Identification and Characterization of Pathogenic *Aeromonas veronii* Biovar Sobria Associated with Epizootic Ulcerative Syndrome in Fish in Bangladesh

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Sparse information is available on the virulence factors of *Aeromonas* strains isolated from diseased fish, from the environment, and from humans. In the present study, 52 *Aeromonas* isolates obtained from epizootic ulcerative syndrome (EUS) lesions in fish, from the aquatic environment, and from children with diarrhea in Bangladesh were identified by biochemical phenotyping (i.e., PhenePlate [PhP] typing) and DNA fingerprinting and then characterized with respect to certain putative virulence factors. The isolates from the fish exhibiting EUS symptoms were identified to be *Aeromonas veronii* biovar sobria by fatty acid methyl ester analysis and amplified fragment length polymorphism fingerprinting. Biochemical phenotyping revealed that all EUS-associated isolates belonged to a unique phenotype which was not identified among more than 1,600 environmental and diarrheal isolates in a previously collected database of PhP types of Bangladeshi *Aeromonas* isolates. The 52 *Aeromonas* isolates were investigated for the production of hemolysin and cytotoxin; for hemagglutination with erythrocytes from fish, human, and rabbit sources; for the presence of a cytolytic enterotoxin gene; and for adhesion to and invasion into fish cell lines. All of the EUS isolates produced all of the virulence factors investigated, as did also some of the environmental isolates, but the isolates from EUS were unique in their ability to agglutinate fish erythrocytes. Our results suggest that a clonal group of *A. veronii* biovar sobria is associated with, and may be a causative agent of, EUS in fish in Bangladesh.

Aeromonas spp. are ubiquitous inhabitants of aquatic ecosystems such as freshwater, coastal water, and sewage (36). They are increasingly being reported as important pathogens for humans and for lower vertebrates, including amphibians, reptiles, and fish (19). These bacteria have a broad host range, and have often been isolated from humans with diarrhea (17), as well as from fish with hemorrhagic septicemia (39). The pathogenesis, pathogenic mechanism, and virulence factors responsible for selected *Aeromonas* infection in different species are not well understood. Strains isolated from the environment do not seem to differ from strains isolated from cases of infection with respect to the prevalence of virulence factors (29). However, it has been shown that certain species are more frequently isolated from patients with diarrhea as well as from diseased fish than from the environment (25).

Epizootic ulcerative syndrome (EUS) is a fish disease characterized by the presence of severe, open dermal ulcers on the head, on the middle of the body, and on the dorsal regions of the fish (33). EUS has been characterized as an epizootic disease of freshwater fish in the Indo-Pacific region since 1980 (8) and was first reported in Bangladesh in 1988 (2). This disease is now frequently occurring in many fish farms in Bangladesh (6); the disease generally develops with ulcers that develop on the fish bodies, and the fish may die within a week of being infected. The disease has caused substantial economic loss to fish farmers and the fisheries sector. The etiological agent(s) of EUS in Bangladesh is still unknown; however, organisms belonging to the potentially fish-pathogenic genera *Aeromonas*, *Vibrio*, *Plesiomonas*, and *Pseudomonas* were often isolated from the lesions and blood samples of infected fish. Representatives of *Aeromonas hydrophila* and *Aeromonas sobria* were recovered most frequently, followed by *Vibrio* and *Plesiomonas* spp. (33).

In light of the increased incidence of EUS and the economic importance of these epizootic diseases and because of possible public health effects, it is of great importance to further study and characterize the etiologic agents of EUS. In the current study, we sought to identify bacteria recovered from EUS infections in Bangladesh and to investigate the virulence factors of the isolates associated with EUS. In addition, we also compared the virulence properties of the isolates from diseased fish to those of human diarrheal and environmental isolates from Bangladesh.

