

Detection and Characterization of the Hemolysin Genes in *Aeromonas hydrophila* and *Aeromonas sobria* by Multiplex PCR

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A multiplex PCR assay was designed to amplify the *Aeromonas hydrophila* and *A. veronii* bv. *sobria* hemolysin and aerolysin genes. The assay was evaluated by using 121 clinical isolates and 7 reference strains of *Aeromonas* spp., and these were divided into five genotypes on the basis of the results of the multiplex PCR. The five genotypes were characterized as type 1 for those carrying the *ahh1* gene only (36% of isolates), type 2 for those carrying the *asa1* gene only (8.5% of isolates), type 3 for those carrying both the *ahh1* and the *asa1* genes (4% of isolates), type 4 for those carrying the *ahh1* gene and the *A. hydrophila* *aerA* (aerolysin) gene (37.5% of isolates), and type 5 for those in which no hemolysin genes were detected (14% of isolates). The most common single hemolysin gene carried among all the *Aeromonas* isolates examined was *ahh1*, with 99 of 128 (77%) of isolates testing positive for this gene either alone or in combination with other hemolysin genes. Phenotypic expression of toxins was evaluated in a Vero cell culture cytotoxicity assay. These results indicated that there is a statistically significant correlation between the cytotoxin titers and the hemolysin genotype. Isolates belonging to genotype 4 (carrying both the *ahh1* gene and the aerolysin and hemolysin *aerA* genes) expressed higher cytotoxin titers than isolates of the other genotypes ($P < 0.001$). These isolates were more cytotoxic in cell culture and may have greater clinical significance.

Aeromonas hydrophila and *A. veronii* bv. *sobria* (otherwise known as *A. sobria*) are gram-negative bacteria of the family *Vibrionaceae* that are often found in association with hemorrhagic septicemia in cold-blooded animals including fish, reptiles, and amphibians (4). However, these organisms have also been implicated as primary pathogens in cases of acute diarrheal disease in immunocompetent humans of all age groups (1, 12, 13, 34). Indeed, Poirier et al. (35) reported a case of pneumonia of nosocomial origin caused by *A. hydrophila* that complicated toxic coma, while both *A. hydrophila* and *A. sobria* have been isolated from humans with sepsis, peritonitis, urinary tract infections, and severe muscle degeneration (12). Hemolytic-uremic syndrome, a life-threatening condition normally associated with infections due to *Escherichia coli* O157:H7, has also been associated with *A. hydrophila* infection (6, 23, 37); and a cytotoxin with homology to Shiga toxin 1 has been identified in both *A. hydrophila* and *A. caviae* (18).

A number of virulence factors derived from *A. hydrophila* and *A. sobria* have been proposed in an effort to explain the pathogenesis of infections due to these organisms. Toxins with hemolytic, cytotoxic, and enterotoxic activities have been described in many *Aeromonas* spp. (12, 32); and while a number of toxins are produced by different species, single isolates often carry the genes encoding multiple toxins. Mutagenesis studies indicated that the hemolytic activity of *A. hydrophila* is related

to both the hemolysin and the aerolysin genes. In addition, a gene encoding the cytolytic enterotoxin (Act) from *A. hydrophila* has been sequenced and shown to possess hemolytic, cytolytic, and enterotoxic activities (11). In an isogenic investigation in which a ligated ileal loop assay was used in a mouse model, it was concluded that the three toxins studied, the cytotoxic enterotoxin (*act* gene product), the heat-labile cytotoxic enterotoxin (*alt* gene product), and the heat-stable cytotoxic enterotoxin (*ast* gene product), all contributed to *A. hydrophila*-induced gastroenteritis. The Act enterotoxin resulted in the highest degree of fluid accumulation (38). Sequence analysis revealed that the *act* gene shared 89 and 93% DNA and amino acid homologies, respectively, with the *A. hydrophila* *aerA* (aerolysin) gene (11, 38). The biological functionality of this gene has been mapped to specific amino acid residues within the protein (14). Distinct hemolysins (hemolysin and aerolysin) have been detected in *A. hydrophila* by both immunologic and genetic methods (20, 22, 26). Beta-hemolytic isolates of *Aeromonas* were found to cause significantly more fluid accumulation in the ileal loops of experimentally infected rabbits than the alpha-hemolytic and nonhemolytic isolates, regardless of their species designation (40). Nacescu et al. (29) postulated a correlation between the pathogenic potential and the hemolytic activity of *Aeromonas* species. A majority of the *A. hydrophila* and *A. sobria* isolates were highly hemolytic, whereas only 11% of the *A. caviae* isolates were capable of lysing sheep erythrocytes (30). Mutagenesis of *A. hydrophila* isolates that carry two hemolysin genes (*hlyA* and *aerA*) revealed that the hemolytic activity of the isolate on horse blood agar was eliminated only following double mutations in the *hlyA* and *aerA* genes (44). A subsequent study suggested that

