1981
Peptone Beef Extract Glycogen Agar (PEG)	Environmental	Na. lauryl sulfate	Glycogen	25-30 °C	McCoy and Pilcher 1974
Rippey-Cabelli Agar (mA)	Environmental	Ampicillin, desoxycholate ethanol	Trehalose	25-30 °C	Rippey and Cabelli 1979
Ampicillin-Dextrin Agar (ADA)	Environmental	Ampicillin sodium desoxycholate	Dextrin	30 °C	Havelaar *et al.* 1987

TABLE 3, continued

Alkaline Peptone Water (APW)	Clinical	pH 8.5		35–37 °C	Millership *et al.* 1983
TSB w/5% Sheep	Clinical	–	Erythrocytes	35–37 °C	Janda *et al.* 1984
Blood Agar + Ampicillin (BA-AMP)	Clinical	Ampicillin	erythrocytes	35–37 °C	Millership *et al.* 1983
Trypticase Soy Broth + Ampicillin (TSBA)	Clinical	Ampicillin		35–37 °C	V. Graevenitz & Bucher 1983
Inositol-brilliant green bile salts (IBG)	Clinical	Brilliant green, bile salts	Inositol	35–37 °C	Schubert 1977
Dextrin-fuchsin-sulfite Agar (DFS)	Clinical, H_2O	Na. sulfite, fuchsin	Dextrin	35–37 °C	Schubert 1967
DNase-toluidine blue + ampicillin (DNTA)	Clinical	Ampicillin	DNase	35–37 °C	v.Graeventiz and Zinterhofer 1970
Salt-starch-Xylose-Lysine desoxycholate Agar (SSXLD)	Clinical	Desoxycholate, citrate, NaCl 1.5%	Lysine, starch, xylose	35–37 °C	Roland 1977
Pril-xylose-ampicillin Agar (PXA)	Clinical	Pril, Ampicillin	Xylose	35–37 °C	Rogol *et al.* 1979
Xylose-desoxycholate-Citrate Agar (XDC)	Clinical	Citrate, desoxycholate	Xylose	35–37 °C	Shread *et al.* 1980

TABLE 3, continued

Cefsulodin-Irgasan-Novobiocin Agars (CIN I and CIN II)	Clinical	Crystal violet I II Cefsulodin 15 mg/L 4 mg/L Irgasan 4 mg/L 4mg/L Novobiocin 25 mg/L 25 mg/L	Mannitol	25 °C	Altorfer *et al.* 1985
MacConkey Agar with 1% Tween 80 (MT80)	Clinical	Bile salts, Crystal violet	Lactose, Tween 80	35-37 °C	Hoban *et al.* 1981
MacConkey Agar with Tween 80 and Ampicillin (AT-80)	Clinical	Bile salts, Crystal violet, Ampicillin	Lactose, Tween 80	35-37 °C	Hoban *et al.* 1983
Gibco Aeromonas Agar	Clinical	Sodium deoxycholate, sodium citrate	Xylose	35-37 °C	Grand Island Biological Co. 1986

[1]Membrane Filter

Aeromonas screening medium of Kaper *et al.* 1979, followed by testing in the API 20 E and finally the scheme of Popoff and Vernon (1979) to accomplish speciation (Table 2).

For specimens with less than 10 cfu/mL, enumeration was accomplished by a most probable number procedure using a modified Rimmler-Shotts medium for initial enrichment. After 24h at 37 °C, tubes showing an acid reaction were streaked onto MacConkey's agar and then taken to further confirmation as described above.

Foods. In a recent study (Stern *et al.* 1987), pig, beef, sheep and turkey fecal specimens were assayed for recovery of inoculated *Aeromonas* sp. by directly plating the samples on five different agar media. Of these, starch-ampicillin was optimal with respect to selectivity and ability to differentiate *Aeromonas* from other resident microflora. Generally, the numbers of inoculated *Aeromonas* sp. recovered on starch-ampicillin agar were similar to those recovered on brain heart infusion and blood ampicillin agar media, and were 10^1 to 10^3 greater than the recovery rate on either MacConkey-ampicillin or cefsulodin-irgasan-novobiocin (CIN) agars. The sensitivity for the direct recovery of *Aeromonas* sp. from inoculated beef feces with naturally contaminating microflora, using streaked starch-ampicillin agar medium, was between 10^2 and 10^3 cells per gram. Using starch-ampicillin agar, the incidence of *Aeromonas* detected from feces was 1/32 (beef), 0/22 (pig), 0/24 (sheep) and 3/21 (turkey). Based upon current taxonomic criteria, the isolate from the beef feces had characteristics consistent with both *Aeromonas sobria* and *Aeromonas caviae*, whereas three isolates from turkey feces were identified as *A. caviae* or *A. hydrophila* (Stern *et al.* 1987).

Many studies have verified the high incidence of *Aeromonas* on various food products (Gray 1984; Kalina 1977; Myers *et al.* 1982; Palumbo *et al.* 1985; Morse and Hird 1984; Stern *et al.* 1987; Callister and Agger 1987; Michel *et al.* 1985). At what stage is *Aeromonas* introduced in the processing of these foods? This question remains to be answered completely. Since this organism is ubiquitous in soil and water, grows abundantly in heterotrophic conditions and at refrigeration temperatures, and is resistant to chlorine to some extent (LeChevallier *et al.* 1982), it is obvious that opportunities are numerous for *Aeromonas* to remain viable, colonize and proliferate.

Identification and Speciation

Following growth on a suitable, agar medium, suspected colonies should be subjected to a schema which will provide eventual identification to the species level (Table 4). Presumptively, there should be growth after 24 h incubation (28–37 °C) and growth on blood agar plates should be oxidase positive and resistant to the vibriostat agent with further confirmation determined by performing

TABLE 4.
AEROMONAS ISOLATION & IDENTIFICATION SCHEMA

- Streak organisms for isolation and incubate 24 h at preferred temperature (28 °C environmental and 35 °C for clinical specimens)
  - 5% Sheep Blood Agar
  - TSA Agar
  - SA Agar
  - CIN
- Analysis of Growth
  - Gram Negative Rods
  - Catalase - positive
  - Oxidase - positive
    (Use growth from Blood Agar)
  - Hemolysis - Beta or gamma
  - Motility - positive
    (Wet drop)
  - Indole - positive
    (use growth from Blood Agar)
- Set-up from 24 h Plate
  - API 20E strip
    (incubate 24 h at 35 °C)
  - Check Vibriostat (0/129)
    (should be resistant)
  - Metabolism
    (TSI should be Fermentative)
  - No Growth in TSB with 6% NaCl

API 20 E strip tests. Final speciation can be accomplished by performing the analyses lited in Table 2.

Concluding Statements

Several media have been proposed. Each investigator, at this point, must evaluate them and decide on a medium of choice. It is clear that a reliable, standard evaluation of available media needs to be done with samples from food, clinical, or environmental sources. Conditions of incubation such as temperature should be seriously considered in such an evaluation.

Considering the presence of DNA relatedness groups, some researchers recommend the terminology, *Aeromonas hydrophila* group, rather than speciation. Our opinion is that reference to the presently existing terminology provides better use of emerging information, allowing researchers to relate that information more specifically. Therefore, we would recommend continued use of the species designation and look forward to clarification of the other groups, be they new species, biotypes or serotypes.

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Aeromonas trota sp. nov., an Ampicillin-Susceptible Species Isolated from Clinical Specimens

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Previous DNA hybridization studies established 12 *Aeromonas* genospecies, from which nine phenotypic species have been proposed: *Aeromonas hydrophila*, *A. sobria*, *A. caviae*, *A. media*, *A. veronii*, *A. schubertii*, *A. salmonicida*, *A. eucrenophila*, and *A. jandaei*. We have delineated a new *Aeromonas* genospecies, *A. trota*, on the basis of 13 strains isolated primarily from fecal specimens from southern and southeastern Asia. All strains were highly related to the proposed type strain, AH2 (ATCC 49657^T): 51 to 100% (60°C) and 49 to 99% (75°C), with 0.2 to 2.2 divergence. AH2 was only 16 to 41% (60°C) related to all other *Aeromonas* type strains and DNA group definition strains. The unique profile of *A. trota* includes negative reactions for esculin hydrolysis, arabinose fermentation, and the Voges-Proskauer test, positive reactions for cellobiose fermentation, lysine decarboxylation, and citrate utilization, and susceptibility to ampicillin, as determined by the broth microdilution MIC method and the Bauer-Kirby disk diffusion method (10 μg). Nine of the *A. trota* strains were from a single study of 165 geographically diverse aeromonads. This finding questions the efficacy of screening fecal specimens for *Aeromonas* spp. with ampicillin-containing media and suggests a previously unrecognized prevalence of this new species.

The genus *Aeromonas* consists of nine validated and/or proposed species: *A. hydrophila*, *A. sobria*, *A. caviae*, *A. salmonicida*, *A. media*, *A. veronii* (biogroups sobria and veronii), *A. schubertii*, *A. eucrenophila*, and *A. jandaei* (1, 2, 7, 13, 14, 16, 18, 23, 24). They are all oxidase-positive, glucose-fermenting, facultatively anaerobic gram-negative rods that are resistant to vibriostatic agent 0/129, reduce nitrates to nitrites, are unable to grow in 6.5% NaCl, and are generally resistant to ampicillin and carbenicillin. All species are motile via a polar flagellum, except for most *A. salmonicida* strains (26).

These organisms are globally distributed and autochthonous to marine, estuarine, and freshwater environments (12, 19). They can tolerate temperatures ranging from 4 to 42°C, accounting for their ability to cause disease in a wide variety of cold- and warm-blooded animals (16). Although they are currently classified in the family *Vibrionaceae*, it was proposed in 1986 by Colwell et al. that, on the basis of 5S rRNA cataloging, 5S RNA sequencing, and RNA-DNA hybridization data, the aeromonads constituted a separate family, *Aeromonadaceae* (10).

All of the more recently described species, with the exception of *A. eucrenophila*, have been associated with human disease (2). The purpose of this study was to establish the taxonomic position and possible clinical significance of an unusual group of ampicillin-susceptible *A. sobria*-like organisms that thus far appear to be predominantly from southern and southeastern Asia.

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MATERIALS AND METHODS

Bacterial strains. The thirteen strains of *A. trota* used in this study are listed in Table 1 along with their geographic distribution, clinical source of isolation, and original phenotypic designation when received. All strains were originally isolated on media that did not contain antibiotics, in particular, ampicillin. The strains were then frozen in 2 ml of tryptic soy broth with 10% glycerol (Remel, Lenexa, Kans.), maintained at −70°C, and subsequently subcultured to tryptic soy agar plates (BBL Microbiology Systems, Cockeysville, Md.). After overnight incubation at 36 ± 1°C, single colonies were subcultured as stated above and used for all analyses. Unless otherwise stated, all analyses were performed at 36 ± 1°C.

Phenotypic analysis. Of the 32 identification tests (see Table 2), 30 were performed either by conventional methods (15, 22) with media and biochemicals from Remel or by the API 20E rapid identification system (Analytab Products, Plainview, N.Y.). Other media used for identification and characterization were modified GCF agar for the detection of H_2S from cysteine (25) and BEAM elastase agar (11).

DNA-related analysis. DNA was extracted and purified by previously described methods (4, 5) from the 13 *A. trota* strains, 1 *Vibrio parahaemolyticus* strain, and all type and reference strains representing all recognized *Aeromonas* DNA hybridization groups. The proposed type strain, ATCC 49657^T (AH2), was nick translated and labeled with ^{32}P (NEK-004; Dupont, NEN Research Products, Boston, Mass.), and hybridization analysis was performed at both the optimal reassociation temperature of 60°C and the stringent reassociation temperature of 75°C. Relatedness was expressed as the relative binding ratio and as the divergence in the melting temperature (dTm) (see Table 3). The relative binding ratio was calculated as a percentage to enable the determination of similarities between strains. The relative

TABLE 1. *A. trota* strains studied

Strain	ATCC no.	Geographic location	Source	Original designation
AH2	49657	India	Stool	*A. hydrophila* or *A. sobria*
MOB/A7		Bangladesh	Stool	*A. sobria*
MOB/A8	49658	Bangladesh	Stool	*A. sobria*
MOB/A9		Bangladesh	Stool	*A. sobria*
MOB/A11		Bangladesh	Stool	*A. hydrophila* or *A. sobria*
MOB/A16		Bangladesh	Stool	*A. hydrophila*
AMC 5439		Maryland	Stool	*A. hydrophila*
NMRI-206		Indonesia	Stool	*A. sobria*
NMRI-208	49661	Indonesia	Stool	*A. sobria*
CHAK 5258		Indonesia	Stool	*A. caviae*
CHAK 5274		Indonesia	Stool	*A. caviae*
AS 66	49660	Thailand	Stool	*A. sobria*
AS 370	49659	California	Appendix	*A. sobria*

binding ratio values of approximately 70% (for reactions at 60°C) and 55% (for reactions at 75°C) and a dTm of ≤5°C were considered the cutoff values for DNA relatedness at the species level (27).

Antimicrobial susceptibility. The 13 *A. trota* strains were tested for their susceptibility to 17 antimicrobial agents by the broth microdilution MIC method with the Gram Negative Flex 1 and Flex 2 panels of the Vitek system (Vitek Systems, Hazelwood, Mo.). MICs for 50% of all strains (MIC_{50}) were calculated (see Table 5). Antibiotic susceptibility was also determined on Mueller-Hinton agar by the disk method of Bauer et al. (3), and percent susceptibility was calculated (see Table 5).

RESULTS AND DISCUSSION

Phenotypic analysis. Nine of the 13 *A. trota* strains were originally part of a large systematic assessment of 165 geographically diverse aeromonads from clinical, environmental, and veterinary sources (6). Cluster analysis by SAS/TAXAN, a numerical taxonomy program, revealed clusters representing the four major mesophilic species, *A. hydrophila*, *A. caviae*, *A. veronii* (biogroups sobria and veronii), and *A. schubertii*, and a small cluster of nine strains that have recently been proposed to be *A. jandaei* (formerly DNA group 9 *A. sobria*) (7). This numerical taxonomy analysis also revealed a second small cluster of nine strains that were distinct from all other *Aeromonas* phenotypic species. They were uniformly negative for esculin hydrolysis, arabinose fermentation, and acetoin utilization (Voges-Proskauer) and uniformly positive for citrate utilization and lysine decarboxylation. They were also generally positive for the production of gas from glucose and H_2S from cysteine on modified GCF media and generally negative for sucrose fermentation (Table 2). The discovery of ampicillin susceptibility in conjunction with the acquisition of four additional such strains (AS 66, AS 370, 5258, and 5274) led to further investigation of this cluster. Additional phenotypic markers, including failure to oxidize gluconate, positive cellobiose fermentation, and susceptibility to carbenicillin, led us to suspect that this cluster represented a new *Aeromonas* phenotypic species.

DNA hybridization. Definitive identification of this cluster as a new species was accomplished in a stepwise process. Strain AH2 was labeled and tested against all the *Aeromonas* type and definition strains and ATCC reference strains

TABLE 2. Biochemical reactions of 13 *A. trota* strains

Test	Cumulative % positive on day[a]				Reaction for type strain ATCC 49657[T] (AH2)[a]
	1	2	3	7	
Indole production	100				+
Voges-Proskauer		0			−
Citrate[b]	100				+
Esculin hydrolysis			0		−
H_2S on TSI[c]				0	−
H_2S on modified GCF media	75		100		+
Lysine (Moeller's)	100				+
Arginine (Moeller's)	100				+
Ornithine (Moeller's)			0		−
Motility (25°C)	92				+
Gelatin hydrolysis	100				+
D-Glucose:					
Acid	100				+
Gas	75	83	83		+
Acid produced from:					
Amygdalin[b]	83				+
L-Arabinose			0		−
Cellobiose	100				+
Inositol[b]	0				−
D-Mannitol	75	83	83		+
Mannose	100				+
L-Rhamnose[b]	0				−
Salicin			0		−
D-Sorbitol[b]	0				−
Sucrose	17	17	17	17	−
Gluconate		0			−
Ascorbate utilization		0			−
Elastase				0	−
Nitrate reduction	100				+
Oxidase	100				+
ONPG[d]	100				+
String test	0				−
O/129 Resistance	100				+
Hemolysis on SBA[e]	54	77	77		+

[a] −, negative at end of appropriate incubation period; +, positive at 24 h or at end of appropriate incubation period.
[b] API 20E result.
[c] TSI, triple sugar iron.
[d] ONPG, *o*-nitrophenyl-β-galactopyranoside.
[e] SBA, sheep blood agar (tryptic soy agar with 5% sheep erythrocytes).

(Table 3) and found to exhibit only 16 to 41% homology. The second run used AH2 as the labeled probe against the other 12 phenotypically similar strains (Table 1). They showed 51 to 100% homology at 60°C and 49 to 99% homology at the more stringent 75°C, with a dTm of 0.2 to 2.2 (Table 3). Although strains A16, AMC 5439, and A7 fell below the 60°C accepted cutoff of 70% homology, their respective values for dTm and percent homology at 75°C taken collectively confirm that they are of the same species (27).

Designation of *A. trota*. We propose the name *Aeromonas trota* for the 13 strains listed in Table 1 (21). The species name (pronounced trō′ta) is derived both from the Greek adjective "trotos," meaning "vulnerable," and the modern (neo) Latin feminine adjective "trota," meaning "vulnerable." This designation is predicated upon the susceptibility of the organisms to ampicillin and carbenicillin, which first led us to suspect that the organisms were a separate group of aeromonads. The type strain of the species is designated ATCC 49657[T] (formerly AH2), and a complete description of all of the strains is provided in Table 2. The unique profile of *A. trota* includes negative reactions for esculin hydrolysis,

TABLE 3. DNA relatedness of the type strain of *A. trota* to other strains of *A. trota* and *Aeromonas* spp.

Strain	Relatedness (%) to labeled DNA of *A. trota* ATCC 49657[T]		
	60°C	dTm[a]	75°C
ATCC 49657[T] (AH2)	100	0.0	100
MOB/A7	67	0.2	71
MOB/A8	81	1.6	79
MOB/A9	100	2.0	93
MOB/A11	73	0.9	73
MOB/A16	69	2.2	68
AMC 5439	51	1.0	49
NMRI-206	100	1.0	98
NMRI-208	82	0.4	88
CHAK 5258	100	2.2	92
CHAK 5274	85	1.7	81
AS 66	82	0.3	89
AS 370	91	1.0	99
V. parahaemolyticus	14	11.1	ND[b]
A. hydrophila (DNA group 1) CDC 9079-89 (ATCC 7966[T])	35	ND	ND
A. sobria (DNA group 7) CDC 9538-76 (CIP 7433[T])	32	ND	ND
A. veronii biogroup sobria (DNA group 8)			
CDC 9080-79 (ATCC 9071)	36	ND	ND
CDC 0437-84	36	ND	ND
A. veronii biogroup veronii (DNA group 10) CDC 1169-83 (ATCC 35624[T])	41	ND	ND
A. hydrophila (DNA group 2) CDC 9533-76	37	ND	ND
A. hydrophila (DNA group 3) CDC 0434-84	27	ND	ND
A. caviae (DNA group 4) CDC 9083-79 (ATCC 15468[T])	30	ND	ND
A. caviae (DNA group 5a) CDC 0862-80	38	ND	ND
A. media (DNA group 5b) CDC 9072-83	33	ND	ND
A. eucrenophila (DNA group 6) CDC 0859-83 (NCMB 74[T])	32	ND	ND
A. jandaei (DNA group 9) CDC 0787-80 (ATCC 49568[T])	35	ND	ND
A. schubertii (DNA group 12) CDC 2446-81 (ATCC 43700[T])	17	ND	ND
Aeromonas group 501 CDC 2478-85	16	ND	ND
Enteric group 77 (DNA group 11) CDC 1306-83	32	ND	ND

[a] dTm, difference in melting temperature between each homologous and heterologous DNA complex.
[b] ND, not determined.

arabinose fermentation, Voges-Proskauer test, and gluconate oxidation. Further, *A. trota* is positive for cellobiose fermentation, lysine decarboxylation, and citrate utilization but is susceptible to both ampicillin and carbenicillin. A comparison of this profile with those of other mesophilic *Aeromonas* species is given in Table 4 (6, 7, 13–15, 17, 23, 26). Negative sucrose fermentation, positive mannitol fermentation, gas production from glucose, production of H_2S from modified GCF media, and hemolysis on tryptic soy agar with 5% sheep blood could also be used as presumptive markers but showed slight variability within the group and were not as definitive as the profile markers described above.

API 20E profile numbers for these strains identified them all as "*A. hydrophila* complex," with an identification level of "good" to "excellent," but with the comment that the negative reaction for mannitol and/or sucrose was considered atypical in their data base. Likewise, these strains were all identified as *A. hydrophila* in the Vitek system with a Gram Negative Identification (GNI) Card at 99% accuracy.

Antimicrobial susceptibility. The antibiogram of *A. trota* generally showed susceptibility to all 17 antimicrobial agents tested, with the exception of moderate susceptibility to cefoxitin and cefazolin and generalized resistance to cephalothin. This resistance to cephalothin is atypical of clinical *A. sobria* (now *A. veronii* biogroup sobria) (17), although the newly proposed *A. jandaei* (formerly DNA group 9 *A. sobria*) also showed generalized resistance to cephalothin (7). This difference may serve as a useful marker for the differentiation of one *A. sobria* biogroup from another.

Most interesting was the uniform susceptibility of *A. trota* to ampicillin and carbenicillin found by both the broth microdilution MIC method and the classic Bauer-Kirby disk diffusion method (Table 5). The absence of plasmids in the type strain, ATCC 49657[T] (8), and in aeromonads in general supports the observation that resistance to ampicillin and carbenicillin, when observed, is most likely chromosomal in nature (9, 20).

The majority of the strains were isolated during the mid-1970s and early 1980s, well before the availability and resultant trend toward the use of ampicillin-containing media for the screening and isolation of aeromonads. This fact suggests that the use of such media may result in a negative selection against *A. trota*, which has apparently been in the environment for quite some time and appears to be capable of causing human disease. Awareness of ampicillin susceptibility is important not only for *A. trota* but also for other aeromonads as well, since results from the previously mentioned numerical taxonomy study at the University of Maryland also revealed the existence of a subset of ampicillin-susceptible *A. caviae* strains from clinical specimens (6).

Geographic distribution. Another interesting aspect of this new species is its geographic distribution. Although it was found in five countries on two continents, a majority of the strains (11 of 13) originated from southern or southeastern Asia (Table 1). Whether this geographic distribution represents an original reservoir for *A. trota* in that area of the world that is now spreading to other areas can only be determined by both accurate biotyping and examination of antibiograms of a larger number of similar isolates from numerous geographic areas.

Clinical significance. *A. trota* was isolated almost exclusively from fecal specimens, with the exception of AS 370, which was isolated from an appendix. Although there is a strong association between aeromonads and gastrointestinal disease, Koch's postulates have not been fulfilled to date.

Most of the isolates in this study were accompanied by very limited patient information (Table 1), but there was a well described case history available on the type strain, ATCC 49657[T] (AH2). The patient was a 28-year-old laboratory worker with no preexisting health problems. The individual was exposed to a brain heart infusion broth suspension of AH2 by accidental ingestion of an 18- to 24-h broth culture (approximately 10^9 organisms) while preparing it for permanent storage and subsequently suffered a severe bout of diarrhea with "rice-water" stools over a period of 2 days. The clinical symptoms arose suddenly, without prodromes, within 24 h of the laboratory accident. The illness was self-limiting and subsided uneventfully after 2 days without any further complications.

A stool culture was performed on two separate occasions during the illness by streaking fecal material on human blood agar, MacConkey agar, and chocolate agar plates and inoculating brain heart infusion broth. After overnight incubation at 30°C, pure cultures of a single type of gram-negative bacterium were observed on all the media. The organism

TABLE 4. Comparison of distinguishing profiles of *A. trota* and other *Aeromonas* spp.

Test	Result[a] for:						
	A. trota	*A. jandaei*	*A. veronii* biogroup sobria	*A. veronii* biogroup veronii	*A. schubertii*	*A. hydrophila*	*A. caviae*
Esculin hydrolysis	−	−	−	+	−	+	+
Voges-Proskauer	−	+	+	+	V	+	−
Cellobiose	+	−	−	+	−	−	V
Gluconate oxidation	−	+	+	ND	−	+	−
Fermentation of:							
Arabinose	−	−	−	−	−	V	+
Mannitol	+	+	+	+	−	+	+
Sucrose	−	·	+	+	−	+	+
Susceptibility to:							
Ampicillin	S	R	R	R	R	R	R
Carbenicillin	S	R	R	R	R	R	R
Cephalothin	R	R	S	S	S	R	R
Colistin[b]	S	R	S	S	S	V	S
Decarboxylase:							
Lysine	+	+	+	+	+	+	−
Arginine	+	+	+	−	+	+	+
Ornithine	−	−	−	+	−	−	−
Indole	+	+	+	+	−	+	+
H_2S on modified GCF media	+	+	+	+	−	+	−
Glucose (gas)	+	+	+	+	−	+	−
Hemolysis (5% sheep erythrocytes)	V	+	+	+	+	+	V

[a] −, negative; +, positive; V, variable; ND, not determined; S, susceptible; R, resistant.
[b] Single-dilution MIC, 4 μg/ml.

isolated on both occasions was oxidase positive, was beta-hemolytic on human blood agar, and was an opaque, non-lactose fermenter on MacConkey agar. Biochemical characterization with the API 20E system identified the organism as *A. hydrophila*, and further identification of the organism to the species level by the criteria of Popoff and Veron (23) assigned it to the *A. sobria* group. These isolates were preserved and subsequently confirmed to be identical to the original AH2 strain that was ingested. This was accomplished with the phenotypic analyses listed in Table 2.

Strain AH2 also exhibited mannose-sensitive hemagglutination and was untypeable by the Sakazaki serotyping scheme. Furthermore, sterile, filtered culture supernatants were found to possess hemolytic activity on human erythrocytes, cytolytic activity on Vero cells, and proteolytic activity on tryptic soy agar with added skim milk.

TABLE 5. Antimicrobial susceptibility of 13 *A. trota* strains

Antibiotic	Broth microdilution MIC method			Bauer-Kirby disk diffusion method	
	MIC		Interpretation	Amt/disk (μg)	% Susceptibility
	Range (μg/ml)	50% (μg/ml)[a]			
Amikacin	<2–8	4	S	30	100
Ampicillin	<0.25–2	<0.25	S	10	100
Aztreonam	<8	<8	S	30	100
Carbenicillin	<16	<16	S	100	100
Cefazolin	<8–>32	16	MS	30	62
Cefotetan	<16	<16	S	30	100
Cefoxitin	<2–16	16	MS	30	92
Ceftazidime	<8	<8	S	30	100
Ceftriaxone	<8	<8	S	30	100
Cefuroxime	<4	<4	S	30	100
Cephalothin	<2–>32	>32	R	30	8
Ciprofloxacin	<0.5	<0.5	S	5	100
Gentamicin	<0.5–25	15	S	10	100
Imipenem	<4	<4	S	10	100
Mezlocillin	<16	<16	S	75	100
Piperacillin	<8	<8	S	100	100
Tetracycline	<1–8	<1	S	30	92
Ticarcillin	<16	<16	S	85	100
Ticarcillin-clavulanate	<16	<16	S	75	100
Tobramycin	<0.05–2	1	S	10	100
Trimethoprim-sulfamethoxazole	<10	<10	S	23.75/1.25	100

[a] 50%, MIC for 50% of all strains tested.

Conclusions. We have reported on the isolation and identification of a new *Aeromonas* species, designated *A. trota*. This species has a biochemical profile of negative reactions for esculin hydrolysis, arabinose fermentation, Voges-Proskauer test, and gluconate oxidation, positive reactions for cellobiose fermentation, citrate utilization, and lysine decarboxylation, and a uniform dual susceptibility to ampicillin and carbenicillin. Rapid identification systems, such as API 20E, and automated identification systems, such as the Vitek system, will identify these isolates as either *A. hydrophila* complex or *A. hydrophila*. However, some of the distinguishing biochemical markers mentioned above are incorporated within these two systems and should lead the clinical microbiologist to suspect the possibility of *A. trota*.

Although *A. trota* appears to be predominantly from southern and southeastern Asia, it may in fact be present worldwide. Documentation of its possible clinical significance and virulence potential for humans, presented here, strongly suggests that this species should be considered in biotyping of aeromonads from clinical and environmental sources.

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Species Identification of *Aeromonas* Strains Based on Carbon Substrate Oxidation Profiles

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Twenty clinical strains each of *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas sobria* were evaluated for their abilities to oxidize one or more of 95 carbon sources on a GN Microplate (BIOLOG, Hayward, Calif.). Nine substrates yielded good, discriminatory values for the three species tested. The panel appears to be useful for the species identification of *Aeromonas* isolates originating from human material.

The genus *Aeromonas* has received increased attention from the medical community as a suspected cause of bacterial gastroenteritis and as the etiologic agent of a number of additional infectious syndromes which include septicemia, cellulitis, and peritonitis (4). Within the genus at least 11 distinct species exist, as determined by DNA hybridization studies; of these 11 species, 7 have taxonomic standing in the literature (6). Recent studies have indicated that a majority (75 to 93%) of *Aeromonas* isolates recovered from clinical material fall into hybridization groups 1, 4, and 8; these hybridization groups correspond to the phenotypically identifiable species of *A. hydrophila*, *A. caviae*, and *A. sobria*, respectively (3, 6). Although these three phenotypic species can be identified by using a conventional battery of eight or more biochemical tests, most commercial identification panels for gram-negative organisms do not have the capability for accurate species identification within the genus *Aeromonas*. Instead, many such systems choose to collectively refer to the mesophilic aeromonad group as either *A. hydrophila*, the *A. hydrophila* complex, or *Aeromonas* spp. Recently, a new gram-negative microplate panel has been commercially introduced (GN Microplate; BIOLOG, Hayward, Calif.) which tests for the ability of bacteria to oxidize one or more of 95 carbon sources in the presence of a redox indicator (tetrazolium dye). We have evaluated this system as a potentially useful tool in the species identification of *Aeromonas* isolates recovered from clinical material.

Sixty *Aeromonas* strains (*A. hydrophila*, $n = 20$; *A. sobria*, $n = 20$; and *A. caviae*, $n = 20$) recovered from clinical material were evaluated in this study; three type or reference strains (namely, *A. hydrophila* ATCC 7966[T], *A. sobria* ATCC 9071, and *A. caviae* ATCC 15468[T]) were additionally included to ensure intra- and interlaboratory reproducibility. Each strain was grown overnight on Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar at 35°C, after which the 18- to 20-h resultant growth was removed and spectrophotometrically standardized (by optical density at 590 nm) in 0.85% NaCl according to the instructions of the manufacturer. These standardized suspensions were then inoculated into individual wells (150-μl volumes) of a 96-well GN Microplate (includes one control well) by using a multichannel pipetter. Inoculated plates were then incubated for 18 to 20 h at 35°C before being read.

Of the 95 carbon substrates screened in this system, one or more of the 60 *Aeromonas* strains were able to oxidize 66 (69%) of the tested compounds. The 29 compounds which yielded uniformly negative results included adonitol, *i*-erythritol, L-fucose, *m*-inositol, maltose, D-melibiose, D-raffinose, xylitol, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, hydroxy-L-proline, L-phenylalanine, L-pyroglutamic acid, DL-carnitine, phenylethylamine, 2-aminoethanol, and 2,3-butanediol. In a similar fashion, 10 other carbon substrates were positive for all 60 strains, including dextrin, glycogen, *N*-acetylglucosamine, D-fructose, α-D-glucose, D-mannitol, β-methylglucoside, sucrose, D-trehalose, and L-asparagine.

Of the remaining 57 carbon substrates, 14 compounds were positive 90% of the time or more with the 60 mesophilic aeromonads evaluated; these compounds included Tween 40 and 80, D-galactose, D-mannose, methyl pyruvate, D-gluconic acid, succinic acid, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-serine, inosine, glycerol, and DL-α-glycerol-phosphate oxidation. Twelve other compounds were positive at a frequency ranging between 1 and 89% and included α-cyclodextrin, D-arabitol, L-rhamnose, turanose, citric acid, formic acid, α- and β-hydroxybutyric acid, propionic acid, L-leucine, α-aminobutyric acid, and putrescine.

The remaining 30 carbon sources that displayed variable positivity rates (between 10 and 90%) were further evaluated for their potential usefulness in phenotypically distinguishing between the three *Aeromonas* species tested. Nine substrates were unquestionably found to have significant discriminatory value in species identification and are listed in Table 1; of these nine substrates, seven (excluding L-arabinose and cellobiose) have not previously been identified as useful biochemical markers (1, 5, 7). In addition, although 90% of the strains studied were positive for the oxidation of D-mannose, all mannose-negative strains ($n = 6$) were identified as *A. caviae*, another property previously found to be useful in other *Aeromonas* identification schemes (5). The remaining 21 carbon substrates exhibiting variable positivity rates overall (10 to 90%) are listed in Table 2; within this latter group only lactose and L-histidine have previously been reported to have discriminatory value in *Aeromonas* species identification (1, 5, 7).

The results of this study suggest that the BIOLOG GN Microplate panel may be useful for species identification within the genus *Aeromonas*, although this system is currently not approved for clinical use. On the basis of the

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TABLE 1. Phenotypic properties useful in *Aeromonas* species identification

Carbon source	% Positive			
	Overall[a]	*A. hydrophila*	*A. caviae*	*A. sobria*
N-Acetylgalactosamine	43	100	5	25
L-Arabinose	65	80	100	15
Cellobiose	33	5	85	10
Gentobiose	17	0	50	0
D-Galacturonic acid	18	0	55	0
DL-Lactic acid	62	95	90	0
Urocanic acid	33	10	90	0
Glucose-1-phosphate	68	100	5	100
Glucose-6-phosphate	67	100	0	100

[a] Based on 60 strains (*A. hydrophila*, $n = 20$; *A. caviae*, $n = 20$; *A. sobria*, $n = 20$).

results listed in Table 1, all 60 *Aeromonas* strains could unambiguously be placed in their correct taxonomic positions. Several carbon substrates (cellobiose, L-arabinose, and lactose) included in the GN panel have previously been found to be valuable in species identification, and comparable results were also obtained in this study. One significant difference noted in this study was L-histidine oxidation, a test originally proposed by Popoff and Veron (7) to be useful in determining the species status of individual *Aeromonas* isolates. Our results were less dramatic (Table 2) than those previously published; however, it should be noted that although the substrates are identical, the methods are entirely different, and each test may not produce equivalent results. Intra- and interlaboratory reproducibilities of test results for mean values were found to be 95 and 94%, respectively; the discrepancies noted were invariably reactions which were visually difficult to determine as weak positives or negatives. The system has additionally incorporated D-mannitol as a carbon substrate, which could facilitate the identification of *A. schubertii* if present (2). Recent studies conducted by Arduino and colleagues at the Centers for Disease Control (M. J. Arduino, F. W. Hickman-Brenner, and J. J. Farmer III, Abstr. Int. Workshop on *Aeromonas* and *Plesiomonas*, p. 37, 1988) have identified 41 biochemical characteristics that can correctly identify 85% of the individual *Aeromonas* strains (and can partially identify an additional 14%) to the correct DNA grouping with the aid of a computer identification program. Although progress has been made in the correct taxonomic (genospecies) assignment of *Aeromonas* strains through phenotypic characterization, much work remains to be accomplished. The number of tests currently required to perform such analysis is prohibitive, necessitates the use of specialized reagents and media, and is not directly applicable to the clinical laboratory. Furthermore, DNA hybridization groups 4, 5, and 6, which phenotypically resemble *A. caviae*, cannot at present be easily separated with this expanded biotyping system. We chose clinical isolates for this study since a vast majority of such strains reside in only three distinct species (hybridization groups 1, 4, and 8), and each corresponds to a particular identifiable phenotype (*A. hydrophila*, *A. caviae*, and *A. sobria*). Because of the apparent success of the present study, it seems possible that the BIOLOG GN panel might be additionally useful in the phenotypic characterization of environmental strains where greater genetic diversity occurs, although the current cost per panel ($5.65) seems unreasonably high for routine laboratory use.

TABLE 2. Variable carbon oxidation profiles of *Aeromonas* species

Carbon source	% Positive			
	Overall[a]	*A. hydrophila*	*A. caviae*	*A. sobria*
Lactose	27	20	55	5
Lactulose	17	10	40	0
Psicose	88	85	100	80
D-Sorbitol	38	20	55	35
Monomethyl succinate	22	35	30	0
Acetic acid	47	30	75	30
cis-Aconitic acid	17	5	5	40
Bromosuccinic acid	87	95	75	90
Succinamic acid	35	10	55	40
Alaninamide	13	0	30	10
D-Alanine	37	30	75	5
L-Alanine	73	50	85	85
L-Alanylglycine	83	70	90	90
Glycyl-L-glutamic acid	80	65	95	80
L-Histidine	45	55	50	30
L-Ornithine	12	5	30	0
L-Proline	18	5	45	5
D-Serine	85	95	70	90
L-Threonine	22	10	55	0
Uridine	87	75	100	85
Thymidine	68	45	100	60

[a] Based on 60 strains (*A. hydrophila*, $n = 20$; *A. caviae*, $n = 20$; *A. sobria*, $n = 20$).

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Aeromonas jandaei and *Aeromonas veronii* Dual Infection of a Human Wound following Aquatic Exposure

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Exudate removed from an infection that developed below the left eye of a 10-year-old male following a previously inflicted wound after aquatic exposure was cultured and revealed two different *Aeromonas* spp. Further characterization showed that one strain was phenotypically identical to *Aeromonas veronii*, while the other strain was confirmed by DNA hybridization analysis to be *Aeromonas jandaei* sp. nov. This is the first report of these more recently described aeromonads, thus far rarely reported from clinical disease, occurring simultaneously in a human infection.

Aeromonads have been isolated from almost every part of the human anatomy including tissue, spinal fluid, and blood (15). To date, of the nine species thus far validated or recommended for validation, five have been isolated from human intestinal infections, extraintestinal infections, or both. There is evidence that *Aeromonas veronii* biogroup sobria (formerly *A. sobria*) may potentially be the most virulent of these five species (8, 12–14). Within the past few years convincing data have accumulated to implicate aeromonads as primary causes of human infections, although the virulence mechanisms of these organisms are not as yet clearly defined. This is in sharp contrast to the historical perspective that aeromonads were associated only with infections in immunocompromised individuals.

The identification of numerous DNA relatedness groups by Fanning et al. (9) and the finding that three to five of these groups are the most commonly found in *Aeromonas*-associated human clinical disease (1, 11, 20, 22) suggest that perhaps we are beginning to penetrate the veil which has enveloped this formerly enigmatic and complex group of organisms.

A. veronii was proposed for separate species designation by Hickman-Brenner et al. (10) because of its ornithine decarboxylase-positive characteristic which distinguishes it from the other aeromonads. Their study was based on nine strains in their collection that resembled *Vibrio cholerae* but that were string test negative and produced gas from glucose. Unlike the halophilic vibrios, *A. veronii* does not require NaCl for growth.

