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Biotyping of *Aeromonas* Isolates as a Correlate to Delineating a Species-Associated Disease Spectrum

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A group of 147 *Aeromonas* isolates from diverse clinical and environmental sources was subjected to the biotyping scheme of Popoff and Veron. Of the 147 isolates biotyped, 137 (93%) could be identified, with *Aeromonas hydrophila* predominating (48%) and equal percentages (25 to 27%) of the other two species (*Aeromonas sobria* and *Aeromonas caviae*). A number of additional biochemical properties were found to be significantly associated with one or more of these three species. These included lysine decarboxylase activity, hemolysis of sheep erythrocytes, lecithinase production, staphylolytic activity, arbutin hydrolysis, and acid production from utilization of various carbohydrates. By incorporating these phenotypic properties into an extended biotyping system, 98% of the isolates were identified. Selective distribution of individual species with respect to certain body sites was noted.

The recognition of *Aeromonas* spp. in the clinical laboratory is increasing as a consequence of improved isolation techniques and a heightened awareness of their pathogenic potential as evidenced by the panorama of infections associated with these vibrio-like organisms. Aeromonads, members of the family *Vibrionaceae*, are primarily recovered from freshwater sources (13, 14) and from inhabitants of these ecological niches such as fish, amphibia, and reptiles (5, 19, 25), which also are subject to their invasive propensity. Human infection with *Aeromonas* may ensue through trauma or after ingestion. Subjects so infected may show syndromes ranging from cellulitis or ulcerative lesions (12, 21) to gastroenteritis (11, 17, 32) or even bacteremia in susceptible (immunocompromised) individuals (16, 18, 23).

Until recently, only two well-described species or biogroups of *Aeromonas* were recognizable. One group, represented by *Aeromonas salmonicida*, is associated with fish furunculosis (7) and is an extremely important cause of economic loss in the fishing industry. *A. salmonicida* is oxidase positive and characterized by lack of motility and indole production, auxotrophy, inability to grow at 37°C, and production of a melanin-like pigment on tyrosine-containing agar. No human infection due to this bacterium has ever been reported.

The second biogroup of *Aeromonas* is comprised of the motile, indole-positive species that grow optimally at 35 to 37°C and have been referred to as the *Aeromonas hydrophila* complex. Over the past 25 years, various investigators have proposed from one to four species within this complex, using designations such as *A. hydrophila*, *Aeromonas punctata*, *Aeromonas caviae*, *Aeromonas dourgesi*, and *Aeromonas proteolytica* to describe these species (6, 9, 22, 29, 30). In 1974, Schubert proposed that two species, *A. hydrophila* and *A. punctata*, each with specific subspecies, be recognized, based primarily upon acetylmethylcarbinol production and aerogenic utilization of glucose (31).

Recent studies based upon numerical taxonomy data (20, 27) and polynucleotide sequence relatedness (26) suggest that motile aeromonads should be comprised of at least three separate species (or biogroups), namely, *A. hydrophila*, *Aeromonas sobria*, and an anaerogenic species, *A. caviae*. Within each species, more than one hybridization group exists, suggesting that the taxonomy is still not clear and that further phenotypic studies are required. Since the initial studies by Popoff and Veron, little justification regarding the practicality or even the necessity of identifying species (or biotyping) of isolates in the clinical laboratory has been forthcoming. The present study was therefore undertaken to assess the usefulness of determining the species of *Aeromonas* isolates and the significance of species determination to clinical microbiologists and infectious disease personnel as a means of more clearly delineating the clinical association(s) with each species.

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MATERIALS AND METHODS

Strains. A group of 147 *Aeromonas* isolates was studied; 39 were recovered from clinical specimens processed at The Mount Sinai Hospital (New York, N.Y.) over an 18-month period, and the remaining 108 were kindly provided by W. G. Barnes, R. C. Bartlett, M. B. Coyle, E. P. Desmond, P. Echeverria, J. C. Fung, G. L. Gilardi, F. W. Hickman-Brenner, M. T. Kelly, T. E. Kiehn, J. M. Matsen, P. R. Murray, T. L. Overman, R. G. Robertson, M. F. Sierra, A. Ternstom, and R. J. Zabransky. All isolates were initially identified as *A. hydrophila* by API 20E analysis (Analytab Products, Plainview, N.Y.) and standard biochemical tests (8), which included resistance to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine). The original sources of the 147 isolates were as follows: gastrointestinal, 52; wounds, 29; respiratory, 12; blood, 18; fluids, 12; genitourinary, 5; miscellaneous, 3; environmental-animal, 13; unknown, 3.