MATERIALS AND METHODS

Isolation and biochemical identification of bacterial isolates. The fish isolates were isolated from different diseased fish (African catfish [*Clarias gariepinus*], rajputi [*Puntius gonionotus*], rui [*Labeo rohita*], catla [*Catla catla*], and shole [*Channa striatus*]) from different fish farms (the Bangladesh Agricultural University [BAU] fish farm; Jhalak Hatchery and Fish Farm; and Dhaka Fisheries, Ltd., Bangladesh, Bangladesh) by the fish disease laboratory at BAU in 1997 and 1998. The disease symptoms were deep hemorrhagic ulcers in the midbody and tail regions. *Aeromonas* selective agar, i.e., *Aeromonas* Medium Base (Oxoid, Ltd.), supplemented with ampicillin SR136 (5 μg ml^{-1}), was used to obtain *Aeromonas* isolates from fish ulcer scraps. Fourteen randomly selected *Aeromonas* isolates (BDF1 to BDF14) were obtained from the BAU fish disease laboratory collection. For comparison, a number of water samples were collected

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from the same ponds as the fish within a 6-month time period. These environmental samples were processed by using the same selective medium, except that no ampicillin was added, due to the low-level ampicillin resistance among environmental isolates. Among the environmental isolates, 26 presumed *Aeromonas* isolates (BDE15 to BDE40) were randomly selected. Furthermore, 12 randomly selected human diarrheal isolates (BDD41 to BDD52) of *Aeromonas* spp. obtained from a previous study (30) on Bangladeshi children with diarrhea were included in the present study.

The presumed *Aeromonas* isolates were confirmed by oxidase and catalase test and by determining the sensitivity to the vibriostatic reagent 0/129 (150 μg ml^{-1}; Sigma, St. Louis, Mo.). The isolates were identified to the species level by traditional biochemical methods (3), including tests for esculin hydrolysis, lysine decarboxylase, arginine dihydrolase, and ornithine decarboxylase; tests for acid production from arabinose, glucose, sucrose, and mannitol; and tests for susceptibility to ampicillin and cephalothine (30 μg ml^{-1}) (4, 20, 21), and, when necessary, by using API 20NE and API 20E biochemical identification strips (bioMérieux, Marcy l'Etoile, France). All isolates were stored in 30% glycerol in brain heart infusion (BHI) broth at $-70°$C until further use, subsequently recultured on BHI agar plates (Becton Dickinson Microbiology Systems), and then incubated overnight at 37°C.

Biochemical phenotyping with the PhP system. Bacterial isolates were typed through a biochemical phenotyping method, PhenePlate (PhP) system (PhPlate Microplate Techniques AB, Stockholm, Sweden), according to the manufacturer's instructions. The PhP typing was performed in previously prepared microplates and is based on the kinetics of fermentation of 48 dehydrated reagents especially selected to discriminate between individual bacterial strains (32, 35). The biochemical reactions of the isolates were compared pairwise, and a similarity matrix consisting of the correlation coefficient between all possible pairs was constructed. The similarity matrix was clustered according to unweighted pair-group method by using average linkages (UPGMA) (38). Isolates with a level of similarity of >0.97 were assigned to the same PhP phenotypes. All data processing, including optical reading and calculation of the correlation coefficient, as well as the clustering and printing of dendrograms, was performed with PhP software (PhPlate Microplate Techniques AB).

Identification of *Aeromonas* spp. by gas-liquid chromatographic analysis of FAME profile and AFLP fingerprinting. Presumptive *Aeromonas* isolates were further identified to the genomic species level by using gas-liquid chromatographic analysis of cellular fatty acid methyl esters (FAMEs) as described previously (14). Unknown FAME profiles were compared to the laboratory-based identification library AER48C (13). Isolates that remained unidentified or yielded unreliable FAME identifications were further subjected to whole-genome fingerprinting by using amplified fragment length polymorphism (AFLP) analysis according to the method of Huys and Swings (15). The AFLP profiles of unknown isolates were compared with the laboratory-based identification library AEROLIB comprising AFLP profiles generated from a collection of well-characterized type and reference strains encompassing all currently recognized *Aeromonas* taxa (12).

Cytotoxic and hemolytic activity. The cytotoxic activity of the isolates was tested on the fish cell line EPC (epithelioma papulosum of carp [*Cyprinus carpio*]) as described previously (9). Briefly, confluent monolayers of the cell were grown in 24-well tissue culture plates (Costar, Corning, N.Y.) in minimal essential medium (MEM; SVA, Uppsala, Sweden) supplemented with 10% fetal bovine serum, 1% (wt/vol) glutamine, and 1% (wt/vol) penicillin-streptomycin. The cells were incubated for 6 h at 18°C with 100 μl of sterile culture supernatant (containing 150 μg of protein ml^{-1} as determined by using the Bio-Rad protein assay) serially twofold diluted in supplemented MEM. The cytotoxic activity was measured as rounding up, detachment, and loss of viability of the cells, as seen under a light microscope within 6 h. The titer was determined as the highest dilution of supernatant affecting at least 50% of the cells. Isolates showing a cytotoxic effect at a dilution of 1/8 (final concentration) or more were regarded as positive.