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TABLE 1. Correlation of hemolytic phenotype with hemolysin gene content as determined by multiplex PCR^a

Organism ^b	Phenotype	No. of isolates with each hemolysin genotype				No. of isolates
		<i>ahh1</i> (genotype 1)	<i>asa1</i> (genotype 2)	<i>ahh1</i> + <i>asa1</i> (genotype 3)	<i>ahh1</i> + <i>A. hydrophila aerA</i> (genotype 4)	
<i>A. hydrophila</i> (87)	Hemolytic	35 ^c		1	46	82
	Nonhemolytic	3 ^d			2 ^d	5
<i>A. veronii</i> bv. <i>sobria</i> (21)	Hemolytic		9	4		14
	Nonhemolytic	2 ^d	1 ^d			7
<i>A. caviae</i> (15)	Hemolytic					1 ^d
	Nonhemolytic	3 ^d				11
<i>A. veronii</i> bv. <i>veronii</i> (1)	Hemolytic		1			1
<i>A. trota</i> (2)	Hemolytic	2				2
<i>A. jandaei</i> (1)	Hemolytic	1				1
<i>A. schubertii</i> (1)	Nonhemolytic					1 ^d
Total		46	11	5	48	128

^a All isolates produced 16S rRNA amplicons.

^b Numbers in parentheses are the total number of isolates examined within each species.

^c Includes positive control strain CA11.

^d DNA hybridization was performed with all of these isolates.

all virulent *A. hydrophila* isolates carried both the *hlyA* and the *aerA* genes (19).

By using methods involving PCR and restriction fragment length polymorphism analysis, the virulence genes of *Aeromonas* spp. were grouped as aerolysins-hemolysins, cytolytic enterotoxins, or cytotoxic enterotoxins (25). A PCR method for the amplification of the aerolysin gene was shown to detect β -hemolysin-positive *A. hydrophila* isolates from patients with diarrhea (36). Control isolates of hemolytic *A. sobria*, isolates of nonhemolytic *Aeromonas* spp., and *A. caviae* isolates did not produce an amplification product with these primers, even when these isolates were capable of producing cytotoxins or enterotoxins (5, 36). PCR techniques for the detection of two distinct hemolysin genes of *A. sobria* have also been developed (39). Alignment of known cytotoxic enterotoxin-aerolysin genes was used to detect sequences conserved throughout this family of genes, and PCR primers whose sequences are complementary to these sequences should detect all known cytotoxin genes (25). This PCR protocol in combination with PCR and restriction fragment length polymorphism analysis or PCR amplicon sequence analysis should partially permit estimation of the distribution of gene variants within different *Aeromonas* spp. and should assist in assessing the virulence of isolates carrying defined subsets of cytotoxin genes (25).

While a number of PCR methods reported previously appear to be effective for detection of a subset of cytotoxin-hemolysin genes, the multiplex PCR assay developed in the present study was designed to amplify both the *A. hydrophila* and the *A. sobria* hemolysin and aerolysin genes. The assay was evaluated with 121 clinical isolates and 7 reference strains of *Aeromonas* spp., and the relationship between the hemolysin genotypes and the virulence phenotypes of clinical isolates was also analyzed. In addition, a scheme for classifying *Aeromonas* spp. into five distinct genotypes is proposed.

