

## Comparison of lethalities in mouse versus goldfish for clinical and food isolates of *Aeromonas hydrophila*

Robert L. Buchanan, Marianne M. Bencivengo and Samuel A. Palumbo

---

### SUMMARY

The lethality of 16 clinical or food isolates of *Aeromonas hydrophila* was assessed by determination of LD<sub>50</sub> (i.p.) in mice and goldfish. In mice LD<sub>50</sub> values for the various *A. hydrophila* strains were similar, ranging from 1.2–21.0 × 10<sup>8</sup> cells/animal. A wider range of LD<sub>50</sub> values, 0.03–11.8 × 10<sup>8</sup> cells/animal, was observed with goldfish. Lethality was not correlated between the two test animals. Further, cytotoxic response in Y-1 adrenal cells did not correlate with lethality in either test animal. It appears that lethality is not a good measure of potential enterotoxigenicity, but may be useful in assessing the invasive character of isolates causing systemic infections in immunocompromised hosts.

---

### INTRODUCTION

*Aeromonas hydrophila* has become increasingly recognized as an important cause of potentially fatal septicemia in immunocompromised patients [16,30]. Likewise, the microorganism has been associated increasingly with human gastroenteritis and wound infections in both immunocompetent and

immunosuppressed individuals [2,7,14,24,28,34]. However, it appears that only some *A. hydrophila* isolates are virulent and that there may be significant host-related factors that influence pathogenicity [4]. Currently, the mechanisms underlying virulence in this species are unknown.

*A. hydrophila* can be isolated from a variety of sources, including being ubiquitously associated with refrigerated fresh foods of both plant and animal origin. Foodborne transmission has been suggested as playing an important role in the dissemination of the microorganism [6,8,22,23]. Determination of the mechanisms of virulence is important;

effective methods are needed to allow differentiation and segregation of potentially pathogenic isolates while avoiding unwarranted concern when only non-pathogenic strains are present in foods. Our laboratory has been comparing cultural and virulence-associated characteristics of strains isolated from clinical and food samples to identify potential means of distinguishing virulent and non-virulent isolates.

One attribute of *A. hydrophila* often overlooked when considering the public health significance of the species is its involvement as a well documented, economically important cause of disease in fish, amphibia and reptiles [12,20,21,27,29]. This suggested that insights into determinants of virulence for human *A. hydrophila* infections could be gained by studying the microorganism's pathogenicity in cold-blooded species. However, only a limited number of studies have evaluated relative virulence of *A. hydrophila* isolates in cold- and warm-blooded animals, although these have suggested some degree of relatedness [3-5,11,25]. The objective of the current investigation was to assess the virulence of clinical and food isolates of *A. hydrophila* in both mice and fish. Additionally, the study served to determine whether goldfish could be used as an alternate to mice as a test animal for evaluating lethality, and whether lethality correlated with cytotoxin production.

## MATERIALS AND METHODS

**Microorganisms.** Sixteen clinical or food isolates of *A. hydrophila* (Table 1) were grown in 50-ml Erlenmeyer flasks containing 20 ml of Brain Heart Infusion (BHI) (Difco) for 16 h at  $28 \pm 1^\circ\text{C}$  on a rotary shaker (140 rpm). The cultures were harvested by centrifugation, washed twice with sterile physiological saline, and serially diluted to appropriate levels in sterile saline. Cell concentrations of each dilution were subsequently confirmed by plating on Trypticase Soy Agar (Difco).

**Animals.** Mice (Swiss Webster, 20-25 g) and goldfish (*Carassius auratus*) (3-9 g) were obtained from local commercial suppliers. Mice were housed

Table 1

Source of *A. hydrophila* strains

Strain	Source
B-3-7	Beef <sup>a</sup>
CJ-2-0	Chicken exudate <sup>a</sup>
F-9-10	Cod fillet, fresh <sup>a</sup>
F-8-7	Flounder, fresh <sup>a</sup>
F-9-7	Cod fillet, fresh <sup>a</sup>
F-11-7	Bluefish, fresh <sup>a</sup>
L-2-0	Lamb <sup>a</sup>
L-1-7	Lamb <sup>a</sup>
SC-1-7	Scallops <sup>a</sup>
SC-1-0	Scallops <sup>a</sup>
V-1-7	Veal <sup>a</sup>
V-2-0	Veal <sup>a</sup>
BA-6	Clinical <sup>b</sup>
C-3518A	Clinical <sup>b</sup>
K-140	Clinical <sup>c</sup>
K-144	Clinical <sup>c</sup>

<sup>a</sup> Ref. 22.