Hemolytic activity of the isolates was measured on 1% (vol/vol) human and rabbit erythrocytes as described earlier (22). Isolates were considered positive for hemolysin production when the culture supernatant at a final concentration of 1/8 lysed at least 50% of the erythrocytes as determined by visual examination. Known positive (BD2 to BD9) and negative (BD12) isolates from an earlier investigation were included as controls for both the cytotoxin and hemolysin assays.

Hemagglutination. Hemagglutination tests were performed on glass slides by mixing a loopful of bacteria with a 3% (vol/vol) suspension of erythrocytes from rabbit, humans, or fish (*Labeo rohita*) in phosphate-buffered saline (PBS). Visible agglutination within 5 min was considered a positive result. The agglutination inhibition test was performed by using a dilution of 1% (wt/vol) D-mannose, D-galactose, and L-fucose sugar in PBS (10).

Adhesion and invasion. Two freshly seeded fish cell lines, EPC and RTG (for rainbow trout gonad), were cultured to monolayers on 24-well tissue culture plates with coverslips in MEM at 18°C and used for both kinds of tests. Adhesion and invasion were expressed as the percentage $\pm$ the standard error of the mean (SEM) of bacteria recovered after a careful washing.

Bacterial strains were cultured in BHI broth overnight at 22 or 37°C, harvested by centrifugation (3,000 rpm, 30 min, 22°C), and washed in PBS, and the concentration was adjusted before the adhesion assay was performed. At 2 h before an adhesion assay was done as described elsewhere (28), the cell medium was replaced by fresh medium without antibiotic. The monolayer was then incubated with a bacterial suspension of 10^7 CFU ml^{-1} for 1 h at 18°C and washed three times with PBS in order to eliminate nonattached bacteria. The coverslip with the monolayer and attached bacteria was removed from the tissue culture plates, fixed in methanol, stained with Giemsa, and examined for evidence of bacterial adhesion by using an inverted microscope. For a quantitative assay, the monolayer and attached bacteria of some of the coverslips were then lysed in 0.1% Triton in sterile water for 10 min. The ability of the bacteria to adhere to the cell lines was determined by spreading the Triton-induced cell lysate on nutrient agar plates and counting the number of colonies after incubation overnight at 37°C.

The invasion capacity of the isolates was determined by the gentamicin protection assay as described elsewhere (34). Briefly, after a 1-h incubation of the confluent cell monolayer with a bacterial suspension, as described above, the monolayer was washed three times with PBS and incubated for another hour in MEM containing gentamicin (50 μg ml^{-1}) in order to kill extracellular bacteria. The monolayer was again washed three times with PBS and lysed with 0.1% Triton to release intracellular bacteria, and a viable count was made as described above. All experiments were repeated five times, and the mean value was calculated. *Escherichia coli* HB101, an adhesion and invasion negative strain, was used as a negative control in all experiments.

An adhesion inhibition assay was performed by using trypsin or vigorous stirring as described by Kirov et al. (24). In brief, bacteria were incubated with trypsin at a final concentration of 1 mg ml^{-1} for 30 min at 37°C, and the residual trypsin was removed by washing the bacteria in PBS. Vigorous stirring was performed in an Omnimixer (Labora AB, Sollentuna, Sweden) at high speed (1,400 rpm) in order to detach the fimbriae from the surface; detached fimbriae were then removed by washing the cells in PBS, and the bacterial concentration was adjusted before the adhesion assay was performed.

