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Isolation and Identification of Autoagglutinating Serogroup O:11 *Aeromonas* Strains in the Clinical Laboratory

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We evaluated the extent to which serogroup O:11 *Aeromonas* strains could be recovered from both clinical and environmental specimens and the cultural parameters that affected the phenotypic marker (autoagglutination) associated with this group. Of over 200 *Aeromonas* strains screened, serogroup O:11 was identified only among the phenospecies *A. hydrophila* and *A. sobria* and was associated with clinical isolates more frequently than with environmental strains. Blood and wound isolates accounted for almost 50% of all O:11 strains identified. The autoagglutination phenotype associated with O:11 strains could be detected in most commercial liquid media, under a wide range of growth temperatures, and within 15 min of incubation at 100°C. The results suggest that clinical laboratories can recognize this important group of *Aeromonas* strains by two simple tests.

Recently, two independent laboratories identified an important group of highly pathogenic *Aeromonas* strains that have been linked to a variety of human infections as well as diseases of fish (2, 3, 7, 9). This subgroup of *Aeromonas* strains has been shown to possess a number of closely associated phenotypic and structural properties which include the ability to autoagglutinate (or autoaggregate) in broth, the presence of a common somatic antigen (Sakazaki and Shimada scheme, serogroup O:11), and the presence of a surface array protein in the form of an S layer on the outermost surface of the bacterium (9). This last feature is shared in common with virulent strains of *Aeromonas salmonicida* which are pathogenic for fish (4), although the two S layers are not identical proteins (9). Since the S layer of *A. salmonicida* has been shown to be the major virulence determinant responsible for pathogenicity in fish (4), it is suspected that this surface array protein may play a similar role for serogroup O:11 *Aeromonas* strains in both human and animal infections.

Serogroup O:11 *Aeromonas* strains can at present be screened for and recognized by two fairly simple tests, autoagglutination (AA) in brain heart infusion broth (BHIB) and, subsequently, serologic reactivity in slide agglutination assays against polyclonal O:11 antisera. Since our original report (7), we have not evaluated how cultural conditions might affect the AA phenotype, nor have we extensively surveyed a large collection of *Aeromonas* strains to determine the frequency and distribution of this serogroup. This study therefore serves as the basis of this report.

MATERIALS AND METHODS

Bacterial strains. A collection of 220 mesophilic *Aeromonas* strains were evaluated in this study (Table 1). All of these strains were initially identified to the genus and then phenospecies level by previously established criteria (5, 6). Approximately one-third of the environmental strains originated from freshwater lakes, rivers, and streams in California; the majority of the remaining strains were obtained from either colonized or infected animals.

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Identification of serogroup O:11. Initially, all *Aeromonas* strains were screened for the AA^+ marker as previously described (7). Briefly, each strain was inoculated in a 6-ml tube of BHIB and incubated at 35°C under static conditions. After overnight incubation (18 to 20 h), each tube was gently vortexed to resuspend any pelleted growth, after which it was immediately placed in a boiling water bath for 1 h. Strains showing visible evidence of precipitation from broth after 1 h were considered AA^+. The cell sediment obtained from duplicate BHIB cultures was assessed for cell viability by inoculation onto sheep blood agar.

All AA^+ and AA^- strains were then screened in slide agglutination assays against somatic antisera previously generated against *Aeromonas hydrophila* NMRI-54 and *Aeromonas sobria* ATCC 9071, both serogroup O:11 (7, 9). To ensure that no false-positive agglutination reactions occurred because of rough strains, somatic antiserum prepared against *A. sobria* 14 (serogroup O:25) was included as a negative control. All strains exhibiting a reaction (range, 3+ to 4+) against NMRI-54 and ATCC 9071 while unreactive against *A. sobria* 14 were considered to belong to serogroup O:11. Eighteen of these O:11 strains were later confirmed as O:11 through the courtesy of R. Sakazaki and T. Shimada (Tokyo, Japan).