<sup>b</sup> Ref. 23.

<sup>c</sup> Ref. 18.

in groups of 5 animals in plastic cages, with food and water being supplied ad libitum. Goldfish were placed in groups of 15 in 10 gallon tanks at  $25 \pm 2^\circ\text{C}$  and fed commercial dry pellets once daily. Dividers were placed in the tanks so that the fish could be divided into subgroups of 5 animals. The mice and goldfish were acclimated to their surroundings for 24 h and 48 h, respectively, prior to initiation of lethality determinations.

**Lethality determinations.** Groups of 5 mice and fish were injected intraperitoneally with 100  $\mu\text{l}$  portions of the appropriate cell dilutions. Uninjected controls and controls receiving 100  $\mu\text{l}$  of sterile physiological saline were included as part of each assay. All animals were then observed periodically for 96 h. Non-specific deaths (1-2 h post-injection) were not observed. LD<sub>50</sub> values were calculated using the method of Reed and Muench [26].

**Cytotoxin assays.** Monolayer cultures of Y-1 mouse adrenal cells (approximately  $5 \times 10^5$ ) were propagated at  $37^\circ\text{C}$  in 96 well plates in Minimal Eagle's Medium with 20% fetal bovine serum

(Flow Labs, McLean, VA). Cell-free supernatants from BHI cultures of *A. hydrophila* strains were prepared by centrifugation, diluted and added in 100 µl portions to the monolayers. After 16 h of incubation, the monolayers were examined with an inverted phase-contrast microscope, with only dilutions causing a complete detachment of the monolayer being recorded as a positive response.

## RESULTS

Most strains of *A. hydrophila* had similar LD<sub>50</sub>'s in mice, 14 of 16 strains having values ranging from 1.24–4.25 × 10<sup>8</sup> cells/animal. The remaining two strains, L-1-7 and F-11-7, had higher values of 10.9 × 10<sup>8</sup> and 21.0 × 10<sup>8</sup>, respectively. A wider range of responses was observed with goldfish; LD<sub>50</sub> values ranged from 0.03–11.8 × 10<sup>8</sup> cells/animal (Table 2). There was a relatively even distribution of the strains over the range of LD<sub>50</sub>'s, with 6, 7 and 3 of the strains having values < 1 × 10<sup>8</sup>, < 5 × 10<sup>8</sup>,

Table 2

LD<sub>50</sub> values and extracellular cytotoxin production observed with various clinical and foodborne strains of *A. hydrophila*

Strain	LD <sub>50</sub> values (cells/animal × 10 <sup>8</sup> )		Cytotoxin (greatest dilution yielding positive response)
	mice	goldfish	
B-3-7	3.86	0.03	100 000
CJ-2-0	1.86	3.46	128
F-9-10	1.89	0.71	256
F-8-7	2.27	0.07	2
F-9-7	1.24	3.32	1 024
F-11-7	21.00	2.52	100 000
L-2-0	1.54	3.89	1 024
L-1-7	10.90	1.18	512
SC-1-7	2.61	3.73	512
SC-1-0	4.01	6.64	1 024
V-1-7	3.01	0.31	64
V-2-0	1.93	0.90	1 024
BA-6	2.98	1.53	1 000
C-3518A	4.25	0.27	100 000
K-140	3.70	11.80	512
K-144	2.90	7.68	512

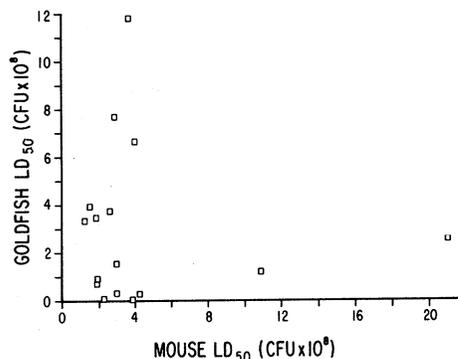


Fig. 1. Scatter diagram of LD<sub>50</sub> (i.p.) values with mice and goldfish for sixteen clinical and food isolates of *A. hydrophila*.

