JOURNAL OF CLINICAL MICROBIOLOGY, May 1989, p. 854–859
0095-1137/89/050854-06$02.00/0
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Vol. 27, No. 5

Characterization of the *Aeromonas hydrophila* Group Isolated from Retail Foods of Animal Origin

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During a recent survey of retail fresh foods of animal origin (fish and seafood, raw milk, poultry, and red meats) for organisms of the *Aeromonas hydrophila* group, we isolated representative strains from the various foods. In this study, we sought to characterize these isolates for biochemical properties and virulence-associated factors and to compare the food isolates with clinical isolates. We identified all food and clinical isolates as *A. hydrophila* and found that all isolates were typical in their biochemical reactions. Examination of the isolates for various virulence-associated factors indicated that most food and clinical isolates were serum resistant, beta-hemolytic, cytotoxin positive (against Y1 adrenal cells), hemagglutinin positive, Congo red positive, elastase positive, and staphylolysin positive. Mouse 50% lethal doses were $\log_{10}$ 8 to 9 CFU for most isolates. All isolates had biotypes identical to those of enterotoxin-positive strains. The public health significance of these organisms in foods is not known at present, although their widespread occurrence and ability to grow competitively in foods kept at 5°C represents a potential hazard.

Aeromonas hydrophila is a pathogen of reptiles, frogs, and fish (7). Organisms of the *A. hydrophila* group (*A. hydrophila*, *A. sobria*, and *A. caviae*) are becoming recognized as important pathogens of humans, in whom they can cause various extraintestinal diseases such as wound infections, meningitis, endocarditis, and osteomyelitis (17, 18, 22, 24, 40, 61) and can be associated with gastrointestinal (diarrheal) symptoms (3, 26–28, 31, 32, 41, 45, 46, 48, 63, 65, 73). Organisms of the *A. hydrophila* group are recognized as opportunistic pathogens in immunocompromised patients and persons with underlying malignancies (29, 36, 42, 47, 66); these organisms are frequently isolated from individuals with traveler's diarrhea, individuals from underdeveloped countries and, particularly, children (4, 5, 9, 12, 13, 20, 28, 50, 57, 64, 70, 71).

Organisms of the *A. hydrophila* group occur widely in the environment, especially in water (30). They are found in both raw and chlorinated water supplies (44) and often exhibit a temporal (seasonal) appearance, their numbers increasing with temperature (77). Previous work from this laboratory (53) determined that organisms of the *A. hydrophila* group occurred widely in retail fresh foods of animal origin (fish and seafood, raw milk, red meats, and poultry). Other surveys indicated that these organisms also occurred in retail fresh produce (11). Although the link between foods and human illness has not yet been established definitely, we sought to characterize the biochemical reactions and virulence-associated factors of these organisms isolated from foods of animal origin (53) and compare their properties with those of organisms of clinical origin.

MATERIALS AND METHODS

Cultures. The origin of the eight clinical isolates was described previously (54); all strains were of fecal origin. The 42 food strains were isolated from retail fresh foods of animal origin (fish and seafood, red meats [beef, lamb, pork, and veal], and poultry) (53).

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Biochemical characteristics. The biochemical reactions of the isolates were determined as described by Pelczar (56), Cowan (15), West et al. (76), and Popoff and Veron (59).

Cytotoxicity. The isolates were grown overnight in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) broth at 37°C. After growth, the cells were harvested by centrifugation (10,000 × *g*, 5°C, 10 min), and the culture supernatant was filter sterilized (0.45-μm pore size; Millipore Corp., Bedford, Mass.) and added to monolayers of Y1 adrenal cells (grown in minimal essential medium plus 20% fetal bovine serum and incubated in an atmosphere of 5% CO_2 at 37°C). The Y1 adrenal cells were observed after 24 h, and a positive reaction was indicated by rounding of the cells and detachment of the monolayer. Serial dilutions of the supernatant were made in the tissue culture medium described above, and data were expressed as the highest dilution that gave a positive response. Controls of BHI broth and heat-inactivated (80°C for 10 min) supernatant were included.

Mouse LD_{50} determination. Cultures were grown overnight in BHI broth at 28°C. The cells were washed twice in sterile physiological saline. Appropriate dilutions of the cultures were prepared in sterile saline for injection into groups of five 20- to 25-g male and female Swiss-Webster mice, and 100 μl of each dilution was injected intraperitoneally. The animals were observed at 24-h intervals for 96 h. There were no nonspecific deaths (1 to 2 h postinjection). The viable counts of the various dilutions were determined by surface plating onto tryptic soy agar (Difco); colonies were counted after 24 h at 28°C. Fifty percent lethal doses (LD_{50}s) were calculated by the method of Reed and Muench (62).

