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Aeromonas hydrophila Isolated from Food and Drinking Water: Hemagglutination, Hemolysis, and Cytotoxicity for a Human Intestinal Cell Line (HT-29)

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Aeromonas hydrophila **isolated from food and drinking water was tested for pathogenicity by studying its hemolysis, hemagglutination, and cytotoxicity. Hemolysis, tested on erythrocytes from six different species, was more frequently seen with water isolates (64%) than with food isolates (48%). Hemagglutination was more frequently encountered with food isolates (92%) than with water isolates (73%). Cytotoxicity, evaluated on seven cell lines, was frequently observed with food isolates (92%) and with water isolates (73%). Heat treatment (56°C for 10 min) of culture supernatant fluids inhibited the toxicity of some but not all toxin-producing isolates. Our results suggest that the human intestinal cell line HT-29 could be a useful complement for testing** ***A. hydrophila*** **exotoxins and for studying the enteropathogenicity of this species for humans.**

Aeromonas hydrophila is a ubiquitous bacterium frequently isolated from food, drinking water, and aquatic environments. It has been increasingly recognized as an enteric pathogen and has been associated with a wide variety of other human infections including septicemia, meningitis, and wound infections. A greater risk of infection is reported in young children, elderly people, and immunocompromised patients (11, 13).

The enteropathogenicity of *Aeromonas* spp. has been ascribed to the production of exotoxins. Clinical and environmental strains of *A. hydrophila* have been reported to produce a heat-labile cytotoxin and a heat-stable cytotonin that have enterotoxic activities (7, 13, 14, 19). Another important attribute of pathogenic *Aeromonas* strains could be the production of adhesins (4). Despite the production of extracellular enzymes and toxins, the importance of each virulence factor in the pathogenicity of *A. hydrophila* in various types of infections remains unclear.

Drinking water and food are reservoirs of aeromonads and therefore may be important sources of human infections. The aim of the present study was to survey the production of important *A. hydrophila* virulence factors in 166 food-borne and water-borne isolates.

Bacterial isolates and statistical treatment of data. A total of 72 *A. hydrophila* food isolates were obtained from raw meat (66 isolates) and ready-to-eat products (6 isolates). These isolates were recovered by previously described methods (18) and tested for oxidase production. A total of 94 *A. hydrophila* isolates were obtained from chlorinated and unchlorinated drinking-water supplies. These isolates were recovered by standard procedures for screening for coliforms in drinking water (1). Atypical (lactose-negative) colonies were tested for oxidase production. Presumptive *A. hydrophila* isolates were confirmed with the API 20E system (Analytab Products). Type strain ATCC 7966 (*A. hydrophila* isolated from milk) and *A. hydrophila* H-1 isolated from a patient with hemolytic-uremic syndrome in the Quebec city area were included in all assays. Statistical significance was evaluated by the χ^2 test; results were considered significant at $P < 0.05$ and highly significant at $P < 0.001$.

Hemolysis and hemagglutination assays. The first part of this study dealt with hemolysis and hemagglutination properties of food-borne and water-borne *A. hydrophila*. For hemolysis assays, erythrocytes were centrifuged (3,400 × *g* for 10 min at 4°C), washed three times, and resuspended in sterile 20 mM phosphate-buffered saline (PBS; pH 7.2) to obtain a 2% (vol/vol) suspension. Bacterial isolates were cultured in 5 ml of Trypticase soy broth supplemented with 0.6% yeast extract and incubated at 35°C for 16 to 18 h. Supernatant fluids were carefully removed after centrifugation at 10,000 × *g* for 30 min at 4°C and mixed with an equal volume (50 μl) of a 2% (vol/vol) suspension of washed erythrocytes in a 96-well microtiter tray. The mixture was incubated for 1 h at 37°C and then 1 h at 4°C. An erythrocyte suspension in PBS was included in each assay as a negative control. Hemolysis was recorded by visual inspection.