and > 5 × 10<sup>8</sup>, respectively. Higher levels of lethality were not restricted to strains originally isolated from fish; the strain most lethal to fish was originally isolated from beef. No relationship between lethality in the two test animals was apparent (Fig. 1). Likewise, there was no apparent relationship between lethality in either test animal and the source (clinical vs. food) of the isolates.

A wide span of cytotoxin production values was observed among the various strains (Table 2), ranging from essentially non-toxic (F-8-7) to positive responses at dilutions of 1:100 000 (B-3-7, F-11-7, C-3518A). No relationship was apparent among cytotoxin production and lethality in either test animal. A range of cytotoxin production values was observed with both clinical and food isolates of *A. hydrophila*.

One advantage of employing cold-blooded species for lethality determinations is the ability to as-

Table 3

Comparison of LD<sub>50</sub> values for five strains of *A. hydrophila* obtained with goldfish held at 25°C versus 7°C

Strain	LD <sub>50</sub> (cells injected × 10 <sup>8</sup> )	
	25°C	7°C
F-8-7	0.07	4.14
L-2-0	3.89	>41.60
B-3-7	0.03	2.19
K-140	11.80	4.47
K-144	7.68	3.63

sess readily the impact of temperature. Five strains that included a range of LD<sub>50</sub> and cytotoxin production values were re-examined for their lethality to goldfish held at 7°C instead of 25°C (Table 3). Three strains had substantially decreased lethalities while the remaining two were essentially unchanged. Interestingly, the strains with decreased lethality were isolated from foods, whereas the strains with unchanged lethalities were both of clinical origin.

## DISCUSSION

The wide range of LD<sub>50</sub> values observed with goldfish was similar to observations by other investigators concerning the relative lethality of *A. hydrophila* isolates [4,19]. The values correspond numerically to the weakly virulent to non-virulent classes described by Mittal et al. [19], though direct comparison is hampered by differences in sites of administration (intraperitoneal vs. intramuscular). The smaller range of LD<sub>50</sub> values observed for mice is also similar to previous reports [4]. The lack of correlation ( $r^2 = 0.004$ ) between strains' lethalities in mice and goldfish suggests that there are substantial differences in the disease process in the two test animals, though lethal infections in both have been reported to involve a toxemia or endotoxemia [5,20]. One possibility is that the difference in the animals' body temperatures is affecting the microorganism's virulence characteristics. Temperature effects on lethality and/or production of virulence-associated cell products have been observed in the current study and by other investigators [9,17]. Alternatively, Brenden and Huizinga [4] suggested that differences in lethality in mice and goldfish may reflect differences in immune responses or specific bacterial-host attachment factors. Whether the observed changes in lethalities with temperature were due to decreased bacterial virulence or increased host resistance will require further research.

Various investigators have concluded that the  $\beta$ -hemolysin produced by strains of *A. hydrophila* is also the microorganism's major cytotoxin and enterotoxin [1,9,13,25,31,33]. Based on cytotoxin levels

it appears that most food isolates have significant enterotoxigenic activity. The lack of correlation between lethality and cytotoxin levels ( $r^2 = 0.284$  and  $0.096$  for mouse vs. cytotoxin and goldfish vs. cytotoxin, respectively) suggests that this cell product is not a major virulence factor involved in lethal infections of the type induced by i.p. injection. The specific virulence factors associated with lethal infections in fish and other animals is not known, though several groups have concluded that  $\beta$ -hemolysin is not a major determinant [4,15,17]. Other cell products proposed as affecting lethality of *A. hydrophila* strains includes  $\alpha$ -hemolysin [15], proteases [32], cell surface characteristics [10,19] and endotoxin [4,5]. Rigney et al. [27] reported that the symptoms of red leg disease in frogs could be produced by using a combination of  $\beta$ -hemolysin and endotoxin from *A. hydrophila*.

