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Further Studies on Biochemical Characteristics and Serologic Properties of the Genus *Aeromonas*

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We characterized a collection of 268 *Aeromonas* isolates from diverse sources (clinical, animal, and environmental sources) for their species and serogroup designations. Overall, 97% of these strains could be identified to the genomospecies level by using an expanded battery of biochemical tests. Members of the *Aeromonas hydrophila* complex (*A. hydrophila*, HG2, and *A. salmonicida*), a group that has previously been difficult to separate biochemically, could easily be distinguished from one another by using a number of recently described phenotypic properties which included utilization of DL-lactate and urocanic acid. Differences in species distributions on the basis of the source of isolation were noted. Serogroup analysis of these 268 isolates plus a number of reference cultures indicated that (i) each genomospecies is serologically heterogeneous and individual serogroups can be found in more than one species, (ii) most type or reference strains for each hybridization group are not serologically representative of the genomospecies at large, (iii) serogroups O:11, O:34, and O:16 predominate clinically (48%), supporting previous studies indicating their importance in human infections, and (iv) most *A. trota* strains do not express the O139 antigen of *Vibrio cholerae*. The collective results suggest that both species and serogroup designations are important factors in establishing which isolates can cause human infections when they are acquired from nonclinical sources (foods, animals, and the environment).

The genus *Aeromonas* is composed of a large number of distinct taxa (9). This group is represented by at least 12 legitimate species, several other nomenspecies of questionable validity (4), and a number of unnamed hybridization groups or clusters (HG2, HG11, and *Aeromonas* group 501). Of the published species, only *A. sobria*, *A. eucrenophila*, and *A. encheleia* have not yet been recovered from clinical material (5, 8). Other species, such as *A. allosaccharophila*, cannot be identified in the clinical laboratory since they do not possess unique biochemical characteristics that enable one to phenotypically separate this group from other mesophilic species (16).

The genus *Aeromonas* is also antigenically diverse. Several different serologic typing schemes based upon the presence of unique heat-stable somatic (O) determinants have been developed. Of the several published schema, the most widely used system to date is that of Sakazaki and Shimada (19), which recognizes 44 serogroups and an additional 52 provisional serogroups within the genus *Aeromonas* (2). By using the typing system of Sakazaki and Shimada (19), several important *Aeromonas* serogroups have been defined. These include serogroup O:11, which is responsible for serious invasive clinical infections such as septicemia, meningitis, and peritonitis (13), and serogroup O:34, which is responsible for human wound infections such as myonecrosis and epizootic outbreaks of septicemia in goldfish (12, 17).

Although there have been a limited number of clinical investigations documenting the association of specific *Aeromonas* serogroups with specific species, those studies have generally been limited by the number of strains studied, the anatomical sites or clinical specimens from which strains were recovered (primarily feces), and the degree to which *Aeromonas* isolates were identified (phenospecies). Recently, using a large collection of strains genetically characterized by DNA-DNA hybridization, Abbott et al. (1) were able to phenotypically identify >98% of these strains to the genomospecies level by using a battery of biochemical tests. We have extended the observations of Abbott et al. by analyzing a large group of more than 200 field isolates of *Aeromonas* from diverse sources for both biochemical and serologic properties in order to determine whether additional correlations exist between genomospecies and serogroup designations.

MATERIALS AND METHODS

Bacterial strains. Two hundred sixty-eight field isolates of *Aeromonas* (clinical isolates, $n = 208$; environmental isolates, $n = 35$; isolates from animals, $n = 25$) were studied in the present investigation. Of the 208 clinical isolates, 104 originated from a previous investigation (11) of 164 strains that had only been identified to the phenospecies level (*A. hydrophila*, *A. caviae*, and *A. sobria*). The remaining 104 strains were from the Microbial Diseases Laboratory collection of strains submitted between 1986 and 1995. All 35 environmental isolates were recovered from freshwater sources. A majority of these strains were recovered from freshwater streams, creeks, and lakes in northern California in 1987 by the late B. Nelson. The 25 animal isolates were received from various investigators between 1981 and 1995. All strains were maintained as working cultures on motility deeps.