Lytic enzymes. Elastase and staphylolytic activity assays were performed as described by Hsu et al. (34). An overnight culture of *Staphylococcus aureus* 196E was used in the staphylolytic activity assay.

Hemagglutination. Hemagglutination tests were performed in 96-well microdilution plates (Nunc, Roskilde, Denmark). Fresh human group O, sheep, and rabbit erythrocytes (RBCs) were purchased from Rockland Laboratories,

Gilbertsville, Pa., and immediately stored at 4°C until use. Before use, the RBCs were washed three times in phosphate-buffered saline (pH 7.4), and a 3% (vol/vol) suspension was prepared in phosphate-buffered saline. The strains were grown statically in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) without glucose or phosphate as described by Atkinson and Trust (6). The cultures were incubated at 28°C for 18 h, and the cells were washed three times in phosphate-buffered saline and suspended in phosphate-buffered saline to a concentration of 10^{10} bacteria per ml. Hemagglutinins were screened for at 4 and 25°C by mixing 50 μl of RBC suspension with 50 μl of bacterial suspension. A phosphate-buffered saline–RBC control was included. Plates were examined for hemagglutination after 30 min at 4°C. If positive, the suspensions were allowed to warm up to room temperature and were examined again to detect eluting hemagglutinins. The hemagglutinin titer was determined with human group O RBCs.

Inhibition of hemagglutination. Microdilution hemagglutination tests were performed as described above except that 50 μl of a 1% (wt/vol) solution of mannose, galactose, or fucose was added to the bacterial suspension prior to the addition of the RBCs. A positive control (50 μl of PBS and 50 μl of both bacteria and RBCs) and a negative control (100 μl of PBS and 50 μl of RBCs) were included.

Cell surface characteristics. Agglutination in acriflavine and stability after boiling were examined by the procedures of Mittal et al. (49). Autoagglutination was determined after 24 h of growth at 28°C as described by Janda et al. (38).

Serum resistance. The isolates were grown overnight in BHI broth at 28°C. After growth, 100 μl of culture containing 10^7 cells was mixed with 100 μl of peptone-glucose medium (1% of each) containing 25% pooled normal human serum (fresh or frozen at −80°C). At intervals, the number of viable cells was determined by surface plating onto tryptic soy agar; the colonies were counted after 24 h at 28°C. Serum-sensitive strains decreased at least one log cycle in viable count over a 4-h incubation; strains showing no decrease in viable number or an increase in viable count were designated serum resistant.

Production of proteins cross-reactive with antisera to *Escherichia coli* and *Vibrio cholerae* enterotoxins. The isolates were grown in casein hydrolysate-sucrose-salts broth and casein hydrolysate-yeast extract-salts broth overnight at 28°C (DR920 reagent instruction sheet; Oxoid Ltd., London, England). After growth, the cultures were centrifuged (10,000 × *g*, 5°C, 10 min), and the supernatant was filtered (0.45-μm pore size) and examined by reverse passive latex agglutination (Oxoid).

Dye binding. The ability to bind dyes was studied with tryptic soy agar containing 100 μg of Congo red (68) per ml or BHI agar containing 100 μg of Coomassie brilliant blue (78) per ml. Plates were incubated at 37, 28, and 5°C.

RESULTS

Biochemical characteristics. The food and clinical isolates were short, motile, gram-negative rods; positive for amylase, oxidase, catalase, DNase, and growth at 37°C (in nutrient broth in 3 days); positive for protease production on skim milk and gelatin agars; positive for esculin hydrolysis (at 28°C); and positive for indole production and ornithine decarboxylase. In addition, all isolates were Congo red positive (100 μg/ml in tryptic soy agar at 28 and 37°C), resistant to vibriostatic agent 0/129 (150 μg/ml), and resistant to ampicillin (10 μg/ml). Furthermore, all isolates were positive for the Voges-Proskauer reaction, lysine decarboxylase, gluconate oxidation, beta-hemolysin (human blood), and gas production, traits observed by Turnbull et al. (74) to correlate with enterotoxin production. However, only 3 of 42 food isolates degraded xanthine, and none of the 8 clinical isolates did. All food and clinical isolates were identified as *A. hydrophila* (58–60).