The DNA group 9 aeromonads are characteristically negative for sucrose, esculin, and cellobiose and do not grow in the presence of KCN (2). The species designation *Aeromonas jandaei* has been recommended for this group (6).

In this report we describe one strain each of *A. veronii* (DNA group 10) and *A. jandaei* (DNA group 9 *Aeromonas*) obtained from the tissue wound of a 10-year-old male who swam in a freshwater pond after a missile-imposed injury was sustained below the left eye.

MATERIALS AND METHODS

Case history. A 10-year-old male was referred to the ophthalmology service for presumed cellulitis overlying the left lacrimal sac. The patient reported being struck in this area by a BB from an air rifle 5 weeks prior to admission. Because there was minimal bruising and bleeding at the time of injury, the patient did not believe that the BB had penetrated the skin and thus did not report this injury to anyone at the time. The patient went to summer camp 2 days after the injury had been inflicted and later swam in a freshwater pond. He subsequently developed erythema and mucopurulent drainage from the skin overlying the left lacrimal sac. The patient was evaluated at a local emergency room, where samples from the wound were obtained for culture. He was treated with cephalothin for presumed cellulitis and was later referred because of continued drainage and subsequent radiographic studies which showed a metallic foreign body in the area of the lacrimal sac. The patient underwent an exploration and excision of the lacrimal sac-cutaneous fistula, removal of the foreign body (BB), and intubation of the proximal nasolacrimal system.

Initially, the culture was found to contain two isolates, one resembling *A. hydrophila* and the other an oxidase-positive, gram-negative rod resembling *V. cholerae*. Further analysis revealed these strains to be two separate *Aeromonas* spp., by which time the patient had been treated postoperatively with intravenous cefotaxime for 3 days and for 7 subsequent days with oral Bactrim (trimethoprim-sulfamethoxazole), with complete resolution of the infection.

Bacterial strains. The strains used in this study, 4658/WRII and 4659/WRI, and the reference strains *A. jandaei* ATCC 49568[T] (6) and *A. veronii* ATCC 35624[T] (10) were quick-frozen in 2 ml of Trypticase soy broth with 10% glycerol (Remel, Lenexa, Kans.), maintained at −70°C, and subsequently subcultured onto Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, Md.) and incubated overnight at 36 ± 1°C.

Phenotypic analysis. Strains were examined for phenotypic

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traits representing a range of morphological, biochemical, physiological, and tolerance tests, which included Gram stain, oxidase activity, glucose fermentation, string test, and resistance to Vibriostat (0/129) (150 μg/ml; Oxoid, Columbia, Md.). They were also characterized with the API 20E system (Analytab Products, Plainview, N.Y.) and further examined for production of diffusible pigment on Trypticase soy agar; indole production; esculin hydrolysis; growth in KCN; fermentation of arabinose, salicin, mannitol, and sucrose; production of gas from glucose; ornithine decarboxylase; motility by wet preparation of growth at 25°C; arbutin hydrolysis; and H_2S production from cysteine by using a modified GCF (gelatin-cysteine-thiosulfate) medium.

Further tests were conducted to differentiate specific biotypes, to detect potential virulence-associated markers, or both. These were lysine decarboxylase, Voges-Proskauer, hemolysis on Trypticase soy agar plates with 5% sheep or 5% horse blood by streaking and stabbing and incubation at 36°C for 24 to 72 h, acriflavine agglutination (21), autoagglutination (17), and pyrazinamidase activity (7).

Unless stated otherwise, all tests were performed at 36 ± 1°C by using conventional methods and media (Remel).

Determination of mole percent G+C content. The mole percent guanine plus cytosine content was determined in duplicate for strains 4658/WRII and 4659/WRI as follows. Preparation of DNA samples was performed as described previously (18), with the following modifications. The cells were washed twice with 10 to 20 ml of TE buffer (10 mM Tris, 1 mM EDTA) with centrifugation at 5,000 rpm in a Sorvall RC-5B centrifuge at 4°C for 20 min. The cell pellets were resuspended in 0.01 M Tris (pH 8.0) with lysozyme (500 μg/ml) and then disrupted by alternating freezing and lysis in dry ice (5 min) and immersion at 55°C. To the mixture, 20 μl of sodium dodecyl sulfate (SDS) (25% [vol/vol]) per ml was added to complete lysis, followed by adjustment of [Na^+] to 1 M by using 5 M NaCl or 5 M sodium perchlorate. The DNA was precipitated and purified, and the base composition (guanine plus cytosine) was obtained by thermal denaturation (23, 24) by using the Gilford system 2600 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). *V. cholerae* ATCC 14035 was included as a control.

DNA relatedness assay. DNA-DNA hybridization was conducted on strain 4658/WRII in duplicate by using the batch hydroxyapatite thermal elution procedure (4, 5). DNA from *A. jandaei* ATCC 45968^T was nick-translated with ^{32}P (NEK-004; Dupont, NEN Research Products, Boston, Mass.) and reacted with unlabeled DNA from 4658/WRII at both the optimal reassociation temperature of 60°C and the stringent incubation temperature of 75°C. Relatedness was expressed as the relative binding ratio and as the divergence in the melting temperature.

Antibiotic susceptibility. Antibiotic susceptibility was determined by using the MIC microtiter method following the standards for interpretation of the National Committee for Clinical Laboratory Standards (25, 26). The MICs of 17 of the most recently prescribed antimicrobial agents were determined in a 96-well microdilution plate, the MicroScan Gram Negative Panel 7 (Baxter Healthcare Corp., MicroScan Division, West Sacramento, Calif.), and the results were interpreted and recorded both manually and with an AutoScan-4 reader, with computer-assisted analysis done with an IBM PS/2 model 60 computer. The antimicrobial agents and concentrations used are listed in Table 3. In addition to these antimicrobial agents, the panel also tested for a "growth" or "no growth" response against a single dilution of colistin (4 μg/ml).

TABLE 1. Biochemical reactions of *A. jandaei* (DNA group 9) and *A. veronii* (DNA group 10)[a] strains

Test	Reaction[b]			
	4659/WRI	*A. veronii* ATCC 35624^T	4658/WRII	*A. jandaei* ATCC 49568^T
Arginine dihydrolase[c]	−	−	+	+
Ornithine decarboxylase (Moeller)	+	+	−	−
Esculin hydrolysis	+	+	−	−
Pyrazinamidase	−	−	−	w+
Fermentation of:				
Salicin	+	+	−	−
Sucrose	+	+	−	−
Cellobiose	+	+	−	−

[a] DNA group 10 is now considered to coincide with DNA group 8.

[b] −, Negative after the appropriate incubation period; +, positive after the appropriate incubation period; w+, weakly positive. The following tests were positive for all of the strains tested: oxidase, indole, Voges-Proskauer, H_2S on GCF, growth at 42°C, lysine decarboxylase (Moeller), motility, acid and gas from glucose, acid from D-mannitol, gelatin hydrolysis, and β-hemolysis on Trypticase soy agar containing 5% sheep or horse blood. The following tests were negative for all of the strains tested: string test, acid from arabinose, brown pigment on the Trypticase soy agar at 36°C, growth in KCN, and sensitivity to Vibriostat (0/129).

[c] A reaction was observed following analysis with the API 20E system.

RESULTS

Biochemical characterization. Both strains 4659/WRI and 4658/WRII were oxidase-positive, fermentative, gram-negative rods which were motile, string test negative, and resistant to Vibriostat; they did not require NaCl for growth, did not grow in the presence of 6.5% NaCl, and reduced nitrates to nitrites.

A comparison of the two test strains with their respective reference strains showed them to be phenotypically identical. For the characteristics listed in Table 1, 4659/WRI and *A. veronii* ATCC 35624^T yielded identical results, while 4658/WRII and *A. jandaei* ATCC 49568^T had disparate results for only 1 of the 28 features tested: pyrazinamidase activity.

The primary distinguishing features among *A. veronii*, *A. jandaei*, and the three major species *A. hydrophila*, *A. veronii* biogroup sobria (formerly *A. sobria*), and *A. caviae* are presented in Table 2. The *A. jandaei* composite profile which distinguished it from the other major groups included negative reactions for sucrose, cellobiose, and salicin fermentation; negative esculin and arbutin hydrolysis; and resistance to cephalothin, cefazolin, and the single dilution of colistin of 4 μg/ml.

The unique differentiating features between *A. veronii* and the major groups were positive ornithine decarboxylase and esculin hydrolysis and negative arginine dihydrolase reactions. Other distinguishing differential features for *A. veronii* which caused it to differ from other aeromonads were as follows. (i) It was different from *A. caviae* because of positive reactions for lysine decarboxylase, gas from glucose, and H_2S from GCF; (ii) it was different from *A. sobria* by virtue of salicin fermentation and esculin and arbutin hydrolysis; and (iii) it was different from *A. hydrophila* because of negative pyrazinamidase activity and susceptibility to cephalothin and cefazolin.

TABLE 2. Distinguishing composite characteristics of *A. veronii* and *A. jandaei* from other *Aeromonas* spp.

Characteristic	Test result[a]				
	A. hydrophila	*A. veronii* biogroup sobria	*A. caviae*	*A. veronii* biogroup veronii	*A. jandaei*
Gas from glucose	+	+	−	+	+
Arginine dihydrolase	+	+	+	−	+
Pyrazinamidase	+	−	+	−	V
Fermentation of:					
Sucrose	+	+	+	+	−
Arabinose	V	−	+	−	−
Salicin	V	−	+	+	−
Cellobiose	−	−	+	+	−
Voges-Proskauer	+	+	−	+	+
Hydrolysis of:					
Esculin	+	−	+	+	−
Arbutin	+	−	+	+	−
H_2S (GCF)	+	+	−	+	+
Lysine decarboxylase	+	+	−	+	+
Ornithine decarboxylase	−	−	−	+	−
Resistance to:					
Cephalothin (>16 μg/ml)	+	−	+	−	+
Colistin (4 μg/ml)	V	−	−	−	+
Cefazolin (>16 μg/ml)	+	−	V	−	+

[a] +, Positive after the appropriate incubation period; −, negative after the appropriate incubation period; V, variable.

Nucleic acid studies. The results of the DNA hybridization analysis showed a close relationship between strain 4658/WRII and *A. jandaei* ATCC 49568[T] (80 to 78% at 60 and 75°C with a divergence in melting temperature of 3.4°C), which confirmed the identification of strain 4658/WRII as *A. jandaei* (6).

The base compositions of the DNAs for strains 4658/WRII, 4659/WRI, and *V. cholerae* ATCC 14035 were 58.2, 57.6, and 48.39%, respectively. These values are well within the previously described ranges for *Aeromonas* spp. and *Vibrio* spp. (3, 10).

Antibiotic susceptibility. All of the strains tested were susceptible to amikacin, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole. Conversely, they were uniformly resistant to ampicillin. However, variable results were seen with cephalothin, in which the *A. veronii* strains were susceptible and *A. jandaei* strains were resistant (Table 3).

DISCUSSION

Reports of multiple infections by two or more *Aeromonas* spp. are rare. Joseph et al. (19) reported in 1979 the simultaneous infection with *A. sobria* and *A. hydrophila* of a leg wound of a diver. This first report of a human with an *A. sobria* infection and a corollary study by Daily et al. (8) catalyzed the present emphasis on species identification of *Aeromonas* spp. from clinical specimens.

The simultaneous occurrence of multiple species, multiple serotypes, or both is probably more common than is realized. The possibility that these organisms could be acting synergistically, either among species or with other genera, thereby enhancing pathogenesis in humans, should not be ignored. By searching for a single pathogen or a simple mechanistic reason for infection, we may be overlooking a more significant complex ecological phenomenon at an infection site.

In this study we identified a dual infection with two aeromonads, *A. veronii* and *A. jandaei*, both of which have recently been described and have rarely been reported to be isolated from wound infections; in fact, they have rarely been reported to date (1, 6, 10, 15, 22). This may be a result of either the low incidence of these organisms in the environment (yet to be determined) or the low virulence or lack of familiarity with the identifying characteristics of these organisms in clinical laboratories. For example, the positive ornithine decarboxylase activity and the absence of an NaCl requirement for growth of *A. veronii* might initially cause one to suspect it to be *V. cholerae*.

Taxonomically, *A. veronii* and *A. sobria* are related. In fact, the DNA relatedness of organisms in DNA group 8 (*A. sobria*) and DNA group 10 (*A. veronii*) suggests that these groups coincide, and they are, in fact, proposed as two biogroups of *A. veronii* (*A. veronii* biogroups sobria and veronii, respectively) (10). Because the phenotypic characteristics of the type strain *A. sobria* CIP 7433 (DNA group 7) are not consistent with the characteristics seen in the majority of clinical isolates, *A. sobria* ATCC 9071 (DNA group 8Y) is considered by most investigators to resemble clinical *A. sobria* more closely. Lee and Bryant (22), in a study of 163 strains from diverse sources, found that *A. sobria* CIP 7433 did not cluster with their strains, most of which fit into the three major phena. *A. sobria* ATCC 9071 more closely resembles the typical clinical *A. sobria* strains isolated thus far with respect to positive reactions for Voges-Proskauer,

TABLE 3. In vitro susceptibilities of 4659/WRI and 4658/WRII and *A. jandaei* and *A. veronii* type strains to 17 antimicrobial agents

Antimicrobial agent	MIC (μg/ml)[a]				
	Range	4659/WRI	*A. veronii* ATCC 35624[T][b]	4658/WRII	*A. jandaei* ATCC 49568[T][c]
Amikacin	≤2–≥16	8 (S)	8 (S)	8 (S)	4 (S)
Ampicillin	≤1–≥16	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Cefazolin	≤2–≥16	8 (S)	8 (S)	>16 (R)	16 (MS)
Cefoxitin	≤2–≥16	<2 (S)	<2 (S)	<2 (S)	<2 (S)
Ceftazidime	≤2–≥16	<2 (S)	<2 (S)	<2 (S)	<2 (S)
Ceftriaxone	≤4–≥32	<4 (S)	<4 (S)	<4 (S)	<4 (S)
Cefuroxime	≤2–≥16	<2 (S)	<2 (S)	<2 (S)	<2 (S)
Cephalothin	≤8–≥16	16 (MS)	<8 (S)	>16 (R)	>16 (R)
Ciprofloxacin	≤1–≥2	≤1 (S)	<1 (S)	>1 (S)	<1 (S)
Gentamicin	≤1–≥6	4 (S)	4 (S)	2 (S)	2 (S)
Imipenem	≤1–≥8	>8 (R)	>8 (R)	>8 (R)	4 (S)
Piperacillin	≤8–≥64	>64 (R)	64 (MS)	>64 (R)	64 (MS)
Tetracycline	≤4–≥8	<4 (S)	<4 (S)	<4 (S)	<4 (S)
Ticarcillin-K clavulanate	≤8–≥64	>64 (R)	32 (MS)	32 (MS)	<8 (S)
Ticarcillin	≤8–≥64	>64 (R)	>64 (R)	>64 (R)	64 (MS)
Tobramycin	≤1–≥6	6 (I)	4 (S)	<1 (S)	<1 (S)
Trimethoprim-sulfamethoxazole	≤0.5/9.5–>2.0/38	<0.5/9.5 (S)	<0.5/9.5 (S)	<0.5/9.5 (S)	<0.5/9.5 (S)

[a] Therapeutic guide based on National Committee for Clinical Laboratory Standards (NCCLS) standard M100-2S (26) as follows: S, susceptible; R, resistant; MS, moderately susceptible; I, intermediate.
[b] Centers for Disease Control definition strain for DNA hybridization group 10.
[c] Centers for Disease Control definition strain for DNA hybridization group 9.

hemolysin, and citrate. The current type strain *A. sobria* CIP 7433 is negative for all of these well-documented characteristics and does not appear to be representative of clinical strains of *A. sobria*. We therefore suggest that investigators working with *Aeromonas* isolates from human specimens use *A. sobria* ATCC 9071 (*A. veronii* biogroup sobria) as the reference strain for this species and *A. veronii* ATCC 35624[T] as the reference strain for *A. veronii* biogroup veronii.

The antibiograms of *A. veronii* and *A. jandaei* showed that the initial therapy used in the case reported here, i.e., cephalothin, was not effective, as was observed clinically, and that the change to cefotaxime and trimethoprim-sulfamethoxazole was an appropriate therapeutic approach.

The *A. veronii* strains were susceptible to all of the cephalosporin-derived antibiotics tested, while the *A. jandaei* strains were resistant to cephalothin and cefazolin. Susceptibility to cephalothin was noted by Janda and Motyl (16) to be a useful marker for *A. sobria*. With the recommended nomenclature of *A. jandaei* proposed for the DNA group 9 aeromonads, the resistance of this species to cephalothin should be considered a useful marker to distinguish it from *A. sobria* (DNA groups 8 and 10, currently recommended to be *A. veronii* biogroups sobria and veronii, respectively).

Because of the sporadic isolation of DNA group 9 strains from human specimens, we elected to hybridize the 4658/WRII strain to the type strain *A. jandaei* ATCC 49568 (6). These data and the mole percent G+C base composition confirmed our presumptive identification.

This report of the isolation of *A. jandaei* contributes to the probability that it is another aeromonad of significance to clinical microbiologists. The distinguishing features of *A. veronii* biogroup veronii and *A. jandaei* are sufficiently distinct from other *Aeromonas* spp. to recommend that they be identified to the species level when *Aeromonas* isolates are studied in the clinical laboratory.

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Aeromonas jandaei (Formerly Genospecies DNA Group 9 *A. sobria*), a New Sucrose-Negative Species Isolated from Clinical Specimens

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A large numerical taxonomy study conducted in 1988 of 165 mostly clinical *Aeromonas* strains from diverse geographic sources produced a cluster (S = 84%, S_{SM}) of four sucrose-negative strains that included the DNA definition strain for DNA group 9 *A. sobria* (CDC 0787-80). These four strains, together with five additional strains received in 1989, were subjected to DNA-DNA hybridization (hydroxyapatite, ^{32}P, 60 and 75°C), and all eight strains were closely related to the ninth labeled DNA group 9 definition strain CDC 0787-80 (73 to 86% relatedness at 60°C and 68 to 80% relatedness at 75°C; percent divergence, 2.0 to 3.5). Type strains and DNA definition strains for all other established *Aeromonas* species were only 35 to 72% related (60°C) to CDC 0787-80. We propose the name *Aeromonas jandaei* for this highly related group of nine strains, formerly known as DNA group 9 *A. sobria*. The type strain was designated ATCC 49568 (CDC 0787-80). The nine strains were examined at 36°C and were found to be resistant to 0/129 (vibriostatic agent) and uniformly positive for oxidase, gas production from glucose, indole, lysine decarboxylase, arginine dihydrolase, *o*-nitrophenyl-β-D-galactopyranoside, motility (25°C), nitrate reduction, citrate utilization, hemolysis on sheep blood agar, and growth in Trypticase soy broth with no added NaCl. They all fermented D-glucose, D-mannitol, and mannose but did not ferment sucrose, cellobiose, L-arabinose, inositol, salicin, or D-sorbitol. They were uniformly negative for esculin and urea hydrolysis, elastase production, ornithine decarboxylation, and the string test. The antibiogram of *A. jandaei* resembled that of other aeromonads (resistance to ampicillin and cephalothin), but it differed from most other aeromonads because of resistance to a single dilution of colistin and differed from clinical *A. veronii* biogroup sobria (formerly *A. sobria*) by its nearly uniform resistance to cephalothin. The esculin-, sucrose-, and cellobiose-negative and colistin-resistant profile distinguished *A. jandaei* from other *Aeromonas* species. These *A. jandaei* strains were isolated from blood (two strains), wounds (two strains), diarrheal stools (four strains), and a prawn (one strain). The blood and wound isolates, in particular, suggest that there is a possible clinical significance for this species and justify identification of and further research on this group of motile aeromonads.

Aeromonads are oxidase-positive, polar flagellated, glucose-fermenting, facultatively anaerobic, gram-negative rods that are resistant to 0/129 (vibriostatic agent) and autochthonous to aquatic environments worldwide (9, 20). Although they currently reside in the family *Vibrionaceae*, Colwell et al. (6) proposed in 1986 that aeromonads constitute a new family, *Aeromonadaceae*, based on 5S rRNA cataloging, 5S rRNA sequencing, and RNA-DNA hybridization data.

There are currently eight recognized phenotypically distinct genospecies distributed among 12 DNA hybridization groups (1). The genus consists of both psychrophiles and mesophiles that are found in soil and aquatic environments and that produce a diverse spectrum of diseases among both warm- and cold-blooded animals (14).

The species *Aeromonas sobria* was first proposed by Popoff and Veron (26) in 1976 and encompasses four DNA hybridization groups: DNA group 7 (which includes the type strain CIP 7433), DNA group 8 (which includes all clinical *A. sobria* isolates hybridized to date but has been proposed as a biogroup of *A. veronii*), DNA group 10, *A. veronii* biogroup veronii (which is phenotypically distinct from but genotypically identical to DNA group 8) (1, 10, 26), and the rare unnamed DNA group 9 aeromonads (7).

The purpose of this study was to establish the taxonomic position of and proper designation for the DNA group 9 aeromonads by using a suitable number of phenotypically and genotypically related strains. Additionally, by examination of case studies, antimicrobial susceptibility patterns, and phenotypic markers, the possible clinical significance of this group of aeromonads was investigated.

MATERIALS AND METHODS

Bacterial strains. The nine strains of *A. jandaei* that were studied are listed in Table 1. All strains were frozen in 2 ml of Trypticase soy broth with 10% glycerol (Remel, Lenexa, Kans.), maintained at −70°C and subsequently subcultured onto Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, Md.), and incubated overnight at 36 ± 1°C. Unless stated otherwise, all analyses were performed at 36 ± 1°C.

Phenotypic analysis. All of the standard identification tests (Table 2) were performed by either conventional methods (16, 19) with media and biochemicals obtained from Remel or by rapid identification with the API 20E system (Analytab Products, Plainview, N.Y.). Media for ascorbate utilization

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TABLE 1. *Aeromonas* DNA group 9 strains studied

A. jandaei strain	Case no.	ATCC no.[a]	Location of sender	Source	Other clinical information
CDC 0787-80	1	49568[T]	Oregon	Stool	Male with diarrhea and dehydration
AS-14	2	49569	New York	Blood	71-yr-old male with multiple myeloma and diabetes
WRII/4658	3	49570	Maryland	Wound, below left eye	Riflegun-imposed BB injury that became infected after subject swam in a fresh H_2O pond
NMRI-6	4	49571	Maryland	Wound, leg	Navy diver with leg wound infection with two *Aeromonas* spp. from Anacostia River, Washington, D.C.
AS-206	5		New York	Stool	<1-yr-old female with gastroenteritis
AS-235	6		New York	Blood	96-yr-old male with acute erythroleukemia
CDC 1081-81 (AS-167)	7	49572	Hawaii	Prawn	Isolated from water off Honolulu
CDC 1530-81	8		Georgia	Stool	Two isolates from the same patient in Children's Hospital
CDC 1531-81 (AS 171)	9		Georgia	Stool	

[a] ATCC, American Type Culture Collection, Rockville, Md.

TABLE 2. Biochemical reactions of nine *A. jandaei* strains

Test	Cumulative % positive on day:				Reaction for ATCC 49568[T] (CDC 0787-80)[a]
	1	2	3	4	
Indole production	100				+
Voges-Proskauer	78	78	89		+
Citrate[b]	100				+
H_2S on triple sugar iron	0	0	0		−
H_2S on modified GCF	11	56	89		+
Urea hydrolysis[b]	0				−
Lysine (Moeller's)	100				+
Arginine (Moeller's)	100				+
Ornithine (Moeller's)	0	0	0		−
Motility (25°C)	100				+
Gelatin hydrolysis[b]	89				+
D-Glucose:					
Acid	100				+
Gas	100				+
Acid from:					
Amygdalin[b]	11				−
L-Arabinose			0		−
Cellobiose			0		−
Inositol[b]	0				−
D-Mannitol	100				+
Mannose	100				+
Melibiose	36	56	56		+
L-Rhamnose[b]	0				−
Salicin	0	0	0		−
D-Sorbitol[b]	0				−
Sucrose			0		−
Gluconate oxidation		78			−
Ascorbate utilization		67			+
Elastase production	0	0	0	0	−
Esculin hydrolysis	0	0	0		−
Nitrate reduction	100				+
Oxidase	100				+
ONPG test[b,c]	100				+
String test	11				−
Sensitivity to 0/129	0				−
Hemolysis on sheep blood	34	100			+

[a] −, Negative at end of appropriate incubation period; +, positive at 24 h or after appropriate incubation period.
[b] API 20E test result.
[c] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.

and H_2S production from GCF (gelatin-cysteine-thiosulfate) were prepared as described previously (8, 29).

DNA relatedness analysis. DNA-DNA hybridization was conducted in duplicate by using the batch hydroxyapatite thermal elution procedure (2, 3). DNA from the definition strain for DNA group 9 aeromonads (CDC 0787-80) was nick translated with ^{32}P (NEK-004; Dupont, NEN Research Products, Boston, Mass.) and reacted with unlabeled DNA from the other eight phenotypically similar strains as well as reference strains for the other *A. sobria* groups at both the optimal reassociation temperature of 60°C and the stringent incubation temperature at 75°C. Relatedness was expressed as the relative binding ratio and as the divergence in the melting temperature (Table 3). The relative binding ratio was calculated as a percentage to enable determination of similarity between strains. Relative binding ratio values of 70% (for reactions at 60°C) and 55% (for reactions at 75°C) were considered to be the cutoff values for DNA relatedness. A divergence in melting temperature of 5°C was considered the cutoff value for DNA relatedness.

Control reactions, in which labeled DNA was incubated in the absence of unlabeled DNA, were included, and the label-only control values were subtracted for all reactions before the relative binding ratio was calculated.

Antimicrobial susceptibility. MICs were determined for 17 antimicrobial agents in a 96-well microdilution plate (MicroScan Gram Negative Panel 7; Baxter Healthcare Corp., MicroScan Division, West Sacramento, Calif.). Panels were inoculated according to the instructions of the manufacturer and incubated for 24 h at 36°C. After incubation, the MIC for each strain against each antimicrobial agent was determined and recorded both manually and with an AutoScan-4 reader, with computer-assisted analysis done by an IBM PS/2 model 60 computer, which provided interpretations in accordance with guidelines of the National Committee for Clinical Laboratory Standards (24, 25). In addition to the 17 antimicrobial agents listed in Table 4, the panel also tested for a "growth" or "no growth" response against a single dilution of colistin of 4 μg/ml.

RESULTS AND DISCUSSION

DNA hybridization results. By DNA hybridization (Table 3), all eight strains of esculin-, sucrose-, and cellobiose-negative *A. jandaei* were closely related to the DNA group 9 definition strain CDC 0787-80 (ATCC 49568[T]) under both the optimal and stringent conditions. The type strain ATCC

TABLE 3. DNA relatedness of the type strain of *A. jandaei* to other strains of *A. jandaei* and other strains of *Aeromonas* and members of the family *Vibrionaceae*

Source of unlabeled DNA	% Relatedness to labeled DNA of *A. jandaei* ATCC 49568[T]		
	60°C	dT_m[a]	75°C
A. jandaei (DNA group 9)			
CDC 0787-80 (ATCC 49568[T])	100	0	100
CDC 1530-81	85	2.0	76
CDC 1531-81 (AS 171)	86	2.7	75
CDC 1081-81 (AS 167)	84	2.3	80
NMRI-6	83	3.5	77
WRII/4658	80	3.4	78
AS-14	73	2.0	68
AS-206	83	2.6	77
AS-235	82	2.8	76
A. sobria (DNA group 7) CDC 9538-76 (CIP 7433[T])	53	7.7	32
A. veronii biogroup sobria (DNA group 8)			
CDC 9080-79 (ATCC 9071)	54	5.3	38
CDC 0437-84	72	7.9	46
A. veronii biogroup veronii (DNA group 10) CDC 1169-83 (ATCC 35624[T])	58	5.7	40
A. hydrophila (DNA group 1) CDC 9079-79 (ATCC 7966[T])[b]	51	9.8	ND[c]
A. salmonicida subsp. *salmonicida* (DNA group 3) CDC 9701-84 (ATCC 33658[T])[b]	47	11.3	ND
A. caviae/punctata (DNA group 4) CDC 9083-79 (ATCC 15468[T])[b]	39	10.3	ND
A. media (DNA group 5b) CDC 9072-83 (ATCC 33907[T])	40	10.9	ND
A. eucrenophila (DNA group 6) CDC 0859-83 (ATCC 23309[T])[b]	47	11.0	ND
A. schubertii (DNA group 12) CDC 2446-81 (ATCC 43700[T])[b]	35	12.6	ND
Vibrio cholerae CDC 9060-79	3	ND	ND

[a] dT_m, Difference in melting temperature between each homologous and heterologous DNA complex.
[b] Data from Fanning et al. (7), with permission of the authors.
[c] ND, Not determined.

49568 was sufficiently distant from the other established *A. sobria*-like reference strains *A. sobria*[T] and *A. veronii* (*A. veronii* biogroups sobria and veronii) to reinforce the validity of DNA group 9 as originally proposed by Fanning et al. (7). Their original data for DNA hybridization between CDC 0787-80 and previously established *Aeromonas* species are presented in Table 3.

Designation of *A. jandaei*. We propose the name *A. jandaei* for the nine strains listed in Table 1. The species name (pronounced jan.dae'i) sp. nov., is derived from the surname of J. Michael Janda, an American microbiologist who has contributed greatly to our knowledge of *Aeromonas* species, in particular, their biotypes and clinical significance and the relative pathogenicities of mesophilic members (11–16). The species name is treated as a modern (neo) Latin genitive noun and was derived after consultation with T. O. MacAdoo, Virginia Polytechnic Institute and State University, Blacksburg. The ending "ei" is appropriate for a male name ending in the vowel "a" and has been proposed and accepted as such in the latest revision of Appendix 9, Orthography, of the *International Code of Nomenclature of Bacteria* (21, 22).

TABLE 4. In vitro susceptibilities of nine *A. jandaei* isolates to 17 antimicrobial agents

Antimicrobial agent	MIC (μg/ml)[a]		
	Range	50%	90%
Amikacin	4–8	4 (S)	8 (S)
Ampicillin	≥16	≥16 (R)	≥16 (R)
Cefazolin	4–≥16	≥16 (R)	≥16 (R)
Cefoxitin	≤2–4	≤2 (S)	4 (S)
Ceftazidime	≤2–4	≤2 (S)	≤2 (S)
Ceftriaxone	≤4	≤4 (S)	≤4 (S)
Cefuroxime	≤2–8	≤2 (S)	4 (S)
Cephalothin	≤8–≥16	≥16 (R)	≥16 (R)
Ciprofloxacin	≤1	≤1 (S)	≤1 (S)
Gentamicin	≤1–2	2 (S)	2 (S)
Imipenem	2–≥8	≥8 (R)	≥8 (R)
Piperacillin	≥64	≥64 (R)	≥64 (R)
Tetracycline	≤4	≤4 (S)	≤4 (S)
Ticarcillin-K clavulanate	≤8–64	16 (S)	64 (MS)
Ticarcillin	32–≥64	≥64 (R)	≥64 (R)
Tobramycin	≤1–2	≤1 (S)	2 (S)
Trimethoprim-sulfamethoxazole	≤0.5/9.5	≤0.5/9.5 (S)	≤0.5/9.5 (S)

[a] 50% and 90%, MIC for 50 and 90% of isolates, respectively; S, R, and MS, susceptible, resistant, and moderately susceptible, respectively, based on guideline M100-2S of the National Committee for Clinical Laboratory Standards (25).

The type strain of the species is designated ATCC 49568 (CDC 0787-80), with a complete description of the species given in Tables 2 and 5. The unique profile of *A. jandaei* is as follows: negative esculin hydrolysis, negative fermentation of sucrose and cellobiose, and resistance to a specific dilution of colistin (4 μg/ml). In previous taxonomic work in 1984, Turnbull et al. (28) noted the presence of a small asaccharolytic group of aeromonads that were sucrose and esculin negative, and even earlier work in 1981 on antimicrobial susceptibility by Richardson et al. (27) established the nearly universal susceptibility of most *Aeromonas* species to colistin.

A. jandaei resides in the genus *Aeromonas* because it is an oxidase-positive, glucose-fermenting, motile gram-negative rod that is resistant to 0/129, is string test negative, reduces nitrate to nitrite, and does not require NaCl for growth. It is nearly uniformly resistant to ampicillin, cefazolin, cephalothin, imipenem, piperacillin, and ticarcillin (Table 4).

Presumptive identification. Rapid identification with the API 20E strip and data base yields an identification of "*Aeromonas hydrophila* complex" with an identification level of "very good" to "excellent," but with the comment that the negative reaction for sucrose fermentation is considered atypical for their data base. The four different profile numbers generated for the nine strains in this study were 7247144, 7247104, 7246144, and 7247145. Additional presumptive markers for *A. jandaei* were a positive Voges-Proskauer reaction (89%), positive H_2S production from GCF (89%), resistance to cephalothin (78%), and utilization of ascorbate (67%).

Clinical significance of *A. jandaei* strains. *A jandaei* was isolated from specimens of diverse geographic origins, including wounds, blood, stools, and a prawn (Table 1). The isolation of these strains, excluding the environmental prawn isolate, suggests that there is a possible clinical significance for this species, and four case studies are given below with references for those that have been published previously.

(i) Case 1, blood (isolate AS-14) (12). The patient was a

TABLE 5. Differentiation of *A. jandaei* from other *Aeromonas* species

Characteristic	*A. jandaei*	*A. veronii* biogroup sobria[a]	*A. veronii* biogroup veronii	*A. schubertii*	*A. hydrophila*	*A. caviae*
Esculin hydrolysis	−[b]	−	+[c]	−	+	+
Acid from:						
Sucrose	−	+	+	−	+	+
Arabinose	−	−	−	−	V[d]	+
Cellobiose	−	V[e]	+	−	−	+
Salicin	−	−	+	−	V[d]	+
Elastase production	−	V	−	−	+	−
Resistance to cephalothin	+	−	−	−	+	+
Resistance to colistin[f]	+[d]	−[d]	−[d]	−[d]	V[d]	−[d]
Gas production from glucose	+	+	+	−	+	−
Voges-Proskauer	+	+	+	V	+	−
Lysine decarboxylase	+	+	+	+	+	−
Arginine dihydrolase	+	+	−	+	+	+
Indole production	+	+	+	−	+	+
Mannitol production	+	+	+	−	+	+
H_2S from modified GCF	+[d]	+[d]	+[d]	−[d]	+[d]	−[d]
Hemolysis on sheep blood	+	+	+	+	+	−

[a] Formerly *A. sobria*.
[b] −, Negative, i.e., positive for ≤30% of isolates.
[c] +, Positive for ≥70% of isolates.
[d] Data from Carnahan et al. (4).
[e] Variable, positive for 31 to 69% of isolates.
[f] 4 μg/ml (MicroScan Gram Negative Panel 7).

71-year-old male with adult-onset diabetes mellitis. Five months prior to this admission he had been diagnosed as having multiple myeloma and had been treated for this condition with combinations of vincristine, cytoxan, and prednisone. During the course of his chemotherapy his leukocyte count had fallen to 1,900 and he had had several septic episodes with *Enterococcus faecalis* and *Escherichia coli*. In August 1981 he presented to the emergency room with acute mental deterioration and a fever of 102°F (39°C); laboratory findings included a leukocyte count of 1,300, an hematocrit of 22, and a blood pressure of 150/60. The impression on admission was gram-negative sepsis. Blood cultures drawn on two separate occasions yielded *Aeromonas* species. The patient was treated with cephalothin and tobramycin but became lethargic, tachypneic, and hypotensive and died 7 days after admission.

(ii) Case 2, wound (isolate WRII/4658) (17). A 10-year-old male who swam in a freshwater pond after an injury had been inflicted below the left eye presented at a hospital emergency room for cellulitis of the left lacrimal sac. Subsequent cultures revealed both *A. veronii* biogroup veronii and *A. jandaei*. Surgery and treatment with cefotaxime and trimethoprim-sulfamethoxazole resulted in a complete recovery.

(iii) Case 3, wound (isolate NMRI-6) (18). Briefly, case 3 was a U.S. Navy diver who had an infection caused simultaneously by two different species of *Aeromonas* (*A. hydrophila* and *A. jandaei*). The infection was of the soft tissue of the leg and was secondary to a puncture wound sustained during diver training operations in the Anacostia River near Washington, D.C.

(iv) Case 4, blood (isolate AS-235) (12). Case 4 was a 96-year-old male who had been diagnosed as having acute myelogenous leukemia 3 years previously by bone marrow biopsy. He had received no chemotherapy but had been transfused with erythrocytes and platelets periodically for episodes of epistaxis. In July 1984 he presented to the hematology clinic with a 1-day history of shaking, chills, and fever to 101°F (38°C). Laboratory findings included a leukocyte count of 3,200, a platelet count of 25,000, and a hemoglobin level of 7.9%. Blood cultures were drawn because of the apparent septic episode and revealed *Aeromonas* species. The patient was treated with tobramycin and cefamandole for 16 days with no recurrence of fever and was discharged 3 weeks after admission.

The motile aeromonads represent a diverse group of potentially pathogenic organisms whose taxonomy has clearly been in a constant state of flux. An explanation of the mode of pathogenesis, particularly in relation to gastroenteritis, awaits either the development of a successful animal model or the completion of a successful human volunteer trial.

Nonetheless, the species of *A. hydrophila*, *A. veronii* (*A. veronii* biogroups veronii and sobria), *A. schubertii*, and even *A. caviae* have been delineated as distinct phenotypic species to be considered by clinical microbiologists. Some investigators have suggested in the literature an association between a particular taxonomic species and specific disease syndromes, i.e., the significance of *A. veronii* biogroup sobria in bacteremia, *A. schubertii* in traumatic wound infections, and *A. caviae* in cases of pediatric gastroenteritis (5, 12, 23).

In this report we described the phenotypic and genotypic distinction of a previously rare DNA group of aeromonads and proposed *A. jandaei* sp. nov. based on these nine strains. This new species appears to have important clinical significance and warrants identification by clinical microbiologists and further investigation by infectious disease researchers. Only by accurate and complete biotyping of clinical aeromonad isolates can we achieve an understanding of the ecology, epidemiology, and pathogenesis of these enigmatic aquatic microorganisms.

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Molecular Studies on the Aerolysin Gene of *Aeromonas* Species and Discovery of a Species-Specific Probe for *Aeromonas trota* Species Nova

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A large group of aeromonads and other enteric microorganisms were assayed for the presence of the aerolysin gene with use of DNA-DNA hybridization. Two DNA fragments corresponding to the regulatory region (*aer*C) and the structural gene (*aer*A) were used as probes for the detection of the aerolysin gene in these strains. Sequences corresponding to the aerolysin structural gene were widespread among *Aeromonas* isolates. In contrast, the *aer*C probe was much more selective, and sequences corresponding to the *aer*C region were detected in only a small subset of strains. Concurrent studies using numerical taxonomy and DNA hybridization with the *aer*C probe on a larger set of strains led to the identification of a distinct cluster of 14 presumed atypical *Aeromonas sobria* strains. These strains have recently been grouped into a new species designated *Aeromonas trota*. Hence, the DNA fragment *aer*C used in the study is a species-specific gene probe for *A. trota*. The ability of the *aer*C probe to detect strains belonging to a single species suggests that there is selection pressure to maintain the clonality of this species. These results have important implications with respect to the evolution of "pathogenic profiles" among these medically important bacteria.