Biochemical tests. Strains were identified to the species level by using an 18-test format based on the protocol of Abbott et al. (1), with the exception that the final results were recorded at 72 h rather than at 7 days. The 18 biochemical tests used were oxidase and indole reactions, gas from glucose fermentation, ornithine and lysine decarboxylase and arginine dihydrolase activities, esculin hydrolysis, production of acetylmethylcarbinol (Voges-Proskauer), hemolysis on sheep blood agar, elastase production, oxidation of potassium gluconate, hydrogen sulfide formation in gelatin-cysteine-thiosulfate medium, and fermentation of L-arabinose, D-mannitol, salicin, mannose, cellobiose, and sucrose. For some isolates the performance of additional tests was required to identify strains to the species level. These tests included fermentation of glycerol, L-rhamnose, and D-sorbitol and utilization of sodium citrate, DL-lactate, or urocanic acid. DL-Lactate was prepared in slants according to the following formula, per liter:

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TABLE 1. Distribution of *Aeromonas* isolates by Source of Isolation

Species or group	No. (%)[a] of isolates		
	Clinical	Animal	Environmental
A. hydrophila	87 (42)	8 (32)	10 (29)
HG2	2 (1)	2 (8)	1 (3)
A. salmonicida	1 (0)	5 (20)	3 (9)
A. caviae	59 (28)	5 (20)	2 (6)
A. media	3 (1)	1 (4)	0
A. eucrenophila	0	2 (8)	0
A. veronii (HG8)	36 (17)	1 (4)	12 (34)
A. veronii (HG10)	2 (1)	0	2 (6)
A. jandaei	8 (4)	1 (4)	1 (3)
HG11	1 (0)	0	0
A. trota	3 (1)	0	1 (3)
Unidentified	6 (3)	0	3 (9)
Total	208	25	35

[a] Percentage of total by source (clinical, animal, and environmental).

DL-lactic acid (60% [wt/vol]; Sigma, St. Louis, Mo.), 2.5 ml; NaCl, 5 g; $NH_4H_2PO_4$, 1 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; agar, 15 g; and 0.2% bromthymol blue, 40 ml. The final pH of the medium was 6.8 (3). The slants were heavily inoculated with the test organisms and were incubated at 35°C; the formation of a deep blue color was an indication of DL-lactate utilization. Determination of urocanic acid utilization was performed as described by Hänninen (6). For characterization of biochemical phenotypes within the *A. hydrophila* complex (*A. hydrophila* [HG1], HG2, and *A. salmonicida* [HG3]), a number of reference strains were included (1).

Serogrouping. All *Aeromonas* isolates were serogrouped according to the antigenic typing scheme of Sakazaki and Shimada (19). Because of the low numbers of field isolates representing some *Aeromonas* genomospecies, a number of reference strains previously characterized by DNA-DNA hybridization were included in this analysis (1).

Slide agglutination assays and immunoblotting. *A. trota* strains were tested for immunologic cross-reactivity against *Vibrio cholerae* O139 antisera (2). Slide agglutination assays were performed as described previously by using heat-killed (boiled, 10 min) suspensions of bacteria (13). Selected strains were further evaluated for cross-reactivity in immunoblot assays by using a 1:100 dilution of polyclonal O139 antisera (14). Two strains of *V. cholerae* O139 (strains 1058 and 1228) served as positive controls for these assays.