PCR detection of the cytolytic enterotoxin gene and/or extracellular hemolysin gene. A cytolytic enterotoxin gene (AHCYTOEN) in *A. hydrophila* has been reported as a multivirulence gene involved in lethality (in mice), hemolysis, cytotoxicity, and enterotoxicity (5), i.e., activities that are established virulence factors of *Aeromonas* spp. The various *Aeromonas* isolates were therefore investigated by using the PCR primer combination strategy of Kingombe et al. (23) with the primers AHCF1 (5'-GAG AAG GTG ACC ACC AAG AAC A-3') and AHCR1 (5'-AAC TGA CAT CGG CCT TGA ACT C-3'). These primers would detect the presence of the cytolytic enterotoxin gene and/or the extracellular hemolysin gene in *Aeromonas* spp . The PCRs used here were slightly modified from those of Kingombe et al. (23) and were performed in a final volume of 50 μl containing 10 μl of DNA, 5 μl of a mixture containing 0.2 mM deoxyribonucleotide triphosphate, 2.5 μl of 50 mM MgCl$_2$ solution, 5 μl of 10$\times$ PCR buffer, 2.5 μl of a 20 μM solution of each primer, 0.3 μl of *Taq* DNA polymerase (Life Technologies) at 5 U/μl, and 22.2 μl of double-distilled sterile water. We used 1 cycle of denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 66°C for 30 s, and extension at 72°C for 30 s, and a final extension round at 72°C for 7 min. The PCR amplicons were separated electrophoretically in a 1.5% agarose gel (Sigma type 1) and visualized after ethidium bromide staining. The specificity of the primer combination was corroborated with negative PCR results obtained by using *E. coli* reference strain DS17 (27).

RESULTS

Species identification and typing of isolates. All 14 EUS-associated fish isolates were identified as *A. veronii* biovar sobria by traditional biochemical tests both in tubes and by using the API 20E and 20NE systems. Furthermore, in the PhP typing (based upon kinetic reading of 48 biochemical reagents) all of the fish isolates clustered with reference strains of *A. veronii* biovar sobria, the identity of which had been previously

TABLE 1. Frequency of putative virulence factors among isolates obtained from fish, from the environment, and from humans with diarrhea in Bangladesh

Source of isolate	n	No. of isolates (%)					
		Cytotoxin	Hemolysin	Toxin gene[a]	Agglutination of erythrocytes from:		
					Fish	Humans	Rabbits
Fish	14	14 (100)	14 (100)	14 (100)	14 (100)	14 (100)	14 (100)
Environment	26	18 (69)	18 (69)	9 (35)	0	14 (54)	9 (35)
Human diarrhea	12	6 (50)	6 (50)	1 (8)	0	2 (17)	2 (17)
Total	52	38 (73)	38 (73)	24 (46)	14 (27)	30 (58)	25 (48)

[a] Cytolytic enterotoxin gene and/or extracellular hemolysin gene.

established by FAME analysis and AFLP fingerprinting (31, 32). Among the environmental isolates ($n = 26$), 42% ($n = 11$) clustered with the *A. veronii* biovar sobria, 19% ($n = 5$) clustered with the *A. veronii* biovar veronii, 31% ($n = 8$) clustered with *A. trota*, 4% ($n = 1$) clustered with the *A. caviae* complex, and one isolate did not cluster with any of the reference strains. These tentative identifications were fully confirmed by the traditional biochemical tests for *Aeromonas* spp.

The PhP typing data of the 14 EUS-associated *Aeromonas* isolates were compared to and clustered with environmental ($n = 26$) and human diarrheal ($n = 12$) *Aeromonas* isolates (Fig. 1). It was found that the EUS-associated *Aeromonas* isolates showed a low level of diversity, and essentially all isolates were of the same PhP type. The environmental isolates, on the other hand, showed a higher diversity and belonged to a wide variety of PhP types. None of the environmental isolates or human diarrheal isolates was of an identical or even similar PhP type compared to the cluster containing the EUS-associated isolates. Furthermore, the PhP types representing the EUS-associated *Aeromonas* cluster were not found in a previously constructed laboratory-based PhP database comprising 1,200 environmental isolates and 400 diarrheal isolates from Bangladesh.

FAME and AFLP identification of fish *Aeromonas* isolates. The FAME identification of the fourteen fish isolates was not conclusive but indicated that the isolates belonged to the species *A. veronii*. Upon AFLP analysis, on the other hand, the isolates were clearly determined to be *A. veronii*. According to the results of API analysis, all isolates lacked ornithine decarboxylase activity, indicating that these isolates belonged to *A. veronii* biovar sobria and not to *A. veronii* biovar veronii (1).