Aeromonads are gram-negative bacteria that are ubiquitous, autochthonous residents of marine, estuarine, and fresh water environments [1, 2]. While *Aeromonas* organisms are clearly recognized as causative agents of wound and extraintestinal infections [3], there has been insufficient information, such as that obtained from a reliable animal model, studies of documented outbreaks, or successful trials with volunteers, that clearly establishes them as causative agents of enterointestinal disease. However, we have recently reported a well-documented single case of a laboratory worker infected with a distinct strain of *Aeromonas* [4] that represents the first case in proof of aeromonas etiology in intestinal disease.

There are currently 10 accepted or proposed phenospecies of *Aeromonas* [4–10] that are among at least 12 hybridization groups [11]. The often-contradictory information obtained from worldwide studies of *Aeromonas* organisms may be the result of inaccurate identification of most isolated aeromonads; this occurrence has led to confusion in the assignment of strains to established species and/or groups, thus making evaluation of pathogenesis and virulence rather inconclusive. Attempts to characterize the virulence features of *Aeromonas* organisms have likewise been somewhat ambiguous. While *Aeromonas hydrophila* and *Aeromonas veronii* biovar sobria are generally regarded as more virulent than *Aeromonas caviae* [3, 12], there have been no specific characteristics other than hemolysin activity and/or production of enterotoxin that seemingly account for this difference [13–18].

We had previously used marker exchange mutagenesis to derive mutants of *Aeromonas sobria* strain AH2 that were deficient in the aerolysin gene. With use of a mouse infection model, it was demonstrated that aerolysin is required for both the initiation and the maintenance of a septicemic infection [19]. The cloning and entire nucleotide sequence of the aerolysin gene from the clinical isolate of *A. sobria* strain AH2 (now classified as *Aeromonas trota*) has been reported [4, 20, 21]. Two regions (denoted *aer*C and *aer*B and located upstream and downstream, respectively, from the *aer*A gene) were found to effect the production of aerolysin. The *aer*C region was specifically shown to contain two divergent nonoverlapping promoters and elements required for the regulation of aerolysin production [21].

With the availability of these recombinants and the independent cloning and sequencing of the aerolysin gene from *A. hydrophila* strain Ah65 and *A. trota* strain AH2 [20, 22], we have begun to use different probes derived from strongly conserved and divergent regions between the two cloned sequences for examination of the prevalence of aerolysin in a large variety of *Aeromonas* isolates that originated from both clinical and environmental sources. In our first studies with strains originating from The Netherlands, we found that

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while the *aer*A gene was present in nearly all of the strains probed, none of the strains harbored sequences homologous to the *aer*C region [23].

This study was done initially with use of a well-defined set of strains that had been previously identified according to species, source of isolation, and virulence potential in a mouse infection model [12]. Our results showed that sequences corresponding to the structural gene *aer*A were restricted to the genus *Aeromonas* and were present in every aeromonad tested in this collection. Furthermore, we discovered two strains that hybridized with the *aer*C gene probe, which contains sequences involved in the regulation of aerolysin [21]. Subsequent analysis led to the discovery of additional strains that hybridized with this gene probe. All of these strains were found to cluster phenotypically and genotypically and were distinct from all other existing phenospecies and genospecies presently recognized and have been recently designated *A. trota* [4].

Materials and Methods

Bacterial strains. The strains used in this study (table 1) were stored at −70°C in trypticase soy broth with 10% glycerol (Remel, Lenexa, KS) and subsequently subcultured on trypticase soy agar plates (BD Microbiology Systems, Cockeysville, MD) at 36°C ± 1°C. Single colonies from these plates were restreaked on a second trypticase soy agar plate, incubated at 36°C ± 1°C for 18–24 hours, and used subsequently for analysis. These strains represented several species of *Aeromonas*, including the newly proposed species *Aeromonas jandaei* and *A. trota* [4, 7]. The diverse geographic distribution and sources of isolation of the strains are listed in table 1.

Phenotypic analysis. The strains were examined for numerous phenotypic characteristics, including morphological, biochemical, physiological, and tolerance traits, as previously described [24]. The phenotypic traits listed in table 2 represent a smaller subset of well-discriminating characteristics that differentiate *A. trota* from other closely related *Aeromonas* species.

Strains for probe analysis. The fourteen *A. trota* strains in addition to other *Aeromonas* strains described in table 1 comprised a group of 52 aeromonads, which were analyzed for the presence of aerolysin with use of the *aer*A and *aer*C probes.

Purification of DNA. Whole cell DNA was isolated by the method of Priefer [25]. The plasmid pHPC 3-1, from which the two probes were derived, has been previously described [20]. The DNA probes that were used are depicted in figure 1. The restriction fragments for the preparation of the probe were purified from vector sequences on an agarose gel as described previously [23].

Dot blot and Southern blot hybridization. Samples were prepared for dot blot hybridization as previously described [23]. We found this method to be superior to colony hybridization since many of the *Aeromonas* strains lysed poorly in situ on nylon strips. Chromosomal DNAs from a laboratory isolate of *Escherichia coli* strain 5K and *A. trota* strain AB3 served as negative and positive controls, respectively, for each filter (Gene Screen Plus, DuPont, Wilmington, DE).

For Southern blot hybridizations, 5 μg of the DNA was digested with the appropriate restriction endonuclease, electrophoresed on 0.7% agarose gels with use of TPE buffer, transferred to nylon sheets (Biodyne B, Pall) by the alkaline transfer method, and processed further with use of the manufacturer's protocol. After prehybridization (50 m*M* Tris, pH 7.5; 10 m*M* EDTA; 1 *M* NaCl; 10× Denhardt's; 0.1 mg of herring sperm DNA/mL; 0.1% sodium pyrophosphate; 1% SDS), the hybridization was performed at 60°C overnight. The filters were washed twice (0.2× standard saline citrate; 1% SDS; 0.1% sodium pyrophosphate) at 60°C for 30 minutes (high stringency conditions). The low stringency conditions were hybridization at 42°C overnight followed by two to three washes for 30 minutes at 42°C (2× standard saline citrate; 1% SDS; 0.1% sodium pyrophosphate). The wet filters were exposed to Fuji RX films (Fuji, Tokyo) for 12–48 hours with intensifying screens.

DNA probes were labeled with [^{32}P]ATP by the random priming technique of Feinberg and Vogelstein [26].

Results

Characteristics of the DNA probes. The DNA probes used for the detection of the aerolysin gene and the 5′ regulatory elements were isolated and purified as described under Materials and Methods. On the basis of the known nucleotide sequence from two independent aerolysin genes [21, 22] the following two gene probes were generated. The probe designated as 1 is a 394-base pair (bp) *Pst* I fragment covering the *aer*C region from *A. trota* strain AH2, and the homology between the two sequences of the aerolysin gene is only 46%; on the other hand, the probe designated as 2 is an internal 337-bp *Bam*HI-*Kpn* I fragment in a region where the homology between the two sequences is highest at 81%. The regions covered by the respective probes are depicted in figure 1. It is apparent from these data that aerolysin may be a relatively heterogenous family of cytotoxic hemolysins present in *Aeromonas* species.

Studies of dot blot hybridization with aerolysin-specific probes. For rapid determination of the presence of homologous sequences to the aerolysin gene in a collection of *Aeromonas* strains, we performed a dot blot hybridization assay using both DNA probes. In initial studies using a colony hybridization assay, we found that many strains of *Aeromonas* gave weak and diffuse signals when either gene probe was used [23]. This occurrence appeared to be due in part to a potent DNase that is produced by virtually all isolates of *Aeromonas*, regardless of species or source of isolation. The

Table 1. Characterization and DNA probe analysis of *Aeromonas* strains studied.

Strain	Phenospecies	Source of isolation	Geographic source	β-Hemolytic activity*	DNA probe used	
					aerC	*aerA*
AH2	*A. trota*	Feces	India	+	+	+
AB3[1]	*A. trota*	Feces	Not applicable	+	+	+
A7	*A. trota*	Feces	Bangladesh	+	+	+
A8	*A. trota*	Feces	Bangladesh	+	+	+
A9	*A. trota*	Feces	Bangladesh	+	+	+
A11	*A. trota*	Feces	Bangladesh	+	+	+
A16	*A. trota*	Feces	Bangladesh	+	+	+
AS66	*A. trota*	Feces	Thailand	+	+	+
AS370	*A. trota*	Appendix	California	+	+	+
NMRI 206	*A. trota*	Feces	Indonesia	+	+	+
NMRI 208	*A. trota*	Human diver	Virginia	+	+	+
5258	*A. trota*	Feces	Indonesia	+	+	+
5274	*A. trota*	Feces	Indonesia	+ (weak)	+	+
AMC5439	*A. trota*	Feces	Maryland	+	+	+
AS14 (ATCC 49569)	*A. jandaei*	Blood	New York	+ (weak)	−	+
NMRI 6 (ATCC 49571)	*A. jandaei*	Wound	Washington, D.C.	+	−	+
WRH/4658 (ATCC 49570)	*A. jandaei*	Wound	Washington, D.C.	+ (weak)	−	+
AS167 (CDC 1081-81)	*A. jandaei*	Prawn	Hawaii	+	−	+
AS171 (CDC 1531-81) (ATCC 49572)	*A. jandaei*	Feces	Georgia	+	−	+
AS206	*A. jandaei*	Feces	New York	+	−	+
AS235	*A. jandaei*	Blood	New York	+	−	+
Muldoon	*A. sobria*	Feces	Maryland	−	−	+
AS202	*A. veronii* biovar sobria	Rectal biopsy	New York	ND	−	+
NMRI 7	*A. hydrophila*	Leg wound	Washington, D.C.	+	−	+
NMRI 16	*A. hydrophila*	River water	Washington, D.C.	+	−	+
NMRI 56	*A. hydrophila*	River water	United States	+	−	+
NMRI 203	*A. hydrophila*	Feces	United States	+	−	+
NMRI 204	*A. hydrophila*	Feces	United States	+	−	+
I 206	*A. hydrophila*	Feces	The Sudan	+	−	+
AMC2052	*A. hydrophila*	Feces	Maryland	+	−	+
NMRI 2	*A. caviae*	River water	Washington, D.C.	+	−	+
NMRI 150	*A. caviae*	Foot wound	United States	−	−	+
NMRI 209	*A. caviae*	Feces	United States	+	−	+
NMRI 214	*A. veronii* biovar sobria	Sputum	United States	ND	−	+

NOTE. These type strains and DNA definition group strains were positive for *aer*A only. *A. hydrophila* strains ATCC 7966[T] (DNA group 1), CDC 9533-76 (DNA group 2), and CDC 0434-84 (DNA group 3); *Aeromonas salmonicida* strain ATCC 33658[T] (DNA group 3); *A. caviae* strains ATCC 15468[T] (DNA group 4), CDC 0862-83 (DNA group 5A), and CDC 0435-84 (DNA group 5B); *Aeromonas media* strain ATCC 33907[T] (DNA group 5B); *Aeromonas eucrenophila* strain NCMB 74[T] (CDC 0859-83) (DNA group 6); *A. sobria* strain CIP 7433[T] (DNA group 7); *A. veronii* biovar sobria strain CDC 0437-84 (DNA group 8X); *A. veronii* biovar sobria strain ATCC 9071 (CDC 9080-79) (DNA group 8Y); *A. jandaei* strain ATCC 49568 (CDC 0787-80) (DNA group 9); *A. veronii* biovar veronii ATCC 35624 (CDC 1169-83) (DNA group 10); *Aeromonas* species ornithine-positive CDC 1306-83 (DNA group 11); *Aeromonas schubertii* strain ATCC 43700[T] (DNA group 12); and *Aeromonas* group 501 ATCC 43946 (CDC 2478-85).

* Determined on trypticase soy agar with 5% horse erythrocytes. ND = not determined.

[1] Nalidixic acid–resistant mutant of *A. trota* strain AH2 [20].

results obtained with the first collection of strains isolated in the United States, Australia, and Indonesia are depicted in figure 1. While most of the *Aeromonas* strains that were tested hybridized strongly with the gene probe from within the structural gene, only two strains (NMRI 206 and NMRI 208) of the 40 tested showed the presence of homologous sequences to the *aer*C gene probe derived from *A. trota* strain AH2. We found that strains unrelated to the genus *Aeromonas* (including *E. coli*, *Shigella dysenteriae*, *Salmonella typhimurium*, *Yersinia* species, *Vibrio cholerae* serogroup O:1, *Vibrio fluvialis*, *Vibrio cholerae* non–serogroup O:1, *Bacillus* species, *Listeria monocytogenes*, and *Enterococcus faecalis*) did not hybridize with either probe under the conditions used, thus demonstrating the specificity of the probe for the genus *Aeromonas*. The data obtained suggested that while the structural gene for aerolysin is present in many *Aero-*

Table 2. Distinguishing characteristics of *A. trota* in comparison with other *Aeromonas* species.

Characteristic	*A. trota*	*A. hydrophila* (DNA group 1)	*A. caviae* (DNA group 4)	*A. jandaei* (DNA group 9)	*A. veronii* biovar sobria (DNA group 8)	*A. veronii* biovar veronii (DNA group 10)	*A. schubertii* (DNA group 12)
Hydrolyzes esculin	–*	+†	+	–	–	+	–
Produces acetylmethylcarbinol from glucose‡	–	+	–	+	+	+	V
Acid from:							
Cellobiose	+	–	V	–	–	+	–
Mannitol	+	+	+	+	+	+	–
Sucrose	–	+	+	–	+	+	–
Oxidates gluconate	–	+	–	+	+	ND	–
Susceptibility to:							
Ampicillin (10 μg)	S§	R‖	R	R	R	R	R
Cephalothin (30 μg)	S	R	R	R	S	S	S
Colistin# (4 μg/mL)	S	V	S	R	S	S	S
Carbenicillin (100 μg)	S	R	R	R	R	R	R
Produces gas from glucose	+	+	–	+	+	+	–
Produces ornithine decarboxylase	–	–	–	–	–	+	–

NOTE. All species were oxidase-positive, glucose-fermenting, gram-negative rods that were resistant to vibriostatic agent (0/129) (150 μg/mL), reduced nitrate, were motile at 36°C, and did not grow in the presence of 6.5% NaCl. Table adapted from [4]. V = variable; ND = not determined.

* Negative reaction after appropriate incubation period (i.e., postive ≤30%).

† Positive reaction after appropriate incubation period (i.e., positive ≥70%).

‡ Voges-Proskauer test.

§ Susceptible by Kirby-Bauer method.

‖ Resistant by Kirby-Bauer method.

\# S = susceptible to single MIC dilution of 4 μg/mL with use of the MicroScan gram-negative Combo Panel (Baxter Healthcare, MicroScan Division, West Sacramento, CA); R = resistant to single MIC dilution of 4 μg/mL.

monas strains, there is considerable diversity in sequences in the *aerC* region of the various strains.

Phenotypic characterization of strains hybridizing with the aerC *gene probe.* In concurrent systematic studies using numerical taxonomy, we discovered that all of the three *Aeromonas* strains detected in this study formed a cluster with six other strains (A7, A8, A9, A11, A16, and AMC5439) that was distinct from those of all other major phenospecies. These presumed atypical *A. veronii* biovar sobria strains were distinguishable from other phenetic clusters based on the reactions for esculin hydrolysis, Voges-Proskauer, sucrose fermentation, and susceptibility to ampicillin [4].

Further screening of *Aeromonas* strains with use of both dot blot hybridization and numerical taxonomy led to the discovery of four similar, atypical *A. veronii* biovar sobria strains (5258, 5274, AS66, and AS370) and three further identifying characteristics: fermentation of cellobiose, inability to oxidize gluconate, and susceptibility to carbenicillin (table 2). Definitive identification of this cluster was accomplished by analysis of total chromosomal DNA against all known definitive strains with use of DNA-DNA hybridization. The proposed name of this group is *A. trota* species nova [4].

Analysis of Aeromonas *strains by means of Southern blot hybridization with the* aerC *and* aerA *probes.* Since several of the strains identified as *A. trota* had been classified only on the basis of numerical taxonomic studies, we wondered how efficacious the *aerC* gene probe might be in the detection of bona fide *A. trota* strains. In addition, we also examined the reference strains comprising all known genospecies for the presence of homologous sequences to *aerC*. Chromosomal DNA from all of the *A. trota* strains was isolated, digested with the restriction endonuclease *Sst* I, subjected to agarose gel electrophoresis, blotted onto nylon-based filters, and probed for homologous sequences to the *aerC* gene probe. For every single strain of *A. trota* tested, a 4-kilobase (kb) hybridizing fragment was observed; for strains 5274 and AMC5439, however, hybridization of the *aerC* gene probe with 5.1-kb and 5.9-kb *Sst* I fragments, respectively, was observed (figure 2). In contrast, no hybridization with chromosomal DNA was observed for any of the other reference strains of non-*trota* genospecies of *Aeromonas* (data not shown). Hence, the *aerC* fragment used in this study is a species-specific probe for *A. trota*.

We have previously commented upon the diverse patterns of restriction fragments seen when hybridization with probes encompassing different regions of the aerolysin gene was performed [23]. In another series of experiments, we used the *aerA* gene probe to observe the sizes of the hybridizing fragments in *Sst* I digests of chromosomal DNA from the different above-mentioned *Aeromonas* strains. A 1.2-kb *Sst* I fragment was found to hybridize with the probe in all strains except 5274, A11, NMRI 206, and AMC5439, which showed 5.1-kb, 1.0-kb, 1.5-kb, and 5.9-kb hybridizing frag-

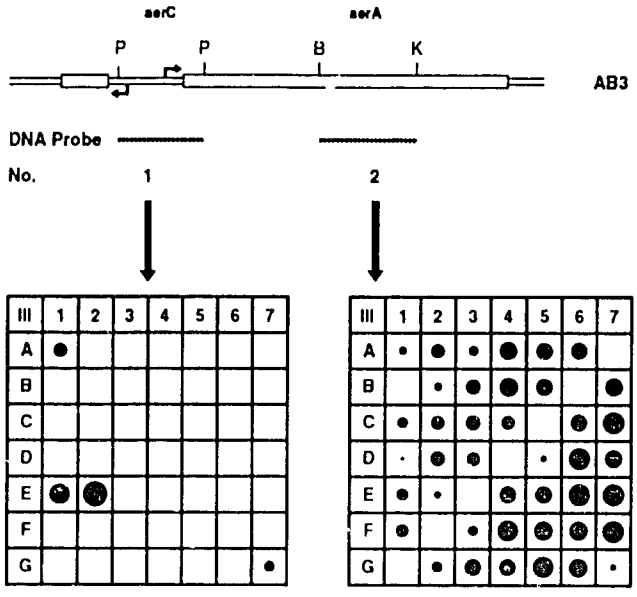

Figure 1. *Top*, overview of the region containing the aerolysin gene (*aer*A) and its regulatory region (*aer*C); the arrows represent the respective promoters [21]. The selected probes are shown and designated as probe 1 and 2. DNA was derived from strains isolated in the United States, Australia, and Indonesia. P = *Pst* I, B = *Bam*HI; and K = *Kpn* I. *Bottom*, Autoradiographs of radiolabeled DNA probes hybridized with undigested genomic DNAs from different *Aeromonas* strains that were spotted onto nylon filters. Positive controls: *A. trota* strain AB3 in spots A1 and G7; negative controls, *E. coli* strain 5K in spots A7, B6, C5, D4, E3, F2, and G1. The strain in spot B1 was a weakly reacting (±) *A. sobria*. NMRI 206 and NMRI 208, in spots E1 and E2, respectively, were later identified as *A. trota* after this initial screening.

ments, respectively (figure 3). In contrast, hybridization with the *aer*A gene probe of *A. trota* strain AB3 was observed only under less stringent conditions of hybridization (see Materials and Methods) for the chromosomal DNA from the other reference strains of *Aeromonas*. Diverse restriction fragment length polymorphisms were observed for these strains; the fragment lengths of DNA ranged from 0.7 to 15 kb (figure 4). These data provide further evidence for the genetic homogeneity of the species now defined as *A. trota*. The cumulative data with respect to hybridization with both probes, hemolytic activity, and source of isolation of all strains studied here are presented in table 1.

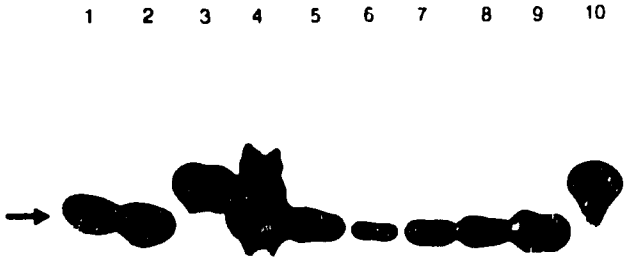

Figure 2. Southern blot hybridization profile of genomic DNAs from *A. trota* strains with use of the *aer*C probe. Lanes: 1, AB3; 2, AH2; 3, 5274; 4, A7; 5, A9; 6, A11; 7, A16; 8, NMRI 208; 9, NMRI 206; and 10, AMC 5439. The arrow indicates the 4-kb *Sst* I DNA fragment of strain AB3, from which the probe was derived.

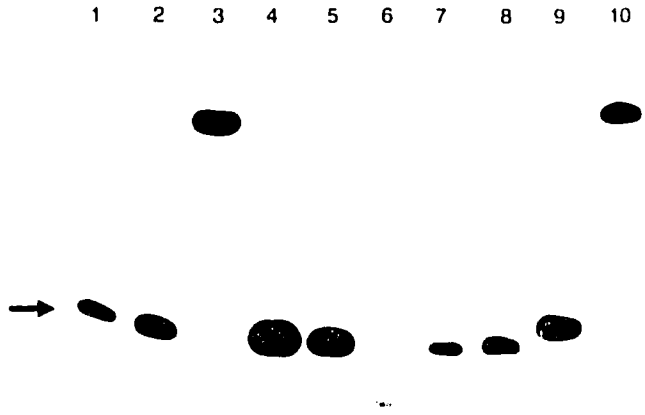

Figure 3. Southern blot hybridization profile of genomic DNAs from *A. trota* strains with use of the *aer*A probe (see figure 2 for identification of lanes). The arrow indicates the 1.2-kb DNA fragment of strain AB3.

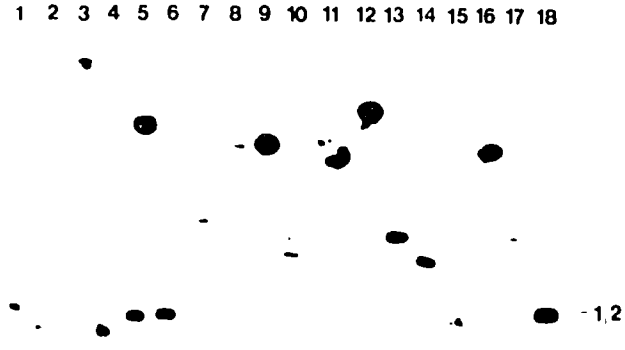

Figure 4. Southern blot hybridization profile of the *aer*A probe with *Sst* I-digested genomic DNA of other *Aeromonas* genospecies. Lanes: 1, *A. hydrophila* strain CDC 9079-79 (DNA group 1); 2, *A. hydrophila* strain CDC 9533-76 (DNA group 2); 3, *A. hydrophila* strain CDC 0434-84 (DNA group 3); 4, *A. salmonicida* strain ATCC 33658 (DNA group 3); 5, *A. caviae* strain CDC 9083-79 (DNA group 4); 6, *A. caviae* strain CDC 0862-83 (DNA group 5A); 7, *A. caviae* strain CDC 0435-84 (DNA group 5B); 8, *A. media* strain CDC 9072-83 (DNA group 5B); 9, *A. eucrenophila* strain CDC 0859-83 (DNA group 6); 10, *A. sobria* strain CDC 9538-76 (DNA group 7); 11, *A. veronii* biovar sobria strain CDC 0437-84 (DNA group 8); 12, *A. veronii* biovar sobria strain CDC 9080-79 (DNA group 8Y); 13, *A. jandaei* strain CDC 0787-80 (DNA group 9); 14, *A. veronii* biovar veronii strain CDC 1169-83 (DNA group 10); 15, *Aeromonas* enteric group 77 CDC 1306-83 (group 11); 16, *A. schubertii* strain CDC 2446-81 (DNA group 12); 17, *A. jandaei* strain CDC 1530-81; and 18, *A. trota* strain AH2 ATCC 49657[T]. The size (in kb) of the restriction fragment of *A. trota* strain AH2 is indicated.

Discussion

The main finding of this study is that all strains hybridizing with the *aerC* probe of *A. sobria* (*A. trota*) strain AH2 cluster biochemically and form a distinct phenetic group that we initially designated as atypical *A. sobria* strains. This cluster was confirmed by whole cell DNA-DNA hybridization, which established these organisms as a distinct species for which the name *A. trota* species nova is now proposed [4]. A summary of the distinguishing features of this new species is listed in table 2. The correlation between phenotype and hybridization with the *aerC* probe was unexpected and suggests that some kind of selection pressure operates to maintain the clonal nature of this species.

Strains belonging to *A. trota* species nova have primarily been isolated thus far from southern and Southeast Asia and have been associated predominately with cases of gastrointestinal disorders (table 1). A comparison of published sequences of two aerolysin genes cloned from *A. trota* strain AB3 (table 1) and *A. hydrophila* strain Ah65 showed that these genes have diverged widely. Indeed, the overall homology of 77% of the nucleotide sequence of the *aerA* gene is lower than the homology of 79% observed, for example, between the sequences of cholera toxin gene (*ctxB*) and the heat-labile toxin gene (*eltB*) of enterotoxigenic *E. coli* [27]. Within the aerolysin gene, sequences encoding the N terminal half of the molecule are less well conserved than those encoding the C terminus (73% vs. 81%). The major differences are confined to the 5′ control region of the aerolysin structural gene. This region showed a homology of only 46% despite conservation of the A+T richness and of the approximately similar length between both sequences. Hence, differing levels of hemolytic activity seen with wild-type isolates of *Aeromonas* may simply reflect differences in expression of the gene.

Southern blot hybridization of chromosomal DNA isolated from *A. trota* with use of the *aerC* probe revealed a very homogenous pattern of hybridizing fragments, regardless of the restriction endonuclease used (figure 2) (authors' unpublished results). There was no correlation between the different hybridizing fragments seen and the hemolytic phenotype for the various *A. trota* strains except strain 5274, which exhibits a weak hemolytic phenotype. Further analysis with Southern blot hybridization using *Sau*3A-digested genomic DNA has indicated alterations in the *aerC* and *aerA* regions of this strain, an occurrence that may be responsible for the weaker hemolytic phenotype (authors' unpublished results).

The extreme specificity of the *aerC* probe for *A. trota* strains was unexpected. The restricted occurrence of homologous regions to *aerC* is reminiscent of recent studies on the genome of several strains of *Pseudomonas aeruginosa* in which insertion elements have contributed to the organization of the upstream regulatory region of the exotoxin A gene [28]. No hybridization with the *aerC* probe was observed with the DNA hybridization group 7 strain (*A. sobria* CIP 7433), the *Aeromonas* group 501 strain ATCC 43946, or DNA group 9 strains (*A. jandaei*). It is of interest that these groups of strains are taxonomically close to *A. trota* and that similarities in the *aerC* region between these strains may have reflected common ecological habitats. Inability to ferment sucrose is a marker for the species *A. trota*, and the ability of strains NMRI 206, 5274, and 5278 to utilize this substrate suggests that these strains may be bridge organisms to the other sucrose-positive organisms, such as *A. hydrophila*, *A. veronii* (biovars sobria and veronii), and *A. caviae*.

The *aerA* probe was found to be specific to the genus *Aeromonas* and showed hybridization with all *Aeromonas* isolates that we tested. An interesting observation was the hybridization of this probe with isolates belonging to the species *A. caviae*, which are supposedly nonhemolytic. This observation confirms and extends data that we have previously obtained with the same probe using a collection of 97 strains comprising several species from The Netherlands [23]. For many of those *A. caviae* strains as well as for two of the three *A. caviae* strains in this study, clear zones of hemolysis on sheep or horse blood agar are noted. This occurrence implies that the species *A. caviae* may be more heterogeneous than has previously been suspected. This result, combined with the finding that *A. caviae* is associated with clinical disease, suggests that hemolysin should be further investigated as a potential virulence feature of this organism [17, 29].

In some cases (strains Muldoon, L206, and AMC2052E) variable expression of hemolytic activity, depending on the type of erythrocyte used in the assay, was observed. All of these strains harbor the aerolysin gene; therefore, this variability in hemolytic activity is likely to be due to differing receptor affinities of the aerolysin molecule for the respective erythrocyte type. For instance, Brenden and Janda [30] have tested aerolysins from different strains of *Aeromonas* that exhibited widely different spectra of hemolytic activity. The divergence of sequences encoding the aerolysin may be a heterogenous family of cytolytic hemolysins present in the genus *Aeromonas*. Further support for this hypothesis comes from the observation that hyperimmune rabbit antisera to purified aerolysin from *A. trota* strain AB3 failed to cross-react with aerolysin produced by some hemolytic strains harboring the *aerA* gene (data not shown).

The pattern of hybridization of the *aerA* gene probe with chromosomal DNA digested with a particular restriction endonuclease revealed unique hybridization patterns for strains belonging to the other genospecies, while *A. trota* strains were extremely homogeneous in the hybridizing fragments. Additionally, homologous sequences to *aerA* in the other reference strains were detected only under lowered conditions of hybridization stringency (figure 4), thereby suggesting divergence of the nucleotide sequences encoding this gene in the various genospecies. These results have important implications with respect to the possibility of distinct

toxins in different *Aeromonas* strains as virulence factors for this genus. Hence, several cytotoxic enterotoxin cross-reactive (CTC) toxins may in fact be aerolysins that have strongly diverged, thus revealing either lack of antigenic cross-reactivity or differences with respect to primary sequence. The molecular weights (49–50 kD) and isoelectric points (range, 4.5–5.5) of the purified "Asao-toxin" and CTC cytolysin are similar to those of aerolysin, and like aerolysin they are also capable of inducing fluid accumulation in ligated rabbit ileal loops. Strong evidence for this hypothesis comes from the finding that the sequence of the first 25 amino acids of the CTC cytolysin is identical to that of the aerolysin produced by *A. hydrophila* strain Ah65 [14, 22]. Thus, although the primary sequences of aerolysin produced by different *Aeromonas* species are considerably different, the three properties of hemolysis, cytotoxicity, and enterotoxicity have been conserved.

The presence of the aerolysin gene in virtually all *Aeromonas* species suggests that the hemolytic/cytolytic activity is a primitive trait in the genus *Aeromonas* and has been retained in the course of evolutionary events that generated genomic diversity of other characteristics. Alternatively, this widespread distribution might be explained by multiple episodes of horizontal transfer and recombination in many toxin-deficient strains. However, there is no evidence to date that suggests that the gene can be transferred by bacteriophage lysogenic conversion or plasmid- or transposon-mediated processes. The detection of allelic variant strains differing in nucleotide and amino acid sequences suggests that the *aer*A gene is evolutionally old.

In conclusion, the data presented herein describe the construction of a gene probe for strains of *Aeromonas* species and a species-specific probe for *A. trota* species nova. Our results point to a wide distribution of the aerolysin gene within the genus *Aeromonas*. In addition, divergence in the primary sequence of both published sequences and the restriction fragment length polymorphism within the aerolysin gene determinant of the definition strains suggest that a family of aerolysin genes may exist in this genus that is similar to that observed for Shiga toxin and the various classes of Shiga-like toxins [31] as well as those for cholera toxin [32, 33]. Our results suggest that a judicious choice of oligonucleotide probes coupled together with in vitro DNA amplification techniques may be a rapid way of revealing the scope of diversity of this important virulence factor in this genus.

We conclude that the aerolysin gene is unique to the genus *Aeromonas* and is present in all known phenospecies. In addition, the *aer*C gene appears to be conserved in and restricted to a small subset of *Aeromonas* strains, *A. trota*, most of which are associated with gastrointestinal disorders.

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Elastolytic Activity Among *Aeromonas* spp. Using a Modified Bilayer Plate Assay

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A total of 166 isolates of Aeromonas, *representing diverse geographical regions and originating from various sources, were evaluated for the ability to produce elastase by using a bilayer elastin agar medium (BEAM) plate assay. The degree of elastase activity of individual strains was roughly assessed by measuring the clear area beneath or peripheral to the colony and recorded as 1+, 2+, or 3+. Of the 166 aeromonads tested, 53 (32%) were found to produce elastase, of which 26 (49%) were 3+, 21 (40%) were 2+, and 6 (11%) were 1+. All but one* A. hydrophila *(n = 45) were observed to produce elastase (98%). One of three* A. schubertii *strains as well as one isolate of* Aeromonas *group 501 were elastase positive. All 3+ elastolytic activity was associated with* A. hydrophila *only. Elastase activity was not detected even after prolonged incubation with* A. veronii *biogroup* sobria *(n = 26),* A. caviae *(n = 57),* A. veronii *biogroup* veronii *(n = 4),* A. media *(n = 1), and* A. eucrenophila *(n = 1). In addition to its value as a reliable indicator of elastase production for eventual use in virulence assays, we have found that the detection of elastase using the BEAM plate serves as a very useful phenotypic marker for the major, clinically important* Aeromonas *spp.*

INTRODUCTION

Aeromonas species are ubiquitous, waterborne microorganisms that have been implicated repeatedly as the causative agents of clinical illness, often serious, ranging from gastrointestinal and wound infections to septicemia (Janda and Duffey, 1988; Joseph et al., 1979; von Graevenitz and Mensch, 1968). In compromised patients, this illness can be fatal (Daily et al., 1981).

A number of extracellular factors associated with bacterial virulence, such as hemolysins, proteases, lecithinases, nucleases, and amylases, are produced by *Aeromonas*, but their roles in the pathogenesis of aeromonad infections require further assessment. Recent studies involving a number of pathogenic microorganisms have indicated that elastase activity (the ability to degrade the native elastin molecule) may be an important virulence factor in relation to disease-producing potential (Hsu et al., 1981; Kothary et al., 1984; Janda, 1986; Morihara and Tsuzuki, 1977; Williams et al., 1988). Elastin is a major structural protein of the lung and comprises a significant proportion of other tissues, such as blood vessel endothelium (arteries) and periodontal ligament.

Clinical studies indicate that primary and secondary wound infections are second only to *Aeromonas*-associated gastroenteritis as the most prevalent form of aeromonad disease (Janda and Duffey, 1988). Most aeromonad infections appear to be the result of colonization due to contact with polluted water following traumatic injury. Once established, aeromonad infection can spread throughout and penetrate deeper into the wound site, causing an increase in severity. Elastase from aeromonads, similar to other bacteria and fungi (Kothary et al., 1984; Morihara and Tsu-

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zuki, 1977), may play a part in the severity of the infection as one of the factors that can contribute to the virulence of the microorganisms. Hsu et al. (1981) found that elastase positive strains of *A. hydrophila* produced lesions and mortality when injected into channel catfish and concluded that motile aeromonads with positive elastase activity were usually highly virulent strains.

The determination of the role played by elastase in any infection requires that the elastase be obtained in a purified state so that its activity can be ascertained without interference from other biochemically active substances. Before attempting purification, the optimal conditions for detecting elastase activity in a plate assay, as well as the optimal conditions for the in vitro production of elastase, should be determined. This report describes the development of a sensitive, semiquantitative bilayer plate assay for elastase production.

MATERIALS AND METHODS

Bacterial Strains

Of the 166 isolates, initially received as *Aeromonas* spp., used in this study, 150 were collected from several countries between 1985 and 1989 and originated from a wide variety of sources (Carnahan et al., 1990). Included were 14 CDC (Centers for Disease Control) definition strains for the DNA hybridization groups 1, 2, 3, 4, 5a, 5b, 6, 7, 8X, 9, 10, 11, 12, and *Aeromonas* group 501 and two ATCC reference strains, *A. media* (ATCC 33907) and *A. sobria* (ATCC 9071) [now considered *A. veronii* biogroup *sobria* (Joseph et al., 1991)]. Also, *Pseudomonas aeruginosa* strains PA 01 and PA 103, both from human clinical cases, were used as positive and negative controls, respectively. All strains were stored at −70°C in tryptic soy broth with 10% glycerol (Difco Laboratories, Detroit, MI) and subcultured to tryptic soy agar plates (Difco) followed by overnight incubation at 36° ± 1°C prior to inoculation to elastin agar medium.

Species Identification

The strains were biotyped to the species level by a numerical taxonomy cluster analysis program, SAS/TAXAN (SAS Institute, Cary, NC) (Carnahan et al., 1990) based on 50 characteristics representing biochemical, morphologic, physiologic, and antimicrobial resistance activity. The typical aeromonads (n = 154) consisted of 46 *A. hydrophila*, 26 *A. veronii* biogroup *sobria* [previously designated *A. sobria* (Joseph et al., 1991)], 57 *A. caviae*, one *A. media*, four *A. veronii* biogroup *veronii* [previously designated *A. veronii* (Joseph et al., 1991)], three *A. schubertii*, one *A. eucrenophila*, four *A. jandaei* (Carnahan et al., 1991a), nine *A. trota* (Carnahan et al., 1991b), one *Aeromonas* group 501, and one *Aeromonas* species, enteric group 77 (ornithine +). *Aeromonas sobria* (CIP 7433) is listed separately in Table 1 because it is not entirely representative of *A. veronii* biogroup *sobria* as described previously (Joseph et al., 1991). Atypical strains were considered as such in that at least two or more tests were inconsistent when compared with the typical strains. There were five atypical *A. hydrophila*-like strains, three atypical *A. caviae*-like, and three atypical *A. veronii* biogroup *sobria*-like (Table 1).

Media for Elastase Production

The ability of selected strains such as *P. aeruginosa* (PA 01 and PA 103), *A. hydrophila* (ATCC 7966^T, NMRI 5, and NMRI 7), *A veronii* biogroup *sobria* (ATCC 9071), and *A. caviae* (ATCC 15468^T) to produce elastase in a variety of different media was investigated initially. The commercially available preparation of porcine pancreatic elastase (type 1, Sigma, St. Louis, MO) was also used as a positive control.

Brain–heart infusion agar (BHIA; Difco) and Columbia agar base (CAB; Oxoid LTD, Basingstoke, England) containing 2.3% peptone had previously been suggested as suitable media for elastase production (Janda, 1986; Williams et al., 1988); therefore, these two media were examined as basal media incorporated with particulate bovine neck ligament elastin (Sigma).

Effect of Elastin Particle Size and Concentration

The effect of elastin particle size on zones of clearance in the agar was ascertained by comparing plates formulated with powdered (ground) elastin particles with those containing larger elastin particles as received by supplementing the media with either 1% (wt/vol) or 0.3% (wt/vol) elastin. Grinding of elastin (Sigma) into powdered form was performed conventionally by using mortar and pestle. After grinding, the required amount (such as 0.3 g/100 ml) of ground elastin was added to the agar base medium.