Most of the biochemical tests mentioned above were incubated at 28°C. In addition, some tests were also performed at 37°C. Many reactions which were positive at 28°C were negative at 37°C, with no clear-cut difference between the two temperatures except for esculin hydrolysis: all strains tested (both food and clinical isolates) were positive at 28°C and negative at 37°C. These observations are in agreement with those of Ewing at al. (23) and Eddy (21), who found differences in biochemical reactions based on temperature of incubation. Since the taxonomy for the genus *Aeromonas* in *Bergey's Manual of Systematic Bacteriology* (58) is based on biochemical tests incubated at 30°C, the results of this study further emphasize the need for careful selection of incubation temperature for determining the species of this genus.

Heat-labile enterotoxin cross-reactivity. The strains were evaluated for the production of extracellular materials cross-reactive with antibodies against heat-labile enterotoxins from *V. cholerae* and *E. coli*. None of the isolates—food or clinical—produced a cross-reactive protein.

Serum resistance. In many gram-negative bacteria, the ability to resist the bactericidal or bacteriolytic activity of complement in normal human serum is a virulence determinant associated with agents causing infections involving tissue damage (72). We found that 37 of 42 food isolates were serum resistant, while only 5 were serum sensitive; of 8 clinical isolates, 4 were serum resistant and 4 were serum sensitive (data not shown).

Dye binding. When the food isolates were incubated at 37, 28, and 5°C on tryptic soy agar containing 100 μg of Congo red per ml, all took up the dye. Our observations agree with those of Statner and George (68) that all strains were positive, but they varied in the amount of the dye uptake; certain strains, particularly at the higher temperature, were a deep orange-red, while at the lower temperature, some strains were only faintly pink even after extended incubation. None of the cultures—food or clinical isolate—were the deep red seen in a virulent culture of *Shigella flexneri* 5348 grown on the same medium as that described for virulent strains of *A. salmonicida* (35).

Wilson and Horne (78) described a medium containing Coomassie brilliant blue for the detection of virulence in *A. salmonicida*. All food and clinical isolates were positive (blue) at 37, 28, and 5°C on their medium.

Cytotoxicity. When the supernatants of the food and clinical isolates were examined for cytotoxicity to Y1 adrenal cells, 41 of 42 food isolates and 8 of 8 clinical isolates were positive (Table 1). The data indicate that certain of the cultures produced a very high titer of cytotoxin and that, as with many other traits, the food isolates were similar to the clinical isolates. Cytotoxic activity was lost upon heating for 10 min at 80°C.

Mouse LD_{50}s. The mouse LD_{50}s for *A. hydrophila* are shown in Table 2. The data indicate that the food isolates are similar to the clinical isolates.

Lytic activities. In addition to previously mentioned lytic activities, the isolates were also tested for elastase and staphylolysin activities; 30 of 42 food isolates were elastase positive, and 40 of 42 were staphylolysin positive. Of the

TABLE 1. Cytotoxicities of food and clinical isolates for Y1 adrenal cells

Highest dilution resulting in a positive reaction	No. (%) of the following isolates that were positive[a]:	
	Food (n = 42)	Clinical (n = 8)
1:8–1:64	5 (12)	—[b]
1:128	5 (12)	1 (12.5)
1:256	8 (19)	1 (12.5)
1:512	7 (17)	3 (37.5)
1:1,000	12 (29)	2 (25)
1:100,000	3 (7)	1 (12.5)

[a] At a dilution of at least 1:8. Total percents positive: 95 for food isolates and 100 for clinical isolates.
[b] —, No isolate tested was positive in this range.

TABLE 3. Hemagglutination reactions of food and clinical isolates of *A. hydrophila*

Pattern	Hemagglutinins detected with RBCs from:	No. (%) of positive strains that demonstrated only one pattern (n = 50)
1	Sheep	2 (4)
2	Rabbit	6 (12)
3	Human[a]	4 (8)
4	Sheep or rabbit	6 (12)
5	Sheep or human	2 (4)
6	Rabbit or human	0 (0)
7	Rabbit, sheep, or human	10 (20)
8	No species (nonreactive)	20 (40)

[a] Human group O RBCs.

eight clinical isolates examined, 7 were elastase positive and 8 were staphylolysin positive. The clinical and food isolates appeared to have similar activities; three food isolates were negative for both lytic activities, while seven of the eight clinical isolates were positive for both. The small number of clinical isolates precluded the determination of significance.

Hemagglutination. A large number of isolates examined, 20 of 50 (40%), did not agglutinate sheep, rabbit, or human type O RBCs (Table 3). Among these, five of eight clinical isolates were hemagglutination negative. The remaining three were represented in hemagglutination group 7, group 4, and group 5 (one isolate each). The next highest percentage of isolates, 10 of 50 (20%), agglutinated all of the species of RBCs tested, followed by 6 of 50 (12%) that agglutinated only sheep or rabbit RBCs. The remaining isolates demonstrated several different hemagglutination patterns (Table 3). However, each of the hemagglutination patterns represented no more than 10% of the total isolates examined. Among the eight possible hemagglutination patterns, no isolate had the ability to react with both rabbit and human RBCs.

