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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.

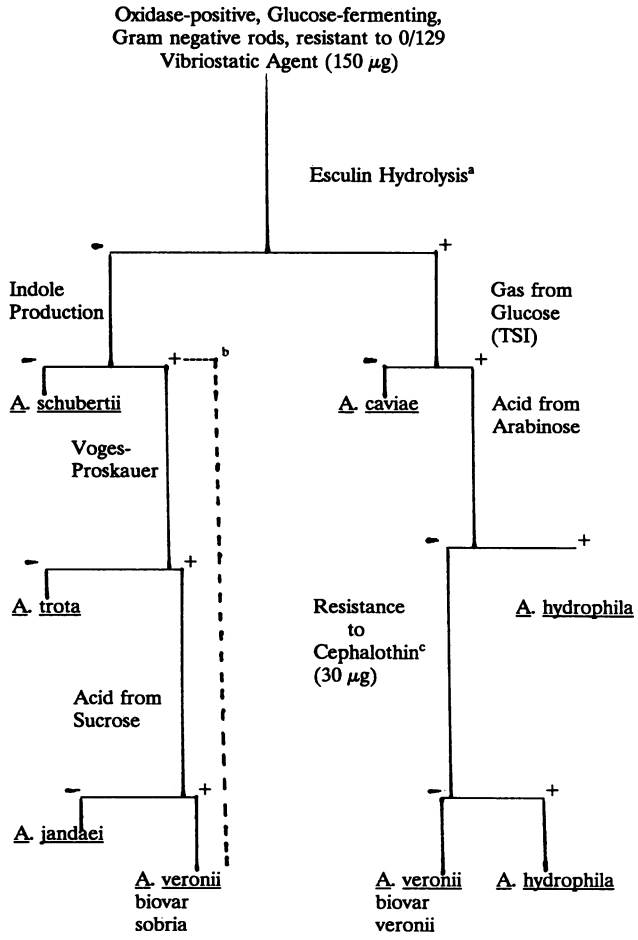

FIG. 1. Aerokey II identification key for clinical *Aeromonas* species. a, agar formulation only; b, Aerokey II can be modified to end here with an identification of *A. veronii* bv. sobria; c, Bauer-Kirby disk diffusion method only.

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 seven 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, 39), 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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