Identification. Species identification was made by the method of Popoff and Veron (27), except for incubation at 37°C rather than 30°C and with a slightly modified eight-test assay (16) including determination of esculin hydrolysis, gas production from glucose, acetylmethylcarbinol formation, acid production from L-(+)-arabinose and salicin, elastase activity, and growth in potassium cyanide (1:13,300 final dilution) broth. Hydrogen sulfide formation was detected by using the semisolid medium (GCF) of Veron and Gasser (33), with positive reactions indicated by a diffuse blackening of the medium radiating from the stab line after 72 h at 37°C. Identification of each isolate was achieved by comparison to the eight ideal phenotypes (seven in the case of *A. sobria*) for each species as described by Popoff and Veron (27) (Table

1). Strains not typable by this test system were further evaluated for nine additional properties of value in the determination to the species level of *Aeromonas* isolates. These nine tests included the following.

(i) Hemolysin and (ii) lecithinase activities (15) were determined on tryptic soy agar plus 5% sheep blood or egg yolk suspension, respectively. (iii) Staphylolytic activity was determined by the method of Satta et al. (28). Briefly, *Staphylococcus aureus* ATCC 25923 was grown to confluency on the surface of nutrient agar. Cells were harvested, washed twice in distilled water, and autoclaved. Heat-killed cell suspensions were then added to tryptic soy agar at a final concentration of 2 optical density units at 540 nm per ml. The solidified medium was then spot inoculated with eight test strains per plate and incubated for 5 days at 37°C. Positive reactions were seen as a clearing (lysis) of the opaque medium around the inoculated aeromonads. (iv) Arbutin hydrolysis was determined by the methodology of Frank and von Riesen (10). Arbutin (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 0.5% to a basal slanted medium consisting of 0.2% tryptone, 0.03% dipotassium phosphate, and 0.5% sodium chloride with agar (1.5%). The slant was heavily streaked with each isolate and incubated at 37°C for 3 days. A positive reaction (hydrolysis) was indicated by a browning (oxidized hydroquinone) of the agar slant. Reduced hydroquinone (colorless), potentially present on slants failing to demonstrate a typical brown color, was detected by addition of 3 drops of a 40% KOH solution to each slant and vigorous shaking. The formation of an immediate brown color was considered a positive reaction.

Acid production from (v) D-(+)-cellulose, (vi) D-(+)-mannose, and (vii) 1-*O*-methyl-α-D-glucopyranoside was detected individually by incorporation of a 1% solution of each carbohydrate into O-F medium. Reactions were recorded after 5 days of incubation at 37°C.

(viii) Acid production from 10% lactose was evaluated in purple agar base medium (Difco Laboratories, Detroit, Mich.) after 3 days of incubation at 37°C.

(ix) Lysine decarboxylase activity was assessed by using both the API 20E system (24 h at 37°C) and Moeller medium (72 h at 37°C).

RESULTS

By using an eight-test primary typing system, 137 of 147 (93%) *Aeromonas* isolates were identifiable to the species level. Approximately half of these isolates were *A. hydrophila*, and there were equal percentages of the other two species (*A. caviae* and *A. sobria*). Of the *Aeromonas* isolates identified in this manner, 70% generated ideal phenotypes. Thirty-nine of 43 isolates (91%) deviated from the ideal profile in only one phenotypic property, and only one strain (*A. caviae*) produced three discrepant reactions. Ten isolates were not identifiable by this typing system and were intermediate in properties between two or all three recognizable species.

Concurrent with the above eight tests, nine supplementary biochemical tests were significantly associated with one or more of the three aeromonad species (Table 1). Hemolysin and lecithinase production and lysine decarboxylase activity were of value in the separation of *A. hydrophila* and *A. sobria* from *A. caviae*. In a similar fashion, the elaboration of a staphylolytic enzyme and the formation of acid from 1-*O*-methyl-α-D-glucopyranoside and mannose enabled distinction of *A. hydrophila* from *A. caviae* and *A. sobria*. The latter species was distinguishable by its inability to hydrolyze arbutin or to produce acid from lactose. Cellobiose utilization was helpful in the separation of any two *Aeromonas* spp.