Type strain ATCC 7966 produced hemolysins against sheep, rabbit, and guinea pig erythrocytes, while the human H-1 strain was hemolytic against rabbit and guinea pig erythrocytes only. A greater ($P < 0.001$) proportion of *A. hydrophila* water isolates (64%) than food isolates (48%) produced hemolysis (Table 1). The highest percentage of hemolytic isolates was obtained against mouse erythrocytes ($P < 0.001$), in agreement with previously reported data (3). There is a noticeable difference between the percentage of hemolytic food isolates observed in this study and reported data from other groups (15–17). Obviously, any environmental factor that influences the growth of the bacteria potentially contributes to such differences. It has not been possible to confirm a seasonal difference in the rate of isolation of hemolysin-producing strains. A previous study by Burke et al. (5) has shown that it is possible to identify 97% of enterotoxigenic *Aeromonas* isolates by biotyping and a hemolysin assay (with rabbit erythrocytes). Therefore, our results indicate that 68% of the water isolates and 41% of the food isolates used throughout this study are potentially enteropathogenic. These data agree with previously reported results by Burke et al. (4). Nevertheless, the type of erythrocyte used in the hemolysis study seems to be an impor-

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TABLE 1. Hemolysis and hemagglutination reactions of food- and water-borne *A. hydrophila* isolates

Erythrocyte source	% of isolates showing:			
	Hemolysis		Hemagglutination	
	Food isolates (n = 72)	Water isolates (n = 94)	Food isolates (n = 72)	Water isolates (n = 94)
Mouse	75	87	96	72
Guinea pig	54	76	95	78
Chicken	55	56	96	74
Rabbit	41	68	87	70
Sheep	44	52	93	72
Human[a]	21	44	87	73
Mean value[b]	48	64	92	73

[a] O-negative human blood.
[b] Mean value for the sum of all assays.

TABLE 2. Cytotoxicity of food- and water-borne *A. hydrophila* culture supernatant fluids tested on various cell lines.

Cell line	% of isolates showing cytotoxicity	
	Food isolates (n = 72)	Water isolates (n = 94)
HT-29	96	74
CHO	95	78
Y-1	96	72
HFF	93	72
HeLa	91	73
A-549	87	73
Vero	87	70
Mean value[a]	92	73

[a] Mean value for the sum of all assays.

tant variable: rabbit, sheep, and human erythrocytes are the most commonly used, and mouse erythrocytes appear to be the most sensitive. The availability of mouse blood may have hampered its use as a standard in *A. hydrophila* hemolysis assays.

Only a limited number of studies were done with a significant number of isolates and multiple types of erythrocytes to study hemagglutination of food and water *Aeromonas* isolates (4, 15, 17). The hemagglutination assay in the present study was performed by modifications of the method of Burke et al. (4). Isolates were streaked on Trypticase soy agar plates and incubated at 35°C for 16 to 18 h. Two loopfuls of bacteria were emulsified in 0.5 ml of sterile PBS, which provided an approximative concentration of 10^{10} to 10^{11} organisms per ml. An equal volume (50 μl) of each sample was mixed with a 6% suspension of washed erythrocytes placed on a microscope slide. A negative control consisting of erythrocyte suspension with PBS was included in each assay. Hemagglutination was recorded positive if the reaction occurred within 10 min. Type strain ATCC 7966 and human isolate H-1 agglutinated all erythrocytes tested. A greater ($P < 0.001$) percentage of food (92%) than water (73%) isolates showed hemagglutination (Table 1). No particular type of erythrocyte was significantly more sensitive to hemagglutination than the others. In comparison with previous data, a higher percentage of food isolates produced hemagglutination (15, 17). This difference could be attributed to culture conditions prior to the hemagglutination assays (10) or simply to the nature or geographical situation, or both, of the samples. Various studies stated that human diarrheal *Aeromonas* isolates and enterotoxigenic strains strongly agglutinated human erythrocytes (2, 4, 10). According to our results, hemagglutination appears to be a frequent feature of food-borne and water-borne *A. hydrophila* isolates.

Cytotoxicity and cytotonicity assays. Production of a variety of extracellular toxins in both clinical and environmental isolates of *A. hydrophila* has been reported (6, 7, 12, 14, 19). The results are often confusing with regard to the contribution of each potential virulence factor to the overall pathogenesis of *Aeromonas* infections. There have been numerous reports on the cytotoxicity of *Aeromonas* isolates from food and water. However, evaluation of cytotoxicity was done on only one or two cell systems and with a limited number of isolates. The present study evaluated the cytotoxicity of 166 *A. hydrophila* isolates to seven different cell lines: human lung carcinoma cells (A-549; ATCC CCL 185), Chinese hamster ovary cells (CHO; ATCC CRL 1793), human epithelioid carcinoma cells (HeLa; ATCC CCL 2), human foreskin fibroblasts (HFF; developed at the Laboratoire régional de virologie), human colon adenocarcinoma cells (HT-29; ATCC HTB 38), African green monkey kidney cells (Vero; ATCC CRL 1587), and mouse adrenal tumor cells (Y-1; ATCC CCL 79). The cells were grown in 96-well microplates in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and antibiotics: gentamicin (Gibco Laboratories), 10 μg/ml; vancomycin (Eli Lilly), 25 μg/ml; and amphotericin B (Flow Laboratories), 1.25 μg/ml. Cell suspension (100 μl) was seeded in every well 24 h before the assay at concentrations allowing the formation of a nearly confluent monolayer at the time the cytotoxicity assay was performed. Each *A. hydrophila* isolate was inoculated into 5 ml of Trypticase soy broth supplemented with 0.6% yeast extract, and the mixture was incubated for 16 to 18 h at 35°C. Following centrifugation at 10,000 × *g* for 30 min at 4°C, 100 μl of the supernatant fluid was carefully removed and immediately delivered to each well of the different cell monolayers, which were then incubated for 7 days at 37°C under a 5% CO_2 atmosphere. Sterile Trypticase soy broth was used as negative control for each cell monolayer. *Escherichia coli* O157:H7 culture supernatant fluid was used as a positive control for each cell line.