Inoculation and Incubation

Each of the seven strains and a purified elastase control were spot-inoculated (four to a plate) to either BHIA, CAB containing 2.3% peptone, or CAB with additional 2.7% peptone (total 5% peptone) that con-

TABLE 1 Elastase Activity of 166 Isolates of *Aeromonas spp.* Using the Bilayer Elastin Agar Medium (BEAM) Plate[a]

Organism	No. Isolates Tested	No. Elastase Positive (%)	Degree of Elastase Activity[b]		
			+ + + (%)	+ + (%)	+ (%)
A. hydrophila	46	45(98)	26(58)	17(38)	2(4)
A. veronii biogroup *sobria*	26	0(0)			
A. sobria (CIP 7433[T])	1	1(100)		1(100)	
A. caviae	57	0(0)			
A. media	1	0(0)			
A. veronii biogroup *veronii*	4	0(0)			
A. schubertii	3	1(33)			1(100)
A. eucrenophila	1	0(0)			
A. jandaei	4	0(0)			
A. trota	9	0(0)			
A. group 501	1	1(100)			1(100)
A. species, enteric group 77 (ornithine +)	1	0(0)			
Atypical,[c] *A. hydrophila*-like	5	4(80)		3(75)	1(25)
Atypical, *A. veronii* biogroup *sobria* like	4	0(0)			
Atypical, *A. caviae*-like	3	1(33)			1(100)
Total	166	53(32)	26(49)	21(40)	6(11)

[a]Performed in duplicate. No significant variation was observed from run to run.
[b]+ = zone of clearing directly beneath the colony; + + = clearing 2–5 mm beyond the edge of the colony; and + + + = clearing more than 5 mm beyond the edge of the colony.
[c]When at least two or more tests were inconsistent in comparison with the typical strains.

tained either 1% (wt/vol) or 0.3% (wt/vol) elastin. They were then incubated under several sets of conditions: either 4 days at 36° ± 1°C, 4 days at room temperature, or for 2 days at 36° ± 1°C followed by 2 days at room temperature.

Then, 166 *Aeromonas* strains and *P. aeruginosa* elastase-positive (PA 01) as well as elastase-negative (PA 103) control strains were inoculated and incubated in the latter manner, that is, 2 days at 36°C followed by an additional 2 days at room temperature using the bilayer elastin agar medium (BEAM) described below. Each of the 166 strains and the control strains was tested at least twice on the BEAM plate in the same fashion.

Development of the BEAM Plate Assay

A comparison was made between the previous method of using a basal agar layer of dispersed elastin particles throughout (Janda and Duffey, 1988; von Graevenitz and Mensch, 1968) with a bilayer agar medium in which elastin particles were incorporated in the top layer only. This bilayer was prepared by first pouring a 10-ml layer of sterile nutrient agar, for example, CAB, into a sterile 15 × 100-mm Petri plate (VWR Scientific, San Francisco, CA) and allowing this layer to harden. This solidified layer was then overlaid with a sterile, 10-ml top layer of the same basal medium incorporated with ground elastin (0.3% wt/vol) particles. This medium must be continuously shaken while pouring the top layer to obtain a homogeneous suspension. The bilayer plates were generally maintained at room temperature overnight before being used or before storage at 4°C.

Detection of Elastase Production

Each inoculated BEAM plate was visually examined daily (24-hr intervals) for evidence of positive elas-

tase activity identified as a clearing of the elastin particles either beneath the colony or peripherally extending from the colony. The degree of elastase activity was measured and scored as follows: clearing of the agar directly beneath the colony only as compared to no clearing in the negative control was 1+, a zone of clearance around the colony that measured 2–5 mm was considered 2+, and a zone of clearance >5 mm was scored as 3+.

RESULTS

Effect of Elastin Particle Size on Elastase Production

Control plates containing ground elastin particles were observed to influence more rapid elastase activity (generally 1 to 2-mm larger zone) than plates in which the elastin was not ground.

Effect of Culture Medium on Elastase Production

Of the three basal media compared (BHIA, CAB containing 2.3% peptone, and CAB with additional 2.7% peptone (total 5% peptone), supplemented with either 1% (wt/vol) or 0.3% (wt/vol) ground elastin, the CAB containing 2.3% peptone incorporating 0.3% elastin was found to permit larger zones of clearing, which appeared more rapidly than other media.

Effect on Incubation Time and Temperature

The incubation of plates at 36° ± 1°C for 2 days followed by 2 days incubation at room temperature produced larger zones of elastase activity and clearing occurred more rapidly, using this set of incubation conditions.

Comparison of the Bilayer Plate (BEAM) with the Monolayer Plate

When comparing the monolayer CAB with 0.3% ground elastin to the BEAM with 0.3% ground elastin in the top layer of 10 ml of CAB, the BEAM plate exhibited greater elastolytic activity. Control strain PA 01 and test strain NMRI 5 often showed positive activity after only 24 hr of incubation on BEAM plates.

Elastase Activity Related to Species

Of the 166 aeromonads screened in duplicate, 53 (32%) were observed to produce elastase activity. Of these 53 positive strains, 26 (49%) were considered 3+, 21 (40%) were 2+, and only six (11%) were 1+. All but one *A. hydrophila* (98%) and the single *Aeromonas* group 501 were positive. The only species or group that produced 3+ elastolytic activity was *A. hydrophila*. Only one of three *A. schubertii* strains was positive (1+). Elastase activity was not detected in any of the isolates of *A. veronii* (biogroups *sobria* and *veronii*), *A. caviae*, *A. jandaei*, and *A. trota* even after a prolonged incubation of 7 days, except for *A. sobria* (CIP 7433). The *A. media* and *A. eucrenophila*-type strains were also negative. The elastase activity was found to be completely reproducible, and no significant variation was observed from run to run in terms of degree of elastase activity of aeromonads on the BEAM plate (Table 1).

Distribution of Elastase-Positive Strains

The 53 elastase-positive strains were recovered from a variety of sources: clinical (n = 37), veterinary (n = 5), environmental (n = 7), and ATCC/CDC (n = 4). They were likewise diversely distributed as to their geographic source of isolation: USA (n = 35), Bangladesh (n = 4), The Sudan (n = 2), Somalia (n = 3), Egypt (n = 4), Puerto Rico (n = 1), and ATCC/CDC (n = 4). The single elastase-negative *A. hydrophila* strain was a diarrheal isolate from Dhaka, Bangladesh. No association has been observed between the elastase-positive aeromonads with their sources and geographic distribution. Elastase reactions for the control strains (PA 01 and PA 103) were also consistent throughout the experiment.

DISCUSSION

Recent attention has focused on the elastolytic activity of bacteria, because of its potential diagnostic and pathogenic significance. Most studies of elastolytic activity have used insoluble, particulate elastin dispersed in a nutrient agar medium with dissolution of elastin particles as a substrate for indication of positive elastase activity. Cultures on such plates usually required prolonged incubation (7–10 days) before dissolution of particles beneath or peripheral to colony growth could be detected (Janda, 1985 and 1986; Williams et al., 1988).

To provide a more efficient assay, we have developed the BEAM by grinding the elastin particles into powder form and incorporating them only in the top layer of our bilayer medium. We used CAB containing 2.3% peptone as our base layer. When compared with BHIA having only 1% peptone, CAB appeared to produce faster and stronger enzymatic activity. This suggests that production of elastase in aeromonads may be media dependent in similar fashion to staphylococci (Janda, 1986). However, use of CAB, with 2.7% peptone added, resulted in a final concentration of 5%, actually causing smaller zones

of clearance. This indicates that there may be an upper limit to the amount of peptone that can be utilized by the organism.

Ground elastin in the top layer could be digested more rapidly, through closer contact between the elastin substrate and the bacterial elastase. Thus, by grinding the elastin before incorporation into the top layer, a larger surface area for bacterial contact was created. This was evidenced by the fact that all of the 3+ elastase-positive strains were recorded as at least 1+ after only 24 hr of incubation. Thus the previous incubation period of 7 days was reduced by 43% to a maximum of 4 days required to record any and all 3+, 2+, and 1+ reactions on the BEAM plate.

Of the 166 *Aeromonas* spp. tested, only *A. hydrophila* (45 of 46) was almost exclusively positive (98%), confirming previously published reports (Janda, 1985; Lee, 1987; Ljungh and Wadstrom, 1985; Popoff, 1984; Renaud et al., 1988). Further, the level of activity was scored as strongly positive or 3+. This leads to the suggestion that wound infections with *A. hydrophila* may be more severe. However, Trust (1986) has emphasized that the contribution of a single enzyme or toxin to bacterial virulence must be confirmed by studies using bacterial isogenic mutants, deficient in the specific exoproducts.

Positive elastase activity was not detected in any of the 26 *A. veronii* biogroup *sobria* isolates, which also confirms previous studies (Popoff, 1984; Renaud et al., 1988), but is contradictory to those that detected some positive elastase activity among this species (Daily et al., 1981; Janda, 1985). It is interesting to note that the single *A. sobria* was the so-called type strain (CIP7433), which is not representative of clinical *A. veronii* biogroup *sobria* thus far isolated (Lee 1987; Joseph et al., 1991). All 57 typical *A. caviae* isolates were negative for elastase activity, also as previously observed (Janda, 1985; Ljungh and Wadstrom, 1985; Popoff, 1984; Renaud et al., 1988).

Five atypical isolates were elastase positive, of which four of five were *A. hydrophila*-like, with the remaining isolate identified as an *A. caviae*-like aeromonad.

We have found the BEAM described herein to be a sensitive, semiquantitative test to measure elastase production among the aeromonads. It is a simple medium to prepare, set up, and read with a shorter incubation period than previously described and could easily be incorporated into routine identification procedures for screening isolates for elastase production.

The development of this reliable and more rapid assay for use in studying elastase as a virulence feature has provided a second important finding, that is, elastase activity can serve as a reliable phenotypic marker for the species identification of motile aeromonads, specifically *A. hydrophila*, *A. veronii* biogroups *sobria* and *veronii*, *A. caviae*, *A. jandaei*, and *A. trota*. The role of elastase as a potential virulence feature or as a reliable phenotypic marker for the more recently described species associated with human disease, *A. veronii* biogroup *veronii*, *A. schubertii*, *A. jandaei*, *A. trota*, and *Aeromonas* group 501, and the nonclinical species, *A. media* and *A. eucrenophila*, warrants further investigation with larger numbers of these particular species.

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I. Taxonomy, ecology, isolation and identification

Aeromonas taxonomy

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Key words. Aeromonas; taxonomy.

The taxonomy of the aeromonads has been the subject of scrutiny and reclassification by several investigators in an attempt to clarify the position of this group of organisms and their relationship to similarly described but obviously separate genera. Recommendations have varied from recognition of one genus comprising three species to the proposal of a complex subspeciation[3,10]. Other than the obvious need for accurate nomenclature and classification, there are pragmatic reasons to seek improvement in the classification of *Aeromonas*, e.g., in clinical medicine, where physicians tend to refer to all aeromonads as *Aeromonas hydrophila* and, in fact, most of the early medical literature concerning *Aeromonas* used this nomenclature only. We now know such simplification to be unrealistic in light of Popoff and Véron's contributions[4], which showed that *A. hydrophila* and *A. punctata* constitute a single species, *A. hydrophila*. A second, previously undescribed group of strains was also described and named *A. sobria*. The human medical significance of *A. sobria* was demonstrated when it was isolated from a scuba diver's wound infection[5], and in a study of the comparative occurrence and virulence of *A. hydrophila* and *A. sobria* from human and environmental sources[2].

The species on which there is agreement is *A. salmonicida*. It differs from other aeromonads as follows: non-motile, no growth at 37°C, produces a brown, water-soluble pigment on nutrient agar. In Bergey's Manual of Systematic Microbiology[7] this species is subspeciated into *salmonicida*, *achromogenes*, and *masoucida*.

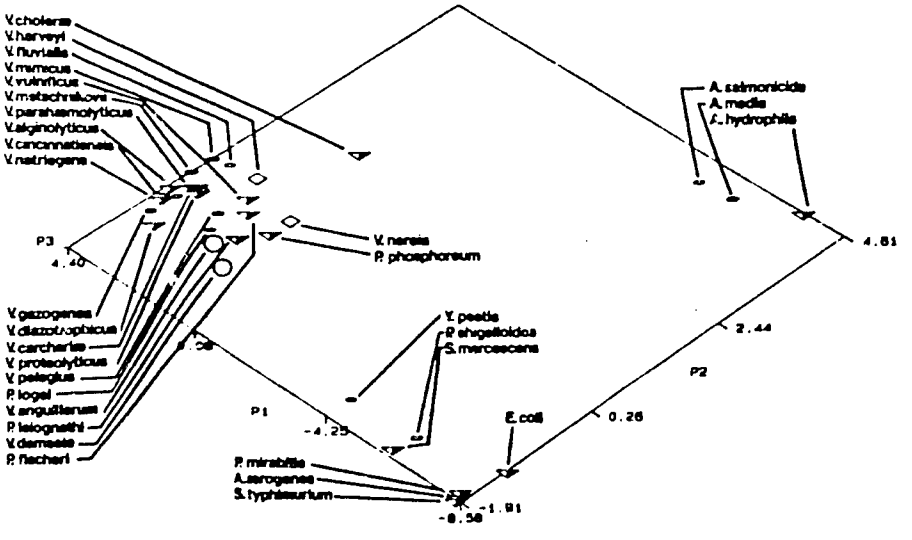
Based upon differential, phenotypic, and genetic characteristics, Popoff and Véron recommended establishment of a third species, *A. caviae*, which is included amongst the motile aeromonads[4]. In summary, five species and three sub-species of *Aeromonas* are recognized at the time of this writing.

Definition

Aeromonas consists of straight cells, rod-shaped and with rounded ends to coccoid. Resting stages are not known. Generally motile by a single flagellum. Some species are non-motile. Metabolism of glucose is both respiratory and fermentative. Oxidase positive and resistant to the vibriostatic agent 2,4,diamino-6,7-diisopropylpteridine (0/129). The mol% G + C of the DNA is 57–63 (Bd,T_m).

Minimal identifying and differential characters

The common characteristics which define the motile aeromonads are possession of motility, morphology, growth in nutrient broth at 37°C, indole production, fermentation of sucrose and



Overhead view of a four-dimensional plot of evolutionary relationships amongst species of the gamma subdivision of the Rhodobacteria[6,11]. In this projection, principal components 1 and 2 are given by the X and Y axes, respectively, while principal component 4 is indicated through the use of symbols. Principal component 3, i.e., the Z axis, is lost in this projection.

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mannitol, breakdown of inositol, and oxidase activity (table 1). Differential characteristics for the motile aeromonads include esculin hydrolysis, growth in KCN broth, L-histidine and L-arginine utilization, L-arabinose utilization, fermentation of salicin, gas from glucose, and H_2S from cysteine (table 2).

DNA analyses

DNA hybridization studies have verified the distinction of the three species, *A. hydrophila*, *A. sobria* and *A. caviae*. Within these species there are at least seven DNA hybridization groups, three in *A. hydrophila*, two in *A. caviae*, and at least two in *A. sobria*. Thus far these DNA-related new groups have not been distinguished phenotypically one from the other[5].

Similarly, Farmer et al.[4], using DNA hybridization (hydroxyapatite, ^{32}P, 60C), found only 5 of 60 *Aeromonas* strains to be highly related to the type strain of *A. hydrophila* (70% or greater). Similarly, the type strain of *A. sobria* was highly related to only two other strains, suggesting that many strains of the motile, mesophilic group of *Aeromonas* belong to species other than *A. hydrophila*, *A. sobria*, and *A. caviae*. There appear to be nine to twelve different DNA hybridization groups.

5S RNA sequence analysis

Results of 5S RNA sequence analysis showed that *Aeromonas* spp. should be included in a family, Aeromonadaceae, separate from the Enterobacteriaceae and the Vibrionaceae[1]. Molecular genetic information compiled to date, including results of 16S ribosomal ribonucleic acid cataloging and 5S ribosomal ribonucleic acid sequence analyses, suggest that *Aeromonas* demonstrate an evolutionary divergence which is significantly at variance with that of other members of the Vibrionaceae[6]. An indication of the phylogenetic diversity among vibrios, aeromonads, and enterics can be gleaned from the figure.

Table 1. Identifying characteristics for motile aeromonads

Catalase	+
Oxidase	−
Motility	−
Morphology	Rods in singles and pairs
Growth in nutrient broth at 37°C	+
Arginine dihydrolase	+
Ornithine decarboxylase	−
Indole production	+
Fermentation:	
sucrose and mannitol	+
dulcitol, rhamnose, xylose,	−
raffinose, inositol, and adonitol	−
Breakdown of inositol	−
NO_3 reduction to NO_2	+
Growth in peptone H_2O without NaCl	−
0/129 resistance	−
Starch, gelatin, ONPG, RNA and DNA hydrolysis	−
Tween 80 esterase	−

Table 2. Minimal identifying characteristics for the motile aeromonads

Characteristics	*A. hydrophila*	*A. sobria*	*A. caviae*
Esculin hydrolysis	+	−	+
Growth in KCN broth	+	−	+
L-histidine utilization	+	−	+
L-arginine utilization	−	−	−
L-arabinose utilization	+	−	+
Fermentation of salicin	+	−	+
Gas from glucose	+	+	−
H_2S from cysteine	+	+	−

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Plesiomonas: Taxonomy, classification and enterotoxin production

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Key words. *Plesiomonas* taxonomy; *Plesiomonas* classification; *Plesiomonas* enterotoxin; *Aeromonas*/*Plesiomonas*.

Encountered in clinical specimens and the natural environment, *Plesiomonas shigelloides* has been implicated as a potential diarrheal agent in man, but has been overshadowed in importance by related bacteria in the genera *Aeromonas* and *Vibrio*, resulting in its neglect. Consequently, a good deal of uncertainty remains with regard to the taxonomy, ecology and pathogenicity of this member of the family Vibrionaceae. In fact, recent phylogenetic data, based on 5S rRNA sequencing, suggest a closer relationship of *P. shigelloides* with the Enterobacteriaceae rather than the Vibrionaceae. The closest relationship amongst the genera of the Enterobacteriaceae for *P. shigelloides* is with *Proteus mirabilis*[2,3]. Furthermore, a serological relationship between *P. shigelloides* and *Shigella* has been reported[6]. To clarify the taxonomy of *P. shigelloides*, phenotypic characterization was carried out, emphasizing extracellular enzyme profiles. Several investigators have reported production of enterotoxin(s) by *P. shigelloides*[1,4]. Enterotoxin of *P. shigelloides* is not believed to be related immunologically to cholera toxin (CT). We have deter-

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BACTERIOLOGY

Enzymatic Characterization of Three *Aeromonas* Species Using API Peptidase, API "Osidase," and API Esterase Test Kits

Amy M. Carnahan, Mark O'Brien, Sam W. Joseph, and Rita R. Colwell

An enzymatic characterization of 16 strains of Aeromonas species including *A. hydrophila* (7), *A. sobria* (5), and *A. caviae* (4) was carried out using API Peptidase (strips numbered 1, 2, 3, 4, 5, and 6); API Esterase and API "Osidase" test strips. A total of 89 substrates was used in the assay and included 59 arylamides (aminopeptides), 10 esters, and 20 carbohydrates. All three species were remarkably uniform in their reactivities. Nineteen (32%) of the arylamide substrates used were hydrolyzed by all three species. Very strong arylamidase activity was displayed by all three species for L-lysine, L-hydroxyproline, L-arginine, L-alanine, L-proline, and L-leucyl-L-alanine. Esterase activity was strongest against caproate (C6), caprylate (C8), nonanoate (C9), and caprate (C10) substrates. Only a limited number of carbohydrate substrates were hydrolyzed; strong N-acetyl-β-D-glucosaminidase activity was given by all strains. Both *A. hydrophila* and *A. caviae* gave strong β-D-glucosidase reactivities, while *A. sobria* appeared to be negative for this enzyme. The results of our preliminary study show that some of the enzymes examined may be useful in the identification and differentiation of these species. The API enzyme assays yielded rapid (4 hr) results. The assays were easy to perform, relatively inexpensive and reproducible. The importance of replicate testing and the inclusion of uninoculated (buffer only) controls as part of the assay is emphasized.

INTRODUCTION

Members of the genus *Aeromonas* are ubiquitous in their distribution; some have been associated with a variety of disease in man and animals (Hubbard 1981; Huizinga et al., 1979; Kaper et al., 1981; McDaniel, 1979; Pitarangsi et al., 1982; Sakazaki and Balows, 1981; von Graevenitz 1985). While the taxonomy of this group is still under revision, significant progress has been made; the contributions of Popoff and Veron (1976) and Popoff et al. (1981) represent key contributions to the taxonomy of *Aeromonas* spp. Clarification of the taxonomy of *Aeromonas* has been provided by Farmer et al. (1986), MacDonell et al. (1986), and Colwell et al. (1986), the latter proposing the establishment of a new family, *Aeromonadaceae*. Recently, Hickman-Brenner et al. (1987, 1988) proposed two new species, *Aeromonas veronii*, and *Aeromonas* Group 501, indicating continuing interest in the systematics of the aeromonads.

Waltman et al. (1982) generated enzymatic profiles of *A. hydrophila*, using API-

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ZYM. More recently, Janda (1985) carried out a biochemical and enzymatic characterization of *A. hydrophila*, *A. sobria*, and *A. caviae* using conventional and rapid micro-assay methods, including APIZYM. Enzyme profiles generated in that study provided useful taxonomic and virulence markers. However, most important is the need to improve the accuracy of identification of *Aeromonas* spp. associated with disease. It is expected that new species, biotypes, and serovars of *Aeromonas* will continue to be recognized. Thus, approaches such as that taken in this study to expand the criteria for identification serve a useful purpose.

To achieve a broader enzymatic characterization of *Aeromonas* spp., the following study was undertaken employing a selected set of strains and three API enzyme test kits: API Peptidase, API "Osidase," and API Esterase. These rapid micro-assay systems have proven effective in the characterization of a related organism, *Plesiomonas shigelloides* (1987). The study was, therefore, extended to include *Aeromonas*.

MATERIALS AND METHODS

Strains

Sixteen strains, representing *Aeromonas hydrophila*, *A. sobria* and *A. caviae* were examined. *A. hydrophila* (Type strain, ATCC 7966), *A. sobria* (Type strain, ATCC 9071), and *A. caviae* (Type strain, ATCC 15468) were included as reference strains in the study. Isolates, other than the type strains, were collected and identified at the International Centre for Diarrhoeal Disease Research in Dhaka, Bangladesh, and were of clinical origin. The latter were identified according to the protocol of Popoff and Veron (1976).

Culture Conditions

All test strains were grown on Tryptic soy agar (TSA) (Difco) for 24–48 hr at 37°C. The TSA plate cultures served as the source of inoculum for all enzyme assays.

Enzyme Profiles

All strains were assayed for arylamidase, esterase, and carbohydrate hydrolase activities, using API Peptidase (strips numbered 1, 2, 3, 4,5, and 6), API Esterase, and API "Osidase," respectively. The API enzyme kits were obtained from API (API System S.A., F-38390 Montalieu, Vercieu, France), and the manufacturer's directions were adhered to, except for slight modifications. The methods are described briefly, as follows.

Preparation of the Test Strips. Each test strip was placed in an incubation chamber containing 5-ml of distilled water to ensure a humidified atmosphere. Each test strip contained two identical "galleries" of 10 tests, except for the API "Osidase" gallery, which consisted of 20 separate tests.

Inoculation and Incubation of the Test Strips. Each microcupule of the test strips was inoculated with 65-μl of bacterial suspension prepared as described for API ZYM (1982). However, sterile phosphate buffer (0.01 M; pH (7.4) was substituted for sterile distilled water, since the test strips used in the present study did not contain buffered substrates (1955). The test strips were incubated aerobically at 37°C for 4 hr.

Reading and Interpretation. For API "Osidase," 1 drop of 0.1 M NaOH was added to each microcupule. A positive reaction was given by a yellow color. Reactions were

recorded as 0 (negative), 1(+/−), 2 (+), or 3 (+ +). Intermediate results (i.e. 1/2 or 3/2) were also recorded.

For API Peptidase strips 1–6 and API Esterase, a different method of scoring the results was employed. Procedurally, 1 drop each of reagent ZYM A and ZYM B was added to each microcupule and the reaction allowed to "develop" for 10 min. To enhance the reaction the test strips were placed in a dark room. The test strips were then exposed to the light of a very strong lamp for approximately 10 sec, which allowed for elimination of the yellow base due to an excess of unreacted Fast Blue BB (reagent ZYM B), and to make the negative reactions colorless. Recording of the reactions was as follows: negative (0), weakly positive (1) and positive (2 or 3) to strongly positive (4 or 5), according to the API ZYM color coded chart supplied by the manufacturer. Intermediate reactions (0/1 or 3/2 etc.) were also recorded.

As a measure of reproducibility, replicate tests were performed by two separate investigators and the data compared. Replicate uninoculated (buffer only) controls were also included. Relative enzyme activity for all of the strains (and their replicates) tested against each of the 89 substrates was calculated as described by Janda (1985) and corrected by reference to the control.

Intermediate results were scored as decimal fractions (e.g., 3/2 = 2.5 or 0/1 = 0.5).

Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (minimum RA value = 0.00; maximum RA value = 5.00).

RESULTS AND DISCUSSION

Enzymatic characterization of many bacterial groups, including *Aeromonas*, have been reported using API ZYM (Frank, 1981; Gray, 1987; Humble et al., 1977; Janda, 1985; O'Brien and Davis, 1982; Waltman et al., 1982). API ZYM is a rapid, semi-quantitative, micro-assay for a small number of broad enzymatic activities including phosphatases, arylamidases, esterases, and carbohydrate hydrolases. API Peptidase, API Esterase, and API "Osidase" are recent developments that augment API ZYM, permitting assay of a broader range of enzyme groups.

Results of tests for arylamidase activities employing API Peptidase are given for the three *Aeromonas* species listed in Tables 1–6. The readings for each strain of all three species were remarkably uniform in their reactivities. Nineteen (32%) of the 59 arylamidase substrates were hydrolyzed by all three *Aeromonas* species examined (Table 7). *A. caviae* was slightly more active than either *A. hydrophila* or *A. sobria*. Single amino acid arylamides and di- and tripeptides were hydrolyzed preferentially by all three species. In comparison, *A. hydrophila* was the only species to hydrolyze the single tetrapeptide substrate, while none of the strains examined were active against the single pentapeptide substrate.

Very strong (RA = 4.00–5.00) activity was demonstrated by all three species for L-lysine ARA, L-hydroxyproline ARA, L-arginine ARA, L-alanine ARA, L-proline ARA, and L-leucyl-L-alanine ARA. None of the three (RA = 0.00) were active when tested with glycyl-L-tryptophane, L-histidyl-L-leucyl-L-histidine, and N-benzoyl-L-alanine-4-methoxy arylamides. All three species showed only very weak (RA = <1.00) activity for several arylamide substrates (Tables 1–6). Based on an RA value of 2.0 as a positive reaction, several enzymes merit further evaluation as possible contributing markers in a speciation schema for these three species, including: γ-glutamyltransferase, glycyl-phenylalanine ARA, L-alanyl-L-phenylalanyl-L-proline ARA, L-alanyl-L-phenylalanyl-L-prolyl-L-alanine ARA, α-L-aspartyl-L-alanine ARA, L-phenylalanyl-L-arginine ARA, and N-CBZ-glycyl-glycyl-L-arginine ARA.

Results of the API Esterase test for *Aeromonas* spp. examined in this study are given in Table 8. Esterase activity was strongest for caproate (C6), caprylate (C8),

TABLE 1. Results[a] for API Peptidase 1 for *A. hydrophila*, *A. sobria*, and *A. caviae*

Arylamidase enzyme[c]	Relative activity[b]		
	A. hydrophila (14)[d]	*A. sobria* (10)	*A. caviae* (8)
1	1.36	1.50	1.38
2	0.07	0.20	0.13
3	1.71	1.90	1.88
4	4.36	4.50	4.19
5	4.25	4.40	4.81
6	1.68	1.95	1.63
7	2.89	3.00	2.81
8	1.50	1.10	1.25
9	4.68	4.30	4.88
10	4.50	4.40	4.56

[a]Number of strains tested (n = 16): *A. hydrophila* (n = 7), *A. sobria* (n = 5), *A. caviae* (n = 4).
[b]Relative activity (RA) was defined as the total numerical value for each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 5.00).
[c]1, L-tyrosine arylamidase ARA; 2, L-pyrrolidone ARA; 3, L-phenylalanine ARA; 4, L-lysine ARA; 5, L-hydroxyproline ARA; 6, L-histidine ARA; 7, glycine ARA; 8, L-aspartate ARA; 9, L-arginine ARA; 10, L-alanine ARA.
[d]Total number of replicates tested.

nonanoate (C9), and caprate (C10). RA activity was variable. In general, *A. hydrophila* and *A. caviae* gave higher RA values than *A. sobria* for the substrates tested, e.g., valerate (C5) EST, caproate (C6) EST, caprylate (C8) EST, and caprate (C10) EST. All strains exhibited at least some activity for all of the esterase substrates examined here, ranging from very weak to very strong activity.

Table 9 summarizes results for carbohydrate hydrolase activities for the *Aero-*

TABLE 2. Results[a] for API Peptidase 2 for *A. hydrophila*, *A. sobria*, and *A. caviae*

Arylamidase enzyme[c]	Relative activity[b]		
	A. hydrophila (17)[d]	*A. sobria* (12)	*A. caviae* (10)
1	1.68	1.95	2.20
2	0.06	0.08	0.05
3	1.15	1.04	1.45
4	1.85	1.75	2.20
5	3.38	3.67	3.35
6	1.65	1.29	2.00
7	NR[e]	NR	NR
8	2.59	2.54	2.50
9	1.06	0.88	1.15
10	0.00	0.00	0.00

[a]Number of strains tested (n = 16): *A. hydrophila* (n = 7), *A. sobria* (n = 5), *A. caviae* (n = 4).
[b]Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 5.00).
[c]1, γ-glutamyltransferase; 2, N-benzoyl-leucine ARA; 3, S-benzyl-cysteine ARA; 4, methionine ARA; 5, glycyl-glycine ARA; 6, glycyl-phenylalanine ARA; 7, glycyl-proline ARA; 8, leucyl-glycine ARA; 9, L-seryl-tyrosine ARA; 10, Control, negative.
[d]Total number of replicates tested.
[e]NR, no result because uninoculated buffer control gave an RA value of 2.00 or more.

TABLE 3. Results[a] for API Peptidase 3 for *A. hydrophila*, *A. sobria*, and *A. caviae*

Arylamidase enzyme[c]	Relative activity[b] *A. hydrophila* (16)[d]	*A. sobria* (12)	*A. caviae* (88)
1	0.19	0.17	0.19
2	2.53	3.00	3.13
3	0.50	0.42	0.69
4	0.16	0.38	0.06
5	1.22	1.58	1.50
6	4.43	4.71	4.75
7	3.38	3.54	3.63
8	0.97	1.25	1.06
9	0.44	0.42	0.44
10	3.16	2.00	3.50

[a]Number of strains tested (n = 16): *A. hydrophila* (n = 7), *A. sobria* (n = 5), *A. caviae* (n = 4).
[b]Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 5.00).
[c]1, N-CBZ-arginine-4-methoxy ARA; 2, L-glutamine ARA; 3, α-L-glutamate ARA; 4, L-isoleucine ARA; 5, L-ornithine ARA; 6, L-proline ARA; 7, L-serine ARA; 8, L-threonine ARA; 9, L-tryptophane ARA; 10, N-CBZ-glycyl-glycyl-arginine ARA.
[d]Total number of replicates tested.

monas species using API "Osidase." The "Osidase" enzymes examined were β-D-galactosidase, phospho-β-D-galactosidase, α-D-glucosidase, β-D-glucosidase, α-maltosidase, N-acetyl-β-D-glucosaminidase, α-D-fucosidase, β-D-lactosidase, α-D-galactosidase, α-L-arabinosidase, β-D-galacturonohydrolase, β-D-glucuronidase, β-maltosidase, N-acetyl-α-D-glucosaminidase, α-L fucosidase, β-D-fucosidase, α-D-mannosidase, β-D-mannosidase, α-D-xylosidase, and β-D-xylosidase. Only a limited

TABLE 4. Results[a] for API Peptidase 4 for *A. hydrophila*, *A. sobria*, and *A. caviae*

Arylamidase enzyme[c]	Relative activity[b] *A. hydrophila* (12)[d]	*A. sobria* (8)	*A. caviae* (7)
1	0.04	0.00	0.00
2	4.13	3.44	4.14
3	2.17	0.56	2.29
4	2.08	1.75	1.36
5	3.38	2.75	4.07
6	1.46	2.00	1.57
7	1.21	1.00	1.29
8	0.04	0.00	0.21
9	0.71	0.50	0.57
10	3.88	4.00	4.00

[a]Number of strains tested (n = 16): *A. hydrophila* (n = 7), *A. sobria* (n = 5), *A. caviae* (n = 4).
[b]Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 5.00).
[c]1, β-alanine ARA; 2, L-alanyl-L-arginine ARA; 3, L-alanyl-L-phenylalanyl-L-proline ARA; 4, L-alanyl-L-phenylalanyl-L-prolyl-L-alanine ARA; 5, L-arginyl-L-arginine ARA; 6, α-L-aspartyl-L-alanine ARA; 7, α-L-aspartyl-L-arginine ARA; 8, α-L-glutamyl-α-L-glutamic ARA; 9, α-L-glutamyl-L-histidine ARA; 10, glycyl-L-alanine ARA.
[d]Total number of replicates tested.

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TABLE 5. Results[a] for API Peptidase 5 for *A. hydrophila*, *A. sobria*, and *A. caviae*

Arylamidase enzyme[c]	Relative activity[b]		
	A. hydrophila (12)[d]	*A. sobria* (8)	*A. caviae* (7)
1	3.33	2.75	2.79
2	0.00	0.00	0.00
3	0.00	0.00	0.00
4	1.42	1.38	1.07
5	4.50	4.63	4.07
6	0.92	0.86	0.79
7	4.38	3.81	3.29
8	3.42	2.94	2.50
9	2.75	1.31	2.14
10	NR[e]	NR	NR

[a]Number of strains tested ($n = 16$): *A. hydrophila* ($n = 7$) *A. sobria* ($n = 5$), *A. caviae* ($n = 4$).
[b]Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 5.00).
[c]1, glycyl-L-arginine ARA; 2, glycyl-L-tryptophane ARA; 3, L-histidyl-L-leucyl-L-histidine ARA; 4, L-histidyl-L-serine ARA; 5, L-leucyl-L-alanine ARA; 6, L-leucyl-L-leucyl-L-valyl-L-tyrosyl-L-serine ARA; 7, L-lysyl-L-alanine ARA; 8, L-lysyl-lysine ARA; 9, L-phenylalanyl-L-arginine ARA; 10, L-phenylalanyl-L-proline ARA.
[d]Total number of replicates tested.
[e]NR, no result because uninoculated buffer control gave an RA value of 1.8 or greater.

number of carbohydrate substrates were hydrolyzed. Strongest (RA = >2.00) enzyme activity was given by all strains examined for N-acetyl-β-D-glucosaminidase. Both *A. hydrophila* and *A. caviae* gave strong β-D-glucosidase reactions, while *A. sobria* appeared to be negative for this enzyme. These particular findings, as well as results for API Esterase and API "Osidase", are in good agreement with those of Janda's API

TABLE 6. Results[a] for API Peptidase 6 for *A. hydrophila*, *A. sobria*, and *A. caviae*

Arylamidase enzyme[c]	Relative activity[b]		
	A. hydrophila (12)[d]	*A. sobria* (8)	*A. caviae* (7)
1	1.50	1.63	1.00
2	2.75	3.00	4.21
3	1.25	1.69	1.57
4	1.96	1.75	1.43
5	0.00	0.00	0.00
6	0.00	0.06	0.00
7	3.21	1.94	4.07
8	1.83	1.06	1.50
9	0.13	0.31	0.29
10	0.46	0.63	0.14

[a]Number of strains tested ($n = 16$): *A. hydrophila* ($n = 7$), *A. sobria* ($n = 5$), *A. caviae* ($n = 4$).
[b]Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 5.00).
[c]1, L-phenylalanyl-L-prolyl-L-alanine ARA; 2, L-prolyl-L-arginine ARA; 3, L-seryl-L-methionine ARA; 4, L-valyl-L-tyrosyl-L-serine ARA; 5, N-benzoyl-L-alanine-4-methoxy ARA; 6, N-CBZ-arginyl-4-methoxy ARA; 7, N-CBZ-glycyl-glycyl-L-arginine ARA; 8, N-acetyl-glycyl-L-lysine ARA; 9, L-histidyl-L-phenylalanine ARA; 10, L-lysyl-L-serine-4-methoxy ARA.
[d]Total number of replicates tested.

TABLE 7. Summary[a] of Results of Arylamidase Activities for the *Aeromonas* Strains Used in the Study

Peptide	No. peptides tested	No. (%) positive			
		A. hydrophila	*A. sobria*	*A. caviae*	All[b] Species
Single	26	8 (31)	8 (31)	10 (39)	8 (31)
Di-	25	11 (44)	11 (44)	12 (48)	10 (40)
Tri-	6	3 (50)	1 (17)	3 (50)	1 (17)
Tetra-	1	1 (100)	0 (0)	0 (0)	0 (0)
Penta-	1	0 (0)	0 (0)	0 (0)	0 (0)
Total	59	23 (39)	20 (34)	25 (42)	19 (32)

[a]Calculated from Tables 1–6. A result of grade 2 or more was considered positive.
[b]Positive (grade 2 or more) against all three *Aeromonas* species.

ZYM studies (Janda, 1985). In particular, Janda (1985) suggested that β-D-glucosidase might be of significant value in species differentiation. Gray (1987), also using API ZYM, further recommended chymotrypsin, β-glucuronidase and B1-phosphohydrolase, incubated at 30°C for 24 hr. The only other "Osidase" that showed some differential ability was the β-D-galactosidase (Table 9).

Reproducibility of all three enzyme assays was good. That is, for API Peptidase and API Esterase, the reactions were consistent on repeated testing, with the exception of minor variations in the intensity of the color reaction.

Uninoculated (buffer only) controls gave consistently negative results in nearly all replicate tests. Interestingly, two substrates, glycyl-proline arylamide (API Peptidase 2) and L-phenylalanyl-L-proline arylamide (API Peptidase 5) gave consistently positive (grade 2 or >) reactions in cupules containing buffer only. This was taken

TABLE 8. Results[a] for API Esterase for *A. hydrophila*, *A. sobria*, and *A. caviae*

Esterase enzyme[d]	Relative activity[c]		
	A. hydrophila (12)[e]	*A. sobria* (6)	*A. caviae* (4)
1	0.63	0.67	0.63
2	2.29	1.50	1.88
3	3.75	1.88	3.63
4	4.57	1.00	4.75
5	4.54	2.60	4.25
6	3.96	1.67	4.13
7	1.50	0.25	1.38
8	0.54	0.25	0.25
9	1.04	0.58	0.75
10	0.07	0.13	0.17

[a]In some replicate tests, a grade of 1 or more was recorded for the corresponding uninoculated (buffer only) control. Results for these replicates were disregarded in the calculation of the RA value.
[b]Number of strains tested (n = 12): *A. hydrophila* (n = 7), *A. sobria* (n = 3), *A. caviae* (n = 2).
[c]Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 5.00).
[d]1, butyrate (C4) esterase EST; 2, valerate (C5) EST; 3, caproate (C6) EST; 4, caprylate (C8) EST; 5, nonanoate (C9) EST; 6, caprate (C10) EST; 7, laurate (C12) EST; 8, myristate (C14) EST; 9, palmitate (C16) EST; 10, stearate (C18) EST.
[e]Total number of replicates tested.