Relative degree of precipitation (RDP). For some experiments, the AA^+ phenomenon was semiquantitated by use of the RDP as previously described (7). The RDP was expressed as the quantitative difference (in units of optical density at 610 nm) in turbidity between the heated (boiled) and unheated tubes of BHIB containing overnight growth of individual strains.

RESULTS

Of over 200 *Aeromonas* strains screened for the AA phenotype, approximately one-fourth were found to be AA^+ (Table 1). AA^+ strains were detected in five of the six species evaluated, the only exception being *Aeromonas media*, of which only two strains were available for study. While 83% (49 of 59) required incubation in boiling water before the AA^+ phenotype was expressed, a number of isolates spontaneously pelleted after overnight growth in BHIB, as previously noted (7). When these strains were

TABLE 1. Frequency of serogroup O:11 among *Aeromonas* species

Phenospecies	No.				
	Tested	Clinical	Environmental	AA$^+$	Serogroup O:11
A. hydrophila	108	72	36	32 (29)[a]	21 (66)[b]
A. sobria	53	40	13	21 (40)	15 (71)
A. caviae	47	38	9	2 (4)	0
A. veronii	6	5	1	1 (17)	0
A. schubertii	4	4	0	3 (75)	0
A. media	2	0	2	0	0
Total	220	159	61	59 (27)[c]	36 (16)[c]

[a] Percentage of total strains of designated phenospecies.
[b] Percentage of AA$^+$ strains of designated phenospecies.
[c] Percentage of total strains analyzed.

resuspended, they quickly precipitated from BHIB upon being placed in boiling water. When all 59 AA$^+$ strains were subsequently screened for reactivity against serogroup O:11 antisera, 36 (61%) were found to belong to this serogroup. Each of these strains gave a reaction (range, 3+ to 4+) in slide agglutination assays against both O:11 polyclonal antisera (NMRI-54 and ATCC 9071) while remaining unreactive against the control O:25 antibodies. No AA$^-$ strains gave positive specific slide agglutination reactions against either O:11 antiserum. Serogroup O:11 strains were found only among the phenospecies *A. sobria* and *A. hydrophila*; 28% (15 of 53) of all the *A. sobria* strains tested were identified as belonging to serogroup O:11.

Of the 36 strains of serogroup O:11 identified, a majority of these (83%) originated from clinical material ($P < 0.025$). The two most common sites from which O:11 strains were recovered were blood ($n = 9$) and wounds ($n = 8$), accounting for almost one-half of all clinical O:11 strains. Wound infections caused by serogroup O:11 were almost always caused by *A. hydrophila*, involved the extremities, and often resulted from trauma to a mucosal surface that had come into contact with an aquatic source containing aeromonads. Fecal strains ($n = 6$) were recovered from individuals with the diagnoses of enterocolitis, colitis, chronic diarrhea, or typhoid fever-like syndrome. Other clinical isolate sources included peritoneal fluid ($n = 4$), gall bladder and bile ($n = 2$), and the throat ($n = 1$). Among environmental strains, four isolates were identified which originated from infectious processes or pathologic specimens involving a frog, fish, hamster, and canary. The sole shellfish isolate was recovered from a clam.

Several of these O:11 strains were then selected in order to evaluate the effects of different growth temperatures on the expression of the AA$^+$ phenotype. All six O:11 *Aeromonas* strains tested gave strong AA reactions when grown in BHIB at 30°C; five of these six strains showed similar tendencies at 35°C. One strain, *A. sobria* 86, gave a moderate response at both 22 and 35°C, and a majority of the strains tested performed similarly at 42°C, which was partially due to their diminished growth capacities at this elevated temperature.

In an attempt to determine the minimum time required to detect AA$^+$ O:11 strains grown in BHIB at 35°C, a time course study was performed on 36 of these isolates (Table 2). More than 90% of all tested strains were AA$^+$ after incubation in a boiling water bath for 20 min; almost 70% of these strains required only 10 min of incubation to express the marker. Rare strains required longer periods of incubation to exhibit AA positivity; this delayed expression appeared to be strain dependent and did not substantially vary from one experiment to the next.