MATERIALS AND METHODS

Bacterial isolates and culture media. A total of 121 clinical isolates of *Aeromonas* spp. from sporadic cases of enteritis across Canada were evaluated and characterized in this study (Table 1). These isolates were submitted to the National Laboratory for Enteric Pathogens (NLEP) by Canadian provincial public health laboratories between 1986 and 1997 as part of the normal reference and enteric disease surveillance efforts in Canada. Reference strains ATCC 7966 (*A. hydrophila*), ATCC 9071 (*A. sobria*), ATCC 35622 (*A. veronii* bv. *veronii*), ATCC 15468 (*A. caviae*), ATCC 49568 (*A. jandaei*), and ATCC 43700 (*A. schubertii*) were also investigated. The hemolytic isolate of *A. hydrophila*, isolate CA11, was obtained from the Center for Food Safety and Applied Nutrition of the Food and Drug Administration, Cincinnati, Ohio, and was used as a positive control. All 128 isolates were grown on Mueller-Hinton agar (Oxoid Ltd., Nepean, Ontario, Canada) containing 5% defibrinated sheep blood erythrocytes and were identified by standardized biochemical protocols (2). Cultures were stored either on slants of Institut Pasteur (IP) maintenance medium in the dark or in brain heart infusion broth containing 15% glycerol at -80°C . Positive hemolytic activity was defined as a zone of hemolysis around colonies on the blood agar plate after 18 to 24 h of incubation at 37°C .

Nucleic acid isolation. Nucleic acids were isolated by lysis of the bacteria in a solution containing 0.5% sodium dodecyl sulfate and 5 mg of lysozyme per ml, followed by extraction with phenol-chloroform (21). Nucleic acid samples were precipitated with ethanol and dissolved in TE buffer (10 mM Tris chloride, 1 mM EDTA [pH 8.0]). The nucleic acid content was quantified by determining the optical density (OD) at 260 nm (OD₂₆₀) and was adjusted to give a final concentration of 200 $\mu\text{g}/\text{ml}$ in TE buffer. Template DNA for PCR was prepared by further dilution in distilled H₂O to a concentration of 2 $\mu\text{g}/\text{ml}$.

Oligonucleotide primers and PCR conditions. The AHH1 primer set was designed to amplify a 130-bp fragment of *A. hydrophila* extracellular hemolysin gene *ahh1* (20). The AH-aerA primer set amplified a 309-bp fragment of the *A. hydrophila* aerolysin gene *aerA* (GenBank accession no. M16495); the nucleotide sequence of this gene had 89% similarity to that of the hemolysin gene of AHH5 (GenBank accession no. X65045). Similarly, the ASA1 primer set was designed to amplify a 249-bp fragment of *A. sobria* hemolysin gene *asa1* (GenBank accession no. X65046). Primers based on the *A. hydrophila* ATCC 7966 16S rRNA sequence (GenBank accession no. X74677) were designed to amplify a portion of the 16S rRNA gene as an internal control (Table 2). DNA samples (5 ng per reaction mixture) were amplified in a 25- μl reaction mixture consisting of 50 mM potassium chloride; 10 mM Tris chloride (pH 8.3); 1.25 mM magnesium chloride; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 2.0 μM (each) AHH1 primers; 1.5 μM (each) AH-aerA and ASA1 primer; 0.05 μM (each) A16S primers (Table

TABLE 2. Primer pairs used for PCR amplification

Primer pair	Sequence (5' to 3')	Target gene	Location within gene	Size of PCR amplicon (bp)	Reference or GenBank accession no.
AHH1F AHH1R	GCCGAGCGCCAGAAGGTGAGTT GAGCGGCTGGATGCGGTTGT	<i>ahh1</i> ^a	961–983 1090–1071	130	20
AH-aerAF AH-aerAR	CAAGAACAAGTTCAAGTGGCCA ACGAAGGTGTGGTTCCAGT	<i>A. hydrophila aerA</i>	1323–1344 1631–1613	309	M16495
ASA1F ASA1R	TAAAGGGAAATAATGACGGCG GGCTGTAGGTATCGGTTTTTCG	<i>asa1</i>	863–883 1111–1091	249	X65046
A16SF A16SR	GGGAGTGCCTTCGGGAATCAGA TCACCGCAACATTCTGATTTG	16S rRNA	1020–1041 1375–1355	356	X74677

^a From strain ATCC 7966.

2); and 1.25 U of FastStart *Taq* DNA polymerase (Roche Diagnostic GmbH, Mannheim, Germany). Amplifications were performed with a model 2400 DNA thermal cycler (Applied Biosystems, Foster City, Calif.). Parameters for the amplification included an initial denaturation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 0.5 min, annealing of the primers at 59°C for 0.5 min, and primer extension at 72°C for 0.5 min. A final extension at 72°C for 7 min was used. Ten microliters of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.5% agarose at 5 V/cm, and the reaction products were visualized with UV light after staining with ethidium bromide. The identities of the amplicons were confirmed by comparison of the amplicon sizes with the predicted sizes, as indicated in Table 2, and by digestion with restriction endonucleases. While the internal control (the amplification product obtained by PCR with the A16S primer set) gave 113- and 243-bp fragments after digestion with *NheI*, the product obtained by PCR with the AH-aerA primer set yielded 141- and 168-bp fragments after *SacI* digestion. The amplification product obtained by PCR with the AHH1 primer set showed 55- and 75-bp fragments following *HinfI* digestion, and the amplicon obtained by PCR with the ASA1 primer set produced 116- and 133-bp fragments after digestion with *PvuII*.