RESULTS

Species identification. Overall, approximately 97% of all *Aeromonas* isolates tested could be assigned to one of the recognized groups or species within the genus (Table 1). With the exception of *A. schubertii*, all other groups with recognizable biochemical markers (except *A. allosaccharophila* and *A. encheleia*) were identified during the course of the investigation. Regardless of the origins of the strains tested, *A. hydrophila* was the single most common genomospecies detected, accounting for between 30 and 40% of all strains identified. Among the clinical isolates tested, the *A. hydrophila-A. caviae-A. veronii* biotype sobria group predominated, accounting for more than 85% of all isolates typed. These same three species accounted for 56% of all animal isolates and 69% of all environmental isolates, although the frequencies of occurrence of the individual species varied somewhat. The remaining 15 to 30% of *Aeromonas* isolates were identified as species less commonly encountered in the microbial biosphere. Among members of the *A. hydrophila* complex, HG2 and *A. salmonicida* were more often recovered from animals or environmental samples (total, 12 to 28%) than from clinical material (<2%). Of the less frequently isolated species recovered from human specimens, *A. jandaei* predominated. Only two isolates of *A. eucrenophila* were identified, both of which were recovered from pork products. All other groups were recovered from at least two different sources.

TABLE 2. Phenotypic characterization of the *A. hydrophila* complex

Character	No. (%) positive		
	A. hydrophila (n = 22)	HG2 (n = 14)	*A. salmonicida* (n = 16)
Fermentation			
L-Rhamnose	5 (23)	10 (71)	0
Salicin	20 (91)	1 (7)	7 (44)
D-Sorbitol	0	0	14 (88)
Utilization of:			
DL-Lactate	19 (86)	0	0
Urocanic acid	2 (9)	13 (93)	16 (100)
Elastase production	13 (59)	0	5 (31)
Gluconate oxidation	15 (68)	2 (14)	4 (25)

Biochemical characterization of the *A. hydrophila* complex. Abbott et al. (1) and Hänninen (6) have recently proposed a series of new tests useful in separating members of the *A. hydrophila* complex (*A. hydrophila*, HG2, and *A. salmonicida*). We evaluated these tests and their usefulness for a series of 52 *Aeromonas* strains which included field isolates and reference strains (Table 2). Although some strain-to-strain variation was noted within each hybridization group, each genomospecies exhibited consistent biochemical properties useful in their separation from phenotypically related taxa. A majority of *A. hydrophila* strains (sensu stricto) utilized DL-lactate (86%), fermented salicin (91%), oxidized potassium gluconate (68%), and produced elastase (59%). Most strains failed to ferment L-rhamnose (23%) or utilize urocanic acid (9%). All *A. hydrophila* strains failed to produce acid from D-sorbitol. In contrast, HG2 strains utilized urocanic acid (93%) but not DL-lactate (0%) and were L-rhamnose positive (71%). Most strains were negative for gluconate oxidation (14%) and the fermentation of salicin (7%); no strain tested produced acid from L-sorbitol or elaborated elastase. *A. salmonicida* strains (mesophilic isolates, indole-positive, and motile) were primarily characterized by their ability to ferment the alcoholic sugar D-sorbitol (88%). Like the HG2 strains, they metabolized urocanic acid (100%) but not DL-lactate (0%). Approximately 50% of the strains tested fermented salicin but not L-rhamnose. A few isolates produced elastase and oxidized gluconate.

Serogroup distribution and frequency. The serogroup (somatic) designations for more than 300 field and reference isolates of *Aeromonas* were determined according to the antigenic typing scheme of Sakazaki and Shimada (19). Approximately 85% of all *Aeromonas* strains were typeable, with the remaining isolates either belonging to unknown serogroups (10%) or being judged to be serologically "rough" (5%). Most ungroupable strains (unknown O antigen) were biochemically identified as *A. caviae*. Of the typeable *Aeromonas* isolates, 56 of the 96 (58%) established or provisional serogroups within this genus were detected. The serogroup distributions of these strains according to their species identifications are listed in Table 3. Each species or group was found to be serologically heterogeneous, and no serogroup was uniquely associated with a single genomospecies or hybridization group. The more common serogroups identified in the major *Aeromonas* species (*A. hydrophila*, *A. caviae*, and *A. veronii*) represented between 20 and 30% of all isolates of each genomospecies. Among the less frequently encountered species, similar trends were observed with two notable exceptions. Five of seven (71%) *A. veronii* biogroup veronii (ODC^+) strains belonged to serogroup O:3.