TABLE 9. Results[a] for API "Osidase" for *A. hydrophila*, *A. sobria*, and *A. caviae*

Enzyme[c]	Relative activity[b]		
	A. hydrophila (6)[d]	*A. sobria* (4)	*A. caviae* (3)
1	2.17	1.50	3.00
2	0.00	0.00	0.17
3	0.17	0.00	0.00
4	2.25	0.00	3.00
5	0.08	0.00	0.50
6	2.92	3.00	3.00
7	0.00	0.00	1.33
8	0.17	0.00	1.00
9–20	0.00	0.00	0.00

[a]Number of strains tested ($n = 11$): *A. hydrophila* ($n = 5$), *A. sobria* ($n = 3$), *A. caviae* ($n = 3$).
[b]Relative activity (RA) was defined as the total numerical value of each replicate of each strain of each species divided by the total number of replicates of strains tested for each species (Minimum RA value = 0.00; maximum RA value = 3.00).
[c]1, "Osidase" enzymes = β-D-galactosidase; 2, phospho-β-D-galactosidase; 3, α-D-glucosidase; 4, β-D-glucosidase; 5, α-maltosidase; 6, N-acetyl-β-D-glucosaminidase; 7, α-D-fucosidase; 8, β-D-lactosidase; 9, α-D-galactosidase; 10, α-L-arabinosidase; 11, β-D-galacturonohydrolase; 12, β-D-glucuronidase; 13, β-maltosidase; 14, N-acetyl-α-D-glucosaminidase; 15, α-L-fucosidase; 16 β-D-fucosidase; 17, α-D-mannosidase; 18, β-D-mannosidase; 19, α-D-xylosidase; 20, β-D-xylosidase.
[d]Total number of replicates tested.

into consideration when reading the actual, inoculated test strip and the values adjusted accordingly. In addition, API Esterase uninoculated controls, containing buffer only, gave a reaction of grade 1 or 2, in a few cases. For example, three out of seven of the corresponding replicate uninoculated controls for C18 esterase gave a score of grade 2. For this reason it is important that the buffer used for API Peptidase and API Esterase must be of the correct molarity and pH, since these factors may seriously affect the results.

From the results presented here, the suggestion can be made that the enzymes examined in this study be considered for inclusion in the panoply of tests employed for the characterization and differentiation of *Aeromonas* spp. Obviously, further work is required with a wide variety of species and a larger number of strains before the value of these tests for taxonomic purposes can be conclusively ascertained.

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Species Identification of *Aeromonas* Strains Based on Carbon Substrate Oxidation Profiles

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Twenty clinical strains each of *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas sobria* were evaluated for their abilities to oxidize one or more of 95 carbon sources on a GN Microplate (BIOLOG, Hayward, Calif.). Nine substrates yielded good, discriminatory values for the three species tested. The panel appears to be useful for the species identification of *Aeromonas* isolates originating from human material.

The genus *Aeromonas* has received increased attention from the medical community as a suspected cause of bacterial gastroenteritis and as the etiologic agent of a number of additional infectious syndromes which include septicemia, cellulitis, and peritonitis (4). Within the genus at least 11 distinct species exist, as determined by DNA hybridization studies; of these 11 species, 7 have taxonomic standing in the literature (6). Recent studies have indicated that a majority (75 to 93%) of *Aeromonas* isolates recovered from clinical material fall into hybridization groups 1, 4, and 8; these hybridization groups correspond to the phenotypically identifiable species of *A. hydrophila*, *A. caviae*, and *A. sobria*, respectively (3, 6). Although these three phenotypic species can be identified by using a conventional battery of eight or more biochemical tests, most commercial identification panels for gram-negative organisms do not have the capability for accurate species identification within the genus *Aeromonas*. Instead, many such systems choose to collectively refer to the mesophilic aeromonad group as either *A. hydrophila*, the *A. hydrophila* complex, or *Aeromonas* spp. Recently, a new gram-negative microplate panel has been commercially introduced (GN Microplate; BIOLOG, Hayward, Calif.) which tests for the ability of bacteria to oxidize one or more of 95 carbon sources in the presence of a redox indicator (tetrazolium dye). We have evaluated this system as a potentially useful tool in the species identification of *Aeromonas* isolates recovered from clinical material.

Sixty *Aeromonas* strains (*A. hydrophila*, $n = 20$; *A. sobria*, $n = 20$; and *A. caviae*, $n = 20$) recovered from clinical material were evaluated in this study, three type or reference strains (namely, *A. hydrophila* ATCC 7966[T], *A. sobria* ATCC 9071, and *A. caviae* ATCC 15468[T]) were additionally included to ensure intra- and interlaboratory reproducibility. Each strain was grown overnight on Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar at 35°C, after which the 18- to 20-h resultant growth was removed and spectrophotometrically standardized (by optical density at 590 nm) in 0.85% NaCl according to the instructions of the manufacturer. These standardized suspensions were then inoculated into individual wells (150-μl volumes) of a 96-well GN Microplate (includes one control well) by using a multichannel pipetter. Inoculated plates were then incubated for 18 to 20 h at 35°C before being read.

Of the 95 carbon substrates screened in this system, one or more of the 60 *Aeromonas* strains were able to oxidize 66 (69%) of the tested compounds. The 29 compounds which yielded uniformly negative results included adonitol, *i*-erythritol, L-fucose, *m*-inositol, maltose, D-melibiose, D-raffinose, xylitol, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, α-hydroxybuytric acid, β-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, hydroxy-L-proline, L-phenylalanine, L-pyroglutamic acid, DL-carnitine, phenylethylamine, 2-aminoethanol, and 2,3-butanediol. In a similar fashion, 10 other carbon substrates were positive for all 60 strains, including dextrin, glycogen, *N*-acetylglucosamine, D-fructose, α-D-glucose, D-mannitol, β-methylglucoside, sucrose, D-trehalose, and L-asparagine.

Of the remaining 57 carbon substrates, 14 compounds were positive 90% of the time or more with the 60 mesophilic aeromonads evaluated; these compounds included Tween 40 and 80, D-galactose, D-mannose, methyl pyruvate, D-gluconic acid, succinic acid, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-serine, inosine, glycerol, and DL-α-glycerol-phosphate oxidation. Twelve other compounds were positive at a frequency ranging between 1 and 10% and included α-cyclodextrin, D-arabitol, L-rhamnose, turanose, citric acid, formic acid, α- and β-hydroxybutyric acid, propionic acid, L-leucine, α-aminobutyric acid, and putrescine.

The remaining 30 carbon sources that displayed variable positivity rates (between 10 and 90%) were further evaluated for their potential usefulness in phenotypically distinguishing between the three *Aeromonas* species tested. Nine substrates were unquestionably found to have significant discriminatory value in species identification and are listed in Table 1; of these nine substrates, seven (excluding L-arabinose and cellobiose) have not previously been identified as useful biochemical markers (1, 5, 7). In addition, although 90% of the strains studied were positive for the oxidation of D-mannose, all mannose-negative strains ($n = 6$) were identified as *A. caviae*, another property previously found to be useful in other *Aeromonas* identification schemes (5). The remaining 21 carbon substrates exhibiting variable positivity rates overall (10 to 90%) are listed in Table 2; within this latter group only lactose and L-histidine have previously been reported to have discriminatory value in *Aeromonas* species identification (1, 5, 7).

The results of this study suggest that the BIOLOG GN Microplate panel may be useful for species identification within the genus *Aeromonas*, although this system is currently not approved for clinical use. On the basis of the

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TABLE 1. Phenotypic properties useful in *Aeromonas* species identification

Carbon source	% Positive			
	Overall[a]	*A. hydrophila*	*A. caviae*	*A. sobria*
N-Acetylgalactosamine	43	100	5	25
L-Arabinose	65	80	100	15
Cellobiose	33	5	85	10
Gentobiose	17	0	50	0
D-Galacturonic acid	18	0	55	0
DL-Lactic acid	62	95	90	0
Urocanic acid	33	10	90	0
Glucose-1-phosphate	68	100	5	100
Glucose-6-phosphate	67	100	0	100

[a] Based on 60 strains (*A. hydrophila*, $n = 20$; *A. caviae*, $n = 20$; *A. sobria*, $n = 20$).

results listed in Table 1, all 60 *Aeromonas* strains could unambiguously be placed in their correct taxonomic positions. Several carbon substrates (cellobiose, L-arabinose, and lactose) included in the GN panel have previously been found to be valuable in species identification, and comparable results were also obtained in this study. One significant difference noted in this study was L-histidine oxidation, a test originally proposed by Popoff and Veron (7) to be useful in determining the species status of individual *Aeromonas* isolates. Our results were less dramatic (Table 2) than those previously published; however, it should be noted that although the substrates are identical, the methods are entirely different, and each test may not produce equivalent results. Intra- and interlaboratory reproducibilities of test results for mean values were found to be 95 and 94%, respectively; the discrepancies noted were invariably reactions which were visually difficult to determine as weak positives or negatives. The system has additionally incorporated D-mannitol as a carbon substrate, which could facilitate the identification of *A. schubertii* if present (2). Recent studies conducted by Arduino and colleagues at the Centers for Disease Control (M. J. Arduino, F. W. Hickman-Brenner, and J. J. Farmer III, Abstr. Int. Workshop on *Aeromonas* and *Plesiomonas*, p. 37, 1988) have identified 41 biochemical characteristics that can correctly identify 85% of the individual *Aeromonas* strains (and can partially identify an additional 14%) to the correct DNA grouping with the aid of a computer identification program. Although progress has been made in the correct taxonomic (genospecies) assignment of *Aeromonas* strains through phenotypic characterization, much work remains to be accomplished. The number of tests currently required to perform such analysis is prohibitive, necessitates the use of specialized reagents and media, and is not directly applicable to the clinical laboratory. Furthermore, DNA hybridization groups 4, 5, and 6, which phenotypically resemble *A. caviae*, cannot at present be easily separated with this expanded biotyping system. We chose clinical isolates for this study since a vast majority of such strains reside in only three distinct species (hybridization groups 1, 4, and 8), and each corresponds to a particular identifiable phenotype (*A. hydrophila*, *A. caviae*, and *A. sobria*). Because of the apparent success of the present study, it seems possible that the BIOLOG GN panel might be additionally useful in the phenotypic characterization of environmental strains where greater genetic diversity occurs, although the current cost per panel ($5.65) seems unreasonably high for routine laboratory use.

TABLE 2. Variable carbon oxidation profiles of *Aeromonas* species

Carbon source	% Positive			
	Overall[a]	*A. hydrophila*	*A. caviae*	*A. sobria*
Lactose	27	20	55	5
Lactulose	17	10	40	0
Psicose	88	85	100	80
D-Sorbitol	38	20	55	35
Monomethyl succinate	22	35	30	0
Acetic acid	47	30	75	30
cis-Aconitic acid	17	5	5	40
Bromosuccinic acid	87	95	75	90
Succinamic acid	35	10	55	40
Alaninamide	13	0	30	10
D-Alanine	37	30	75	5
L-Alanine	73	50	85	85
L-Alanylglycine	83	70	90	90
Glycyl-L-glutamic acid	80	65	95	80
L-Histidine	45	55	50	30
L-Ornithine	12	5	30	0
L-Proline	18	5	45	5
D-Serine	85	95	70	90
L-Threonine	22	10	55	0
Uridine	87	75	100	85
Thymidine	68	45	100	60

[a] Based on 60 strains (*A. hydrophila*, $n = 20$; *A. caviae*, $n = 20$; *A. sobria*, $n = 20$).

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Characterization of *Aeromonas schubertii* Strains Recently Isolated from Traumatic Wound Infections

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Recent studies have resulted in the proposal of a new species, *Aeromonas schubertii* (mannitol, sucrose, and indole negative), formerly termed Enteric Group 501, on the basis of the study of seven strains isolated from the southeastern and southwestern United States and Puerto Rico. We have isolated two phenotypically similar *A. schubertii* strains from infected human wounds sustained in the Chesapeake Bay area. Their identification was confirmed by DNA-DNA hybridization to the Centers for Disease Control definition strain 2446-81 (ATCC 43700) for group 12. The strains were further examined for the presence of virulence-associated markers: hemolysin, hemagglutinins, cytotoxin production, agglutination in acriflavine, resistance to normal human serum, and autoagglutination phenotype. Both strains were positive for hemolysin by the plate assay, cytotoxin production at 1:10, and DNase and protease. They were resistant to human serum and negative for acriflavine agglutination, and only one of the strains was autoagglutination positive. Both strains were negative for cell-free hemolysin, hemagglutinins, pectinase, and chitinase. These isolations of *A. schubertii* further extend its previously described geographic distribution and reinforce its role as a primary causative agent of cellulitis with possible increased antimicrobial resistance.

The aeromonads are autochthonous inhabitants of aquatic environments ranging from 0 to 3% salinity (14). They are generally distinguished as being glucose-fermenting, facultatively anaerobic gram-negative rods that are oxidase positive, resistant to O 129 (Vibriostat), and motile by means of a polar flagellum. In the last 10 years, *Aeromonas* spp. have been implicated as the causative agents in a variety of human wound infections ranging from cellulitis to osteomyelitis. Additionally, a strong association between several of the *Aeromonas* spp. and gastroenteritis has been established (11). However, until recently most studies involving *Aeromonas* spp. have shown an absence of thorough phenotypic confirmation to the species level.

In fact, systematic classification of *Aeromonas* spp. has been in a state of flux since the earliest isolations by Zimmerman in 1890 and Sanarelli in 1891 (4). In 1976, Popoff and Veron (20), using the method of numerical taxonomy, proposed the species *Aeromonas hydrophila* (with biovars X_1 and X_3) and *Aeromonas sobria* sp. nov. This proposal was followed in 1981 by the study of Popoff et al. (21), who used DNA-DNA homology to show that three species were distinguishable: *A. hydrophila*, *A. sobria*, and *Aeromonas caviae* (formerly the X_2 biovar of *A. hydrophila*). However, it was also determined that each of these three species contained at least two or three distinct genotypes or hybridization groups. This observation indicated that there were additional species that could not be identified by current methods of phenotypic analysis. Fanning et al. (G. R. Fanning, F. W. Hickman-Brenner, J. J. Farmer III, and D. J. Brenner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C116, p. 319) confirmed and expanded Popoff's original hybridization results to include 9 to 12 DNA hybridization groups. Newly described species represent some of these groups; e.g., *Aeromonas media* (1) is associated with group 5A, *Aeromonas veronii* (6) is associated with groups 8 and 10 (6, 15), and *Aeromonas schubertii* (5) is associated with group 12.

The most recent of these proposed new species is *A. schubertii* (mannitol, sucrose, and indole negative), formerly termed Enteric Group 501. The proposal to recognize *A. schubertii* is based on the study of seven strains isolated from the southeastern and southwestern United States and Puerto Rico (5). We have isolated and characterized two phenotypically similar *A. schubertii* strains from infected human wounds sustained in the Chesapeake Bay area. Their identification was confirmed by DNA-DNA hybridization to the Centers for Disease Control definition strain for group 12 (ATCC 43700). These strains were additionally evaluated for the presence of potential virulence-associated traits.

MATERIALS AND METHODS

Case history. The two strains of *A. schubertii* examined in this study, AMC 1108-87 and AMC 4396-87, were isolated at Maryland Medical Laboratory, Inc. (Baltimore, Md.), in August 1987, from leg wounds of a 57-year-old male and a 46-year-old female, respectively, whose exposures to aquatic environments could not be determined. Strain AMC 1108-87 was isolated as the predominant organism of a mixed culture that also had a moderate growth of *Enterobacter cloacae*, while strain AMC 4396-87 was isolated in pure culture.

A detailed case history was available for only one of the patients, a 46-year-old female, who presented at a health clinic on 11 August 1987 with no preexisting conditions other than an injury to her leg sustained during a fall. Her condition was diagnosed as cellulitis, and dicloxacillin (semisynthetic penicillin) was prescribed. However, this treatment proved ineffective, and the patient subsequently returned to have the wound debrided and a specimen cultured. The laboratory results were reported as heavy growth of

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TABLE 1. Biochemical reactions of three *A. schubertii* strains

Test[a]	Reaction[b]		
	AMC 1108-87	AMC 4396-87	ATCC 43700
Oxidase	+	+	+
Sensitivity to O/129	−	−	−
String test	−	−	−
Growth in nutrient broth plus:			
0% NaCl	+	+	+
6.5% NaCl	−	−	−
Motility	+	+	+
ONPG[c]	+	+	+
Citrate (Simmons)[c]	+	+	+
Nitrate reduced to nitrite[d]	+	+	+
H_2S on triple sugar iron	−	−	−
Indole production	−	−	−
VP (O'Meara)	−	+	−
VP[c]	−	+	−
VP[d]	−	+	−
Growth in KCN	−	−	−
Esculin hydrolysis	−	−	−
Gelatin hydrolysis[c]	+	+	+
Lysine (Moeller)	+	+	+
Ornithine (Moeller)	−	−	−
D-Glucose			
Acid	+	+	+
Gas	−	−	−
Acid produced from:			
Adonitol[d]	−	−	−
L-Arabinose	−	−	−
myo-Inositol[c,d]	−	−	−
D-Mannitol	−	−	−
Raffinose[d]	−	−	−
L-Rhamnose[d]	−	−	−
Salicin	−	−	−
D-Sorbitol[d]	−	−	−
Sucrose	−	−	−

[a] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside; VP, Voges-Proskauer.
[b] −, Negative at end of appropriate incubation period; +, positive at 24 h or end of appropriate incubation period.
[c] Results obtained with API 20E.
[d] Results obtained with MicroScan Gram Negative Panel 7.

A. hydrophila, and an oral cephalosporin (500 mg two times daily) was prescribed. The patient returned 4 days later for Iodoform packing and dressing and 6 days later for a final debridement and sterile gauze redressing. Upon completion of the 15-day regimen of oral cephalosporin treatment, the patient was completely recovered.

Bacterial strains. The two strains of *A. schubertii* were quick-frozen in 2 ml of Trypticase soy broth with 10% glycerol (Remel, Lenexa, Kans.) and maintained at −70°C and subsequently subcultured to Trypticase soy agar slants (BBL Microbiology Systems, Cockeysville, Md.) and maintained at room temperature (18 to 28°C). Unless stated otherwise, all analyses were performed at 36 ± 1°C.

Phenotypic analysis. All of the standard identification tests (Table 1) were performed either by conventional methods (7, 17, 20) with media and biochemicals obtained from Remel or by rapid identification methods with API 20E (Analytab Products, Plainview, N.Y.), MicroScan Gram Negative Combo Panel 7 (Baxter Healthcare Corp., MicroScan Division, West Sacramento, Calif.), and Biolog GN Microplate (Biolog, Inc., Hayward, Calif.).

DNA relatedness analysis. DNA-DNA hybridization was conducted in duplicate by using the batch hydroxyapatite thermal elution procedure (2, 3). DNA from the definition strain of *A. schubertii* (ATCC 43700) was nick translated with ^{32}P (NEK-004; Dupont, NEN Research Products, Boston, Mass.) and reacted with unlabeled DNA from representative *Aeromonas* isolates at both the optimal reassociation temperature of 60°C and the stringent incubation temperature of 75°C. Relatedness was expressed as the relative binding ratio (RBR) and as the divergence in the melting temperature (dTm) (Table 2). RBR was calculated as a percentage to enable determination of similarity between strains. RBR values of 70% (for reactions at 60°C) and 55% (for reactions at 75°C) were considered the cutoff values for DNA relatedness.

TABLE 2. DNA relatedness of the type strain of *A. schubertii* to strains AMC 1108-87 and AMC 4396-87

Source of unlabeled DNA	Relatedness (%) to labeled DNA of *A. schubertii* ATCC 43700		dTm (°C)[a]
	60°C	75°C	
A. schubertii			
2446-81[T] (ATCC 43700)	100	100	0
1108-87	97	90	2.8
4396-87	96	100	1.2
V. parahaemolyticus (ATCC 27969)	2		

[a] dTm, Difference in melting temperature between each homologous and heterologous DNA complex.

The dTm, expressed in degrees Celsius, is the difference in melting temperature between each homologous and heterologous DNA duplex. A dTm value of 5°C was considered the cutoff value for DNA relatedness.

Control reactions, in which labeled DNA was incubated in the absence of unlabeled DNA, were included, and the label-only control values were subtracted for all reactions before the RBR was calculated.

DNA from *Vibrio parahaemolyticus* (ATCC 17802) was used as a negative control for the DNA-DNA hybridizations.

Potential virulence-associated assays. (i) Autoagglutination and acriflavine agglutination. The determination as to whether these strains spontaneously pelleted in broth (SP[+]) or pelleted only after boiling (PAB[+]), as well as slide agglutination tests with acriflavine and with prepared O:11 antisera (16) (kindly provided by J. M. Janda, Berkeley, Calif.), were done by the method of Janda et al. (12).

(ii) Exoenzyme analysis, serum resistance, and cytotoxin assays. The strains were examined for the presence of the extracellular enzymes DNase, protease, pectinase, and chitinase by methods previously described (8, 13). Serum resistance was determined as previously described (19) by challenge of 6×10^6 to 3×10^7 CFU of each strain against 65% pooled human serum. Cytotoxin assays were performed on HEp-2 monolayers as previously described (12).

(iii) Hemolysin assay. This was performed by both the plate and tube methods. In the former, each strain was spot inoculated to Trypticase soy agar plates with 5% sheep blood and Trypticase soy agar plates with 5% horse blood (Remel) and incubated at both 37 and 22°C for 2 days. The strains were also tested for cell-free hemolysin. This involved growth in brain heart infusion broth overnight at 37°C in both static and shaken culture conditions. The shaken cultures were rotated on an orbital shaker at 100 rpm, followed by low centrifugation and filtration of the supernatants through a 0.45-μm-pore-size Acrodisc filter (Gelman Sciences, Inc., Ann Arbor, Mich.). The supernatants were then tested in serial twofold dilutions in phosphate-buffered saline against 1% (vol/vol) rabbit cells with incubation at 37°C for 1 h,

TABLE 3. In vitro susceptibility of three *A. schubertii* strains to 17 antimicrobial agents

Antimicrobial agent	MIC (μg/ml) (susceptibility)[a]			
	Range	ATCC 43700	AMC 1108-87	AMC 4396-87
Amikacin	≤2–≥16	4 (S)	8 (S)	>16 (R)
Ampicillin	≤1–≥16	>16 (R)	16 (MS)	>16 (R)
Cefazolin	≤2–≥16	16 (MS)	8 (S)	>16 (R)
Cefoxitin	≤2–≥16	<2 (S)	<2 (S)	<2 (S)
Ceftazidime	≤2–≥16	<2 (S)	2 (S)	<2 (S)
Ceftriaxone	≤4–≥6	<4 (S)	<4 (S)	<4 (S)
Cefuroxime	≤2–≥16	<2 (S)	<2 (S)	8 (S)
Cephalothin	≤8–≥16	<8 (S)	<8 (S)	<8 (S)
Ciprofloxacin	≤1–≥2	<1 (S)	<1 (S)	<1 (S)
Gentamicin	≤1–≥6	2 (S)	4 (S)	4 (S)
Imipenem	≤1–≥8	<1 (S)	<1 (S)	2 (S)
Piperacillin	≤8–≥64	<8 (S)	<8 (S)	<8 (S)
Tetracycline	≤4–≥4	<4 (S)	<4 (S)	<4 (S)
Ticarcillin-potassium clavulanate	≤1–≥6	<8 (S)	<8 (S)	<8 (S)
Ticarcillin	≤8–≥64	<8 (S)	<8 (S)	64 (MS)
Tobramycin	≤1–≥6	4 (S)	6 (I)	>6 (R)
Trimethoprim-sulfamethoxazole	<0.5/9.5–>2.0/38	<0.5/9.5 (S)	<0.5/9.5 (S)	<0.5/9.5 (S)

[a] Therapeutic guide based on National Committee for Clinical Laboratory Standards M100-2S: S, susceptible; R, resistant; MS, moderately susceptible; I, intermediate.

followed by refrigeration overnight before the final reading was made.

Hemagglutinins. Strains tested for the presence of hemagglutinins against rabbit, sheep, and rat (glutaraldehyde-fixed) erythrocytes were grown overnight on colonization factor agar at 35°C and suspended in phosphate-buffered saline to a concentration of ca. 10^{11} to 10^{12} CFU/ml. Then, 20 μl of this suspension was added to 20 μl of 3% (vol/vol) erythrocytes and mixed on a rocked-tile plate for 10 min at 60 rpm. The controls were *Escherichia coli* LY-72 and LY-91 hemagglutinin type 2 (J. Wong, M. A. Miller, and J. M. Janda, unpublished method).

Antibiotic susceptibility tests. Antibiotic susceptibility was determined by using the MIC microtube method following the standards for interpretation of the National Committee for Clinical Laboratory Standards. We determined the MICs of 17 of the most recently prescribed antimicrobial agents in a 96-well-microdilution plate, the MicroScan Gram Negative Combo Panel 7, and the results were interpreted and recorded both manually and with an AutoScan-4 reader with computer-assisted analysis by an IBM PS-60. The antimicrobial agents and concentrations are listed in Table 3.

RESULTS AND DISCUSSION

Continuing interest in the aeromonads has intensified the efforts towards classification of this group. Including the work of Hickman-Brenner et al. (6) and Schubert and Hegazi (22), there are now seven recognized or proposed species: *A. hydrophila*, *A. sobria*, *A. caviae*, *A. media*, *Aeromonas salmonicida*, *A. veronii*, *A. schubertii*, and *Aeromonas eucrenophila*.

The newest species, *A. schubertii*, is represented by DNA hybridization group 12 and to date has been isolated from abscesses, wounds, skin, pleural fluid, and blood (5). In comparison with other *Aeromonas* species, *A. schubertii* is negative for the fermentation of D-mannitol, sucrose, L-arabinose, and salicin; negative for indole production; and negative for esculin hydrolysis (5). The two clinical strains described herein further support the clinical significance of this particular species. Additionally, their isolation from the Chesapeake Bay area extends the geographic range previously described for this species.

Presumptive identification. Our strains, AMC 1108-87 and AMC 4396-87, showed the minimal characteristics for the genus *Aeromonas*, i.e., fermentative, motile gram-negative rods, oxidase positive, resistant to O/129, string test negative, not requiring NaCl for growth, not growing in 6.5% NaCl, and reducing nitrate to nitrites.

The strains were presumptively identified as *A. hydrophila* group on API 20E, but with an identification of "doubtful profile" for AMC 1108-87 and "good likelihood but low selectivity" for AMC 4396-87 by the API computer data base because of the presence of negative reactions for indole, mannitol, and sucrose. The profile numbers generated were 7206004 for both AMC 1108-87 and ATCC 43700 and 7207004 for AMC 4396-87. However, it should be noted that the API 20E data base is constructed only for *A. hydrophila*, *A. sobria*, *A. caviae*, and *A. salmonicida*.

The MicroScan Gram Negative Combo Panel 7 system gave an identification of *A. hydrophila* (probability, 99.9% correct) for only one of the strains, AMC 4396-87 (biotype no. 4000015-1), whereas it identified both AMC 1108-87 and the ATCC 43700 strain as *Vibrio mimicus* (probability, 99.9% correct) with the biotype no. 4000405-1. It should be noted that the MicroScan Data Management System did underscore the negative indole reaction for these latter two strains as being atypical. Likewise, the MicroScan Data Management System includes *A. hydrophila*, *A. sobria*, and *A. caviae* only.

Further characterization of these strains by the taxonomic schema of Popoff and Veron (20) and Janda et al. (13) and comparison with published results for the type strain *A. schubertii* (5) revealed the three strains to be essentially the same biochemically. Conversely, only AMC 4396-87 was Voges-Proskauer positive with the three different methods for detection of acetoin (Table 1). The Biolog Panel was used to analyze these strains for utilization of D-arabitol, cellobiose, *i*-erythritol, D-galactose, glycerol, *myo*-inositol, lactose, maltose, D-mannose, melibiose, and trehalose as sole carbon sources. Of these, D-galactose, glycerol, maltose, D-mannose, and trehalose were utilized by all three strains while the remainder gave negative results.

Both of these strains were weakly beta-hemolytic on Trypticase soy agar with 5% sheep blood and strongly

beta-hemolytic on Trypticase soy agar with 5% horse blood after 24 h of incubation at both 35 and 22°C. ATCC 43700 has been described as gamma-hemolytic on Trypticase soy agar with 5% sheep blood which we confirmed but further noted that it was beta-hemolytic on Trypticase soy agar with 5% horse blood after 24 h at 36°C.

Confirmed identification. The results of the DNA hybridization work showed a close relatedness between these two strains and the DNA group 12 definition strain ATCC 43700 (96 to 100%), which confirmed the identification of these strains as *A. schubertii* (Table 2).

Virulence-associated assays. Prior studies have shown a variation in virulence between species of *Aeromonas* (9, 10). For this reason, it was considered appropriate to investigate these strains for the presence of virulence-associated markers. The results for cell-free hemolysin, hemagglutinins, cytotoxin, pectinase, and chitinase were essentially negative except that both AMC 4396-87 and AMC 1108-87 were positive for cytotoxin production at a 1:10 dilution when grown under aerated conditions.

However, it is noteworthy that both strains were positive for hemolysin by the plate assay and were positive for DNase and protease. They were resistant to serum, with AMC 4396-87 showing a slightly higher serum resistance value than AMC 1108-87 (0.82 and 0.42, respectively). Also of interest is the negative acriflavine agglutination reaction which usually correlates with virulent strains (18).

Finally, the autoagglutination results revealed AMC 1108-87 to be AA⁻ (autoagglutination negative), ATCC 43700 to be SP⁺, and AMC 4396-87 to be PAB⁺. Testing of AMC 4396-87 against the O:11 somatic antisera previously described resulted in 4⁺ agglutination with all three antisera and led to the conclusion that this strain is most likely a rough strain. It is therefore unlikely that this strain possesses the S layer that has been associated with invasive disease in certain populations of *A. hydrophila* and *A. sobria* (12).

Antibiotic susceptibility. The two strains had antibiograms typical of other *Aeromonas* strains, i.e., resistance to ampicillin (strain AMC 1108-87 was actually considered moderately susceptible) and susceptibility to gentamicin and tetracycline (Table 3). Additionally, AMC 4396-87 was resistant to amikacin, tobramycin, and cefazolin; therefore, it was the most resistant of the three strains tested. Otherwise, the two strains as well as ATCC 43700, were very susceptible to the majority of the cephalosporins tested (cephalothin, cefoxitin, ceftriaxone, and cefuroxime), as well as the newer beta-lactam-inactivating drug ticarcillin-potassium clavulanate and the quinolone ciprofloxacin.

In summary, we have confirmed that *A. schubertii* should be considered when an organism resembling the aeromonads is isolated from a wound infection. Furthermore, its range of isolation has been extended up the mid-Atlantic coast region to the Chesapeake Bay.

A. schubertii will most likely be identified as *A. hydrophila* group or *A. hydrophila* by rapid identification systems such as the API 20E or MicroScan, respectively. However, the atypical reaction profile (mannitol, sucrose, and indole negative) should alert the clinical microbiologist that further identification tests are indicated.

Although many of the virulence-associated markers examined were absent in these strains, it is apparent that, at least in the case of AMC 4396-87, *A. schubertii* is capable of causing primary cellulitis. AMC 4396-87 appears to be somewhat more resistant than other *A. schubertii* strains described to date in that it is susceptible to gentamicin but resistant to tobramycin and amikacin.

A final consideration is that these strains of *A. schubertii* represented 2 of 50 *Aeromonas* species isolated in the same laboratory over a 12-month period from various clinical specimens and may suggest a higher frequency than might be suspected otherwise.

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AEROMONAS AND *PLESIOMONAS* IN THE ENVIRONMENT: VALUE OF DIFFERENTIAL BIOTYPING OF AEROMONADS

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Abstract

Environmental parameters of *Aeromonas* and *Plesiomonas* are reviewed and comparisons are made within and between the two genera. Because of the presumed disparity between 'so-called' environmental and clinical aeromonads, the two groups are reviewed particularly in relation to observations made by investigators studying these organisms using various biotyping methods. A retrospective analysis of clinical and environmental strains from one of our original studies is presented after utilizing the most recently available taxonomic criteria. Identifications are at variance in some instances with previous designations.

Aeromonas and *Plesiomonas* are autochthonous to the aquatic environment. While their respective niches in the environment have not been clearly elucidated, both have been implicated as aetiological agents in human and animal disease.

The aeromonads are oxidase-positive, motile non-motile, facultatively anaerobic, Gram-negative rods that have undergone several generations of taxonomic reclassification. Currently, *Aeromonas hydrophila, A. sobria, A. caviae, A. salmonicida, A. media, A. veronii, A. schubertii* and *A. eucrenophila* are validated or proposed species. Most of these species are associated with fresh and estuarine waters (1), sediment, soil and sludge (2). *A. hydrophila, A. sobria, A. caviae, A. veronii* and *A. schubertii* have been isolated from human infections. Reports of *Aeromonas* isolation from various domestic and wild animals, warm- and cold-blooded, are fairly common, including isolations of aeromonads from many avian species. Diverse forms of disease in bird populations, some epidemic, have been attributed to *Aeromonas* spp. (3, 4). Because of the frequency of isolation of *Aeromonas* from various types of birds, it has been conjectured that these avian populations may serve as a significant reservoir for aeromonads in aquatic environments, especially in open municipal water supplies (5). Aeromonads constitute a large proportion of the total microbial population in river water samples, particularly in highly polluted (not necessarily faecal) environs (6, 7). The resilience of these organisms is further illustrated by the isolation of *A. sobria* from chlorinated drinking water supplies (8).

A. hydrophila is pathogenic for fish at particular temperatures in water exhibiting temperature gradients (9, 10, 11). *A. salmonicida* virulence in fish appears to be associated with an A-layer (cell surface virulence associated factor) (12), and possibly with the ability to exist in a dormant phase outside of fish (13). Atypical groups of *A. salmonicida* have been revealed via DNA relatedness (14) and heme requirement studies (15). Dooley and Trust (16) proposed that strains of *A. hydrophila*, which are most virulent for fish, appear to possess a surface array protein (S-layer).

The frequent isolation of *Aeromonas* from

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commercial beef, pork, poultry, fish and produce from retail stores (17, 18, 19) and the increased suggestion of its significance in human diseases mandate studies that would facilitate identification of virulent strains in these food sources and in well water (20) and municipal drinking water (21, 22). Likewise, the increasing number of reports of *Plesiomonas* outbreaks (23, 24), especially in relation to raw oyster consumption and sporadic disease (25), suggest the need for similar studies with this organism.

Plesiomonas is an oxidase-positive, Gram-negative rod that has previously been considered to have some of the attributes of *Shigella*, thereby accounting for the singular species designation *shigelloides*. Formerly designated as either Paracolon C 27, *Aeromonas shigelloides* or *Pseudomonas shigelloides*, this organism is recognised as an autochthonous bacterium in fresh and estuarine waters and as part of the opportunistic, common flora of finfish (26), shellfish, and reptiles, particularly in tropical and sub-tropical climates. Outbreaks of infection in humans indicate that *Plesiomonas* associated with oysters is an attributable risk factor in diseases of this nature (23). *Plesiomonas* has been isolated from healthy human carriers (0.78%) at very low rates, and from dogs (3.8%), and cats (10.2%) in Japan (27). Similarly, Van Damme and Vandepitte (28) have isolated *P. shigelloides* from cattle, pigs, dogs, and birds. It can also be isolated from fresh water (29) and on occasion from sea water (30). Serotyping of *Plesiomonas* has been accomplished by Aldova (31), who recently used 34 O and 19 H sera to test 169 strains of diverse geographic origin and found 017 to be the most frequent serovar in OH serovar combinations 017:C, 017:d and 017:f.

In contrast to *Aeromonas*, *P. shigelloides* does not multiply in temperatures below 8°C, which probably accounts for seasonal variation of isolations from river water in temperate climates. Miller and Koburger (32) found that both environmental and clinical strains could grow in Trypticase Soy Broth supplemented with 4% NaCl at pH 4.5-8.5, in which condition only 22% of the strains were able to grow at 8°C and 25% at 45°C. Huq and co-workers (33) found isolation rates of *Plesiomonas* from the water samples of the Buriganga River and Dhanmondi Lake in Bangladesh to be much higher when incubated at 44°C. *Aeromonas* spp., the usual confounding organism at 37°C incubation, was inhibited at this higher temperature.

Attempts thus far to biotype aeromonads and to separate environmental (so-called avirulent) strains from human virulent strains have met with varying success and there is presently no conclusive means of separating the two groups. In comparing biochemical characteristics and virulence factors of 147 clinical strains from patients with diarrhoea and 94 strains from metropolitan water supplies, Burke *et al.* (34) found that many of the strains from water had properties identical with those of clinical isolates suggesting that such strains were potential enteric pathogens. Haemagglutination patterns appear to differ between the two groups (35), as do virulence features based on suckling mouse assays (36). Nishikawa and Kishi (37) found that haemagglutination patterns in diarrhoeal stools did not seem to correlate with source although some agreement was found according to species, with *A. sobria* being positive more frequently (43%) than *A. hydrophila* (20.5%) and *A. caviae* (8.3%).

Picard and Goullet (38) used enzyme electrophoresis to distinguish and compare *Aeromonas* isolates from human clinical specimens and tap water in a hospital environment. Although they were unable to show consistent similarities among strains in the two groups, prophylactic measures were implemented and appeared to reduce the incidence of nosocomial illness resulting from *Aeromonas* infections.

By use of phage lysotyping, Geiger *et al.* (39) were able to show an epidemiological relationship between the strains of *Aeromonas* isolated from the aquatic environment and those of human clinical origin. Using 95 bacteriophage strains, they were able to establish correlations between 80% of the strains from both sources, thus concluding that these same strains could be present in aquatic and clinical environs. They surmised that these results substantiated the supposition that the aquatic environment is an important reservoir of potentially pathogenic aeromonads.

Although piliation seems to be at least one of the important features which dictate adherence, there

is some difference of opinion as to the frequency and type of piliation of clinical and environmental strains (36, 40, 41). Carello (41) found 0/29 environmental strains to be adherent to HEp-2 cells (>20 bacteria/cell) although all were heavily piliated. They found that environmental strains generally have an S-type pilus while clinical strains seem to have a more adherent L-type pilus, explaining that the S-type pilus may play a role in the survival of *Aeromonas spp.* in environmental habitats. Conversely, Kirov *et al.* (36) found a marked absence of pili on clinical strains as compared with environmental isolates. This was in contrast to the findings of Daily *et al.* (40), who found pili to be present more frequently in clinical strains.