Cell culture cytotoxicity assay. Vero cell cultures were grown for 48 h to give confluent monolayers of cells in 96-well cell culture plates. The cell culture plates contained minimum essential medium with heat-inactivated 10% fetal bovine serum (Sigma-Aldrich, St. Louis, Mo.), 40 U of penicillin-streptomycin (Invitrogen, Burlington, Ontario, Canada) per ml, 40 µg of gentamicin (Invitrogen) per ml, 0.1 mM nonessential amino acids (Invitrogen), and 2 mM L-glutamine (Invitrogen). The bacterial cultures were grown in 5 ml of brain heart infusion broth at 37°C on a rotating drum for 24 h, and the organisms were removed by centrifugation. Supernatants were filtered through a 0.22-µm-pore size syringe filter (Millipore, Nepean, Ontario, Canada), and 20-µl aliquots of each filtrate were diluted twofold in the cell culture medium and added in duplicate to the Vero cell monolayers for the toxin assay. After incubation at 37°C in a humid atmosphere containing 5% CO₂, the cytotoxic activity of the culture supernatants was assessed microscopically after 24 and 48 h of incubation. At the end of the 48-h incubation period, all monolayers were stained with 2% crystal violet (Sigma-Aldrich) diluted 1/80 with buffered formalin (Fisher, Nepean, Ontario, Can-

ada). After 30 min the detached and dead cells in all of the wells were removed by rinsing the wells with distilled H₂O. The stained live cells were solubilized in situ in buffer containing 0.3% acetic acid and 0.5% sodium dodecyl sulfate. The OD₆₃₀ of the plates were read in a Dynatech MRX plate reader (Dynex Technologies, Chantilly, Va.) by using Revelation software, and the cytotoxicities were determined by comparing the OD values for the test wells with those for the control wells to which only phosphate-buffered saline had been added. The percent cytotoxicity was determined as follows: $100 - (T_{OD}/NC_{OD}) \times 100$, where T_{OD} is the OD₆₃₀ of a test well, and NC_{OD} is the average OD₆₃₀ for the negative controls. The inverse of the dilution that resulted in what was equal or closest to 50% cytotoxicity was determined to be the endpoint titer. The data were subjected to statistical evaluation by the *t* test.

DNA hybridization. On the basis of the data obtained from the PCR and the hemolysin and cytotoxin tests, a total of 14 isolates were further investigated for the presence of the *ahh1*, *A. hydrophila aerA*, and *asa1* genes by DNA hybridization with an enhanced chemiluminescence direct nucleic acid labeling and detection system according to the instructions of the manufacturer (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) under high-stringency conditions. These samples included 11 isolates of genotypes 1, 2, and 4 that were phenotypically hemolysin negative and 3 isolates of genotype 5 that were positive for cytotoxicity (Table 1; see Table 3). The probes were prepared from the amplification products obtained by PCR with the AHH1, AH-aerA, and ASA1 primer sets, respectively.

RESULTS

The sizes of the amplification products obtained by the multiplex PCR were identical to those predicted from the design of the primers (Fig. 1; Table 2). Amplicons from the control isolates were subjected to further characterization by digestion with restriction endonucleases with known cleavage sites

TABLE 3. Evaluation of degree of cytotoxicity expressed in Vero cells compared with genotype results

Genotype	Hemolysin-positive isolates			Hemolysin-negative isolates		
	No. of isolates	CT ^a range	Mean ± SE ^b CT	No. of isolates	CT range	Mean ± SE CT
1 (<i>ahh1</i>)	38	2–256	32.37 ± 8.97	8	0–16	3.00 ± 2.10
2 (<i>asa1</i>)	10	2–16	9.20 ± 1.96	1	0	0
3 (<i>ahh1</i> and <i>asa1</i>)	5	4–32	12.0 ± 5.06	0	0	0
4 (<i>ahh1</i> and <i>A. hydrophila aerA</i>)	46	0–256	79.48 ± 13.38 ^c	2	0–16	8.00 ± 8.0
5 (no hemolysin genes detected)	4 ^d	0–16	5.0 ± 3.79	14 ^e	0–2	0.14 ± 0.14

^a CT in Vero cells. Values are the titers obtained in cell culture assays.