TABLE 3. Serogroup distribution among *Aeromonas* species or groups[a]

Species or group	Type or reference strain (serogroup)	Other serogroups (no. of isolates)
A. hydrophila[b]	ATCC 7966^T (O:1)	O:11 (30), O:3 (22), O:16 (8), O:23 (5)
HG2	CDC 9533-76 (O:48)	O:16 (2), O:22 (2), O:11, O:21, O:23, O:25, O:78, O:83, O:87, OUK (1 each)
A. salmonicida[c]	ATCC 33658^T (O:14)	O:16 (2), O:21 (2), OUK (2), O:1, O:18, O:23, O:25, O:49, O:50 (1 each)
A. caviae[b]	ATCC 15468^T (O:11)	O:16 (16), OUK (12), O:3 (7), O:39 (5)
A. media	ATCC 33907^T (R)	O:16 (3), O:14 (2), O:3, O:15, O:40 (1 each)
A. eucrenophila	ATCC 23309^T (R)	O:21 (2), O:34 (2), OUK (2), O:3, R (1 each)
A. veronii, DNA group 8[b]	CDC 437-84 (O:15)	O:11 (17)
A. jandaei	ATCC 49568^T (R)	O:11 (2), R (2), O:12, O:16, O:24, O:25, O:35, O:62 (1 each)
A. veronii, DNA group 10	ATCC 35624^T (OUK)	O:3 (5), O:78 (1)
A. schubertii	ATCC 43700^T (OUK)	O:11 (6), O:25 (2), O:9, OUK (1 each)
A. trota	ATCC 49657^T (O:11)	O:26 (2), R (2), O:5, O:11, O:12, O:16, O:32, O:80, O:86, OUK (1 each)

[a] Abbreviations: OUK, O group unknown; R, rough isolate.
[b] Only serogroups with five or more isolates are listed.
[c] Only mesophilic HG3 (*A. salmonicida*) strains were screened.

For *A. schubertii*, 6 of 11 strains (55%) were found to belong to serogroup O:11. The type or reference strains for 5 of the 11 hybridization groups studied were either rough or belonged to somatic groups not previously recognized. None of the 11 type or reference strains contain somatic antigens typical of the more common or predominant serogroups represented by other field or reference isolates of that species.

Nine serogroups (O:11, O:16, O:34, O:3, O:23, O:5, O:14, O:39, and O:83) accounted for 68% of all typeable strains. Three *Aeromonas* serogroups, O:11 (24%), O:16 (14%), and O:34 (10%), predominated. Variations in the frequency distribution of strains of each of these predominant serogroups was noted. Serogroup O:11 strains were more commonly associated with extraintestinal sites (25 to 48%) than the gastrointestinal tract (10%), while O:16 strains were more often observed in association with cases of septicemia and gastroenteritis (17 to 18%) than with wound infections or miscellaneous sources (6%). Serogroup O:34 strains were most often associated with wounds or other miscellaneous sites (17 to 18%) rather than with blood or feces (7 to 8%).

***A. trota* and *V. cholerae* O139.** Recently, Albert and others (2) have described a series of *A. trota* isolates that exhibit immunologic cross-reactivity with the O139 antigen of *V. cholerae*. To determine whether or not this characteristic was common among *A. trota* strains, we screened 15 strains of *A. trota* for the presence of the O139 antigen. By using polyclonal antisera, only two of the 15 *A. trota* strains tested reacted with O139 sera in slide agglutination assays, giving 3+ to 4+ reactions. These strains were NMRI-208 (serogroup O:12) and MOB A16 (unknown O antigen). Both strains also reacted against somatic antisera previously prepared against select *Aeromonas* serogroups, indicating a possible nonspecific reaction (rough antigen, partial cross-reactivity). To confirm the lack of immunologic cross-reactivity between *A. trota* and *V. cholerae* O139, lipopolysaccharide was prepared from select *A. trota* strains (including NMRI-208 and MOB A16) and immunoblotted against O139 antisera at a 1:100 dilution by using two strains of *V. cholerae* O139 as controls. Only the two *V. cholerae* O139 strains reacted against the polyclonal O139 antisera (data not shown).