The nine supplementary biochemical characteristics were used in an attempt to identify the 10 isolates that were not typable by using the primary eight-test system. Of the 10 isolates, 7 could be identified by using an extended biotyping system. Three were identified as *A. sobria*, two as *A. hydrophila*, and two as *A. caviae*. Three strains, one from a throat and two from pork products, could not be definitively placed into one specific species, although all three fell into the *A. hydrophila-A. caviae* group, being biochemically distinct from *A. sobria*.

By utilizing the data from the 144 *Aeromonas* isolates determined to the species level and the frequency of positive reactions for each of the eight primary tests overall and for each species (Table 1), several biochemical reactions were found to deviate from the ideal phenotype for each species. These were, for *A. hydrophila*, acid production from arabinose (10 negative reactions); for *A. caviae*, acid production from salicin (six negative reactions); and for *A. sobria*, growth in KCN broth (eight false-positives). Six reactions gave consistent results: esculin hydrolysis and growth in KCN broth by *A. hydrophila*, lack of acid production from salicin and H_2S formation by *A. sobria*, and failure to produce elastase or gas in glucose broth by *A. caviae*.

Irrespective of species, the gastrointestinal tract was the most common source of the 141 *Aeromonas* isolates, accounting for almost 40% of all isolations. *A. hydrophila* was most commonly recovered from the gastrointestinal tract and wounds (50%) as was *A. caviae* (72%), whereas *A. sobria* was more often associated with the gastrointestinal tract and bacteremia (68%). *A. hydrophila* and *A. sobria* accounted for 88% (16 of 18) of all blood isolates; *A. caviae* was rarely recovered from blood. Equal distribution of the three species with respect to fluids, the genitourinary tract, and environmental specimens was observed (Table 2).

DISCUSSION

In 1976, Popoff and Veron (27) analyzed 68 strains (58 from fish, amphibia, and water and 10 from humans) by using numerical taxonomy data and suggested that the current *A. hydrophila-A. punctata* group be reorganized into two separate species, namely, *A. hydrophila* and *A. sobria*. These species were separable based upon seven pertinent biochemical properties. Later this scheme was expanded (26) to include a former biovar of *A. hydrophila* as a third species, *A. caviae*. This anaerogenic species was identifiable by these authors on the basis of four biochemical tests that separated it from *A. hydrophila*.

Since their original taxonomic work, only one major study has been published on the use of this typing system. Wakabayashi and colleagues (34) attempted to determine to the species level 291 motile *Aeromonas* isolates, primarily originating from fish and water. Unlike the study of Popoff and Veron, however, Wakabayashi characterized only 104 of 391 (36%) of the isolates tested. Of the 104 species determined, *A. hydrophila* (42%) and *A. sobria* (50%) predominated. In our study, greater than 90% of the clinical isolates tested were identifiable to the species level by using a modified eight-test typing scheme. Species determination improved to 98% when a nine-test supplementary battery (Table 1) was applied to isolates untypable by the primary identification system. These data, along with the primary biochemical profiles and the nine additional phenotypic traits (Table 1) utilized, support the original work of Popoff and Veron on

TABLE 1. Phenotypic properties of value in identifying clinical isolates of *Aeromonas* spp.

Test	No. positive (%)			
	Total	*A. hydrophila*	*A. caviae*	*A. sobria*
Primary[a]				
Esculin hydrolysis	106 (74)	68 (100)	34 (94)	4 (10)
KCN	110 (76)	68 (100)	34 (94)	8 (20)
Arabinose	96 (67)	58 (85)	35 (97)	3 (8)
Salicin	92 (64)	61 (90)	31 (86)	0 (0)
Glucose (gas)	105 (73)	66 (97)	0 (0)	39 (98)
Voges-Proskauer	104 (72)	65 (96)	2 (6)	37 (93)
H_2S production	108 (75)	67 (99)	1 (3)	40 (100)
Elastase	84 (58)	64 (94)	0 (0)	20 (50)
Supplementary[b]				
Hemolysin	89 (74)	55 (95)	6 (18)	28 (93)
Lecithinase	87 (72)	47 (81)	12 (36)	28 (93)
Staphylolytic enzyme	36 (30)	35 (60)	0 (0)	1 (4)
Lysine decarboxylase	88 (73)	57 (98)	1 (3)	30 (100)
Arbutin hydrolysis	92 (76)	58 (100)	33 (100)	1 (4)
Acid from cellobiose	45 (37)	6 (10)	29 (88)	10 (33)
Acid from mannose	73 (60)	57 (98)	8 (24)	8 (24)
Acid from 1-*O*-methyl-α-D-glucopyranoside	51 (42)	45 (78)	1 (3)	5 (17)
Lactose (10% purple agar base)	39 (32)	20 (34)	17 (52)	2 (7)