^b SE, standard error of the mean.

^c *P* < 0.001 compared to the mean CTs for the other genotypes.

^d None of the four hemolysin-positive isolates produced a hemolysin-aerolysin amplicon in the multiplex PCR assay; one isolate each of *A. caviae* and *A. sobria* was cytotoxic.

^e One isolate (*A. schubertii*) showed a low level of cytotoxicity.

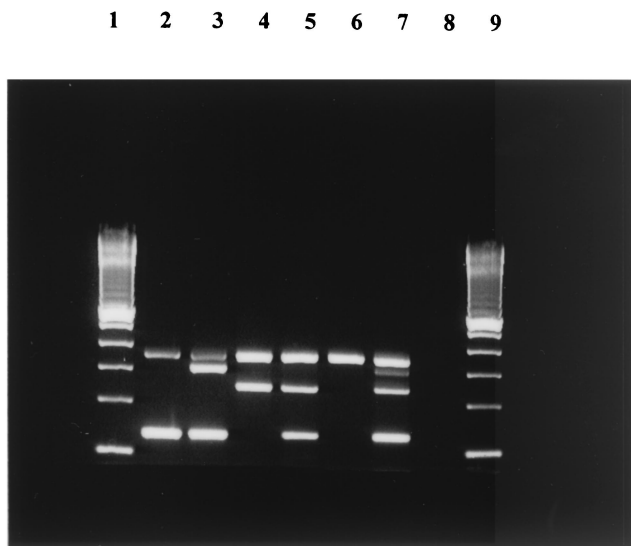


FIG. 1. Detection and identification of *Aeromonas* hemolysin and aerolysin genes by amplification of fragments in the multiplex PCR assay. Lanes 1 and 9, 100-bp ladder (Invitrogen); lane 2, *A. hydrophila* genotype 1 showing the *ahh1* and 16S rRNA genes (130- and 356-bp fragments, respectively); lane 3, *A. hydrophila* genotype 4 showing the *ahh1*, *aerA*, and 16S rRNA genes (130-, 309-, and 356-bp fragments, respectively); lane 4, *A. sobria* genotype 2 showing the *asa1* and 16S rRNA genes (249- and 356-bp fragments, respectively); lane 5, *A. sobria* genotype 3 showing the *ahh1*, *asa1*, and 16S rRNA genes (130-, 249-, and 356-bp fragments, respectively); lane 6, 16S rRNA internal control showing a 356-bp fragment only; lane 7, PCR positive control with a DNA mixture showing PCR amplification fragments for all four genes, *ahh1*, *aerA*, *asa1*, and 16S rRNA (130-, 309-, 249-, and 356-bp fragments, respectively); lane 8, PCR negative control.

within the amplified sequence. Restriction fragments of the anticipated sizes were obtained in each case.

Strains of each of the *Aeromonas* phenotypes carried the hemolysin genes at different frequencies (Table 1). Of the 128 *Aeromonas* spp. isolates tested, 99 (77%) were *ahh1* positive; these included 87 of 87 (100%) of the *A. hydrophila* isolates, 6 of 21 (29%) of the *A. sobria* isolates, 3 of 15 (20%) of the *A. caviae* isolates, 2 of 2 (100%) of the *A. trota* isolates, and the single *A. jandaei* isolate examined. By PCR with the AH-*aerA* primer set, amplicons were produced only by *A. hydrophila* isolates (48 of 87 [55%]). Sixteen of the 128 *Aeromonas* isolates (12.5%) were *asa1* positive, including 14 of 21 (67%) of the *A. sobria* isolates, the single *A. veronii* bv. *veronii* isolate examined, and 1 of 87 (1%) of the *A. hydrophila* isolates (NLEP A1607). Hemolysin genes were not detected in 18 of 128 (14%) of the isolates overall. Most *A. caviae* isolates (12 of 15 [80%]) produced no hemolysin gene amplicons when they were subjected to the multiplex PCR, although one isolate did produce detectable hemolysis on agar containing sheep erythrocytes (Table 1). In addition, hemolysin genes were not detected in 5 of 21 (24%) of the *A. sobria* isolates or in the single isolate of *A. schubertii* tested. All *Aeromonas* isolates were PCR positive for the 16S rRNA internal positive control.