Production of cytotoxin and hemolysin. A total of 73% (38 of 52) of the isolates showed cytotoxic activity to EPC cell lines and also produced hemolysin active on rabbit and human erythrocytes (Table 1). All *A. veronii* biovar sobria isolates from EUS (14 of 14) were positive for cytotoxin and hemolysin, whereas 69% (18 of 26) of the environmental isolates, including 9 of 11 *A. veronii* biovar sobria isolates, and 50% (6 of 12) of the human diarrheal isolates were also positive.

PCR for cytolytic enterotoxin gene and/or extracellular hemolysin gene. Among the isolates, 46% (24 of 52) isolates were positive in the PCR test for cytolytic enterotoxin gene and/or the extracellular hemolysin gene (Table 1). However, all of the 14 *A. veronii* biovar sobria isolates from fish were positive both for production of cytotoxin and in the PCR assay, and 7 of 11

environmental *A. veronii* biovar sobria isolates were also positive (Fig. 1).

Hemagglutination. *Aeromonas* isolates from fish, the environment, and humans with diarrhea were tested for the ability to agglutinate fish, human, and rabbit erythrocytes. In all, 25 isolates (48%) from various sources showed agglutination with human and rabbit erythrocytes (Table 1). However, fish erythrocytes were only agglutinated by the EUS-associated fish isolates and not by any of the human or environmental isolates. All of the erythrocyte agglutinations were inhibited by D-mannose but not by D-galactose. The reaction of four of the environmental isolates was inhibited by L-fucose, indicating different mechanisms for the agglutination reactions among the isolates.

Adhesion and invasion. One representative of the 14 EUS-associated isolates (isolate BDF4) that showed high cytotoxic activity against EPC cells was also tested for adhesion and invasion to fish cell lines. The values for adhesion and invasion experiments were averages from at least five independent experiments. A dose-dependent adhesion was observed, a poor adherence ($0.8\% \pm 0.08\%$) was observed at 10^5 CFU ml^{-1}, but increasing concentrations significantly increased the degree of adherence to a maximum of $5.3\% \pm 0.21\%$ recovered at 10^8 CFU ml^{-1}. However, at $>10^8$ CFU ml^{-1} the high concentration of bacteria caused a detachment of the cells from the chamber bottom, resulting in a lowered degree of adherence.

The adhesion of the BDF4 isolate was found to be similar in both cell lines, although a better adhesion was observed when bacteria were grown at 22°C compared to bacteria grown at 37°C (Table 2). Isolate BDF4 could also invade both fish cell lines (Table 2). The adhesion and invasion displayed by the strain were 140- and 85-fold greater, respectively, than that shown by the nonadherent *E. coli* HB101 strain used as control. Thus, the data indicate that the level of adhesion and invasion seemed to depend on the bacterial growth temperature, the concentration of the bacteria, and the cell line employed. Bacterial adhesion was inhibited by $20\% \pm 1.6\%$ by vigorous stirring and inhibited by $24\% \pm 1.0\%$ by trypsin treatment, irrespective of the cell line used (data not shown).

DISCUSSION

In the present study, several of the *Aeromonas* isolates examined from various sources (i.e., from EUS in fish, from the environment, and from humans with diarrhea) were able to