Since lethality did not correlate with cytotoxin production, determination of LD<sub>50</sub> (i.p.) values would not appear to be a suitable means of assessing the virulence of *A. hydrophila* isolates as potential agents for gastroenteritis. However, determinations of this type, particularly with goldfish, might prove useful for assessing the invasive character of isolates in relation to systemic infections in immunocompromised patients. It would be of interest to assess *A. hydrophila* isolates from such patients to determine whether they also have enhanced lethality in fish.

## REFERENCES

- 1 Asao, T., Y. Kinoshita, S. Kozaki, T. Uemura and G. Sakaguchi. 1984. Purification and some properties of *Aeromonas hydrophila* hemolysin. *Infect. Immun.* 46: 122-127.
- 2 Bhat, P., S. Shanthakumari and D. Rajan. 1974. The characterization and significance of *Plesiomonas shigelloides* and *Aeromonas hydrophila* isolated from an epidemic of diarrhea. *Ind. J. Med. Res.* 62: 1051-1060.
- 3 Boulanger, Y., R. Lallier and G. Cousineau. 1977. Isolation of enterotoxigenic *Aeromonas* from fish. *Can. J. Microbiol.* 23: 1161-1164.
- 4 Brenden, R.A. and H.W. Huizinga. 1986. Susceptibility of normal and X-irradiated animals to *Aeromonas hydrophila* infections. *Curr. Microbiol.* 13: 129-132.