The sensitivity of the *A. hydrophila* hemagglutinins to different sugars was determined only with human type O RBCs. Of the 16 isolates examined, 11 were sensitive to mannose. The remaining five were resistant to mannose as well as galactose and fucose (data not shown). Among the eight clinical isolates examined, two agglutinated human type O RBCs. These demonstrated the predominant carbohydrate sensitivity observed among the food isolates: both were mannose sensitive and resistant to both galactose and fucose. Additionally, only two food isolates were sensitive to both mannose and galactose. No other multiple sensitivities were noted among all of the isolates examined.

Of 50 isolates grown in BHI broth (18 to 24 h), 13 (26%) autoagglutinated (data not shown). This phenomenon was associated with hemagglutination (10 of 13; 76.9%). In addition, 11 of the 13 (84.6%) autoagglutinating isolates also precipitated after being boiled. Precipitation after boiling was present in 17 of the 50 isolates examined. This characteristic may also be associated with hemagglutination; i.e., of the 17 isolates which precipitated after being boiled, 16 also hemagglutinated one or more species of RBCs. Two of eight clinical isolates included in this study autoagglutinated; one of these two isolates also hemagglutinated and precipitated after being boiled, while the other was negative for both characteristics.

TABLE 2. Mouse LD_{50}s for food and clinical isolates of *A. hydrophila*

LD_{50} ($\log_{10}$ CFU)	No. (%) of the following isolates with the indicated LD_{50}:	
	Food (n = 42)	Clinical (n = 8)
6–7	2 (5)	—[a]
7–8	3 (7)	1 (12.5)
8–9	31 (74)	6 (75)
9–10	6 (14)	1 (12.5)

[a] —, No isolate tested had values in this range.

An additional cell surface characteristic, acriflavine agglutination, was examined, with 34 of 50 isolates (68%) being positive. Acriflavine agglutination appeared to occur irrespective of hemagglutination, autoagglutination, or precipitation after boiling. Five of eight clinical isolates were acriflavine positive.

DISCUSSION

The goal of this study was to characterize cultures of the *A. hydrophila* group isolated from foods of animal origin and to compare their characteristics with those of clinical isolates. The general biochemical traits of the food and clinical isolates indicated that they were typical of the *A. hydrophila* group. On the basis of the biochemical profiles described by Popoff and Veron (58, 59), all isolates were *A. hydrophila*. With few exceptions, the biochemical characteristics of the food isolates were similar to those of *A. hydrophila* isolated from the environment (10, 74). Our findings are in contrast to those of Okrend et al. (51), who also isolated *A. sobria* and *A. caviae* from raw meat and poultry products. Callister and Agger (11) reported the isolation of both *A. caviae* and *A. hydrophila* from retail fresh vegetables. The reasons underlying these differences are not known.

Both Callister and Agger (11) and Okrend et al. (51) as well as Abeyta et al. (1) examined their isolates for various virulence-associated factors, particularly cytotoxin (Y1 adrenal cells) and hemolysin production. Our results for cytotoxin production, 98% positive (Table 1), agree with those of Callister and Agger (11), Okrend et al. (51), and Abeyta et al. (1), who found that 100, 92.8, and 85% of their isolates from vegetables, raw meat and poultry, and oysters, respectively, were cytotoxin positive. All of our food isolates were beta-hemolytic on human blood, while Okrend et al. (51) observed that 93% of *A. hydrophila* isolates from raw meat and poultry were beta-hemolytic. Callister and Agger (11) observed that 90% of their cytotoxic isolates produced hemolysin. Thus, virtually all food isolates characterized so

far are capable of producing both cytotoxin and hemolysin. While cytotoxin per se has not been correlated with virulence, hemolysin is recognized as a major virulence factor in *A. hydrophila* (69).

The lack of isolates cross-reactive for cholera enterotoxin agrees with the results of Okrend et al. (51) (none of their isolates were positive). Production of a choleralike toxin appears to be a rare property among *A. hydrophila* isolates (10, 14, 33, 67, 79).