Five genotypes were defined on the basis of the results of the multiplex PCR (Table 1). The reproducibility of the hemolysis data for all isolates was demonstrated in triplicate on agar plate assays containing sheep blood. In addition, the findings

for 29 gene-positive isolates were further confirmed by using horse blood agar. Of these 29 isolates, 22 were positive for hemolysis on both sheep blood agar and horse blood agar, while the remaining 7 isolates (2 *A. hydrophila*, 2 *A. caviae*, and 3 *A. sobria* isolates) were negative on both types of blood agar (data not shown). Of the 27 *Aeromonas* isolates that expressed no hemolytic activity, 8 isolates (3 *A. hydrophila*, 2 *A. sobria*, and 3 *A. caviae* isolates) were genotype 1, 1 isolate (*A. sobria*) was genotype 2, 2 isolates (both of which were *A. hydrophila*) were genotype 4, and 16 were negative for hemolysins in the multiplex PCR assay. All 128 isolates were further analyzed for cytotoxins by the Vero cell culture assay (Table 3). A higher proportion of high cytotoxicity titers (CTs) were obtained for isolates of genotype 4 than for isolates of the other genotypes tested, suggesting a possible relationship between the hemolysin genes present and the degree of cytotoxicity produced by each genotype. However, the ranges of CTs were very similar for genotype 1 and 4 isolates, as were the mean titers for each population of bacteria. Cytotoxicity was detected in three isolates that were negative for the hemolysins targeted by the multiplex PCR assay. Two of these isolates (*A. caviae* A1833 and *A. sobria* A1432) were positive for both hemolytic and cytotoxic activities, and the third one (*A. schubertii*) was weakly cytotoxic only. DNA hybridization showed a stronger signal for *A. sobria* A1432 (with the ASA1 probe) and for both *A. caviae* A1833 and *A. schubertii* (with the AH-*aerA* probe). Among the 11 nonhemolytic isolates, which included 5 *A. hydrophila* isolates, 3 nonhemolytic *A. sobria* isolates, and 3 nonhemolytic *A. caviae* isolates, all of which were positive for one or more hemolysin genes by PCR, consistent positive DNA hybridization results were obtained with the corresponding PCR probe. In addition, two genotype 1 *A. hydrophila* isolates also showed a stronger signal with the AH-*aerA* probe; these were isolates A1915 (CT = 16) and A1556 (CT = 0). Hemolysis was always found in genotype 3 isolates, which carried both the *ahh1* and the *asa1* genes. However, 8 of 46 (17%) genotype 1 isolates, 1 of 11 (9%) genotype 2 isolates, and 2 of 48 (4%) genotype 4 isolates were nonhemolytic, while 2 of 18 (11%) isolates that did not have hemolysin or aerolysin genes detectable by PCR were phenotypically hemolytic (Table 1). There was a statistically significant correlation between the Vero cell CT and the hemolysin genotype ($P < 0.001$). This was demonstrated by a tendency for isolates carrying both the *ahh1* and the *A. hydrophila aerA* genes to have higher CTs than isolates with other genotypes or no hemolysin genes (Table 3). Indeed, of the 110 isolates with at least one hemolysin gene, 99 of 110 (90%) were cytotoxic in Vero cells and showed hemolytic activity (Table 3).

DISCUSSION

Hemolysin genes did not appear to be distributed randomly among the different *Aeromonas* species. All the *A. hydrophila* isolates had the *ahh1* toxin gene, while some also carried the aerolysin gene. However, none of the *A. hydrophila* isolates were positive only for the *A. hydrophila aerA* gene. A PCR and Southern hybridization survey indicated that all virulent *A. hydrophila* isolates were both *hlyA* (AHH1) and *aerA* (aerolysin) positive. Of the 61 *A. hydrophila* isolates investigated, only 2 carried the aerolysin gene alone, and both of these were isolated from healthy fish (19). Although a majority of the *A.*

veronii bv. *sobria* isolates also carried hemolysin genes, the most prevalent gene detected by the multiplex PCR was *asa1*. In contrast, most *A. caviae* isolates tested did not produce an amplicon in the multiplex PCR developed in this study, and the few that did carried only the *ahh1* gene. Indeed, the most common hemolysin gene carried by *Aeromonas* isolates overall was *ahh1* (99 of 128 [77%] isolates). Johnson and Lior (24) previously reported that 80.7% of *A. hydrophila* isolates from stools were cytotoxic. Of 445 *Aeromonas* isolates screened for the presence of aerolysin by PCR in a second study, 79% carried the aerolysin gene and 83% showed cytotoxicity in Vero cell culture assays (33). The investigators found a high frequency of aerolysin gene carriage among all *Aeromonas* spp. tested, including *A. caviae*. Results from other toxin surveys (16, 25) supported the distribution of cytotoxic enterotoxins (hemolysins and aerolysins) noted in this study.