Type strain ATCC 7966 and human isolate H-1 were cytotoxic for all cell lines tested. The overall percentage of food isolates showing cytotoxic activity (92%) was greater ($P < 0.001$) than that of water isolates (73%) (Table 2). These results agree with previously reported data (8, 16, 17). No particular cell line was significantly more sensitive to the cytotoxic effect of both food and water isolates of *A. hydrophila*.

At this point, the cytotonic effect was not uniformly observed among the different cell lines, since the cytotoxic effect was predominant. For this reason, the toxicity of culture supernatant fluids was evaluated following heat treatment at 56°C for 10 min (Table 3). Thermal denaturation had a significant impact on the toxicity response of the cell lines tested. Both cytotoxic and cytotonic alterations of the cell monolayers were still observed, but with a decreased number of bacterial isolates showing a toxic effect ($P < 0.05$). Type strain ATCC 7966 produced a cytotonic effect on CHO, Y-1, and HT-29 cells but produced a cytotoxic effect on HT-29 cell line only. Human strain H-1 produced a cytotonic effect on HT-29 cells only. A greater proportion of food isolates than water isolates produced both types of toxicity ($P < 0.05$). The cytotoxic effect of food isolates was observed most frequently on the Y-1 and CHO cell lines ($P < 0.05$). The cytotoxic effect of water isolates was observed most frequently on the Y-1 and HT-29 cell lines ($P < 0.05$). The cytotonicity of food and water isolates was

TABLE 3. Toxicity of food- and water-borne *A. hydrophila* culture supernatant fluids tested on various cell lines after heat treatment[a]

Cell line	% of isolates showing:			
	Cytotoxicity		Cytotonicity	
	Food isolates (n = 72)	Water isolates (n = 94)	Food isolates (n = 72)	Water isolates (n = 94)
HT-29	40	47	81	38
CHO	58	38	34	20
Y-1	75	60	37	20
HFF	34	32	38	8
HeLa	32	22	52	20
A-549	22	13	23	2
Vero	16	13	78	37
Mean value[b]	40	32	49	20

[a] 56°C for 10 min.
[b] Mean value for the sum of all assays.

observed most frequently on HT-29 and Vero cell lines ($P < 0.05$).

To our knowledge, this study is the first one to report the use of the HT-29 cell line for testing the toxic effect of *Aeromonas* exotoxins. Its human intestinal origin could present a valuable advantage in the study of the enteropathogenicity of *A. hydrophila* exotoxins. It has been reported that a 5-min heat treatment at 56°C is sufficient to inactivate the cytolytic and enterotoxic β-hemolysin (13). Our results demonstrated that some cytolytic effect still remains after thermal denaturation; this could be attributed to the cytotonic enterotoxin, which is thermostable after treatment at 56°C for 20 min when tested with CHO cell lines (9). However, there is also evidence that many *A. hydrophila* strains produce at least four or five proteases, one of them being thermostable (13). The role of each protease in human pathogenesis is still unclear, but proteolytic activity might be responsible in part for the observed damage.

This survey confirmed that many food and water isolates produced different virulence factors. The use of the HT-29 cell line instead of the currently used CHO and Y-1 cell lines increased by twofold the frequency of finding cytotonic activity ($P < 0.05$) in both food and water isolates. The high isolation frequency of *Aeromonas* strains in food and water samples and the production of numerous virulence factors allow us to hypothesize that *A. hydrophila* strains are actually underevaluated as causative agents of different types of infections. Further work is needed to assess the clinical significance of the virulence factors in both food and water isolates.

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