One of the areas most characterized for motile *Aeromonas* spp. is the possible relationship between biotype (certain biochemical reactions) and enterotoxin production (10, 27, 74). Biotyping is important, since the various tests for enterotoxin are tedious and labor intensive, while biochemical tests are relatively easy to perform. The biotype patterns observed in the current study were similar among clinical and food isolates and matched those previously reported to correlate with enterotoxin production. An exception was xanthine degradation, which Turnbull et al. (74) reported as strongly correlating with enterotoxin production. The lack of xanthine degradation among both clinical and food isolates in the current study is in direct conflict with the results of Turnbull et al. (74) but does agree with the findings of Travis and Washington (73). Clarification of the importance of xanthine degradation as a virulence marker will require further investigation.

The ability to resist the bactericidal action of normal human serum is an important virulence-associated factor possessed by many bacteria (72). Janda et al. (37) observed that a majority of *Aeromonas* spp. they studied (mostly of clinical origin) were serum resistant, as were most of the food isolates. They reported that *A. caviae* was the least resistant and that *A. sobria* was the most resistant.

For many organisms, mouse LD_{50}s represent a convenient means of determining virulence. Most food and clinical isolates had mouse LD_{50}s of $\log_{10}$ 8 to 9 CFU (Table 2); the range of mouse LD_{50}s was similar to those described for clinical and environmental isolates (38). Stern and Kazmi (N. J. Stern and S. U. Kazmi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, P46, p. 283) also observed mouse LD_{50}s in this range for various raw meat and poultry isolates. Janda et al. (37) were able to differentiate among *A. sobria*, *A. hydrophila*, and *A. caviae* on the basis of mouse lethality, with *A. sobria* being the most virulent and *A. caviae* being the least. They only inoculated the mice with one level of cells (10^7), so LD_{50}s could not be calculated; however, mortalities were similar to those observed in the current study.

In many bacteria, various surface properties, such as Congo red binding (35, 55), hemagglutination (39), autoagglutination (19, 38, 43, 75), agglutination in acriflavine, and precipitation after boiling (49), are associated with virulence and pathogenicity. As with the biochemical and other reactions, no clear-cut distinction was observed between the food and clinical isolates. Similar to the findings of Statner and George (68), all isolates were Congo red positive; however, Statner and George observed that Congo red uptake did not correlate with the Voges-Proskauer reaction or lysine decarboxylase production. In our study, 60% of the isolates were hemagglutination positive. Adams et al. (2) examined 320 isolates of *A. hydrophila* and observed that 87% were positive, while Crichton and Walker (16) observed that 97.4% of theirs were positive. Whether the higher percentages of positive isolates oberved by these two groups of investigators was due to the use of a larger number of RBC species or to the use of factors and culture conditions which enhance adhesion expression (16) was not determined. Some investigators have been able to relate hemagglutination patterns to the source of the *Aeromonas* isolates and species (8). Our observations of autoagglutination, agglutination in acriflavine, and precipitation after boiling for both food and clinical isolates were similar to those of other investigators for both environmental and clinical isolates of *A. hydrophila*. Certain biotypes, such as autoagglutination negative and precipitation positive, appear to be associated with disease (38). The adhesive capabilities of the motile *Aeromonas* spp. (25, 52) may be of importance for the virulence of these organisms because they may determine whether adhesion can occur. If the organism can recognize specific receptors, it will have the ability to colonize the host, with subsequent elaboration of tissue-damaging toxins that will facilitate penetration into the host and the establishment of infection. Therefore, not only adhesive factors but also enzymatic capabilities are essential for colonization, as observed in both clinical and food isolates (e.g., elastase, amylase, DNase, enterotoxin, and proteases).

Among other enzymatic properties examined in this study, we observed both elastase and staphylolytic activities in both clinical and food isolates. Hsu et al. (34) examined 127 strains of *A. hydrophila* for extracellular enzymatic activity (elastase, caseinase, gelatinase, and staphylolysin). They found that elastase-positive strains produced lesions and mortality when injected into channel catfish. Hsu and coworkers also observed that elastase activity correlated closely with staphylolysin activity. Both food and clinical isolates possessed these two activities.

In summary, *A. hydrophila* isolated from foods of animal origin (1, 51, 53) and from vegetables (11) possesses various biochemical markers and factors associated with virulence in other species and implicated as pathogenicity-associated factors in *A. hydrophila*, e.g., serum resistance, cytotoxicity, and hemolysin, among others. The public health significance of these organisms in foods is not known at present; however, these organisms possess many of the virulence determinants that are associated with the pathogenicity of other agents of gastroenteritis. It is possible that host resistance factors play a significant role in a manner similar to that observed with foodborne *Listeria monocytogenes*. It is likely that certain groups, such as patients who are suffering from underlying malignancies or who are immunocompromised, have a substantially greater susceptibility to *A. hydrophila* and that foods could serve as a source of the bacterium.

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