[a] Based on 68 *A. hydrophila* isolates, 36 *A. caviae* isolates, and 40 *A. sobria* isolates.
[b] Based on 58 *A. hydrophila* isolates, 30 *A. caviae* isolates, and 33 *A. sobria* isolates.

the separation of three distinct species (or biotypes) and the feasibility of this typing system.

In addition to the high number (70%) of ideal phenotypes for each recognizable species, most isolates can be presumptively identified by using a somewhat more limited number of biochemical properties. Untypable strains by both the primary and supplementary test systems may represent extremely rare variants of a given species or may be genetic (new species) clusters. Since our study did not include a sufficient number of environmental strains, it is difficult to determine whether this typing scheme would work for isolates recovered from these sources, particularly if such strains were phenotypically more diverse.

There are several reasons to justify determination to the species level of *Aeromonas* strains. As *A. sobria* and *A. hydrophila* accounted for almost 90% of all bacteremic isolates (Table 2) (most likely originating from prior gastrointestinal tract colonization), it may be of paramount importance to determine the species of stool isolates, particularly those from immunocompromised or cirrhotic patients, who are more at risk for invasive disease. Indeed, based upon 50% lethal dose studies in mice and exoenzyme production, Daily et al. (4) postulated that *A. sobria* may be a more virulent species. In contrast, the decreased frequency with which *A. caviae* was associated with blood infections supports a less pathogenic, and hence less invasive, role for this species. Kou (19) and Wakabayashi et al. (34), who used the loach fish model for pathogenicity tests, also attributed an attenuated virulence to the anaerogenic aeromonad.

TABLE 2. Distribution by source of *Aeromonas* spp.

Source	No. isolated (%)[a]			
	Total	*A. hydrophila*	*A. caviae*	*A. sobria*
Gastrointestinal tract	52 (37)	18 (26)	17 (47)	17 (46)
Wounds	29 (21)	16 (24)	9 (25)	4 (11)
Blood	18 (13)	8 (12)	2 (6)	8 (22)
Fluids	12 (9)	7 (10)	2 (6)	3 (8)
Respiratory tract	11 (8)	9 (13)	2 (6)	0 (0)
Genitourinary tract	5 (4)	3 (4)	1 (3)	1 (3)
Miscellaneous	3 (2)	1 (1)	1 (3)	1 (3)
Environmental	11 (8)	6 (9)	2 (6)	3 (8)

[a] Percent distribution of all *Aeromonas* strains recovered from each source or the percent distribution of each individual species related to site of isolation.

Identification of *Aeromonas* isolates to species level has associated a cholera-like illness (2) with *A. sobria* and gastroenteritis in children (11) with *A. hydrophila*. Furthermore, in each instance, certain species could also be correlated with the presence of particular enterotoxins or specific phenotypic properties. Thus, distinct clinical syndromes referable to the bowel may in fact be species mediated when of *Aeromonas* etiology, and as with *Escherichia coli*, this may reveal inherent properties of enteroinvasiveness or enterotoxigenicity or both.

Finally, several outbreaks or clusters of *Aeromonas* infections have recently been reported (1, 3, 24). From an epidemiological standpoint it is important to determine whether these isolations represent a common source exposure (single strain) or random acquisition of nonidentical isolates. Species identification will help to resolve such matters, especially in the absence of other more definitive epidemiological tracers for *Aeromonas*.

From the data presented herein, it is apparent that aeromonads, including the three recognizable species, can be recovered from virtually every body site. Until recently, few studies or clinical reports documenting *Aeromonas* infections had accompanying species data. If further delineation of the pathogenic potential of these microorganisms is to be uncovered, identification and biotyping must be undertaken to elucidate correlates of the clinical significance, pathogenesis, and epidemiology of *Aeromonas* species.

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