Virulence of Starved *Aeromonas hydrophila* to Cyprinid Fish

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The survival of *Aeromonas hydrophila* was studied in a variety of waters containing different concentrations of salts. The survival of *A. hydrophila* for a period of 120 h was found to be optimal at 0.85 and 0.35% NaCl, 1% KCl, 0.2% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ or 0.4% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, respectively. Among the formulated salt water solutions examined, the best survival was found in that containing 0.60% NaCl, 0.50% KCl, 0.10% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.20% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in distilled water (FW_2). Virulence of the bacterium starved for 24 h in FW_2 and 0.85% NaCl was compared to that of the bacterium cultured in nutrient broth to carp and goldfish by intraperitoneal injection. The median lethal dose obtained by the infection verified that the bacterium starved in both salt solutions had obviously higher virulence than the cultured bacterium.

Key words: *Aeromonas hydrophila*, survival, starvation, virulence, cyprinid fish

Aquatic environments are the primary habitat of *Aeromonas* species (Hazen *et al.*, 1978; Hazen and Esch, 1983). The wide distribution of *Aeromonas* species is probably a consequence of its high capacity to adapt different aquatic environment (Mateos *et al.*, 1993). It seems that these bacteria have basically survival capacity in different qualities of waters (Kersters *et al.*, 1996). The survival of the pathogen for a long period of time in the environmental water is thought to contribute to an outbreak of the disease; however, infectivity of the pathogen surviving in the water has not been elucidated. This knowledge will help fish culturists to take the necessary measures of prevention and control against the infections (Chowdhury and Wakabayashi, 1988). Wakabayashi and Egusa (1972) reported that *Flexibacter columnaris* could survive for a long time in tap water presuming that the different salts dissolved in natural waters, Na^+ , K^+ , Ca^+ and Mg^{++} might have influenced the survivability and virulence of *F. columnaris*. The similar phenomena will be observed in *A. hydrophila*.

A. hydrophila is the causative agent of haemorrhagic septicaemia (Haley *et al.*, 1967) and ulcerative disease of fishes (Karunasagar *et al.*, 1995). There are many informations of pathogenicity of *A. hydrophila* (Austin and Austin, 1993; Thune *et al.*, 1993; Angka *et al.*, 1995), however these results were obtained by using the bacteria which are cultured in nutritious media. It is not likely

that infecting bacteria are always in a nutritious conditions but mostly they exist in a starved condition outside of the host fish. Therefore it is essential to determine if the starved bacteria sustain pathogenicity enough to infect fish. From this view point, the present study aimed to compare the pathogenicity of cultured and starved *A. hydrophila*.

Materials and methods

Test waters

Eight kinds of waters were prepared for testing survival of the bacterium; (1) distilled water (DW), (2) river water, (3) tap (well) water, (4) NaCl solution in DW, (5) KCl solution in DW, (6) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution in DW, (7) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ solution in DW, and (8) formulated water (FW) composed of combinations of above salts. The initial concentration of Na^+ , K^+ , Ca^+ and Mg^{++} in the test waters were based on gross sea water and the four successive dilutions were made *i.e.* 3.5–0.0085% of NaCl, 1–0.001% of KCl, 0.2–0.0002% of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.4–0.0004% of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. Formulated waters were designed from the results of survivability of the bacteria in single salt solutions, such as; FW_1 containing 1.20% NaCl, 1% KCl, 0.20% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.40% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in distilled water; FW_2 containing 0.60% NaCl, 0.50% KCl, 0.10% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.20% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; FW_3 containing 0.30% NaCl, 0.25% KCl, 0.05% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.10%

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$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in distilled water; FW_4 containing 0.15% NaCl, 0.13% KCl, 0.025% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.05% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in distilled water. All experimental water solutions were sterilized at 121°C for 15 min before used.