Notermans *et al.* (42) analyzed a large group of environmental and clinical strains representing the three major species (*A. hydrophila, A. sobria,* and *A. caviae*) for "Asao" toxin and found virtually no difference between aeromonads from human sources and those of environmental origin.

Daily *et al.* (40) also showed that a significant number of strains from the environment were potentially pathogenic biotypes based on the presence or absence of numerous virulence-associated features. Based on prevailing taxonomy at the time, the strains were identified to the species, *A. hydrophila* and *A. sobria*, only. The majority of the clinical isolates were *A. sobria*. There seemed to be two subsets of organisms representing these two species; one which was virulent and the other, avirulent. These conclusions did not consider the possibility of additional species, biotypes or genotypes.

We have noted that much of the early literature on *Aeromonas* contains either minimal or no species designation or identifies all isolates as *A. hydrophila*, although some reports have provided biotype information, e.g. where positive lysine decarboxylase (LDC) and Voges-Proskauer (VP) reactions appeared to correlate with cytotoxin production (43). We now know that these are characteristics of *A. hydrophila* and *A. sobria*, since *A. caviae* does not generally yield positive reactions for VP or LDC.

Using recent information on classification of *Aeromonas* and improved methodologies, we (SWJ, RW and DR, coinvestigators in the present article) have retrospectively re-examined many of the strains from the study noted above (40), the purpose being to update the classification of these strains, and based on the results, to re-assess our original conclusions.

Materials and methods

Bacterial strains

The strains for this study (Table I) were maintained at -70°C in Trypticase Soy Broth with 10% glycerol added.

Strain characterization

Strains which were oxidase-positive and resistant to vibriostat (0/129) (150μg/ml) were presumptively placed in the "*Aeromonas hydrophila*-complex" with the API 20E (Analytab, Inc., Plainview, NY).

Identification to species was done using conventional methods (44, 45, 46) and media (Remel, Lenexa, KS). These tests included haemolysin and indole production, reaction on TSI, inhibition by KCN, esculin and bile esculin hydrolysis, acetoin production (VP), production of gas from glucose, lysine and ornithine decarboxylase production, and fermentation of arabinose, salicin, mannitol and sucrose. Unless noted otherwise, all of these tests were performed at 36°C±1°.

Results and discussion

Our earlier work (40) showed that subsets of *A. hydrophila* and *A. sobria* were either avirulent or less virulent when inoculated intraperitoneally in mice (lethal dose 50%, $LD_{50} \geq 10^7$) than the potentially pathogenic group ($LD_{50} < 10^7$). Since this study was accomplished before *A. caviae* was commonly considered for identification purposes, it was of interest to determine how many of these strains, particularly in the $LD_{50} \geq 10^7$ group, were of this species. Current information suggests that *A. caviae* is a less virulent organism than either *A. hydrophila* or *A. sobria*. Five of the fifteen, which were re-examined, are different species than the original designation and four others are atypical in two or more of the features characterizing the

TABLE I — PHENOTYPIC CHARACTERISTICS OF 15 *AEROMONAS* SPP. FROM CLINICAL AND ENVIRONMENTAL SOURCES

Strain No. (Daily *et al.*, 1981)	Lab No.	Source	Original Identification	LD_{50}[a]	Oxidase	Vibriostat (0/129)	TSI[b]	Indole	Haemolysin	KCN	Esculin	Bile Esculin	VP[c]	Glucose (Gas @ 37°C)	Lysine	Ornithine	Arabinose	Salicin	Mannitol	Sucrose	Present Identification
2	NMRI 203	G.I. tract	*A. sobria*	4.1	+	R	A/A	+	ß	w+	+	+	+	−	+	−	+	+	+	+	*A. hydrophila*
3	NMRI 204	Sputum	*A. sobria*	4.3	+	R	K/A	+	ß	+	+	+	+	+	+	−	+	−	+	+	*A. hydrophila*
4	NMRI 208	G.I. tract	*A. sobria*	4.4	+	R	K/A	+	wß	−	−	−	−	−	+	−	−	−	+	−	Atypical *A. sobria*[d]
5	NMRI 7	Leg wound	*A. hydrophila*	4.5	+	R	A/A	+	ß	+	+	+	+	−	+	−	+	−	+	+	Atypical *A. hydrophila*
6	NMRI 54	Environment	*A. sobria*	4.5	+	R	A/A	+	ß	+	+	+	+	+	+	−	+	−	+	+	*A. hydrophila*
7	NMRI 6	Leg wound	*A. sobria*	4.6	+	R	A/A	+	ß	−	+/−	+/−	+	+	+	−	−	−	+	−	Atypical *A. sobria*
8	NMRI 55	Environment	*A. hydrophila*	4.6	+	R	A/A	+	ß	+	+	+	+	+	+	−	+	−	+	+	*A. hydrophila*
10	NMRI 3	Environment	*A. hydrophila*	5.4	+	R	A/A	+	ß	+	+	+	w+	+	+	−	+	+	+	+	*A. hydrophila*
12	NMRI 206	G.I. tract	*A. sobria*	5.9	+	R	A/A	+	wß	−	−	−	−	+	+	−	w+	−	+	+	*A. sobria*
13	NMRI 56	Environment	*A. hydrophila*	6.5	+	R	A/A	+	wß	+	+	+	+	+	+	−	+	+	+	+	*A. hydrophila*
15	NMRI 5	Lung	*A. hydrophila*	6.9	+	R	A/A	+	ß	+	+	+	w+	+	+	−	+	+	+	+	*A. hydrophila*
16	NMRI 4	G.I. tract	*A. sobria*	7.3	+	R	A/A	+	ß	w+	−	−	+	+/−	−	−	−	−	+	+	Atypical *A. sobria*
17	NMRI 209	G.I. tract	*A. sobria*	7.5	+	R	A/A	+	α	+	+	+	−	−	−	−	+	+	+	+	*A. caviae*
22	NMRI 16	Environment	*A. hydrophila*	8.2	+	R	A/A	+	ß	+	+	+	+	+	+	−	+	−	+	+	*A. hydrophila*
23	NMRI 2	Environment	*A. hydrophila*	8.4	+	R	A/A	+	α	+	+	+	−	−	−	−	+	+	+	+	*A. caviae*

[a] LD_{50} = lethal dose 50%

[b] Gas exhibited by all strains except nos. 2, 4, 5 and 17

[c] VP = Voges-Proskauer

[d] Presumptive Group 9 genotype

assigned designation (Table I). Six remained unchanged (Table I). In fact, 2/15 proved to be *A. caviae*, which had given LD_{50}'s of $10^{7.5}$ and $10^{8.4}$, respectively. However, two other strains with $LD_{50} \geq 10^7$, No. 4 and No. 16, were not *A. caviae* and are correct as originally identified. However, we now refer to No. 4 as atypical *A. sobria* because of the atypical reactions shown in Table II. This strain, as well as No. 7, was unable to ferment sucrose. Based on the studies of Arduino *et al.* (Arduino, MJ, Hickman-Brenner FW, Farmer JJ, III. Phenotypic analysis of 132 *Aeromonas* strains representing 12 DNA hybridization groups. 2nd International Workshop on *Aeromonas* and *Plesiomonas*, 1988:37), we suspect that strain No. 4 is a member of DNA hybridization Group 9 of Fanning *et al.* (Fanning GR, Hickman-Brenner FW, Farmer JJ, III, Brenner DJ. DNA relatedness and phenotypic analysis of the genus *Aeromonas*. *In:* Abstr. Ann. Mtg. Amer. Soc. Microbiol, C116, 1988:319). Strains No. 7 and No. 16 are now regarded as atypical *A. sobria*, while No. 5 is an atypical *A. hydrophila* (Table II). Although strains No. 3 and No. 6 are salicin-negative, *A. hydrophila* is the most appropriate designation for these two strains, rather than the original identification, *A. sobria*.

Of the 15 strains, 9 were from clinical and 6 from environmental sources. In contrast to our earlier speculation that *A. sobria* was the predominant clinical aeromonad, our current findings show that there are 4 *A. sobria*, 4 *A. hydrophila*, and 1 *A. caviae* from clinical sources, thus approximating the frequency observed in some recent reports (47) (Table III). One of the *A. sobria* strains, No. 4, is presumptively identified as belonging to hybridization Group 9, pending confirmation by DNA hybridization analysis. *A. hydrophila* comprises 5 of the 6 environmental strains with *A. caviae* being the remaining strain.

In a study of a diver's wound infection in 1979 (48) strains No. 5 and No. 7 were isolated simultaneously and identified as *A. hydrophila* and *A. sobria*, respectively. At the time, it was considered unusual to find two species of *Aeromonas* occurring together and was the first occasion for *A. sobria* to be reported from a human wound. It is interesting that now No. 5 is identified as an atypical *A. hydrophila* and No. 7, as an atypical *A. sobria*.

This study considers the changing dynamics of *Aeromonas* taxonomy in the last decade and exemplifies the importance of correct identification and biotyping if we are to compile accurate epidemiological evidence related to aeromonads. It is encouraging that more investigators are becoming interested in this subject, and with additional input, many of the existing questions of taxonomy should be resolved, thus allowing us to move on more definitively to the question of virulence amongst the numerous genotypes of *Aeromonas*.

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TABLE II – LIST OF ATYPICAL *AEROMONAS* SPP.

Strain No.	Identification	Atypical reactions
4[a,b]	Atypical *A. sobria*	VP, Gas from glucose, sucrose
5[c]	Atypical *A. hydrophila*	Gas from glucose, salicin
7	Atypical *A. sobria*	Esculin, sucrose
16	Atypical *A. sobria*	KCN, lysine

[a]Phenotypically resembles DNA hybridization Group 9 of Fanning *et al.* (Fanning GR, Hickman-Brenner FW, Farmer JJ, III, Brenner DJ. DNA relatedness and phenotypic analysis of the genus *Aeromonas*. *In:* Abstr. Ann. Mtg. Amer. Soc. Microbiol, C116, 1988: 319)

[b,c]These strains produced gas from glucose after 24 hours when incubated at 22°C.

TABLE III – RECAPITULATION OF STRAINS AND SOURCES

Present identification	Number of strains (N=15)	Strain number	Sources
A. caviae	2	23	Environmental
		17	G.I. Tract
A. sobria	1	12	G.I. Tract
A. hydrophila	8	10,22	Environmental
		15	Lung (Morgue)
		6,8,13	Environmental
		2	G.I. Tract
		3	Sputum
Atypical *A. sobria* (Presumptive group 9)	1	4	G.I Tract
Atypical *A. sobria*	2	16	G.I. Tract
		7	Leg wound
Atypical *A. hydrophila*	1	5	Leg wound

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Pyrazinamidase Activity as a Phenotypic Marker for Several *Aeromonas* spp. Isolated from Clinical Specimens

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Negative pyrazinamidase activity was significantly associated with *Aeromonas sobria*, and positive pyrazinamidase activity was associated with *A. hydrophila* and *A. caviae* (χ^2, $P < 0.0001$). The absence of pyrazinamidase activity may be a potentially significant phenotypic marker for *A. sobria*.

Aeromonads are ubiquitous gram-negative, aquatic microorganisms that can cause a wide variety of diseases in humans as well as cold- and other warm-blooded animals (4). In the last decade, there has been increasing research centered on their relative pathogenicity for humans, especially with respect to species-associated virulence factors (5).

However, with *Aeromonas* taxonomy currently in a state of flux, i.e., having 12 or more DNA-DNA hybridization groups (genotypes) within the genus (G. R. Fanning, F. W. Hickman-Brenner, J. J. Farmer III, and D. J. Brenner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C116, p. 319) and 8 proposed and/or recognized species to date, virulence is difficult to assess. For example, Kuijper et al. (9) have shown that only 5 of the 12 genotypes were found in the examination of 142 diarrheal clinical strains. Further, it was shown that the current scheme of classification is not sufficiently discriminating, in that phenotypically similar *A. hydrophila* strains were genetically heterogeneous and that a suspected *A. sobria* could belong to either DNA hybridization group 8 (*A. sobria*) or group 10 (*A. veronii*).

Because we have been investigating several biochemical characteristics for phenotypic classification of the different DNA groups, we decided to examine pyrazinamidase activity, which has been extensively studied in *Mycobacterium* spp. (8) and considered a differentiating feature in the classification of *Corynebacterium* spp. (11), *Rhodococcus* spp., and *Nocardia* spp. (12) and a virulence-associated marker in the genus *Yersinia* (7).

Bacterial strains. There were 119 *Aeromonas* strains used for this study, including *A. hydrophila*, *A. sobria*, *A. caviae*, *A. veronii*, and *A. schubertii*. The strains were collected from 1985 to 1989 in several countries, including the United States ($n = 56$), Northeast Africa ($n = 45$), Bangladesh ($n = 11$), Indonesia ($n = 1$), and South America ($n = 1$), and originated primarily from various clinical sources ($n = 98$). The remaining aeromonads ($n = 21$) were from environmental, veterinary, and miscellaneous sources. The definition strains for the DNA hybridization groups 1, 4, 10, and 12 and a clinical American Type Culture Collection (Rockville, Md.) strain representing DNA hybridization group 8Y were included as controls (Table 1). The strains were maintained at −70°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) with 10% glycerol added.

Strain characterization. Strains which were oxidase positive and resistant to Vibriostat (0/129; 150 μg/ml; Oxoid, Columbia, Md.) were presumptively placed in the *A. hydrophila* complex with the API 20E (Analytab Products, Plainview, N.Y.). Identification to the species level was accomplished by using conventional methods (2, 6, 10) and media (Remel, Lenexa, Kans.). Ornithine decarboxylase production, mannitol fermentation, and indole production were included to screen for the newer species, *A. veronii* and *A. schubertii*, respectively. All tests were incubated at 36°C ± 1°C. Pyrazinamidase activity was assayed by using a supplied medium that contained tryptic soy agar (15 g; Difco), yeast extract (1.5 g; Sigma Chemical Co., St. Louis, Mo.), and Tris-maleate (0.2 M, pH 6, 500 ml) buffer and pyrazinamide (0.5 g; Sigma) and was dispensed in 5-ml aliquots into screw-cap tubes (16 by 150 mm), autoclaved at 121°C for 15 min, and slanted (Remel). These slants were inoculated with 18- to 24-h bacterial growth taken from tryptic soy agar (Difco) and incubated for 48 h at 36°C. One milliliter of 1% (wt/vol) freshly prepared ferrous ammonium sulfate (aqueous) solution was flooded over each slant, and a positive (pinkish rust color) or negative reaction (colorless) was recorded after 15 min. Positive pyrazinamidase activity indicated the presence of pyrazinoic acid resulting from the action of the enzyme pyrazinamidase (7).

Increasing interest in the aeromonads as disease-causing microorganisms has resulted in numerous efforts to identify these microorganisms definitively (4). Because of the current state of taxonomic confusion surrounding aeromonads, we considered it imperative to start with strains that were well characterized to the species level. We screened 119 well-characterized *Aeromonas* strains representing five species from various geographical locales for pyrazinamidase activity. Of the 37 *A. hydrophila* isolates tested, 35 (95%) were positive. Both of the negative strains were environmental isolates from the United States. Similarly, 49 of 53 (93%) *A. caviae* were positive. Negative pyrazinamidase activity was detected in 23 of 24 (96%) *A. sobria* and in 2 of 2 (100%) *A. veronii* and 3 of 3 (100%) *A. schubertii* strains tested. The one positive *A. sobria* isolate and four negative *A. caviae* isolates were human fecal strains from North Africa.

Using the chi-square goodness-of-fit test, it was established that the proportion (percentage) of positives was different among the three species *A. hydrophila*, *A. sobria* and *A. caviae*. Further examination of the data implied that *A. sobria* was distinct from the other two species (χ^2, $P < 0.0001$).

Our findings suggest that pyrazinamidase activity could be a valuable phenotypic marker to assist in the differentiation of *A. sobria* from *A. hydrophila* and *A. caviae*. We propose

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TABLE 1. Sources of *Aeromonas* isolates

Organism (*n*)	No. of isolates						
	ATCC[a]	Blood	Fecal	Wound	Miscellaneous	Environmental	Veterinary
A. hydrophila (37)	1	0	15	12	2	5	2
A. sobria (24)	1	0	20	2	0	0	1
A. caviae (53)	1	1	41	4	1	2	3
A. veronii (2)	1	0	0	1	0	0	0
A. schubertii (3)	1	0	0	2	0	0	0

[a] DNA hybridization group strains used were *A. hydrophila* ATCC 7966 (group 1), *A. sobria* ATCC 9071 (group 8Y), *A. caviae* ATCC 15468 (group 4), *A. veronii* ATCC 35624 (group 10), and *A. schubertii* ATCC 43700 (group 12).

that this test should be included in future biotyping scheme for mesophilic aeromonads. It is simple to perform, and positive and negative reactions are easily interpreted.

The results obtained with a limited number of *A. veronii* and *A. schubertii* strains suggest that this test may also prove to be useful for identifying these organisms. However, analysis of a larger sample of these strains is indicated before a final decision can be reached.

It has been proposed that *A. sobria* may indeed be the most virulent of the aeromonads causing human disease (1, 3). These observations, combined with the success obtained using pyrazinamidase activity as a test for virulence of clinical *Yersinia enterocolitica* strains (G. P. Carter, I. K. Wachsmuth, V. L. Miller, S. Falkow, and J. J. Farmer III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, C147, p. 356), suggest that negative pyrazinamidase activity might also be a new virulence-associated marker specific for *A. sobria* and the two most recently described human pathogens *A. veronii* and *A. schubertii*. Further studies are warranted to determine the nature of this association, i.e., whether or not negative pyrazinamidase activity is related to the presence of a virulence plasmid or some other as yet undetermined property of these species.

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I. Taxonomy, typing and isolation

Aeromonas update: New species and global distribution

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Summary. There are currently eight proposed or validated *Aeromonas* spp. of which five have been implicated in human disease: *A. hydrophila, A. sobria, A. caviae, A. veronii,* and *A. schubertii.* Recent studies have extended the geographic distribution and source of isolation of the newer species and resulted in the possibility of two new species, *A. jandaei* and *A. trota,* from diarrheal, wound, blood and environmental sources.
Key words. Aeromonas; taxonomy; biotyping; clinical.

Aeromonads are oxidase-positive, polarly flagellated, glucose-fermenting, facultatively anaerobic gram negative rods that are resistant to the vibriostatic compound 0/129 and autochthonous to aquatic environments worldwide [3,9]. There are currently eight proposed or validated phenotypic species or phenospecies that reside among at least 12 DNA hybridization groups or genospecies [2]. The genus consists of psychrophiles and mesophiles from aquatic and soil environments encompassing a diverse disease spectrum among many warm- and cold-blooded animals [8].

The earlier literature focused on all aeromonads as *Aeromonas hydrophila,* but several large studies have shown that the majority of clinical isolates fall within three species [2,10]: *Aeromonas hydrophila* (group 1), *A. sobria* (group 8), and *A. caviae* (group 4). However, in recent years two new species, *A. veronii* (group 10) and *A. schubertii* (group 12), were proposed as possible causes of human disease [5,6] and one new environmental species, *A. eucrenophila* (group 6), was delineated [11].

A. veronii (group 10) was originally published as a new DNA group of ornithine decarboxylase-positive, esculin-positive, and arginine dihydrolase-negative aeromonads that may cause diarrhea [5]. However, they were later found to be genetically identical to DNA group 8 *A. sobria.* This group includes nearly all clinical *A. sobria* isolates hybridized to date. Since the type strain for *A. sobria* (CIP 7433) resides in DNA group 7, it is now considered that DNA group 8 *A. sobria* is an ornithine-negative, esculin-negative, and arginine-positive biogroup of *A. veronii* (*A. veronii* biogroup *sobria*).

A. schubertii (group 12) is also a new DNA group of mannitol-negative, indole-negative aeromonads isolated from traumatic wounds and blood [6]. *A. eucrenophila* was proposed as a new species with the type strain ATCC 23309 and is actually an aerogenic, psychrotrophic isolate from fresh water formerly identified as an *A. caviae* [11].

Research on a large number of geographically diverse *Aeromonas* strains at the University of Maryland has extended the source of isolation and geographic isolation of *A. veronii* biogroup *veronii* and *A. schubertii* with the isolation of strains from traumatic wounds incurred in and around the Chesapeake Bay area of the United States (A. Carnahan, S. Behram, A. Ali, D. Jacobs and S. W. Joseph. Systematic assessment of geographically diverse *Aeromonas* spp. as a correlate to accurate biotyping. Abstr. Annu. Meet. ASM 1990).

Further, the existence of two new possible species was determined from this numerical taxonomy study. The first was the delineation of a cluster of nine 'esculin and sucrose-negative' *A. sobria.* These were found on both the East and West Coast of the United States and Hawaii and were isolated from wounds, blood, feces, and prawn. They all hybridized with the definition strain for DNA group 9 *A. sobria* and are proposed as *Aeromonas jandaei* [4].

The second possible new species arose from a second cluster of thirteen 'esculin-hydrolysis and Voges-Proskauer-negative, and cellobiose-positive' *A. sobria* that were, in addition, sensitive to ampicillin and carbenicillin, an extremely unusual trait for clinical aeromonads. They were found on both the East and West Coast of the United States, in Bangladesh, Indonesia, and India, and all but one were isolated from human diarrheal specimens. The nomenclature strain, AH2, was hybridized against all existing DNA group definition strains and found to represent a new and distinct DNA hybridization group of aeromonads isolated from clinical specimens and are proposed as *A. trota* (unpublished data).

Previous studies have suggested that species-related disease syndromes may exist among aeromonads, such as an association between *A. sobria* and bacteremia [7] and *A. caviae* and pediatric diarrhea [1]. Therefore, it cannot be overstressed that accurate biotyping of all aeromonad isolates, regardless of source and location, should be attempted using standard methodology and media. Only then can the true significance of these newer species be determined and used to evaluate the pathogenic mechanisms and virulence potential of these engimatic, aquatic microorganisms.

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Methods for the identification of DNA hybridization groups in the genus *Aeromonas*

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Summary. Among the 269 substrates tested in assimilation tests we found some that may help in the identification of DNA hybridization groups in the genus *Aeromonas*. In addition, isoenzyme analysis and ribotyping seem to be accurate although not routine procedures that allow discrimination between genetic species.
Key words. *Aeromonas*; DNA hybridization groups; assimilation; ribotyping; isoenzyme analysis.

According to Bergey's Manual of Systematic Bacteriology[13] the genus *Aeromonas* contains the three mesophilic species, *A. hydrophila*, *A. caviae* and *A. sobria*, as well as the psychrophilic species *A. salmonicida* with the three subspecies *salmonicida*, *achromogenes* and *masoucida*. Since then, several new entities have been described: *A. media*[1], *A. veronii*[8], *A. eucrenophila*[15], *A. schubertii*[7], and *A. salmonicida* subsp. *smithiae*[4]. However, these species still do not reflect the genetic complexity of the genus and they do not always correlate with genetic data, e.g. DNA hybridization values[14] (and F. W. Hickman-Brenner, G. R. Fanning, M. J. Arduino, D. J. Brenner and J. J. Farmer III, Abstr. Int. Workshop on *Aeromonas* and *Plesiomonas*, p. 51, 1988). The currently known DNA hybridization groups (DHGs) are made up of strains classified into the following phenotypic species[3,10]: DHG 1, *A. hydrophila*; DHG 2, *A. hydrophila*; DHG 3, *A. hydrophila* and *A. salmonicida*; DHG 4, *A. caviae*; DHG 5A, *A. caviae*; DHG 5B, *A. caviae* and *A. media*; DHG 6, *A. eucrenophila*; DHG 7, *A. sobria*; DHG 8, *A. sobria*; DHG 9, *A. sobria*; DHG 10, *A. veronii* (DHG 8 and DHG 10 have been shown to share enough DNA homology to be included in the same DNA hybridization group, DHG 8/10); DHG 11, *A. veronii*-like strains; DHG 12, *A. schubertii*. Two strains phenotypically similar to *A. schubertii* but indole-positive and lysine-negative have been found and were referred to as '*Aeromonas* group 501'[7]. Most probably, there are still other as yet not described DNA hybridization groups.

Phenotypic characteristics allow identification of the named species[1,3–5,7,8,10,13,15] but only few tests have been described that may aid in the separation of the various DHGs within these species[3]. We have, therefore, evaluated three completely different methods for their ability to separate the various DHGs in the genus *Aeromonas*.

Assimilation tests

We have analyzed 168 *Aeromonas* strains, belonging mainly to those DHGs that occur in clinical specimens (only strains of DHG 6 and DHG 7 have not been found in clinical specimens so far), for their ability to grow on minimal medium supplemented with different substrates. For this purpose, commercialized as well as research galleries in the form of either the API 50 or the API ATB strips were kindly provided by D. Monget (API SA, La Balme-les Grottes, France). Incubation was at 29°C for 48 h.

Binary Input Microbial

A PC-Based Procedure For Automated Classification and Identification of Populations With Binary Characteristics[a]

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[a]The FLOABN system may be licensed from the University of Maryland, College Park, MD. For more technical information contact Dr. S. W. Joseph at the address above. For licensing information contact the Office of Technology Liason, Attention: Wayne Swann, University of Maryland, College Park, MD 20742, Telephone (301) 405-4208, Fax (301) 314-9871.

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ABSTRACT

An integrated software system for taxonomic studies has been devised and tested using a database of mesophilic *Aeromonas* isolates. The software generated dichotomous keys and provided an alternative simple matching algorithm for the identification of unknown isolates. The software may be updated by the addition of analytical modules, the modification of the existing database, or the integration of new databases from other sources. The automated procedures are not limited to microbial taxonomy and may be adapted to a wide variety of other uses.

INTRODUCTION

Microbial taxonomic studies often involve large volumes of simple mathematical operations ideally suited for computers. The advent of relatively inexpensive desk-top computing devices will allow even the smallest research laboratories to input, document, process and analyze large volumes of taxonomic information. Although there are a variety of data management programs available, none offer the integration or flexibility necessary for true taxonomic research. Popular spreadsheet programs and data-managers such as Lotus 1-2-3 (Lotus Development Corporation, Cambridge, MA) and Microsoft Excel (Microsoft Corporation, Redmond, WA), offer superb data-management utilities but lack the programmability necessary in performing complex taxonomic analyses. Various other taxonomic software such as the SAS/TAXAN (SAS Institute, Cary, NC) clustering program (Maryland Sea Grant College, College Park) provide powerful statistical and graphical tools for taxonomy, but are deficient in database management and do not allow for modular expansion. Rapid identification systems such as the API system (Analytab Products, Plainview, NY) and other automated systems assist in automatic identification based on particular characteristics of the organisms, but the identification is based on a

limited and usually non-modifiable database. There is a clear need for a unified system which incorporates all of these elements to meet all of the requirements of taxonomists.

METHODS

An integrated system for taxonomic studies has been devised and was successfully tested on an IBM PS/2 model 50Z running under DOS 4.01. The system, called F.L.O.A.B.N. (For Lack Of A Better Name), is modular in design with each module being an independent program written in BASIC (Fig. 1). Each F.L.O.A.B.N. module or procedure (proprietary technology) could share information with any other module, allowing for complete versatility and expansion (Fig. 2). As new taxonomic or statistical procedures require assimilation, an earlier generation system can be modified or a new modular program can be written and integrated into the existing system.

Application

This taxonomic software was first used at the University of Maryland in an *Aeromonas* taxonomic study, for which a clinically useful identification schema was developed (Carnahan *et al.*, 1991). The study involved geographically and clinically diverse aeromonads (n=152) along with the appropriate ATCC Type strains and Centers for Disease Control (CDC) DNA group reference strains (n=15), for a total of 167 Operational Taxonomic Units (O.T.U.s). Seventy five phenotypic characteristics were studied, but only 50 of these phenotypic labels showed sufficient capacity for differentiation to include them for analysis by numerical taxonomy. The resulting matrix was analyzed by independent software which yielded 26 categories at a similarity level of 85% (Carnahan *et al.*, 1991).

Data Management

All of the information, including the test names, the O.T.U. designations and their cluster affiliations, was entered into the data editor module of the F.L.O.A.B.N. system (Fig. 3). All of the test data was binary in nature and, although the system could support unknown test results, there were no unknown values reported in the matrix (Table 1).

Since we were interested in separating certain clinically relevent clusters, the database was modified to create a special sub-set of selected O.T.U. clusters. In order to ultimately generate an identification scheme using this new database, it was necessary to generate frequency tables for each O.T.U. cluster (Table 2). This was done automatically through the F.L.O.A.B.N. software. Each frequency table combined the test results for all the O.T.U.s with the same cluster affiliation. The resulting averaged test results for each cluster could be regarded as those of a Hypothetical Taxonomic Unit (Austin and Priest, 1986). The differences in the profiles between the Hypothetical Taxonomic Units were used to generate a dichotomous identification key separating the O.T.U. clusters.

Dichotomous Keys

For purposes of discussing dichotomous keys, it is useful to develop a coordinate system for identifying different points on the dichotomous tree. We designated the downward progression of the tree as the "vertical number," and to its lateral distribution as its "horizontal number," as measured from left to right (Fig. 4). For ease of reference, these are designated as a "grid number," i.e. (Vertical Number, Horizontal Number). Thus, any point on the tree can be uniquely identified by specifying the grid number coordinates. The maximum horizontal number in a given key would always be equal to the total number of O.T.U.s in the tree. The relationship between the maximum horizontal number and the maximum vertical number would be:

Maximum Horizontal Number = 2 (Maximum Vertical Number -1)

Figure 5 summarizes the logical procedure used by F.L.O.A.B.N. to derive the identification key. The procedure begins by counting the total number of Hypothetical Taxonomic Units to be differentiated and determines the test character with the greatest discriminatory power. This is the test which best splits the total number of clusters into two, roughly equal numbers of clusters. Such a test forms the first branch of the identification tree. This procedure for character selection is repeated until all of the clusters are successfully separated.

More than one test may have the same discriminatory power. Depending on which run-mode the user has selected, the program may prompt the experimentor to choose from among the best tests, or it may select a single test from the group automatically. Once the identification key is complete, the program automatically tabulates the total number of unique tests required for the complete identification of all the clusters.

Upon completion of the identification tree, the program re-runs the entire analysis, allowing for different choices at each branch point of the identification key, where such choices are possible. This could result in a different identification scheme which may or may not use fewer tests than the previous run. This process is repeated, but a record of the shortest run is always stored on disk. Although it's unlikely that the computer could realistically exhaust all the combinations and permutations possible, this procedure does present a novel approach for the rapid generation of workable identification strategies.

Visual Data Display

The Visual Data Display (V.D.D.) is an independent F.L.O.A.B.N. module which enables the graphic visualization of the Hypothetical Taxonomic Units (Fig. 6). This permits the operator to scan large volumes of data visually and to detect trends and patterns in the frequency information. Furthermore, this module permits a completely different method for identification other than the dichotomous key approach already discussed. V.D.D. contains a simple matching algorithm for matching an unknown O.T.U. with the closest cluster from the database. The operator may substitute unknown characters where test results are not known or are not available.

Simple matching identification differs from dichotomous key identification in that the latter employs a small sub-set of the total number of tests and weighs each test differently. While this may be useful in a clinical setting, there may be times when this weighing may be undesirable. The simple matching algorithm is an alternative method which takes all of the tests into consideration, equally. V.D.D. permits the comparison of O.T.U. data entered from the keyboard or a file, with the Hypothetical Taxonomic Units of any other database. In Figure 6, the information regarding the unknown O.T.U is entered on the top of the screen, as represented by the long blocks. A black block represents a positive test result and a white block represents a negative test result. There is also a red block to represent unknown test characters (not shown). The frequency information is represented in a matrix, including four grades to denote averaged H.T.U character values from 0.0 to 1.0. This module will rank the clusters according to their similarity to the O.T.U. being identified and will report these to the operator, accordingly.

Once the identity of an unknown O.T.U. becomes known with confidence, the O.T.U. can be entered into the original database and the database can be re-compiled. It is even possible to generate another dichotomous identification key based on the new information.

The current version of the F.L.O.A.B.N. system was used to generate two identification keys: Aerokey I and Aerokey II, containing 17 and seven clusters, respectively (Carnahan *et al.*, 1991).

RESULTS

Aerokey II was evaluated in a single-blind trial of 60 arbitrarily chosen clinical aeromonads from an independent laboratory as described by Carnahan, et al., 1991, and further tested using additionally acquired ATCC and reference strains of some recently proposed species (n=18). The reference strains obtained included the following phenospecies: *A. veronii* bv. veronii (n=4), *A. schubertii* (n=3), *A. jandaei* (n=5), and *A. trota* (n=6).

Of the 60 randomly selected mesophilic aeromonads used in the trial of Aerokey II, 97% (n=58) were correctly identified to the species level. Furthermore, all 18 of the ATCC/ DNA group reference strains were correctly identified (Carnahan *et al*, 1991).

DISCUSSION

Data Management

F.L.O.A.B.N. provides special sub-routines for the rapid generation of taxonomic sub-sets from any database already entered into the system. There are a great number of approaches by which a database could be modified, and it was our intention to develop

automated routines which would greatly reduce the keystroking necessary for the repetitive or complex tasks inherent to taxonomic data management. It was decided that a menu approach to data-editing would be too cumbersome and too complicated for the user and we opted for the creation of a unique, interactive, macro-editing procedure. This routine permits the user to select from among appropriate command and action words which would be used to construct an English command phrase for execution. This simple approach to data management is shown in Fig. 7 and was used to create a special sub-set of clinically relevent clusters for analysis.

Identification Keys

Although there have been published computer procedures for the generation of identification keys based solely on mathematical principles (Matousek and Schindler, 1989), such procedures are often inadequate for the production of clinically useful identification strategies. For example, some of the 50 tests we used in the classification of our *Aeromonas* species would not be practical for clinical identification because of reproducibility or expense. We felt it critical to allow for the experimentor to be able to override the program's choices of key tests so as to insure a workable and pragmatic identification solution.

F.L.O.A.B.N. also provides a valuable expert mode for the construction of dichotomous keys. In this mode, the computer keeps track of the tests which have already been used in the identification key, and uses them again, whenever possible. The overall effect would be to construct a conservative dichotomous key which would require fewer different tests for the identification of all the O.T.U.s (Fig. 8).

Simple Matching Algorithm

This approach to identification would be most useful in a situation where the experimentor has performed a selected number of tests and wishes to know which other tests are absolutely necessary for the identification of the organism. V.D.D. can use the information from the tests already performed to generate a list of the most likely cluster affiliations for the unknown O.T.U. V.D.D. can even offer the second or third closest matches. Thus, the user can generate a list of the likely identities of the unknown organism. Using the macro-editing feature of the F.L.O.A.B.N. data editor, the experimentor may create a sub-set of the original database which will only contain the clusters which were displayed by the V.D.D. algorithm. The analytical sub-routines will attempt to devise a dichotomous key for the small sub-set of clusters, thus, revealing the tests the experimentor should perform to delineate the identity of the unknown O.T.U.

System Flexibility

The F.L.O.A.B.N. analytical module, in devising dichotomous keys, allows for four different modes of operation, ranging from a fully manual character selection to a fully automated mode of test selection. In the latter three operating modes, the software must use a strategy for selecting important tests for separating the clusters. A "Greedy Algorithm," written in Pascal, has been reported to offer one solution to this type of identification problem by placing importance on choosing the "best test" at each branch point (Matousek and Schindler, 1989). Our approach uses a similar method but differs in two ways. First, F.L.O.A.B.N. allows an "expert" mode which reduces the total number of tests required and, second, it permits using tests which fail the "best test" criteria.

The standardized F.L.O.A.B.N. system also provides a method for the rapid transmission and integration of information from one laboratory to another. We are currently working

on a protocol for the sharing of taxonomic information via computer disks, modem links, and even UPC-like labels. We also hope to develop a "neural net" identification module to supplement the existing identification schemes in F.L.O.A.B.N.

Clinical Laboratory Utility

The success of the two identification strategies suggests that the validity of the procedure is dependent on both the automated portion and on the intuition and the experience of the scientist. It is important for any useful clinical identification strategy to embrace practicality and for the automated software to reflect this need. Integrated taxonomic software, such as F.L.O.A.B.N., offer the taxonomist a dynamic tool for exploring taxonomic and evolutionary relationships between different taxa. The software, and the numerical taxonomic procedures in general, are in no way restricted to the field of microbiology. In fact, any field or discipline which depends upon sorting, organizing and retrieving information for identification can benefit from these computer technologies.

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Program Name	Description
Micron	Responsible for management and integration of all F.L.O.A.B.N. modules. Permits user to operate different programs without exiting to DOS.
F.L.O.A.B.N. Editor/ Analyzer	Allows for creating / loading / saving binary taxonomic databases. Provides data-editing and data-management routines. Formulates frequency tables. Formulates identification strategy based on "best test" criteria.
Flowchart Graphics Viewer	Permits graphical representation of identification key formed by the F.L.O.A.B.N. Editor/ Analyzer.
Visual Data Display	Permits graphical representation of the frequency computations by the F.L.O.A.B.N. Editor/ Analyzer. Provides an alternative identification procedure involving "simple matching."
Initial Info Files	Auxiliary files necessary for the proper function of Micron.
Data	Selected database information plus all associated files.

Figure 1 Description of selected modules of the F.L.O.A.B.N. system.

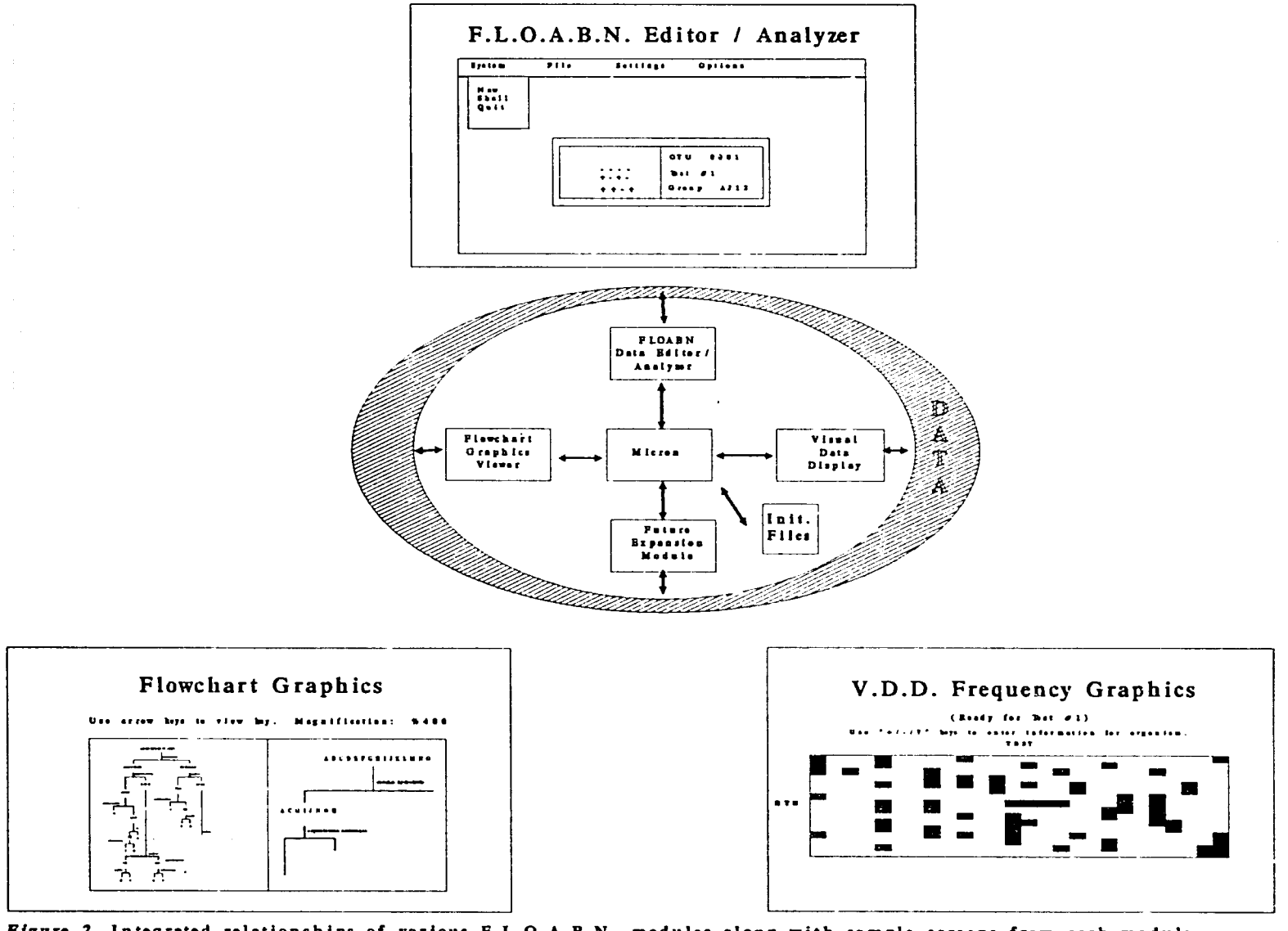

Figure 2 Integrated relationships of various F.L.O.A.B.N. modules along with sample screens from each module.