Correlation coefficient

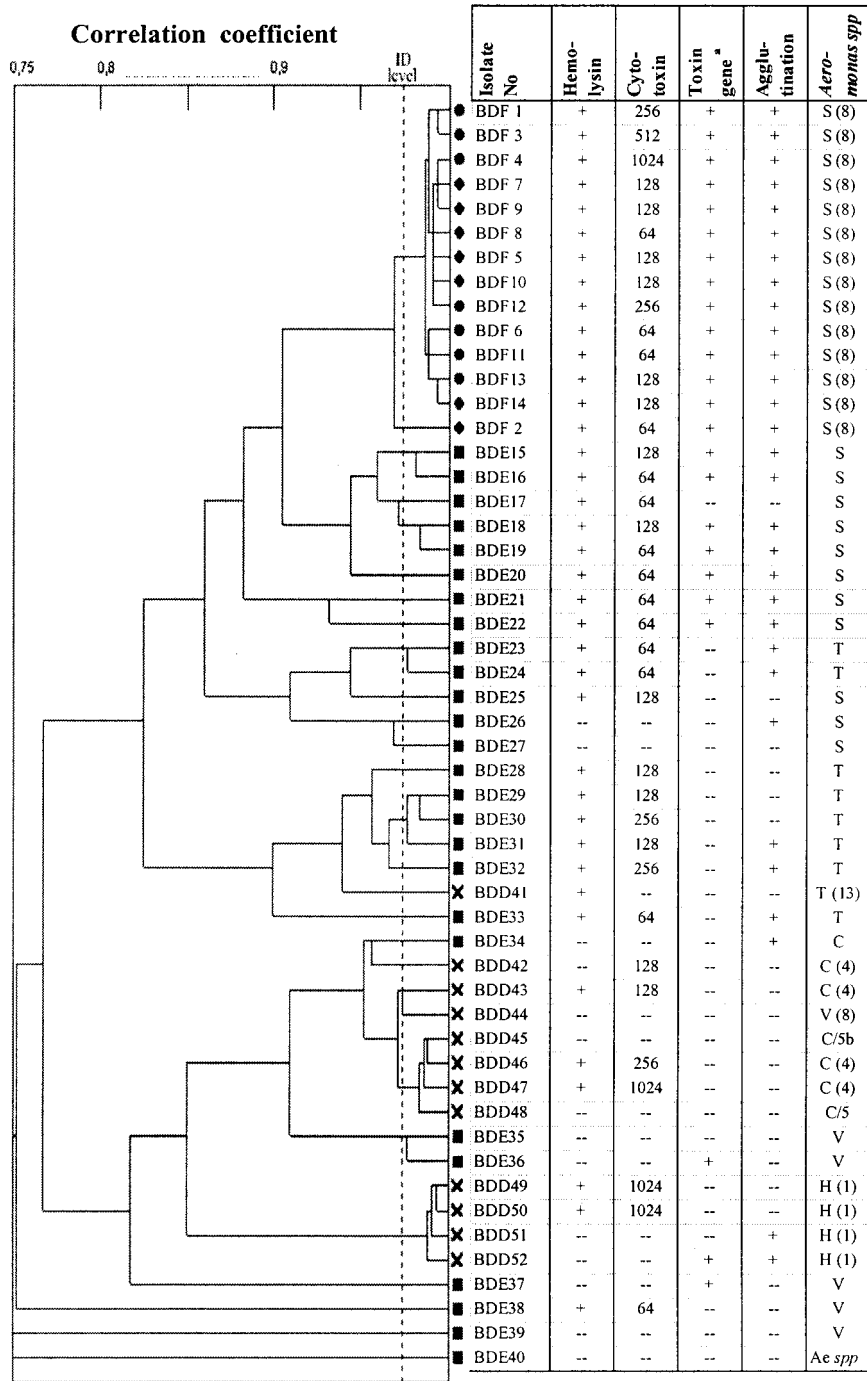| Isolate No	Hemo-lysin	Cyto-toxin	Toxin gene [a]	Agglu-tination	Aero-monas spp
● BDF 1	+	256	+	+	S (8)
● BDF 3	+	512	+	+	S (8)
● BDF 4	+	1024	+	+	S (8)
◆ BDF 7	+	128	+	+	S (8)
◆ BDF 9	+	128	+	+	S (8)
◆ BDF 8	+	64	+	+	S (8)
◆ BDF 5	+	128	+	+	S (8)
◆ BDF 10	+	128	+	+	S (8)
● BDF 12	+	256	+	+	S (8)
● BDF 6	+	64	+	+	S (8)
● BDF 11	+	64	+	+	S (8)
● BDF 13	+	128	+	+	S (8)
◆ BDF 14	+	128	+	+	S (8)
◆ BDF 2	+	64	+	+	S (8)
■ BDE 15	+	128	+	+	S
■ BDE 16	+	64	+	+	S
■ BDE 17	+	64	--	--	S
■ BDE 18	+	128	+	+	S
■ BDE 19	+	64	+	+	S
■ BDE 20	+	64	+	+	S
■ BDE 21	+	64	+	+	S
■ BDE 22	+	64	+	+	S
■ BDE 23	+	64	--	+	T
■ BDE 24	+	64	--	+	T
■ BDE 25	+	128	--	--	S
■ BDE 26	--	--	--	+	S
■ BDE 27	--	--	--	--	S
■ BDE 28	+	128	--	--	T
■ BDE 29	+	128	--	--	T
■ BDE 30	+	256	--	--	T
■ BDE 31	+	128	--	+	T
■ BDE 32	+	256	--	+	T
✕ BDD 41	+	--	--	--	T (13)
■ BDE 33	+	64	--	+	T
■ BDE 34	--	--	--	+	C
✕ BDD 42	--	128	--	--	C (4)
✕ BDD 43	+	128	--	--	C (4)
✕ BDD 44	--	--	--	--	V (8)
✕ BDD 45	--	--	--	--	C/5b
✕ BDD 46	+	256	--	--	C (4)
✕ BDD 47	+	1024	--	--	C (4)
✕ BDD 48	--	--	--	--	C/5
■ BDE 35	--	--	--	--	V
■ BDE 36	--	--	+	--	V
✕ BDD 49	+	1024	--	--	H (1)
✕ BDD 50	+	1024	--	--	H (1)
✕ BDD 51	--	--	--	+	H (1)
✕ BDD 52	--	--	+	+	H (1)
■ BDE 37	--	--	+	--	V
■ BDE 38	+	64	--	--	V
■ BDE 39	--	--	--	--	V
■ BDE 40	--	--	--	--	Ae *spp*