Genotype 2 was characteristic of most hemolytic *A. sobria* isolates and was also found in an atypical *A. hydrophila* isolate, NLEP A1607. This isolate displayed weak hemolytic activity but was positive for both the *asa1* and the *ahh1* genes. On the basis of the results of biochemical assays, this isolate was classified as *A. hydrophila* (esculin test positive, salicin test negative); however, sequence analysis of NLEP A1607 (GenBank accession no. AF410466) confirmed that it had 96% identity with the *A. sobria* hemolysin gene (*asa1*; GenBank accession no. X65046). Furthermore, the partial 16S rRNA sequence of *A. hydrophila* isolate NLEP A1607 (GenBank accession no. AF410780) shared 99% similarity with the 16S rRNA sequence of *A. sobria* isolate ATCC 9071. It is possible that isolate NLEP A1607 represents a novel variant or a new subspecies of *A. hydrophila*.

Immunologic studies have demonstrated that the hemolysins produced by *A. hydrophila* can be divided into two major groups (26). A comparison of the nucleotide sequences of the extracellular hemolysin and aerolysin genes detected only 23.8% nucleotide sequence homology. It is believed that the evolutionary origins of these two genes may differ. Many isolates characterized in this study carried more than one hemolysin: *ahh1* and either *A. hydrophila aerA* or *asa1*. Previous investigations have also found *A. hydrophila* isolates that produced more than one hemolytic toxin (19, 44). Inactivation of both hemolytic toxin genes in an isolate of *A. hydrophila* was necessary to completely attenuate the virulence of the isolate in a mouse model of infection, suggesting that assessment of the toxin gene contents of clinical isolates may be critical for prediction of the virulence potentials of the organisms.

It was difficult to predict the hemolytic or cytotoxic phenotype that an isolate would express solely on the basis of the hemolysin genotype alone. Among the various *Aeromonas* spp. tested, eight isolates carrying the *ahh1* gene, one isolate carrying the *asa1* gene, and two isolates carrying both the *ahh1* and the *A. hydrophila aerA* genes were nonhemolytic on sheep blood agar. Both of the last two isolates were *A. hydrophila*. Although sheep erythrocytes appear to be less sensitive than erythrocytes from other mammals (7, 26), the standardization of the hemolysis assay for all isolates used in the present study suggests that the differences among isolates have a biological basis and are not likely solely due to the sensitivity of the assay. It is possible that the nonhemolytic isolates carried hemolysin genes either that could not be expressed or that had mutations

affecting domains responsible for the hemolytic phenotype. Hemolysin production is significantly correlated ($P < 0.05$) with enterotoxin production, as measured in suckling mouse assays (9, 40). Both phenotypes may be subject to regulation at the phenotypic level, in that all *Aeromonas* isolates that failed to produce enterotoxin in the initial test (44.2%) showed enterotoxic effects after one to three consecutive passages through rabbit ileal loops, suggesting expression of previously repressed toxin genes (40). Little is known about the regulatory mechanisms responsible for these effects and whether they operate in all *Aeromonas* isolates. The hemolytic activities expressed by *A. caviae* and *A. veronii* bv. *sobria* isolates lacking detectable amplicons in the multiplex PCR most likely resulted from the expression of alternate cytotoxin and hemolysin gene variants (43). Recently, it has been confirmed that lecithinase (phospholipase C [PLC]) cloned from *A. hydrophila* was clearly cytotoxic in Vero cells but showed no hemolytic activity (28).