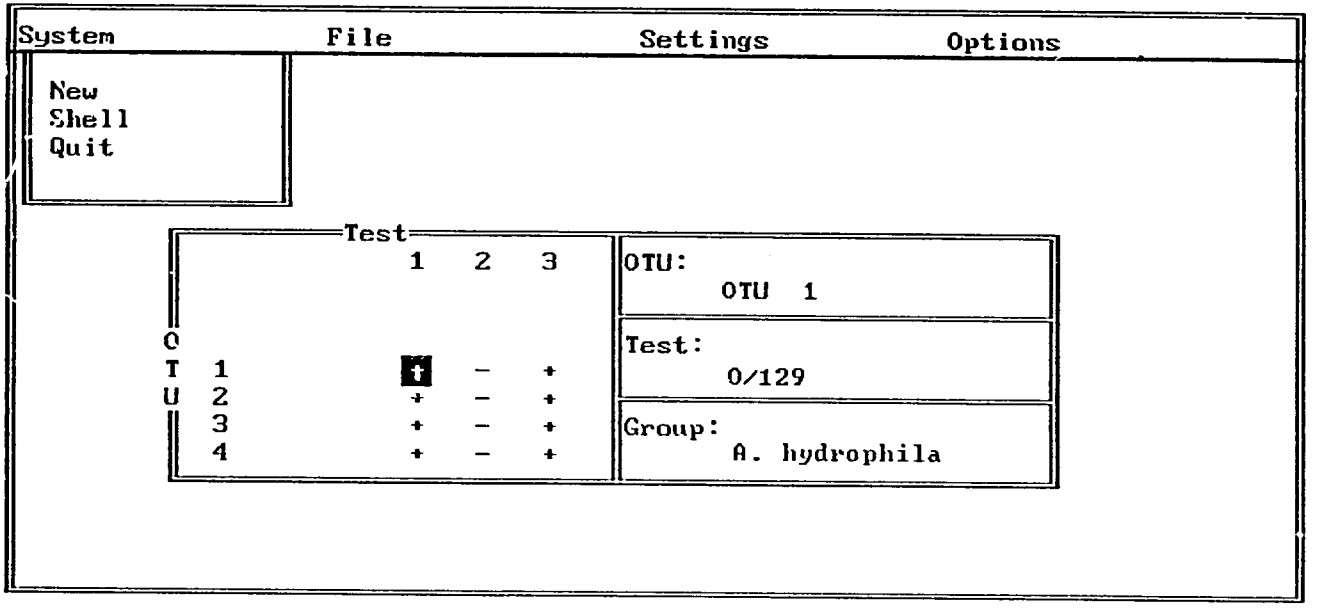

Figure 3 Data-entry screen used to view and edit the database. The selected piece of information is highlighted in the left viewport while information regarding the O.T.U. is displayed at the right.

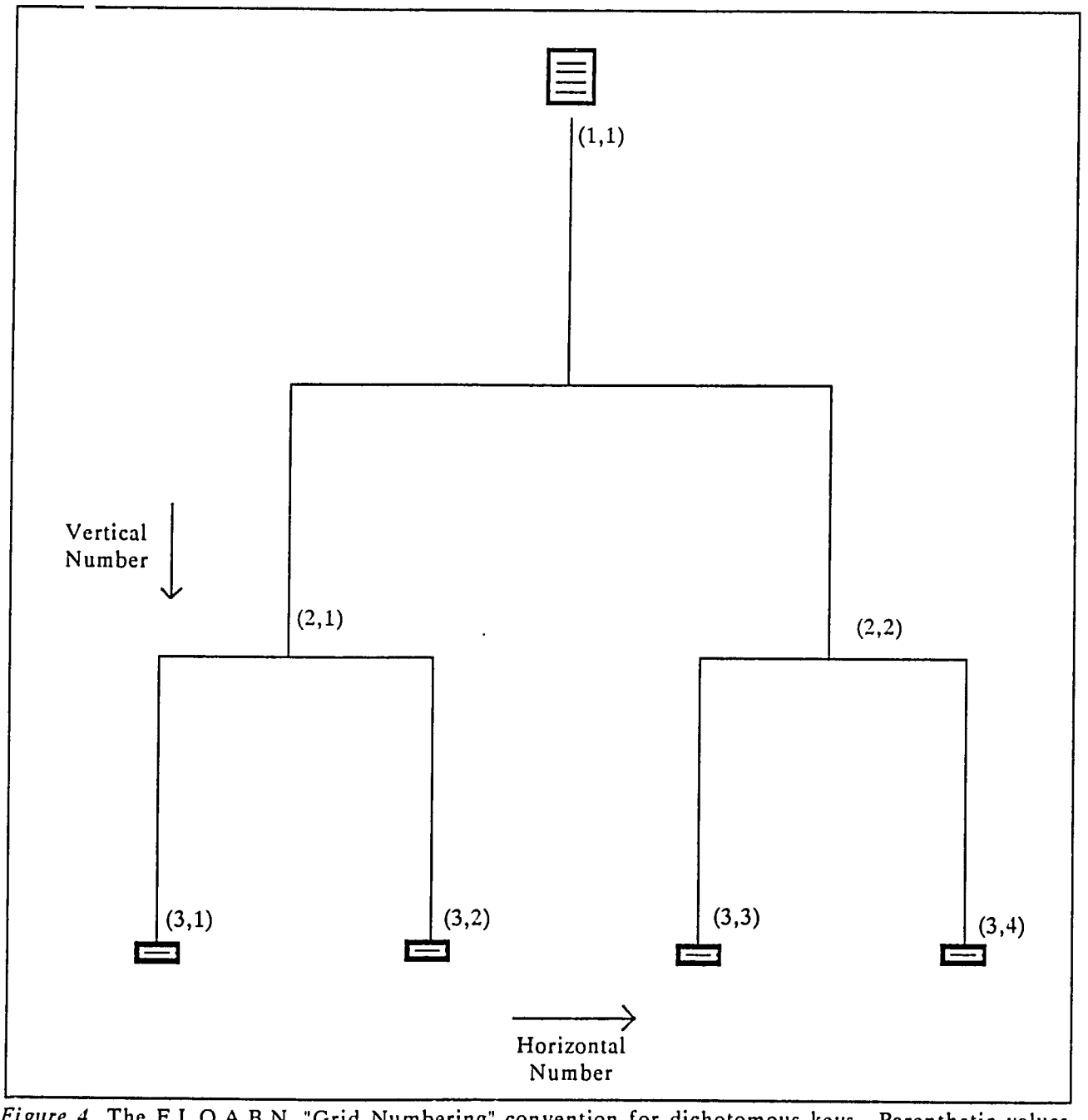

Figure 4 The F.L.O.A.B.N. "Grid Numbering" convention for dichotomous keys. Parenthetic values are in (Vertical Number, Horizontal Number) format.

1. Determine size of matrix

    1a. Count number of tests.
    1b. Count number of O.T.U.'s.
    1c. Count number of clusters.

2. Compute averaged test results for each cluster.

3. Begin generation of key.

    3a. Count all clusters at current Grid Number.
    3b. Scan all tests for "best test."
    3c. Allow for user selection -or- select automatically.
    3d. Split the clusters according to test selected.
    3e. Increment pointer to next Horizontal Number (H.N.).
    3f. If less than the Maximum H.N., then go to "3a."
    3f. Reset H. N. to one and increment Vertical Number (V.N.).
    3g. Go to "3a" until Maximum V. N. and Maximum H. N. are reached.

4. Tally total number of tests used for identification key.

5. Save key to disk.

6. If shorter than best run, save as best run.

7. Repeat "3" until interrupted by user.

Figure 5 Summary of steps used by F.L.O.A.B.N. to generate dichotomous keys.

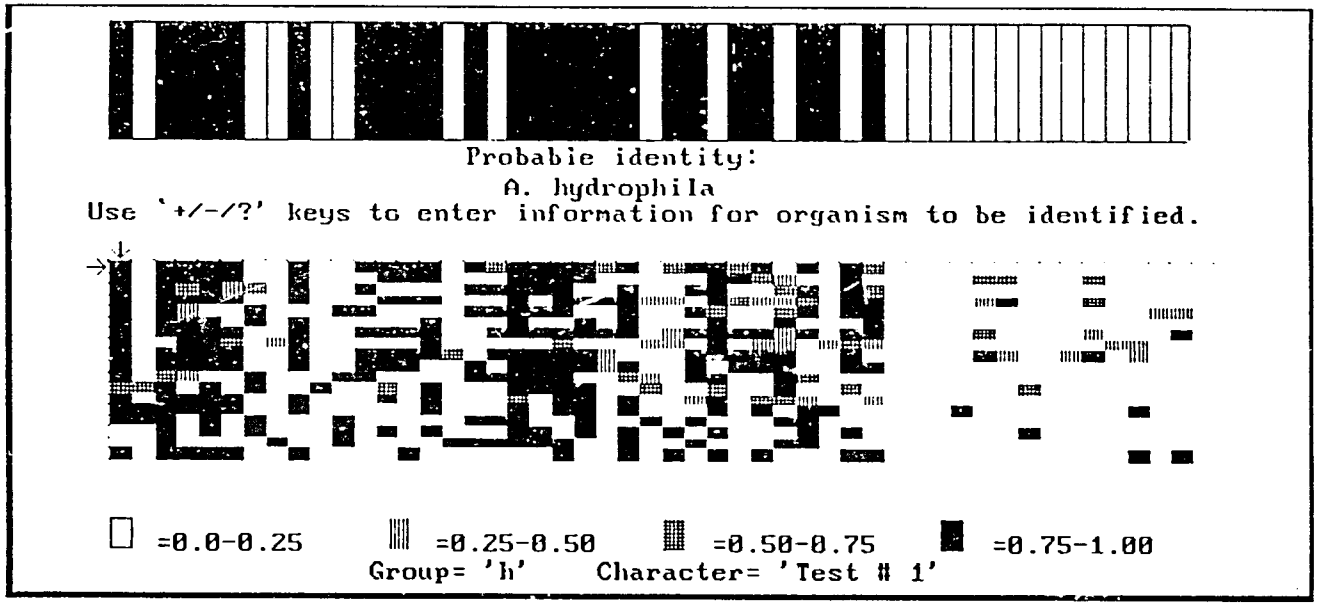

Figure 6 Sample screen from V.D.D. module comparing new O.T.U. information (top) with the H.T.U. patterns (below). The name of the closest match is displayed.

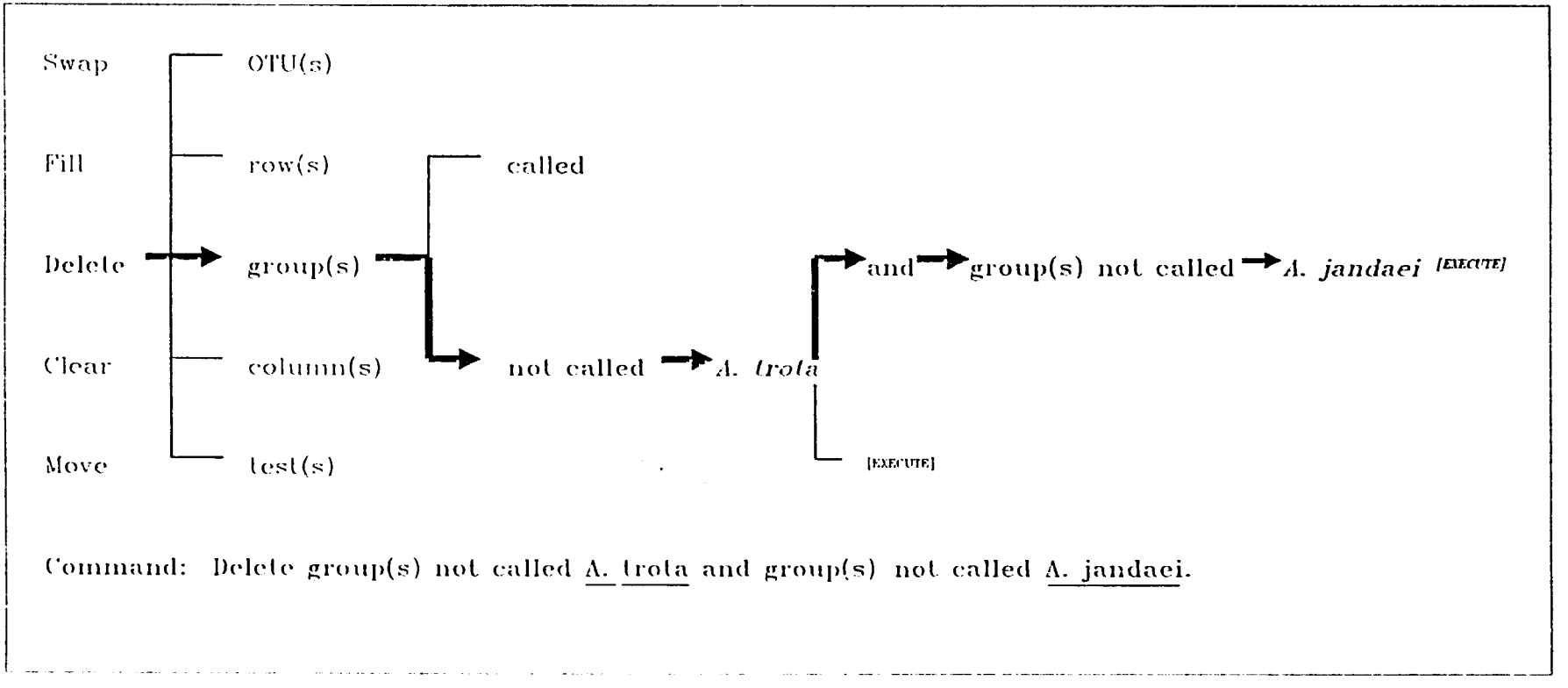

Figure 7 An example of a plain-English directive used to generate a smaller sub-set of the database containing only two clusters. The language interface provides the user with appropriate keywords and action words with which a command phrase can be formed.

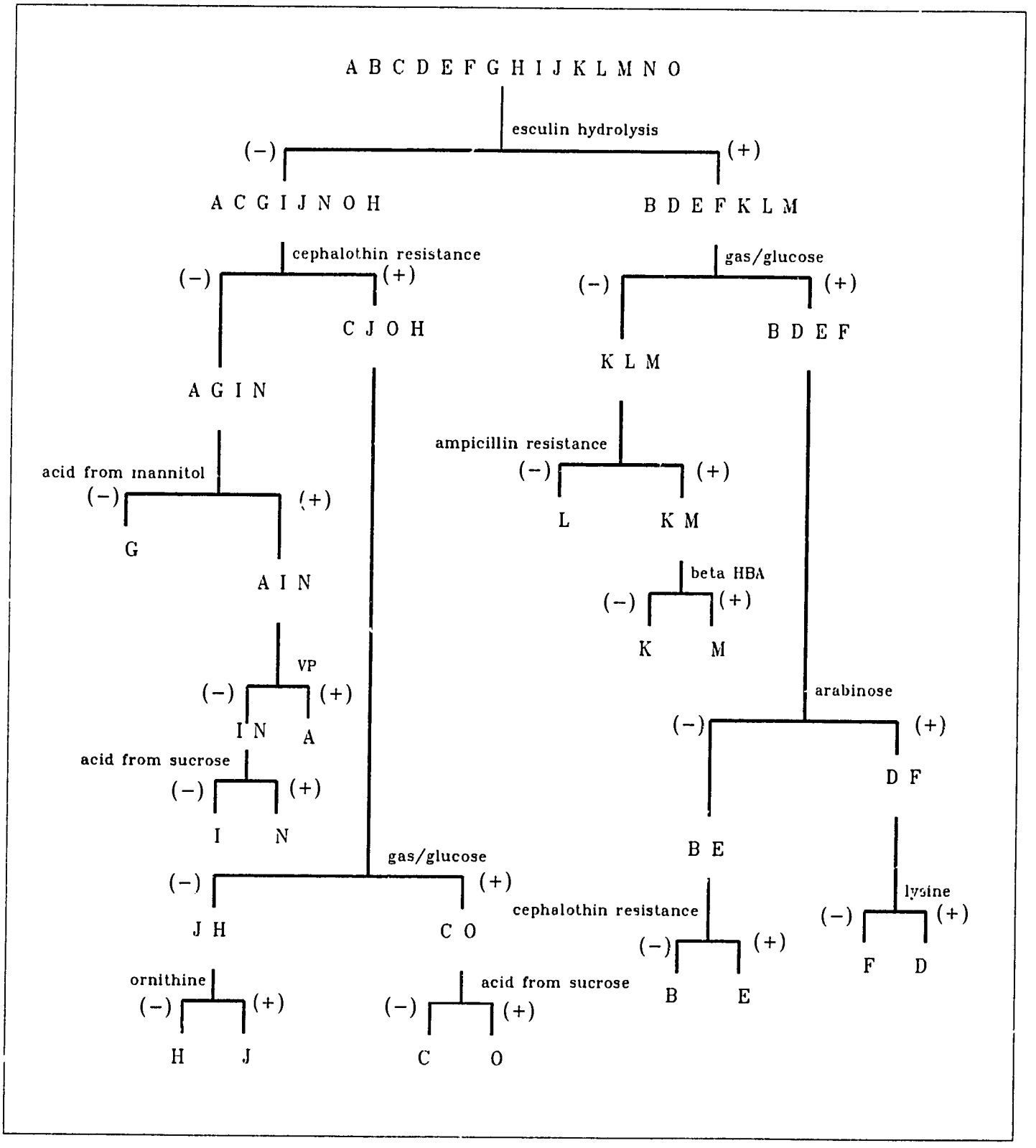

Figure 8 A dichotomous key demonstrating complete identification of 15 hypothetical clusters, using only 11 tests. Note that some tests appear at more than one branch, minimizing the total number of unique tests required. Clusters represent fictional data and are not statistically valid.

Table 1 Partial listing of raw *Aeromonas* data as entered into F.L.O.A.B.N. Actual matrix dimensions were 160 x 50.

O.T.U.	Test																
	O/129	String Test	Indole	ß stab SBA	ß Stab HBA	ß on SBA	Alpha on SBA	Gamma on SBA	ß HBA	Alpha on HBA	Gamma on HBA	Esculin	Gas/ Glucose	VP	Lysine	Orni-thine	Arabi-nose
1	+	–	+	+	+	+	–	–	+	–	–	+	+	+	+	–	+
2	+	–	+	+	+	+	–	–	+	–	–	–	+	+	+	–	+
3	+	–	+	–	+	–	+	–	+	–	–	+	–	–	–	–	+
4	+	–	+	+	+	+	–	–	+	–	–	+	+	–	+	–	+
5	+	–	+	+	+	+	–	–	+	–	–	–	+	+	+	–	–
6	+	–	+	–	–	–	+	–	–	–	+	+	–	–	–	–	+
7	+	–	+	+	+	+	–	–	+	–	–	+	+	+	+	–	+
8	+	–	+	+	+	–	+	–	+	–	–	+	–	–	–	–	+
9	+	–	+	+	+	–	+	–	+	–	–	–	–	–	+	–	–
10	+	–	+	+	+	+	–	–	+	–	–	+	+	–	+	–	–
11	+	–	+	+	+	+	–	–	+	–	–	+	+	+	+	–	–
12	+	–	+	–	+	–	+	–	+	–	–	+	–	–	–	–	+
13	+	–	+	+	+	+	–	–	+	–	–	+	+	+	+	–	+
14	+	–	+	+	+	+	–	–	+	–	–	+	+	–	+	–	–
15	+	–	–	+	+	+	–	–	+	–	–	–	–	–	+	–	–
16	+	–	+	–	+	–	+	–	+	–	–	+	–	–	–	–	+
17	+	–	–	+	+	+	–	–	+	–	–	–	–	–	+	–	–
18	+	–	+	+	+	+	–	–	+	–	–	+	+	+	+	–	–

Table 2 Partial listing of the frequency table compiled from 18 clusters from the *Aeromonas* database using the F.L.O.A.B.N. automated procedures. Actual matrix dimensions were 18 x 50. All values denote fraction positive.

Cluster	Test										
	O/129	String Test	Indole	ß stab SBA	ß stab HBA	ß on SBA	Alpha on SBA	Gamma on SBA	ß HBA	Alpha on HBA	Gamma on HBA
h	1.00	0.07	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
b	1.00	0.00	0.96	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
x	1.00	0.00	0.96	0.60	0.96	0.39	0.66	0.00	0.87	0.12	0.00
f/g	0.75	0.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
v	1.00	0.06	1.00	0.31	0.18	0.06	0.93	0.00	0.00	0.00	1.00
o	1.00	0.00	1.00	1.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00
i	1.00	0.10	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
k/l	1.00	0.00	0.00	1.00	1.00	0.66	0.00	0.33	1.00	0.00	0.00
c	1.00	0.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
j	1.00	0.20	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
w	1.00	0.12	0.50	0.37	0.12	0.00	1.00	0.00	0.12	0.12	0.75
y	0.50	0.50	1.00	0.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00
n	1.00	0.14	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
p	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00
q	1.00	1.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	1.00
z	0.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	1.00
t	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	1.00
m	1.00	0.00	1.00	1.00	1.00	1.00	0.00	0.00	1.00	0.00	0.00

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Aerokey II: A Flexible Key for Identifying Clinical *Aeromonas* Species

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A small subset (n = 18) of highly discriminatory tests was derived from the feature frequency of 50 tests used in the study of 167 predominantly clinical *Aeromonas* strains. Seven of these eighteen tests were used to construct a flexible, dichotomous key, Aerokey II, for identifying clinical aeromonads: esculin hydrolysis, gas from glucose, acid from arabinose, indole production, acid from sucrose, Voges-Proskauer reaction, and resistance to cephalothin (30 μg). This schema was initially evaluated in a single-blind trial of 60 well-characterized clinical *Aeromonas hydrophila* (n = 21), *A. caviae* (n = 19), and *A. veronii* bv. sobria (n = 20) strains from an independent laboratory. Of the 60 strains tested, 58 (97%) were accurately identified to the species level. Aerokey II was further evaluated with 18 additional American Type Culture Collection and reference strains representing the more recently proposed taxa *A. veronii* bv. veronii, *A. schubertii*, *A. jandaei*, and *A. trota* and accurately identified all of these strains.

During the past 20 years there has been a trend toward the recognition of mesophilic aeromonads as causative agents of human disease (27). Concomitantly, there has been an increase in the numbers of clinical cases reported, originating with sporadic case reports of aquatic wound infections in immunocompromised individuals and expanding to numerous associations with disseminated disease (septicemia, meningitis, and osteomyelitis), gastroenteritis, and wound infections among pediatric and adult populations, both immunocompromised and otherwise healthy (17, 44). This accumulation of information has attracted the interest of not only the medical community but also systematists and infectious disease researchers, as reflected by the publication of four review articles on aeromonads in just the last three years (2, 6, 27, 30).

The taxonomy of aeromonads has also been constantly changing. Major taxonomic studies conducted over the last 5 years have provided some clarification of the systematics of aeromonads with respect to the number of DNA hybridization groups (genospecies) and phenotypic species (phenospecies) which currently exist among aeromonads (3, 4, 7, 24, 31). There are now at least seven established or recently proposed taxa of the genus *Aeromonas* associated with human disease (Table 1).

However, often the new additions to the genus *Aeromonas* have actually contributed to and even exacerbated the existing confusion in the taxonomy. A case in point is the species *Aeromonas sobria*. Originally proposed by Popoff and Veron in 1976 on the basis of a study of 68 mostly environmental strains, this species was found to encompass at least three separate DNA hybridization groups (DNA groups 7, 8, and 9) with the type strain, CIP 7433, residing in DNA group 7 (14, 39, 40). However, later taxonomic studies of large numbers of clinical isolates revealed that all clinical isolates that resembled the *A. sobria* phenotype were found by DNA hybridization to actually reside in DNA group 8 (3, 4, 7, 14, 24, 31).

Subsequent to this work, a new species, *A. veronii* (ornithine decarboxylase positive) was proposed as DNA group 10, since it did not hybridize with any of the known *Aeromonas* type strains, including that of *A. sobria* (DNA group 7) (22). However, the authors revealed in an addendum in proof to this proposal that clinical *A. sobria* (DNA group 8) was genetically identical to *A. veronii* (DNA group 10). The dilemma is that although the species *A. sobria* (DNA group 7) is indeed a valid one, it is represented by only a small number of environmental strains, including the type strain, CIP 7433. To date, all clinical strains resembling *A. sobria* belong to DNA group 8, not DNA group 7, and since DNA group 8 is genetically identical to DNA group 10 *A. veronii*, what we have previously identified as clinical *A. sobria* strains must now be considered as strains of a biovar of *A. veronii*.

We have proposed the names *A. veronii* bv. veronii for the ornithine decarboxylase-positive strains and *A. veronii* bv. sobria for those clinical strains that we formerly considered *A. sobria* in an effort to alleviate the inherent confusion and more accurately reflect what has been discovered at the genospecies level (8). While it may seem confusing to use the epithet sobria as part of the nomenclature for two distinct genospecies, it appears to be at present the best solution to an already complex situation. Only in this way will clinical microbiologists and physicians be aware that clinical strains formerly identified as *A. sobria* are now a biovar of *A. veronii*. It is because of the strong association between these formerly identified *A. sobria* strains and bacteremia and because of their possession of markers for increased virulence (26, 29) that this change in taxonomic designation must reflect what has already been presented in the literature.

Although a majority of publications and most rapid identification systems still use the nomenclature *A. hydrophila* or *A. hydrophila* group for any clinical *Aeromonas* isolate, the tide of taxonomic research clearly suggests that we have the means to identify clinical aeromonads to the species level (3, 7, 9, 25, 39). There is also sufficient evidence to indicate that there are possibly species-related disease syndromes, such as the previously mentioned bacteremia with *A. veronii* bv. sobria (26), pediatric diarrhea with *A. caviae* (1, 36), aquatic wound infections with *A. schubertii* (11, 21), and wound infections with *A. hydrophila* following the use of medicinal

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TABLE 1. Current taxonomic status of the genus *Aeromonas*

Genotype (DNA group)	Phenotype (phenospecies)[a]
1[b]	*A. hydrophila* (ATCC 7966[T])
2	*A. hydrophila*
3	*A. salmonicida*
4[b]	*A. caviae* (ATCC 15468[T])
5	*A. media*
6	*A. eucrenophila* (ATCC 23309[T])
7	*A. sobria* (CIP 7433[T])
8/10[b]	*A. veronii* bv. sobria[c] (ATCC 9071)
9[b]	*A. jandaei* (ATCC 49568[T])
8/10[b]	*A. veronii* bv. veronii (ATCC 35624[T])
11	*Aeromonas* spp. ornithine decarboxylase positive (ATCC 35941)
12[b]	*A. schubertii* (ATCC 43700[T])
13	*Aeromonas* group 501 (ATCC 43946)
14[b]	*A. trota* (ATCC 49657[T])

[a] Strains in parentheses are type ([T]) or representative strains.
[b] Genospecies isolated from clinical specimens (3, 4, 8, 10, 21, 22, 24).
[c] Formerly identified as clinical *A. sobria*, but genetically identical to *A. veronii* (3, 8, 22).

leeches (42). This variability in pathogenicity, coupled with the existence of different antibiograms among the species, e.g., resistance to cephalothin (28) or susceptibility to ampicillin (10), could affect a physician's subsequent successful treatment of a patient. However, no previous studies have resulted in a workable identification schema for the mesophilic *Aeromonas* species associated with human disease. Hence, most clinical isolates are still identified as either *A. hydrophila* or *A. hydrophila* group whether conventional methods or rapid identification systems are used.

The objective of this research was to examine a large number of clinical *Aeromonas* isolates from diverse clinical and geographic sources and use the frequency matrix of test results from a numerical taxonomy analysis to develop a highly discriminatory subset of tests. These tests (n = 18) were then used to construct a flexible dichotomous identification key, Aerokey (proprietary technology), that with only seven tests allows a clinical laboratory to accurately identify *Aeromonas* isolates to the species level.

(Part of this work was conducted by A. M. Carnahan in partial fulfillment of the requirements for an M.S. from the University of Maryland, College Park, and was subsequently presented at the 3rd International *Aeromonas/Plesiomonas* Symposium, Helsingør, Denmark, 5 to 6 September 1990.)

MATERIALS AND METHODS

Bacterial strains. One hundred fifty-two strains, initially received from 1985 to 1989 as *Aeromonas* spp., were used for this study. They were collected in several geographical regions, including the United States (n = 78), northeastern Africa (Somalia, the Sudan, and Egypt) (n = 50), Bangladesh (n = 19), India (n = 1), Indonesia (n = 3), and Puerto Rico (n = 1). The majority of the strains came from a variety of clinical sources (n = 131), but some were veterinary (n = 8) or environmental (n = 13) strains. All major American Type Culture Collection (ATCC) type strains, the Centers for Disease Control reference definition strains for DNA hybridization groups 1, 2, 3, 4, 5a, 5b, 6, 7, 8X, 9, 10, 11, and 12, and ATCC reference strains for *A. veronii* bv. sobria (DNA group 8Y) and *Aeromonas* group 501 (DNA group 13) were included as controls.

The strains were stored at −70°C in Trypticase soy broth with 10% glycerol (Remel, Lenexa, Kans.) and subsequently subcultured to tryptic soy agar (TSA) plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and incubated overnight at 36°C. Unless stated otherwise, all analyses were performed at 36 ± 1°C.

Presumptive identification. All strains were initially screened by using the following tests: Gram stain, oxidase activity (1% solution of *p*-aminodimethylaniline oxalate; Difco, Detroit, Mich.), glucose fermentation using a triple sugar iron (TSI) slant, motility by "wet mount" after incubation at 25°C, and resistance to O/129 (a vibriostatic agent) (150 μg/ml; Oxoid, Ogdensburg, N.Y.). Only those strains that were motile, oxidase positive, glucose-fermenting, O/129-resistant, gram-negative rods were considered aeromonads. They were then presumptively identified as belonging to the *A. hydrophila* group with an API-20E strip (Analytab, Inc., Plainview, N.Y.).

Biochemical phenotypic markers. Each strain was further examined for production of diffusible pigments on TSA; indole production (both Kovacs's and Ehrlich's methods); esculin hydrolysis; growth in KCN; acid production from arabinose, salicin, mannitol, and sucrose; production of gas from glucose; production of ornithine decarboxylase; String test (20); arbutin hydrolysis (16); and H_2S production from cysteine with a modified gelatin-cysteine-thiosulfate (GCF) medium (43). Additional phenotypic tests that have been associated with specific biotypes or used as potential virulence-associated markers were also evaluated. These were lysine decarboxylase production, the Voges-Proskauer reaction, hemolysis on TSA plates with 5% sheep blood by streaking and stabbing, hemolysis on TSA plates with 5% horse blood by streaking and stabbing, acriflavine agglutination (32), autoagglutination (29), production of a CAMP-like factor (aerobically and anaerobically) (15), cephalothin susceptibility (28), and pyrazinamidase activity (9).

Excluding the tests on the API-20E strip and those referenced above, all tests were performed by conventional methods (13, 34) based on the Janda modification (25) of the original schema of Popoff and Veron (39) with media and reagents supplied by Remel. Both positive and negative control *Aeromonas* ATCC strains were included for each test (Table 1, footnote *b*).

Comparison of test methods. Several of the tests (n = 11) used in the initial phenotypic analysis of these strains were performed by two or sometimes three different methods, or with different media formulations, in an effort to determine whether the results were comparable to each other. These results are listed in Table 2 as percent positive feature frequencies (percentage of strains giving positive results) for the different methods or media used.

Antibiotic resistance markers. MICs were determined for 17 antimicrobial agents in a 96-well microdilution plate (Gram Negative Panel 7; MicroScan Division, Baxter Healthcare Corp., West Sacramento, Calif.) per the manufacturer's instructions. In addition to these 17 antimicrobial agents, the MicroScan panel tested for a "growth" or "no growth" response against the antimicrobial agents colistin, nitrofurantoin, kanamycin, and penicillin in single-dilution wells. The MIC results were recorded both manually and with an AutoScan-4 reader with computer-assisted analysis by an IBM PS/2 Model 60 which provided interpretations in accordance with the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (37). The antimicro-

bial agents whose MICs were determined were amikacin, ampicillin, cefazolin, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, cephalothin, ciprofloxacin, gentamicin, imipenem, piperacillin, tetracycline, ticarcillin-potassium clavulanate, ticarcillin, tobramycin, and trimethoprim-sulfamethoxazole.

Cephalothin susceptibility was also tested by the Bauer-Kirby agar disk diffusion method (5) using a 30-μg disk and Mueller-Hinton agar (Becton Dickinson Microbiology Systems) per NCCLS standards.

Numerical taxonomy analysis. Examination of the percent positive frequency results for all of the strains examined revealed those phenotypic tests, both biochemical and antimicrobial, whose results varied from strain to strain at a level greater than 5% but less than 95%. Consequently, only the results for those 50 tests were used in the numerical taxonomy analysis. The data were examined with the SAS/TAXAN (SAS Institute, Cary, N.C.) clustering program (Maryland Sea Grant College, College Park) on an IBM 4381 mainframe computer by using the simple matching (S_{SM}) coefficient at a similarity level of 85% to delineate clusters of strains that represented distinct phenospecies (7). Only those clusters which represented the established and proposed clinical species were used to construct Aerokey II, and each cluster contained the appropriate ATCC or DNA definition group strain for that phenospecies. These clusters were composed of *A. hydrophila* (n = 46), *A. caviae* (n = 33), *A. veronii* bv. sobria (n = 26), *A. veronii* bv. veronii (n = 4), *A. schubertii* (n = 3), *A. jandaei* (n = 4) and *A. trota* (n = 7). Once the phenotypic clusters were determined, a frequency matrix was constructed from all of the test results (percent positive results of each of the clusters) on an IBM PS/2 Model 50 computer using a FREQ program written in BASIC by one of the authors of this article (S. Behram). Next, the data base was searched for those tests whose frequencies of positive results were above 70% or below 30% for these specified clusters. Only these tests were used in constructing the identification keys.

FLOABN, a greedy algorithm program. Another computer program, FLOABN (For Lack of a Better Name), which used the frequency data from the SAS/TAXAN analysis, was written in BASIC by S. Behram as a modified version of a recently published Pascal program (33). The FLOABN (proprietary technology) program compared the SAS/TAXAN-derived clusters for the clinical phenotypic species and, using the frequency matrix of results outlined above, constructed a dichotomous identification key by solving a simple test selection problem with a "greedy algorithm." FLOABN was written with a semiautomatic mode that allowed the operator, while constructing the dichotomous key, to select at each level from a number of equivalent, first-choice tests based on media and methods available in clinical laboratories. For this study, several different runs were conducted with many different equivalent first-choice tests at each level, and the result was a small subset (n = 18) of highly discriminatory tests (Table 3).

Aerokey I. The clusters initially examined by FLOABN were those that encompassed all of the DNA hybridization groups and ATCC type and reference strains (n = 17). This resulted in a dichotomous key, Aerokey I (7), that uses 12 of the original 50 tests to separate all currently recognized genospecies (DNA hybridization groups) except DNA groups 2 and 3 (data not shown). Because the collection of strains often had only a single strain to represent the rare environmental genospecies, e.g., *A. eucrenophila* and *A. media* (7), Aerokey I should not yet be considered statistically valid for the identification of every genospecies.

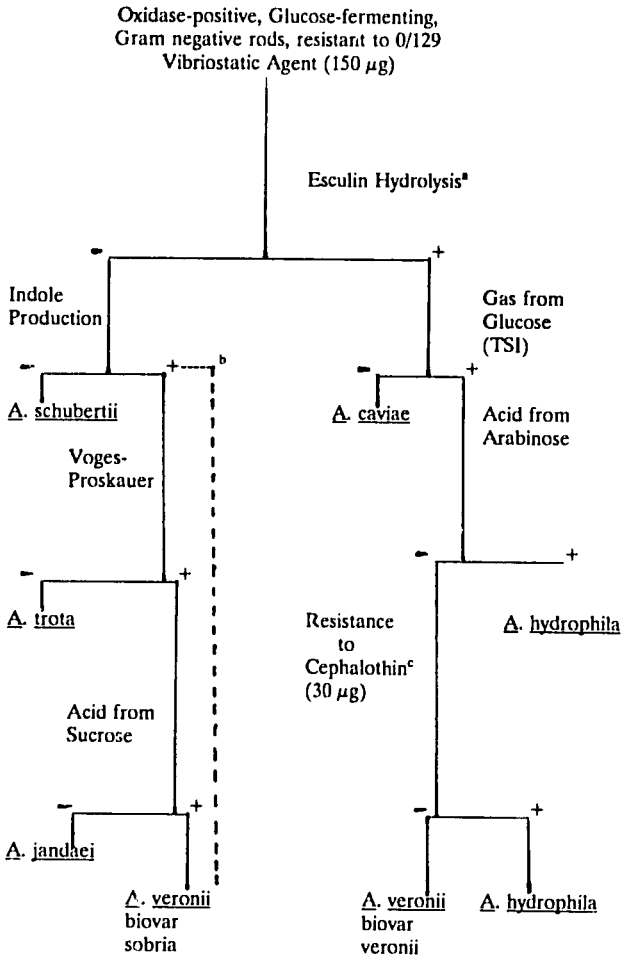

FIG. 1. Aerokey II identification key for clinical *Aeromonas* species. a, agar formulation only; b, Aerokey II can be modified to end here with an identification of *A. veronii* bv. sobria; c, Bauer-Kirby disk diffusion method only.