FIG. 1. Dendrogram showing UPGMA clustering of PhP typing data obtained from 52 *Aeromonas* isolates. Circles indicate isolates from fish, squares indicate isolates from the environment, and crosses indicate isolates from humans with diarrhea. The results from assays of putative virulence properties are shown. The last column indicates the species codes. Some isolates were identified by FAME analysis, in which case the hybridization group (HG) is given, e.g., S(8) = *A. veronii* biovar sobria (hybridization group). Other isolates were identified by biochemical test: S, *A. veronii* biovar sobria; T, *A. trota*; C, *A. caviae* complex; H, *A. hydrophila*, V, *A. veronii* biovar veronii, etc. The column heading "toxin gene" refers to the cytolytic enterotoxin gene and/or extracellular hemolysin gene.

produce putative virulence factors, as reported earlier (18, 29, 30). It is thus possible that the environment contains reservoirs of *Aeromonas* strains that are capable of causing human and animal infections. So far, a major problem has been the lack of knowledge concerning the primary virulence factors causing

EUS and the identification of the bacterial groups that cause the disease. It is not known, for example, whether these virulence factors are produced by all members of a given taxon or only by one or more groups of pathogenic clones within a given taxon. One major achievement would be the development of

TABLE 2. Adhesion and invasion of *Aeromonas* sp. strain BDF4 to different cell lines at different temperatures

Adhesion or adherence	Fish cell line	Temp (°C)	Mean % bacteria ± SEM	
			Aeromonas sp. strain BDF4	*E. coli* HB101 (control)
Adherence	EPC	22	3.1 ± 0.19^a	0.022 ± 0.004
		37	2.8 ± 0.21	NT
	RTG	22	3.0 ± 0.18	0.03 ± 0.003
		37	2.8 ± 0.25	NT
Invasion	EPC	22	0.27 ± 0.030^b	0.003 ± 0.0004
		37	0.24 ± 0.027	NT
	RTG	22	0.23 ± 0.031	0.004 ± 0.0004
		37	0.29 ± 0.030	NT

[a] Mean percent bacteria (±SEM) recovered after washing. NT, not tested.
[b] Mean percent bacteria recovered after gentamicin treatment.

an inexpensive but accurate identification and typing methodology to allow the detection of pathogenic *Aeromonas* clones.

Phenotyping with the PhP system allowed us to detect that the *Aeromonas* isolates associated with EUS formed a separate and very homogeneous phenotypic cluster. The PhP type of the EUS-associated isolates was not identical to any of the environmental and human diarrheal *Aeromonas* isolates included in our previous and ongoing studies. We have previously reported that *Aeromonas* isolates from children with diarrhea, compared to isolates from other sources, showed a low level of diversity, indicating that these diarrheal isolates most probably represent a limited number of clonal groups. Similar to the previous study, wherein a specific PhP type was strongly associated with diarrhea (30), we conclude here that the EUS-associated isolates belong to a clonal group that is possibly pathogenic to fish. We assume that this clonal group carries specific, as-yet-uncharacterized properties that makes it a good colonizer of fish.