DISCUSSION

In a previous study from the Microbial Diseases Laboratory (1), we were able to identify >99% of a large collection of genetically characterized *Aeromonas* strains to the genomospecies level. The present investigation, performed with a collection of clinical, animal, and environmental isolates, has generated a similar identification rate (97%). Collective results suggest that most mesophilic aeromonads can be identified to the genomospecies level (hybridization groups) by using appropriate biochemical reactions. Two groups which have been notoriously difficult to separate biochemically have been members of the *A. hydrophila* (*A. hydrophila*-HG2-*A. salmonicida*) and *A. caviae* (*A. caviae-A. media-A. eucrenophila*) complexes. Recently, a number of new tests useful for separating members of the *A. hydrophila* complex have been proposed, including utilization of DL-lactate by Altwegg et al. (3) and urocanic acid by Hänninen (6). Results from the present study suggest that both of these tests, in addition to others previously proposed by our laboratory (1), are useful in separating members of this complex (Table 2). Because there are strains within each group that have patterns that vary from the ideal profiles, a series of phenotypic tests should be used to identify each isolate to the species level rather than relying on one or two properties only.

The distribution of *Aeromonas* species based upon the source of isolation suggests some possible important differences (Table 1). We noted that higher percentages of HG2-*A. salmonicida* and *A. media-A. eucrenophila* recovered from animal-environmental sources as opposed to clinical material. These findings are similar to those recently published by Hänninen and Siitonen (7), who found each of these groups to be associated with nonclinical sources. Since it has been previously established that >85% of all clinical infections due to *Aeromonas* are caused by *A. hydrophila-A. caviae-A. veronii* biotype sobria (8), the results seem to indicate that infection resulting from environmental exposure to aeromonads (water, soil, and food) is not a random event but requires specific colonization or infection with strains from genomospecies that have the necessary pathogenic properties to cause disease.

As reported, the genus *Aeromonas* is antigenically diverse, being composed of more than 96 distinct serogroups on the basis of the presence of unique somatic antigens. These serogroups are not species specific, and the type and reference strains for each hybridization group are often not representative of the group as a whole (Table 3). In the present study of *Aeromonas* strains isolated in the United States, three serogroups predominated. The high incidence of serogroups O:11, O:16, and O:34 recovered in the present investigation is similar to the incidence obtained by Sakazaki and Shimada (19) in their study of 307 *Aeromonas* strains and is identical to that found by Misra and colleagues (18) in their study of 118 *Aeromonas* isolates recovered from diverse sources in India. In a

study of more than 1,200 *Aeromonas* strains serogrouped by the Central Public Health Laboratory in Colindale, England (20), O:11, O:16, and O:34 were again found to be common serogroups, although a previously unrecognized somatic group (designated AX1) predominated in that study. This indicates that although there are differences in serogroup distribution related to geographic locale, certain serogroups appear to predominate globally. Serogroup O:11 has previously been reported to cause extraintestinal infections in humans and animals, and the topology of its bacterial surface includes a unique lipopolysaccharide side chain architecture, possession of a paracrystalline surface layer (S layer), and possession of a capsule (8, 14, 15). Serogroup O:34 isolates, which also possess a capsule (15), have been associated with severe wound infections and sepsis in humans and have been reported to be the etiologic agent of an outbreak of septicemia in cultured goldfish (17). *Aeromonas* isolates bearing the O:16 somatic antigen have been reported to be important causes of septicemia in adults (10). Whether the serogroup designation is actually a virulence determinant or simply a marker for strains with other cell-associated or enzymatic properties remains to be determined. Some differences noted previously, such as the sharing of partial antigenic identity with isolates of *V. cholerae* O139 by *A. trota* isolates (2), appears to be an unusual finding and not representative of the genomospecies at large. Further studies aimed at identifying *Aeromonas* isolates to the species and serogroup levels should provide important new clues on the ecology, environmental distribution, and host tropism of individual genomospecies.

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