- 5 Brenden, R.A. and H.W. Huizinga. 1986. Pathophysiology of experimental *Aeromonas hydrophila* infection in mice. J. Med. Microbiol. 21: 311-317.
- 6 Buchanan, R.L. and S.A. Palumbo. 1985. *Aeromonas hydrophila* and *Aeromonas sobria* as potential food poisoning species: a review. J. Food Safety 7: 15-29.
- 7 Burke, V., M. Gracey, J. Robinson, D. Peck, J. Beaman and C. Bundell. 1983. The microbiology of childhood gastroenteritis: *Aeromonas* species and other infective agents. J. Infect. Dis. 148: 68-74.
- 8 Callister, S.M. and W.A. Agger. 1987. Enumeration and characterization of *Aeromonas hydrophila* and *Aeromonas caviae* isolated from grocery store produce. Appl. Environ. Microbiol. 53: 249-253.
- 9 Cumberbatch, N., M.J. Gurwith, C. Langston, R.B. Sack and J.L. Brunton. 1979. Cytotoxic enterotoxin produced by *Aeromonas hydrophila*: relationship of toxigenic isolates to diarrheal disease. Infect. Immun. 23: 829-837.
- 10 Dooley, J.S.G., R. Lallier and T.J. Trust. 1986. Surface antigens of virulent strains of *Aeromonas hydrophila*. Vet. Immunol. Immunopathol. 12: 339-344.
- 11 Geiger, D.W., R. Peduzzi and A. Demarta. 1986. Comparaison au moyen de la lysotypie entre souches d'*Aeromonas* de pisciculture et souches isolées de l'homme. Schwiez. Z. Hydrol. 48: 161-170.
- 12 Glorioso, J.C., R.L. Amborski, G.F. Amborski and D.D. Culley. 1974. Microbiological studies on septicemic bullfrogs (*Rana catesbiana*). Am. J. Vet. Res. 35: 1242.
- 13 Hostacka, A., I. Cizenaar, B. Korych and J. Karolcek. 1982. Toxic factors of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. Zent.bl. Bakteriol. Parasitenkd. Infekt.krankh. Hyg. Abt. 1 Orig. Reihe A 252: 525-534.
- 14 Janda, J.M., E.J. Bottone, C.V. Skinner and D. Calcaterra. 1983. Phenotypic markers associated with gastrointestinal *Aeromonas hydrophila* isolates from symptomatic children. J. Clin. Microbiol. 17: 588-591.
- 15 Kanai, J. and Y. Takagi. 1986.  $\alpha$ -hemolytic toxin of *Aeromonas hydrophila* produced in vivo. Fish Pathol. 21: 245-250.
- 16 Ketover, B.P., L.S. Young and D. Armstrong. 1973. Septicemia due to *Aeromonas hydrophila*: clinical and immunologic aspects. J. Infect. Dis. 127: 284-290.
- 17 Lallier, R., R. Bernard and G. Lalonde. 1984. Difference in the extracellular products of two strains of *Aeromonas hydrophila* virulent and weakly virulent to fish. Can. J. Microbiol. 30: 900-904.
- 18 Ljungh, A., M. Popoff and T. Wadstrom. 1977. *Aeromonas hydrophila* in acute diarrheal disease: detection of enterotoxin and biotyping of strains. J. Clin. Microbiol. 6: 96-100.
- 19 Mittal, K.R., G. Lalonde, D. Leblanc, G. Olivier and R. Lallier. 1980. *Aeromonas hydrophila* in rainbow trout: relation between virulence and surface characteristics. Can. J. Microbiol. 26: 1501-1503.
- 20 Miyazaki, T. and Y. Jo. 1985. A histopathological study of motile aeromonad disease in ayu. Fish Pathol. 20: 55-59.
- 21 Miyasaki, T. and N. Kiage. 1985. A histopathological study on motile aeromonad disease in crucian carp. Fish Pathol. 21: 181-185.
- 22 Palumbo, S.A., F. Maximo, A.C. Williams, R.L. Buchanan and D.W. Thayer. 1985. Starch ampicillin agar for quantitative detection of *Aeromonas hydrophila*. Appl. Environ. Microbiol. 50: 1027-1030.
- 23 Palumbo, S.A., D.R. Morgan and R.L. Buchanan. 1985. Influence of temperature, NaCl, and pH on the growth of *Aeromonas hydrophila*. J. Food Sci. 50: 1417-1421.
- 24 Pitarangsi, C., P. Echeverria, R. Whitmire, C. Tirapat, S. Formal, G.J. Dammin and M. Tingtalapong. 1982. Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*: prevalence among individuals with and without diarrhea in Thailand. Infect. Immun. 35: 666-673.
- 25 Rahim, Z., S.C. Sanyal, K.M.S. Aziz, M.I. Hug and A.A. Chowdhury. 1984. Isolation of enterotoxigenic, hemolytic and antibiotic-resistant *Aeromonas hydrophila* strains from infected fish in Bangladesh. Appl. Environ. Microbiol. 48: 865-867.
- 26 Reed, L.J. and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27: 493-497.
- 27 Rigney, M.M., J.W. Zillinsky and J.W. Rouf. 1978. Pathogenicity of *Aeromonas hydrophila* in red leg disease in frogs. Curr. Microbiol. 1: 175-179.
- 28 Rosenthal, S.G., H.F. Bernhart and J.A. Phillips. 1974. *Aeromonas hydrophila* wound infections. Plast. Reconstr. Surg. 53: 77-79.
- 29 Shotts, E.B., Jr., J.L. Gaines, Jr., L. Martin and A.K. Prestwood. 1972. *Aeromonas*-induced deaths among fish and reptiles in an eutrophic inland lake. J. Am. Vet. Med. Assoc. 161: 603-607.
- 30 Sirinavin, S., L. Likitnukull and S. Lolekha. 1984. *Aeromonas* septicaemia in infants and children. Pediatr. Infect. Dis. 3: 122-125.
- 31 Stelma, G.N., Jr., C.H. Johnson and P. Spaulding. 1986. Evidence for the direct involvement of  $\beta$ -hemolysin in *Aeromonas hydrophila* enteropathogenicity. Curr. Microbiol. 14: 71-77.
- 32 Thune, R.L., T.E. Graham, L.M. Riddle and R. Amborski. 1982. Extracellular products and endotoxin from *Aeromonas hydrophila*: effects on age-0 channel catfish. Trans. Am. Fish. Soc. 111: 404-408.
- 33 Turnbull, P.C.B., J.V. Lee, M.D. Miliotis, S. Van de Walle, H.J. Koornhof, L. Jeffery and T.N. Bryant. 1984. Enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* species. J. Clin. Microbiol. 19: 175-180.
- 34 Von Gravenitz, A. and A.H. Mensch. 1968. The genus *Aeromonas* in human bacteriology: report of 30 cases and review of the literature. N. Engl. J. Med. 278: 245-249.