Aeromonas spp. have previously been isolated from EUS-diseased fish in the Indo-Pakistan region by Iqbal et al. (16). They found that 27% (12 of 44) *Aeromonas* isolates from fish with EUS in Malaysia, Thailand, and Bangladesh belonged to *A. veronii* biovar sobria and that 6 of the 11 isolates from Bangladeshi fish belonged to this species. In agreement with these findings, our EUS-associated fish isolates were also genotypically identified as *A. veronii* biovar sobria, indicating that this *Aeromonas* species may constitute an important causative agent of EUS in this geographic area.

We found that the production of cytotoxin and hemolysin was prevalent in more than 50% of the *Aeromonas* isolates of various species and various origins, as reported by several other investigators (29). However, the EUS-associated isolates were all hemolysin and cytotoxin positive. Furthermore, most of the environmental *A. veronii* biovar sobria isolates were also positive, which is in agreement with our previous report on environmental isolates in Bangladesh (30).

Recent studies have shown that the virulence genes of *Aeromonas* spp., e.g., the genes for cytolytic enterotoxin, aerolysin, and hemolysin, have 70 to 99% homology. By using primers from conserved region, it was possible to study the epidemiology of these genes (23). By a similar strategy, we found that all our isolates from EUS were positive for cytolytic enterotoxin

and/or extracellular hemolysin genes. As expected, the production of cytotoxin and that of hemolysin were highly correlated, but the PCR assay did not detect all hemolysin- and cytotoxin-positive isolates (Table 1). Thus, of 38 *Aeromonas* isolates that were positive for hemolysin and cytotoxin production, only 24 possessed the target gene sequence. The prevalence of toxin gene-positive isolates (24 of 52 [46%]) is in agreement with recently published data of Kingombe et al. (23), who reported that of 61 *Aeromonas* reference isolates tested, only 30 (49%) were positive for the same primer combination (AHCF1-AHCR1). Using a different set of primers, Wang et al. (40) studied 41 isolates exhibiting hemolytic activity, and only 6 of these isolates possessed the target cytotoxin gene.

A majority of the *Aeromonas* isolates in the present study agglutinated human and rabbit erythrocytes. This finding is in agreement with previous reports by other investigators (17, 37). Interestingly, only the EUS-associated fish isolates could agglutinate fish erythrocytes. It is plausible that a specific ligand-receptor interaction is important for colonization in fish. The agglutination was inhibited by D-mannose, indicating that glycoproteins, such as bacterial cell lectins, may be involved in the adhesion mechanism, a phenomenon that has been described previously (7). The agglutination of erythrocytes by four of the environmental isolates was also inhibited by L-fucose, which indicates that a different adhesive component might be present in these isolates. Since many different agglutination patterns of *Aeromonas* spp. have been reported (11), these bacteria might have various colonization factors that are antigenically diverse and play important roles in evading the host immune system (26).

In the present study we also demonstrated the presence of adhesion and invasion ability in the EUS-associated isolates that may play a role in the pathogenesis at an early stage in the disease process. The adhesion and invasion appeared to be more pronounced when bacteria were grown at 22°C than when grown at 37°C, a finding that agrees with the observation that adhesive fimbriae are better expressed at 22 than at 37°C. Adhesion was inhibited by trypsin or vigorous stirring, which is in agreement with the observations of Kirov et al. (24) that fimbriae and other filamentous structures might play important roles in colonization.

Since the varieties of fish considered in the present study live in a vast body of water and represent hosts for various opportunistic bacteria and other microorganisms, it may be very difficult to define a single causative agent of EUS. However, our results suggest that the *Aeromonas* bacteria isolated from EUS in Bangladeshi fish likely belong to a certain clonal group of *A. veronii* biovar sobria and that this clonal group is not common in other sources, including humans with diarrhea or the environment. Isolates belonging to this clonal group displayed several putative virulence factors, including the unique property of being able to agglutinate fish erythrocytes, and so this clonal group can be considered a possible causative agent of EUS in fish in Bangladesh.

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