Rather, it was constructed to serve as an experimental guideline for those laboratories attempting to identify large groups of aeromonads from clinical, veterinary, and environmental sources for taxonomic, epidemiological, or virulence-related studies (unpublished data).

Aerokey II. Subsequently, the FLOABN program was used to examine those clusters that represented all even clinical *Aeromonas* taxa: *A. hydrophila*, *A. caviae*, *A. veronii* bv. sobria, *A. veronii* bv. veronii, *A. schubertii*, *A. jandaei*, and *A. trota*. This process generated Aerokey II, which contains seven tests for identification to the species level (Fig. 1).

The validity of Aerokey II was first evaluated for the identification of the three mesophilic *Aeromonas* species most commonly encountered in clinical laboratories. This was accomplished through a single-blind trial of 60 arbitrarily chosen clinical *Aeromonas* isolates from the private collection of an independent laboratory (Table 4). The strains had already been extensively phenotyped, but for this study they were identified by code number only and accompanied by the results for the seven tests used in Aerokey II. These tests were for esculin hydrolysis, gas from glucose, acid from arabinose, indole production, acid from sucrose, the Voges-Proskauer reaction, and resistance to cephalothin

TABLE 2. Results of comparative test methods and media

Test and medium	No. (%) of strains testing positive (n = 167)
Esculin hydrolysis (EHA)	114 (68)
Bile esculin hydrolysis (BEHA)[a]	114 (68)
Gas from glucose	
TSI	91 (54)
1% Andrade's broth with Durham vial	90 (54)
Cephalothin resistance	
Bauer-Kirby method	119 (71)
MicroScan MIC method	122 (73)
Lysine decarboxylase	
API-20E	102 (61)
Moeller's method	97 (58)
Ornithine decarboxylase	
API-20E	11 (7)
Moeller's method	5 (3)
Indole production	
API-20E	158 (95)
Kovacs's reagent on TSA with 5% sheep blood	156 (93)
Voges-Proskauer	
API-20E	95 (57)
MR-VP[b] broth	87 (52)
Acid from arabinose	
API-20E	92 (55)
1% Andrade's broth	96 (57)
Acid from mannitol	
API-20E	156 (93)
1% Andrade's broth	159 (95)
Acid from sucrose	
API-20E	145 (87)
1% Andrade's broth	146 (87)
H_2S Production	
Modified GCF medium	102 (61)
TSI agar stab	5 (3)
API-20E	0 (0)

[a] EHA, esculin hydrolysis agar; BEHA, bile esculin hydrolysis agar.
[b] MR-VP, methyl red–Voges-Proskauer.

(Fig. 1). Acid from arabinose is present at two branches in the key because of the possible existence of two distinct biovars of *A. hydrophila* within the same genospecies (7). Because of the rare isolation to date of the more recently proposed taxa *A. veronii* bv. veronii, *A. schubertii*, *A. jandaei*, and *A. trota*, 18 additional ATCC and reference strains for these species were obtained and tested with Aerokey II.

RESULTS

Comparison of test methods and media. The ability of aeromonads to hydrolyze esculin was evaluated with both esculin hydrolysis agar and bile esculin hydrolysis agar. Both media were incubated at 36°C for 72 h. This was done to determine the possibility of an inhibitory effect on the growth of aeromonads by the bile in bile esculin hydrolysis agar, which would preclude a positive test result on this medium. Bile esculin hydrolysis agar was found to be equivalent to esculin hydrolysis agar in sensitivity and specificity, with no false positives or false negatives occurring on the bile formulation medium (Table 2). It can be concluded that bile esculin hydrolysis agar, available in most clinical laboratories for the identification of *Enterococcus* spp., can be used for identifying aeromonads. However, it should be noted that the evaluation of two different rapid identification systems' esculin broth formulation, using these same strains, resulted in a large number of false-negative reactions (no black precipitate), and therefore the broth formulation is not considered comparable to the agar formulation pending further studies (7).

The production of gas from glucose fermentation was evaluated by examination of an 18-h TSI slant as well as the conventional tube method using Andrade's broth with 1% dextrose and a Durham vial for detection of gas. The TSI slant and butt results were determined after 18 to 24 h at 36°C, while the Andrade's broth was incubated for 72 h at 36°C. All but one of the 167 strains tested yielded identical results in the two tests, with one strain being considered positive with TSI only, suggesting that the use of TSI for gas production is as sensitive as Andrade's broth with a Durham vial.

Susceptibility to cephalothin was determined by both the MIC dilution method (MicroScan) and the Bauer-Kirby agar disk diffusion method, and the only discrepancy was that three strains shown by the MIC method to be resistant to cephalothin were shown by the disk diffusion method to be susceptible. However, if resistance to cephalothin is to be used in Aerokey II as a differential test, only the standard Bauer-Kirby method should be employed pending further evaluation. This recommendation is also made because of the possibility of other major discrepancies in the detection of cephalothin resistance by inducible β-lactamases of *Aeromonas* spp. when an MIC microdilution method (Vitek) is used (41).

The following tests were performed both by conventional methods and with an API-20E strip: lysine and ornithine decarboxylase (API-20E versus Moeller's method); indole production (API-20E versus the conventional spot indole test on a 24-h culture grown on TSA with 5% sheep blood and Kovacs's reagent, but with all negatives confirmed by a conventional tryptone broth with xylene extraction and Ehrlich's reagent); Voges-Proskauer test (API-20E versus methyl red–Voges-Proskauer broth with the modified O'Meara method); and acid from arabinose, mannitol, and sucrose (API-20E versus Andrade's broth with 1% carbohydrate).

The results for the two decarboxylase enzymes were in close agreement for the two different methods, as were the results for indole, the Voges-Proskauer test, and carbohydrate fermentation (Table 2). The API-20E formulation was often more sensitive, e.g., in the case of ornithine decarboxylation. This suggests that results obtained in these tests with API-20E strips (per the manufacturer's instructions) correlate very well with the results obtainable with conventional test media for identifying aeromonad species.

Because production of H_2S by aeromonads has been a subject of controversy (18, 25, 31, 32), three different methods were evaluated: a 72-h TSI agar stab, a 24-h H_2S test on an API-20E strip (per the manufacturer's instructions), and a test using semisolid medium, GCF medium. This medium, a modification of an original Veron and Gasser medium, was inoculated by stabbing and then was incubated for 72 h at 36 ± 1°C and read at 24-h intervals. (43). The differences in the percentage of strains positive by these three test methods were readily apparent, in that none of the 167 strains gave a

TABLE 3. Comparison of distinguishing profiles of mesophilic clinical *Aeromonas* species

Characteristic	Result[a] for:						
	A. hydrophila (n = 46)	*A. veronii* bv. sobria (n = 26)	*A. veronii* bv. veronii (n = 8)	*A. caviae* (n = 33)	*A. schubertii* (n = 6)	*A. jandaei* (n = 9)	*A. trota* (n = 13)
Esculin hydrolysis	+	−	+	+	−	−	−
Voges-Proskauer reaction	+	+	+	−	V	+	−
Pyrazinamidase activity	+	−	−	+	−	−	−
CAMP-like factor (aerobic only)	+	+	+	−	−	V	−
Fermentation							
Arabinose	V	−	−	+	−	−	−
Mannitol	+	+	+	+	−	+	+
Sucrose	+	+	+	+	−	−	−
Susceptibility							
Ampicillin	R	R	R	R	R	R	S
Carbenicillin	R	R	R	R	R	R	S
Cephalothin	R	S	S	R	S	R	R
Colistin[b]	V	S	S	S	S	R	S
Decarboxylase							
Lysine	+	+	+	−	+	+	+
Ornithine	−	−	+	−	−	−	−
Arbutin hydrolysis	+	−	+	+	−	−	V
Indole	+	+		+	−	+	+
H_2S[c]	+	+	+	−	−	+	+
Glucose (gas)	+	+	+	−	−	+	+
Hemolysis (TSA with 5% sheep erythrocytes)	+	+	+	V	+	+	V

[a] +, positive for >70% of isolates; −, negative, i.e., positive for <30% of isolates; V, variable; R, resistant, S, susceptible.
[b] MIC (single dilution), 4 μg/ml.
[c] H_2S from GCF medium.
[d] Modified from Table 4 of reference 10 with permission.

positive result for H_2S production with the API-20E strip and only 5 (3%) gave a positive result with the TSI slant (Table 2). With the modified GCF medium, 102 (61%) of the 167 strains were positive for H_2S production. Further, the results obtained supported the H_2S production test as one that can differentiate between the species, as originally stated by Popoff and Veron (39) and later supported by Janda (25). That is, all of the SAS/TAXAN-phenotyped *A. hydrophila* and *A. veronii* bv. sobria strains were positive (100%) for H_2S, while all of the *A. caviae* strains were negative. Therefore, production of H_2S can be a useful test for identifying clinical aeromonads if modified GCF medium is used.

Another controversial test is growth in KCN broth as first proposed by Popoff and Veron (39). Although our results confirmed those of the initial study (39) as well as the later work of Janda (25), we found this test to be very subjective in its interpretation and both hazardous and labor intensive in its preparation (7). The inherent discrepancies associated with the interpretation of growth in KCN broth and the detection of H_2S production may explain, in part, the variance of our results compared with those of George et al. (18) and Kuijper et al. (31) concerning the identification of clinical *Aeromonas* species.

Finally, the method of Figura and Guglielmetti for the CAMP-like test for the identification of *Aeromonas* species was evaluated (15). We found that only the aerobic CAMP-like test could accurately differentiate *A. hydrophila* and *A. veronii* bv. sobria from *A. caviae* (Table 3). In the anaerobic case, we often observed a positive CAMP-like "arrow" with both *A. hydrophila* and *A. veronii* bv. sobria, not just *A. hydrophila* as originally stated (15). This variance between our findings and those originally published may be due to the fact that the original work was conducted with only a small number of strains. Also, a taxonomic study of a large number of clinical strains by Altwegg et al. found 16% of the *A. veronii* bv. sobria strains tested to be positive when the CAMP-like test was conducted anaerobically (3).

Evaluation of Aerokey II. When Aerokey II was used to identify the 60 coded clinical strains received from the independent laboratory, 58 of 60 (97%) of the strains were correctly identified to the species level. Only two highly atypical *A. veronii* bv. sobria (esculin hydrolysis positive and cephalothin resistant) strains were incorrectly identified as *A. hydrophila* (Table 4). It is interesting to note that one strain identified as *A. jandaei* by Aerokey II had been

TABLE 4. Evaluations of Aerokey II

Phenospecies	No. of strains tested	No. (%) correctly identified
Clinical strains (single-blind trial) (n = 60)[a]		
A. hydrophila	21	21 (100)
A. caviae	19	19 (100)
A. veronii bv. sobria	19	17 (90)
A. jandaei	1	1 (100)
ATCC or DNA reference strains (n = 18)		
A. veronii bv. veronii	4	4 (100)
A. schubertii	3	3 (100)
A. jandaei	5	5 (100)
A. trota	6	6 (100)

[a] Sources of clinical strains: feces (n = 31); wounds (n = 12); blood (n = 10), bile (n = 3); sputum, nares, urine, and cerebrospinal fluid (n = 1 each).

originally phenotyped as *A. veronii* bv. sobria but was subsequently confirmed by DNA-DNA hybridization to indeed be *A. jandaei* (formerly DNA group 9 *A. sobria*) (8). The evaluation of Aerokey II with 18 additional ATCC or DNA-hybridized reference strains of the more recently proposed taxa *A. veronii* bv. veronii, *A. schubertii*, *A. jandaei*, and *A. trota* resulted in all 18 (100%) being correctly identified to the species level (Table 4).

DISCUSSION

While it is quite clear that aeromonads can cause a myriad of infections in human hosts, controversy as to whether we can identify clinical, mesophilic *Aeromonas* isolates to the species level still exists. Some researchers express concern that there is not a sufficient quantity of discriminatory tests to distinguish between the clinical species or that the tests available are not sufficiently accurate (18, 22, 24). However, taxonomic studies of large numbers of clinical isolates have repeatedly shown that the majority of clinical *Aeromonas* isolates reside in DNA groups 1, 4, and 8, which are phenotypically characterized as *A. hydrophila*, *A. caviae*, and *A. veronii* bv. sobria (2, 4, 7, 24, 31). Additionally, biochemical studies of large numbers of *Aeromonas* isolates have provided a number of tests that can clearly separate the clinically significant species (3, 4, 7, 9, 25, 28, 39). The more recently proposed taxa *A. veronii* bv. veronii, *A. schubertii*, *A. jandaei*, and *A. trota*, although isolated in smaller numbers thus far, do have unusual biochemical markers that facilitate screening for these taxa in clinical laboratories (8, 10, 21, 22).

However, there was no single identification schema for identifying clinical *Aeromonas* isolates to the species level that incorporated all of these findings. The previous taxonomic studies of large numbers of clinical aeromonads focused almost exclusively on fecal isolates, usually from just one geographic location (3, 4, 24, 31), whereas our research encompassed a variety of clinical isolates from very diverse geographic locations with a smaller number of veterinary and environmental isolates as well. It was only when a large number of diverse strains were examined by an extensive number of tests using standard methods of identification with well-defined media and reagents that a workable identification system was developed. When Aerokey II is implemented in many different laboratories, we can begin to measure the true frequency and distribution of *Aeromonas* species from clinical isolates both within and between laboratories.

The ability of Aerokey II to correctly identify 97% of the 60 coded clinical isolates from an independent laboratory and 100% of the reference strains to the species level qualifies Aerokey II as a reliable and accurate system for the identification of the *Aeromonas* taxa isolated from clinical specimens to date. Additionally, use of the other discriminatory tests listed in Table 3 in conjunction with Aerokey II allows microbiologists to substitute tests at each step as desired; e.g., resistance to cephalothin could be replaced with either pyrazinamidase activity or ornithine decarboxylase to differentiate between *A. veronii* bv. veronii and *A. hydrophila*. Finally, Aerokey II can be modified to end on the left side of the dichotomous key with a positive reaction for indole production leading to an identification of *A. veronii* bv. sobria (dashed line in Fig. 1), should a clinical laboratory choose not to screen for the newer species at this time.

Aerokey II has already been incorporated at the bench level of the hospital laboratory of one of the authors of this article (A. M. Carnahan) and involves setting up the following for each *Aeromonas* isolate: an API-20E strip, a TSI slant, a bile esculin hydrolysis agar slant, an O/129 disk (150 μg/ml), and a Bauer-Kirby antimicrobial susceptibility panel that includes cephalothin (30 μg). Because of the good correlation between results derived by conventional methods and those derived by the API-20E strip method (Table 2), four of the seven tests needed for Aerokey II can be taken from the API-20E strip: Voges-Proskauer reaction, acid from arabinose, acid from sucrose, and indole production (negative indole reactions should be confirmed by Ehrlich's method). However, it is imperative that all isolates be presumptively identified as *Aeromonas* spp. before Aerokey II is used, i.e., they must be oxidase-positive, glucose-fermenting, gram-negative rods that are resistant to O/129 (150 μg/ml). Aeromonads are also unable to grow in a 6.5% NaCl broth. It is equally important that all tests based on Aerokey II are set up from a pure overnight culture of the *Aeromonas* isolate taken from a single colony. This procedure is recommended because biochemical tests set up directly from a colony growing on an inhibitory primary plate such as cefsulodin irgasan novobiocin agar (CIN) or MacConkey agar may carry over minute colonies of organisms such as *Enterococcus* spp. that would give a false-positive result for esculin hydrolysis at the start of Aerokey II. Finally, we reiterate that the esculin hydrolysis test must be set up with an agar formulation only and that the determination of resistance to cephalothin must be accomplished by the Bauer-Kirby method only.

Because of the absence of large outbreaks of disease caused by *Aeromonas* spp. and a single, unsuccessful human volunteer trial, we have thus far been unable to consider *Aeromonas* spp. definitively as singular, significant causative agents of human gastroenteritis (35). Although Koch's postulates have not been fulfilled, promising research on animal models (19, 38) and virulence features such as hemolysin and pili (12, 23) is being done. Perhaps, when accurate identification to the species level is combined with ongoing and future studies on pathogenesis and epidemiology, we can begin to determine why only certain genospecies appear to predominate in clinical specimens, whether they are equally distributed in the environment, and most importantly what is distinguishable about those subsets of aeromonads within each species that appear to be capable of causing disease in humans. Such information will be invaluable in understanding the role of the immune status of the host and, perhaps, culminate in successful human volunteer trials.

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Low Incidence of *Aeromonas* sp. in Livestock Feces

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ABSTRACT

Pig, beef, sheep and turkey fecal specimens were assayed for recovery of inoculated *Aeromonas* sp. by directly plating the samples on five different agar media. Of these, starch-ampicillin was optimal with respect to selectivity and ability to differentiate from other resident microflora. Generally, the numbers of inoculated *Aeromonas* sp. recovered on starch-ampicillin agar were similar to those recovered on brain heart infusion and blood ampicillin agar media, and were 10^1 to 10^3 greater than the recovery rate on either MacConkey-ampicillin or cefsulodin-irgasan-novobiocin agars. The sensitivity for the direct recovery of *Aeromonas* sp. from inoculated beef feces with naturally contaminating microflora, using streaked starch-ampicillin agar medium, was between 10^2 and 10^3 cells per gram. Using starch-ampicillin agar, the incidence of *Aeromonas* detected from feces of beef, pig, sheep and turkey held at the Beltsville Agricultural Research Center was one of 32, none of 22, none of 24 and three of 21, respectively. Based upon current taxonomic criteria, the isolate from the beef feces had characteristics consistent with both *Aeromonas sobria* and *Aeromonas caviae*, whereas three isolates from turkey feces were identified as *A. caviae* or *Aeromonas hydrophila*. The organism was isolated from five of five packages of ground beef from retail sources. The discrepancy in the consistent presence of the organism in retail meat suggests that many of the food isolates are probably not of fecal origin.

Aeromonas hydrophila and *Aeromonas sobria* have become increasingly implicated as causative agents of human gastroenteritis and important causes of wound infections and septicemia (*2,9,10*). The primary source is thought to be water (*12*), with contaminated waters (*3,13*) and fish (*11*) serving as likely vehicles for human gastrointestinal infections.

Aeromonas sp. are psychrotrophic bacteria (*18*) and can be recovered from chlorinated drinking water samples (*14*). In addition, *A. hydrophila* is a common contaminant in foods of animal origin (*4,6-8,16*). Palumbo et al. (*17*) recovered the organism from virtually all finfish, shellfish, raw milk, chicken, veal, beef and lamb that they sampled. The only product failing to yield *Aeromonas* sp. was fresh sausage, and this was likely due to the high salt content of the product.

Because human gastroenteritis caused by the organism has primarily been associated with vehicles of aquatic origin, it seemed curious that such a large number of foods of animal origin not associated with the aquatic environment also carried *Aeromonas* sp. Yet, to our knowledge, no outbreaks associated with these foods have been described in the literature. Because gram-negative human enteropathogens are frequently transferred from the feces of livestock animals to meat tissue, which can then serve as a vehicle for the organism, we became interested in the rate of fecal carriage among food animals. The purpose of this investigation was to perform an abbreviated assessment on the frequency of isolation in fecal specimens from these animals after optimizing our ability to detect *Aeromonas* sp. by directly plating livestock feces.

MATERIALS AND METHODS

Media

Five agar media were used in this study: (a) starch-ampicillin agar (SA) as described by Palumbo et al. (*17*); (b) MacConkey-ampicillin agar (MA) consisting of MacConkey agar (BBL, Cockeysville, MD) supplemented with 10 mg ampicillin (Sigma, St. Louis, MO) per L; (c) brain heart infusion agar (BHI; Difco, Detroit, MI); (d) BHI-blood-ampicillin (BBA) consisting of BHI agar supplemented with 50 ml of defibrinated sheep blood (Otisville Biotechnology, Inc., Otisville, NY) and 10 mg ampicillin per L; and (e) cefsulodin-irgasan-novobiocin (CIN) agar (Gibco, Madison, WI) as described by Feeley and Schiemann (*5*).

Cultures

The following isolates of *Aeromonas* sp., supplied by M. Janda (Mt. Sinai Hospital, New York), were used in these studies: *A. hydrophila* (MSH 11), *A. sobria (MSH 24), and A. caviae* (MSH 05). Cultures were streaked onto BHI agar and identity confirmed by the use of API 20E (Analytab, Plainview, NY) analyses and taxonomic criteria described by Popoff (*18*). All agar plates in the isolation and enumeration studies were incubated at 35°C.

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Methods

Fecal materials were collected from sheep, cattle (6 to 9 months old), pigs and turkeys located on the Beltsville Agricultural Research Center, Beltsville, MD. Samples were gathered from fresh droppings, except for occasional cattle specimens collected directly in the abbatoir, in sterile petri plates and returned within 15 min to the laboratory for autoclaving (15 min, 15 psi) or for unmodifed use in isolation studies. Feces (1 g) was suspended by vortexing with 9.0 ml of physiological saline solution (PSS) containing dilutions of overnight cultures of *Aeromonas* sp. grown on BHI agar and harvested into PSS. Serial decimal dilutions were made in PSS and 0.1 ml was spread plated onto the five agar media described above. After overnight incubation, numbers of colonies on countable plates were determined and three to five typical colony types per plate were tested for positive catalase, oxidase and starch hydrolysis activities. Subsequently, the isolates were characterized as *Aeromonas* sp. based upon API 20E analysis, and the component species were determined by KCN, salicin, VP, glucose/gas and esculin tests. Reactions which are characteristic of each species are shown in Table 1, and are consistent with the taxonomic criteria described by Popoff (*18*).

The influence of a particular medium on the quantitative recovery of the three inoculated *Aeromonas* sp. cultures was assessed both with sterile feces and with feces containing naturally occurring microflora. This approach was used to determine which of the media produced optimal quantitative recovery, and that level at which the organism could be consistently detected by direct plating. Uninoculated fecal samples from the above mentioned livestock species were assessed for presence of *Aeromonas* sp. using SA medium, which was determined the most useful medium. To further verify our capability in recovering the organism, we analyzed five packages of ground beef, which has been reported to harbor *Aeromonas* sp. (*17*), from five retail outlets for the presence of the organism.

RESULTS AND DISCUSSION

Data indicating the capability of the five selected media to support growth of the three clinical isolates of *Aeromonas* sp. which were suspended in fecal materials from four livestock species are shown in Table 2. With the exception of *A. sobria* in beef feces, the trends observed for aeromonad response were similar irrespective of the type of fecal specimen tested. In general the source of fecal materials had little effect on the ability of the organism to produce colonies. However, both the bacterial species and the media used for enumeration did have substantial influence on quantitative recoveries (Table 2). BBA medium generally supported the best growth of *Aeromonas* sp. yielding an average of 7.61 $\log_{10}$ CFU per g of feces. The next most productive medium, BHI, had an average of 7.27 $\log_{10}$ CFU per g of feces. With each of the other media assessed, there was reduced recovery of *A. caviae* or *A. sobria*. The diminished recovery of *A. caviae* and *A. sobria* on these four media may be explained by strain differences or may be characteristic of these species.

A. sobria was recovered at consistently high levels on BBA. BHI and SA recovered slightly less of the inoculated organisms, MA fewer yet, and CIN did not permit recovery of *A. sobria* at the levels tested. From the sterile livestock feces inoculated with *A. hydrophila*, all of the media tested recovered similar numbers of microorganisms.

The efficacy of the five media tested for selectivity and sensitivity in the recovery of *Aeromonas* sp. from feces is shown in Table 2. In general, BHI and BBA

TABLE 1. *Incidence and classification of* Aeromonas *sp. recovered from livestock feces using starch ampicillin agar for recovery.*

Fecal source	Recoveries/Fecal samples assayed
Beef	1/32
Pig	0/22
Sheep	0/24
Turkey	3/21
Total	4/99

Aeromonas species	Taxonomic criteria used (*18*)				
	KCN	Salicin	V-P	Glucose/gas	Esculin
hydrophila	+[a]	+	+	+	+
caviae	+	+	−	−	+
sobria	−[b]	+	d[c]	+	−

Isolate	Classification of fecal isolates					Tentative identification
Beef	−	+	−	d	−	*A. sobria/caviae*
Turkey 1	+	+	−	d	+	*A. caviae*
Turkey 2	+	+	+	d	+	*A. hydrophila*
Turkey 3	+	+	−	d	+	*A. caviae*

[a] +, positive reaction for indicated test.
[b] −, negative reaction for indicated test.
[c] d, differential or inconclusive reaction for indicated test.

media yielded the greatest recoveries of the three bacterial species tested, with SA and MA supporting slightly fewer numbers, and CIN being the least productive in recovering aeromonads. BHI was nonselective and, therefore, not particularly useful for recovery of the target bacteria. BBA, although productive, was not differential. While both MA and CIN were selective, they were moderately and substantially inhibitory to *Aeromonas* sp., respectively. In contrast, SA medium was highly selective and yielded consistently high recoveries. Generally, the selective honey-yellow colony color surrounded by a yellow zone facilitated presumptive identification of the isolates after 16 to 24 h of incubation. Occasionally, if too lengthy an incubation time was allowed, the colony color would turn red. With this caveat we proceeded to use SA medium for further isolation work.

We determined the sensitivity for recovering inoculated *A. sobria* and *A. hydrophila* from fecal materials by direct plating. At levels between 2 and 3 $\log_{10}$, we were able to consistently recover the organism. Below this level of inoculation, the organism was diluted out, and the bacteria could not be recovered. The limit of the direct plating detection method is acknowledged, and an enrichment method was subsequently developed after our work was initiated (*1*). We did not attempt to isolate all *Aeromonas* sp. present in livestock feces. Rather, we wanted to determine whether the organism was prominent and widely distributed among those animals which we sampled. Because *Aeromonas* sp. are consistently found on meat tissues, we used methods adequate for detecting the large numbers which need to be present in feces to provide this distribution. It also seems reasonable to anticipate fewer aeromonad isolations and inconsistent contamination of meats if only low numbers are present in the fecal materials.

The low incidence of *Aeromonas* sp. isolated from fecal materials of the animals sampled is shown in Table 1. Only four of 99 fecal specimens sampled yielded indigenous *Aeromonas* sp. By comparison, Gray found 11.8% of 459 fecal specimens from horses, pigs, sheep and cows to contain *Aeromonas* sp. (*8*), whereas Morse and Hird (*15*) isolated *A. hydrophila* from 5.7% of 280 slaughter swine lymph nodes. The low incidence of *Aeromonas* sp. in red-meat animals suggests that variation in excretion of the organism among herds can be expected and, perhaps, even controlled. On the other hand, in the present study, turkeys had a comparatively high incidence of *Aeromonas* sp., and the potential public health significance remains to be confirmed.

Based upon the observations of Palumbo et al. (*17*) who have consistently found the organism present in foods of animal origin, we attempted to confirm our methodology and sampled five ground beef packages from retail stores for the presence of the organism. *Aeromonas* sp. was isolated from each of the packages, using the same method that was used for the fecal specimens.

During poultry processing, fecal matter with human enteropathogens may contaminate meat products by being distributed through common water rinses. Consequently, a few contaminated birds can transmit the organism to large numbers of uncontaminated carcasses. In the case of red meat slaughter, each carcass may be subjected to contamination by its own intestinal content, but comparatively minimal transfer is made between

TABLE 2. *Quantitative recovery of* Aeromonas *sp. inoculated into sterile or non-sterile livestock feces as influenced by selective media and/or competing flora.*

Fecal source	*Aeromonas* species	Sterile fecal milieu					Non-sterile fecal milieu				
		Recovery media[a]					Recovery media				
		BHI	CIN	SA	MA	BBA	BHI	CIN	SA	MA	BBA
	A. sobria										
Beef		5.96[b]	<4.00	5.98	<4.00	7.19	7.04	<4.00	6.55	<4.00	7.10
Pig		6.77	<4.00	6.80	5.72	7.12	6.58	<4.00	6.90	5.79	7.16
Sheep		6.94	<4.00	6.35	6.03	7.34	6.61	<4.00	6.83	6.29	7.45
Turkey		8.05	<4.00	7.25	7.22	7.91	8.29	4.51	7.90	7.28	8.14
	A. hydrophila										
Beef		7.57	7.22	7.44	7.34	7.87	7.52	7.01	8.19	7.16	7.54
Pig		7.94	7.60	7.79	7.57	7.78	7.91	7.56	7.89	7.85	7.89
Sheep		7.82	8.00	8.06	8.03	8.09	8.32	7.93	8.27	7.94	8.07
Turkey		8.66	8.37	8.37	8.35	8.63	8.62	8.30	8.61	8.28	8.63
	A. caviae										
Beef		4.99	<4.00	4.88	4.75	6.65	7.19	<4.00	5.53	5.27	6.63
Pig		6.78	<4.00	6.80	6.51	7.48	7.43	<4.00	7.15	6.80	7.80
Sheep		5.59	<4.00	6.13	5.37	7.55	6.09	<4.00	6.10	5.03	6.77
Turkey		7.79	<4.00	7.73	7.55	7.72	7.96	5.80	7.94	7.52	8.06

[a]BHI, brain-heart infusion agar; CIN, cefsulodin-irgasan-novobiocin agar; SA, starch ampicillin agar; MA, MacConkey ampicillin agar; BBA, BHI-blood-ampicillin agar.

[b]Mean $\log_{10}$ colony-forming units per g of feces for duplicate samples.

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carcasses. The intestinal carriage of *Aeromonas* sp. in poultry may or may not be the source of *Aeromonas* contamination of poultry meat products which has been reported by others. The very low carriage rate of *Aeromonas* in red meat animals and the processing techniques used cannot explain the high distribution of *Aeromonas* sp. on red meat products. An alternative explanation is needed regarding the source of these organisms on these foods. Also, means for discriminating between those *Aeromonas* sp. which are a potential public health hazard and those which are less capable of causing disease in humans is needed. A possible explanation lies in previous observations (*10,19*). *Aeromonas* sp. is a major component of the total bacterial population in aquatic environments, especially those containing chlorine and those which are heavily polluted. It follows then that in environments where carcasses are processed with chlorinated waters, aeromonads have an opportunity to thrive in large numbers, consequently leading to colonization of meat products.

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Spotlight on *Aeromonas* - A Human Pathogen of Aquatic Origin

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Aeromonads are ubiquitous oxidase-positive, polar flagellated, glucose-fermenting, facultatively anaerobic, gram-negative rods that are autochthonous to aquatic environments worldwide.[1] Although recognized for over 100 years, interest in these organisms has steadily increased mainly in the last 10 years as evidenced by an almost exponential increase in literature citations. Aeromonads were initially considered "opportunistic pathogens" primarily in immunocompromised individuals with a possible link to enteric disease.[2] However, aeromonads have been isolated from a variety of both cold- and warm-blooded animals, including immunocompetent human individuals of all age groups. Specimen sources include blood, bone, tissue, spinal fluid, pleural fluid, bile, urine, sputum, animal bites, aquatic-related wounds and stools.[1]

Several studies representing various worldwide distributions have presented a strong case for some *Aeromonas* species as enteric pathogens,[3,4] despite an unsuccessful human volunteer trial or fulfillment of Koch's postulates. Although *Aeromonas* possesses various virulence mechanism(s), which may contribute to the cause of disease (e.g., hemolysin, cytotoxin, elastase, protease, siderophore production, S-layer),[5] there is still a lack of understanding as to why some aeromonaos may be more pathogenic than others.

While there is some uncertainty surrounding *Aeromonas* causation in enteric disease, identification of *Aeromonas* to the species level is recommended. Although *Aeromonas* taxonomy was in a constant state of flux for many years, in the last 5 years at least nine different phenotypic species (phenospecies) have been established from among at least 14 different DNA hybridization groups (genospecies).[5] Only seven of these phenospecies have been implicated in human disease: *A. trota, A. jandaei, A. veronii* biogroup *sobria, A. veronii* biogroup *veronii, A. schubertii, A. hydrophila* and *A. caviae.*

Importantly, there is evidence for the presence of species-related diseases such as the association between bacteremia and *A. veronii* biogroup *sobria* (formerly *A. sobria*),[6] pediatric diarrhea and *A. caviae*,[7] aquatic wound infections and *A. schubertii*[8] and wound infections with *A. hydrophila* following the use of medicinal leeches.[9]

The isolation of aeromonads is very straightforward with over 25 different media having been proposed to date for the detection of aeromonads.[10] Most of these media were developed to enhance the isolation of aeromonads from stool specimens. However, aeromonads do grow quite readily on most laboratory media at temperatures ranging from 4 to 42°C. They may be confused initially with *Escherichia coli*, since they often ferment lactose.

Trypticase® Soy Agar with 5% Sheep Blood is an excellent medium for isolating aeromonads, whether used in a stool set-up or as part of a routine set-up for wound, respiratory or miscellaneous cultures.[11] This permits screening for oxidase activity and testing for indole production. Further, observing for the presence or absence of different types of hemolysis may indicate the presence of more than one species of *Aeromonas*.

There are several media available for isolation of aeromonads that include 10 µg of ampicillin to inhibit normal enteric flora and facilitate the isolation of aeromonads, which are generally considered to be resistant to ampicillin. However, these media should be used recognizing that there are numerous references to the isolation of ampicillin-susceptible *Aeromonas* strains. The most recently proposed species, *A. trota*, is susceptible to this concentration of ampicillin and, of those isolated to date, almost all were isolated exclusively from stool specimens.[12]

If a selective medium is desired for isolation of aeromonads from stool specimens, CIN Agar containing cefsulodin, Irgasan® (Ciba-Geigy) and novobiocin is recommended since *Aeromonas* grows quite readily on it at both 25°C and 37°C. Also, CIN Agar allows for the simultaneous screening of stool specimens for *Yersinia*.[13] Although primary enrichment is not recommended for the isolation of *Aeromonas* from routine stool specimens at the present time, enhancement of low numbers of aeromonads can be accomplished using Alkaline Peptone Water.[10] This procedure enables detection of those patients who may be either chronic carriers or may have a subclinical case of *Aeromonas* gastroenteritis.

After initial isolation of the organism, the single most important test result for presumptive identification is the determination of a positive oxidase reaction (Table 1). This test must be performed on a nonselective medium, such as **Trypticase** Soy Agar with 5% Sheep Blood, since false-positive reactions can occur in media where acid production from carbohydrates has resulted in a lowered pH; e.g., as observed on MacConkey Agar.[14] The other tests listed in Table 1 are useful to differentiate *Aeromonas* from the two most closely related genera, *Plesiomonas* and *Vibrio*.

Although nearly all rapid identification systems can easily recognize an isolate as *Aeromonas hydrophila* Group or *Aeromonas hydrophila* Complex, most are unable to identify to the level of species for several reasons. First, there is a lack of sufficient discriminatory tests to determine inter-genus and inter-species differences. For example, *A. caviae* is frequently identified as *V. fluvialis* and either a salt tolerance test or demonstration of resistance to Vibriostatic Agent 0/129 must be done (Table 1). Secondly, *A. hydrophila* and *A. veronii* biogroup *sobria*, indistinguishable from each other in most rapid systems, require additional analysis (Table 2). Finally, there is a lack of correlation between accepted results for tests using conventional media as

TABLE 1 Salient Features for the Identification of *Aeromonas*

	Aeromonas	*Plesiomonas*	*Vibrio*
Gram reaction	-	-	-
Oxidase activity	+	+	+
Resistance to 0/129*			
10 µg	+	+/-	+/-
150 µg	+	-	-
Growth in 6% NaCl	-	-	+
Acid from			
Glucose	+	+	+
Inositol	-	+	-
Mannitol	+/-	-	+
Sucrose	+/-	-	+/-
Gelatin			
liquefaction	+	-	+

*Vibriostatic Agent (2,4-diamino-6, 7-diisopropylpteridine)

(continued on page 2)

(continued from page 1)

compared to modifications used in the rapid ID systems; i.e., esculin hydrolysis and decarboxylase reactions.[15]

However, there are discriminatory biochemical and antimicrobial tests that can be employed to accurately biotype those species of *Aeromonas* isolated from clinical specimens (Table 2).[12]

It is suggested that clinical laboratories make every attempt to biotype their *Aeromonas* isolates to the level of species. There are inherent differences in pathogenicity between species and differences in antimicrobial susceptibility, such as resistance to cephalothin, that may influence the physician's subsequent successful treatment of the patient.[16]

Finally, it is only with accurately biotyped clinical *Aeromonas* isolates that the necessary follow-up studies can be conducted to resolve the etiologic paradox of these aquatic microorganisms; thereby establishing or abrogating them as *bona fide* enteric pathogens.

TABLE 2 Comparison of distinguishing profiles of clinical *Aeromonas* species*

	A. trota	*A. jandaei*	*A.veronii* biogroup *sobria*	*A.veronii* biogroup *veronii*	*A. schubertii*	*A. hydrophila*	*A. caviae*
Esculin hydrolysis	-	-	-	+	-	+	+
Voges-Proskauer	-	+	+	+	V	+	-
Cellobiose	+	-	-	+	-	-	V
Gluconate oxidation	-	+	+	ND	-	+	-
Fermentation:							
Arabinose	-	-	-	-	-	V	+
Mannitol	+	+	+	+	-	+	+
Sucrose	-	-	+	+	-	+	+
Susceptibility:							
Ampicillin	S	R	R	R	R	R	R
Carbenicillin	S	R	R	R	R	R	R
Cephalothin	R	R	S	S	S	R	R
Colistin[a]	S	R	S	S	S	V	S
Decarboxylase:							
Lysine	+	+	+	+	+	+	-
Arginine	+	+	+	-	+	+	+
Ornithine	-	-	-	+	-	-	-
Indole	+	+	+	+	-	+	+
H_2S[b]	+	+	+	+	-	+	-
Glucose (gas)	+	+	+	+	-	+	-
Hemolysis (5% sheep erythrocytes)	V	+	+	+	+	+	V

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- = negative; + = positive; V = variable, S = susceptible, R = resistant; ND = no data.
a = MIC single dilution, 4 µg/ml
b = H_2S from GCF medium (Gelatin Cysteine Thiosulfate).

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Editor's Note: **Trypticase** Soy Agar with 5% Sheep Blood with Ampicillin is available from Becton Dickinson Microbiology Systems in a package of 20 plates (Cat. No. 97346); CIN Agar is available in a package of 10 plates (Cat. No. 21848); Alkaline Peptone Water is available in packages of 10 and 100 tubes (Cat. No. 97814 and 97421, respectively).

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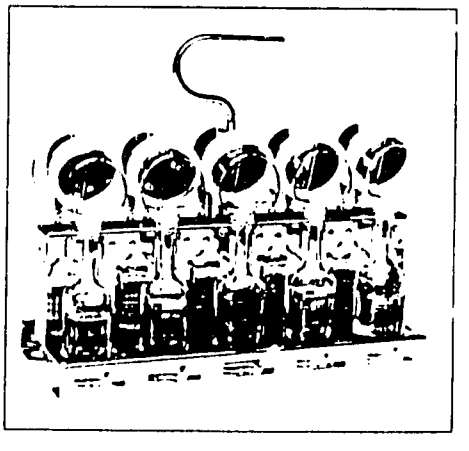

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