The high degree of correlation between the Vero cell CTs and the hemolysin genotype was striking. The range of cytotoxicity values for each genotype (Table 3) suggested that the cytotoxicity in *Aeromonas* spp. is multifactorial and may involve the products of a number of different genes acting either alone or in concert. Those described in the literature include α and β hemolysins (40); aerolysin (8, 16); Act, Alt, and Ast (38); PLC (28); cholera toxin-like factor (42); and proteases and RNase (15). The Vero cell CTs for isolates carrying both the *ahh1* and the *asa1* genes were quite low, while those for those isolates positive for the *ahh1* gene together with the *A. hydrophila aerA* gene were high. One intriguing possibility is that interactions between different hemolysin and aerolysin genes affect the expression of these genes in *A. hydrophila* isolates. Alternatively, mutagenesis studies have suggested that both the hemolysin and the aerolysin toxins act by inducing pore formation in the membranes of affected cells and that the effect of these together is likely to be synergistic (19, 44). In addition, gene variation, plasmid-mediated gene regulation, quorum sensing-dependent regulation, and toxin protein production as well as secretion might all affect cytotoxin expression levels (8, 10, 15, 17, 41). The present data were in agreement with previous observations demonstrating a very close relationship between hemolytic activity and cytotoxicity for all *Aeromonas* spp. tested (33, 43). Similarly, purified hemolysin from *A. hydrophila* was found to cause fluid secretion in infant mouse assays and cytotoxicity in Vero cell assays (3). In the present study, however, Vero cell cytotoxicity did not correlate absolutely with the hemolysin gene content, as determined by PCR. One isolate each of *A. caviae*, *A. sobria*, and *A. schubertii* did not amplify any of the hemolysin genes tested in the multiplex PCR but showed weak hemolytic or cytotoxic phenotypic activity, suggesting that other virulence traits exist. Indeed, factors such as the export of aerolysin and activation of the protoxin may also affect the ability to detect cytotoxicity (8). Furthermore, DNA hybridization data indicated that all three isolates possessed both hemolysin and aerolysin genes; hence, sequence variations in the target DNA may offer one explanation for a lack of PCR amplification products and for the low level of cytotoxicity detected.

Although the production of hemolytic cytotoxins has been regarded as strong evidence of pathogenic potential in *Aeromonas* spp., nonhemolytic aeromonads have been implicated

as human pathogens (31). Cytotoxic enterotoxins also contribute to the pathogenesis caused by *Aeromonas* spp. (38). The presence of these toxins may also need to be assessed, especially in *A. caviae* isolates with no detectable cytotoxins. It has been shown that many enzymes such as lipase, PLC, protease, and RNase are putative virulence factors for *Aeromonas* spp. (15, 28) and that cytotoxicity may be affected by a number of environmental factors or growth conditions. Indeed, Granum et al. (16) confirmed that the optimal growth temperature for toxin production by *A. caviae* was 30°C rather than 37°C, while Mateos et al. (27) reported that *Aeromonas* spp. isolated from humans showed higher levels of toxin production at 37°C. These findings suggest that as yet undefined factors exist. In the present study, one *A. hydrophila* isolate, one *A. sobria* isolate, and eight *A. caviae* isolates showed no cytotoxicity at either 30 or 37°C, while one *A. hydrophila* isolate expressed relatively low levels of cytotoxic activity at 30°C compared to that at 37°C and three other isolates showed higher levels of activity at 30°C compared to that at 37°C (data not shown). A recent study on serine protease activity in *A. hydrophila* confirmed that protease production at 22 and 30°C was under quorum-sensing control but was inhibited at 37°C in a quorum sensing-independent fashion (41). Positive DNA hybridization results for the three genotype 5 isolates (one isolate each of *A. sobria*, *A. caviae*, and *A. schubertii* [Table 1]) suggested that PCR-based detection may be more specific than DNA hybridization, although it may have a lower sensitivity. One genotype 1 isolate (isolate A1556) that was phenotypically both hemolysin and cytotoxin negative tested positive with the AH-aerA probe, further indicating that other factors affect gene expression (8, 10, 15, 17, 41).

Together, these observations suggest that an evaluation of *Aeromonas* virulence requires the assessment of virulence phenotypes and complete virulence gene sets. Phenotypic methods may not detect the presence of toxins. *A. caviae* isolates that did not produce detectable cytotoxic or hemolytic activity were found to regain the ability to express toxins after animal passage, only to lose it again upon subsequent subculture (40). Screening for specific cytotoxin and hemolysin genes appears to be the most effective way of detecting and characterizing *Aeromonas* virulence factors. The multiplex PCR assay described in this study should prove to be a useful method for the identification of hemolysin and aerolysin genotypes when screening for the major species-specific isolates of *Aeromonas* species.

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