TABLE 2. Effect of length of boiling on AA reaction of serogroup O:11 *Aeromonas* strains

Length of boiling (min)	No. of strains AA$^+$ (% of total)
5	17 (47)
10	9 (25)
15	6 (17)
20	1 (3)
30	1 (3)
45	1 (3)
50	1 (3)

To determine whether the growth medium significantly affected expression of the AA$^+$ phenotype, we chose 10 AA$^+$ O:11 strains (including 1 that required 20 to 25 min of boiling to be revealed as AA$^+$) for study in five different broths (Table 3). Of the five broths tested at 35°C, four (all except Penassay) yielded good results. All 10 strains were found to be AA$^+$ in Trypticase soy broth within 20 min of incubation in a boiling water bath; three other liquid media (BHIB, Mueller-Hinton, and veal infusion) were able to detect 9 of the 10 strains in a similar interval, the only exception being the single strain that consistently required 20 to 25 min of incubation in BHIB for phenotype expression. The best growth for all 10 strains was observed in BHIB and was reflected in the higher RDP values; however, regardless of the overt growth index (optical density at 610 nm), each of the four media yielding good results (which did not include Penassay) exhibited a similar percentage (70 to 79%) of reduction in turbidity after boiling.

DISCUSSION

Investigations by Trust and colleagues (2, 3) and our laboratory (6, 7) have simultaneously identified an important subgroup of *A. hydrophila* and *A. sobria* strains involved in a wide range of infections in the animal kingdom. These strains of aeromonads share a number of features, including an unusual phenotype (AA in broth), a common somatic antigen (serogroup O:11), and possession of a surface array protein in the form of an S layer. This group is distinct from the suicide phenomenon described by Namdari and Bottone (8), as all 220 strains were viable upon subculture of their BHIB cell sediments after 18 to 20 h of growth at 35°C. In animals, O:11 infections in fish, frogs, pigs, otares, hamsters, and birds have been documented. In humans, while O:11 has been recovered primarily from individuals with

TABLE 3. Effect of growth medium on AA of 10 selected serogroup O:11 *Aeromonas* strains boiled for 20 min

Broth	No. AA$^+$	RDP[a]
BHIB	9	1.19 (79)
Mueller-Hinton	9	0.89 (73)
Penassay	6	0.63 (61)
Trypticase soy	10	0.81 (70)
Veal infusion	9	0.98 (77)

[a] In units of optical density at 610 nm (percent reduction in total growth after boiling).

septicemia or peritonitis, these bacteria have also been recovered from wound infections and the gastrointestinal tract. On the basis of the results of a serologic study by Sakazaki and Shimada (10) and the present investigation, serogroup O:11 is more commonly associated with human infections, occurring in 10 to 30% of the *A. hydrophila* and *A. sobria* strains studied to date. In contrast, O:11 has been found at a threefold-lower rate in environmental specimens, suggesting both an enhanced pathogenicity for humans and a greater genetic diversity of *Aeromonas* species known to occur in nature. However, in both the study of Sakazaki and Shimada (10) and that of Cheasty et al. (1), 12% of the O:11 strains recognized were identified as *Aeromonas caviae*. The fact that these organisms add to the increasing list of human pathogens known to contain S layers, such as *Campylobacter fetus* subsp. *fetus*, *Mycobacterium bovis*, and *Clostridium botulinum*, suggests an important role for this structure in the disease process (11).

In the clinical laboratory, this important group of *Aeromonas* strains can be recognized by use of the AA marker and a subsequent serologic screen to detect the O:11 serogroup. Results from this study indicate that many commercial media are suitable to screen for AA^+ bacteria, and most isolates in these broths can be detected within 15 min of incubation at 100°C. Since the AA^+ phenotype is expressed over a wide range of temperatures, subtle changes in cultural conditions from one laboratory to another should not significantly affect the overall isolation rate of O:11. Presently, it appears that this group should be screened for in the clinical laboratory when either *A. hydrophila* or *A. sobria* is recovered from a noninvasive anatomical site, in view of the predilection of the group for causing serious systemic and extraintestinal disease. Further studies of this group by other investigators will help to define its range, frequency, and disease spectrum in the animal kingdom.

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