Bacteria

A. hydrophila strain A-3500 originally isolated from an eel was passed in colored (golden) carp *Cyprinus carpio* several times to increase the pathogenicity before starting experiment. Reisolated bacteria were lyophilized and stocked in 10% skimmed milk at -80°C until used.

Preparation of cultured bacteria

The bacteria were cultured in nutrient broth "Nissui" at 25°C for 24 h. Bacterial cells were harvested by centrifugation at $3,000 \times g$ for 20 min and washed twice in physiological saline (0.85% NaCl, PS). After washed, suitable concentrations of bacteria for inoculation or injection were made.

Preparation of starved bacteria

Washed bacterial cells were suspended in PS and FW_2 then incubated at 15°C for 24 h. After one day starvation in PS and FW_2 , suitable concentrations of bacteria for inoculation or injection were made.

Survival test

One hundred milliliter of each kind of water was autoclaved and cooled at room temperature (about 25°C). After cooled bacterial suspension was diluted by 10 fold dilution in PS to make a concentration of about 10^7 CFU/ml then 0.1 ml of bacterial suspension was inoculated into each water solution and incubated at 25°C for 24 h. Bacterial viable count was made on nutrient agar at appropriate intervals by using the agar spread method. Slide agglutination test was carried out to confirm purity of the bacteria using a rabbit antiserum against *A. hydrophila* A-3500 strain.

Pathogenicity test

Three types of cyprinid fish; 2 races of carp "ougon" (golden colored carp) and "kouhaku" (red and white colored carp) and goldfish *Carassius auratus* were used to compare pathogenicity of cultured and starved bacteria. Fish weighing 10-20 g were obtained from a fish farm in Kochi Prefecture, Japan. The fish were kept in 600 l tanks with well aerated flow water until used. The fish were fed commercial food pellets. Different concentrations of starved and cultured bacteria were made in PS by 10 fold dilution, then injected intra-

peritoneally into 5 ougon carp groups, each consisting of 5 fish, 5 kouhaku carp groups, each consisting of 8 fish and 5 goldfish groups, each consisting of 6 fish. Injected fish were reared for 15 days between 20 to 25°C and mortality was recorded. Infetion was confirmed by reisolating the bacteria from the kidney of dead fish using nutrient agar and slide agglutination test on the reisolated bacteria. Finally the median lethal dose (LD_{50}) was calculated by the method of Reed and Muench (1938).

Results

Survival of bacteria in various water solutions

The survival of *A. hydrophila* in distilled, river and tap waters are shown in Fig. 1. The viable bacteria in tap and river waters increased in number slightly at 48 h and 72 h post-inoculation (P.I.) and then decreased until the end of experimental period. On the other hand the viable bacteria in distilled water decreased in number at 24 h P.I. and was not detected thereafter.

Out of 6 NaCl solutions the highest survival was observed in 0.85 and 0.35% NaCl (Fig. 2A). The bacteria decreased in number at the first day then increased towards 48 h and a static survival was maintained until 120 h. But the bacteria decreased in 3.5, 0.035, 0.085 and 0.0085% NaCl solutions from the initial through 120 h. In case of KCl the highest survival was obtained in 1% solution (Fig. 2B). The bacteria

Fig. 1. Survival of *Aeromonas hydrophila* strain A-3500 in river (●), tap (▲) and distilled waters (○). detection limit.

Fig. 2. Survival of cultured *Aeromonas hydrophila* strain A-3500 in various salt solutions. A: NaCl (○, 3.5%; ▲, 0.85%; ●, 0.35%; □, 0.085%; △, 0.035%; ■, 0.0085%); B: KCl (▲, 1%; ●, 0.1%; ○, 0.01%; △, 0.001%); C: CaCl₂ (▲, 0.2%; ●, 0.02%; ○, 0.002%; △, 0.0002%); D: MgCl₂ (▲, 0.4%; ●, 0.04%; ○, 0.004%; △, 0.0004%). , detection limit.

decreased in 3 h and then increased until 96 h. The bacteria decreased in 0.1, 0.01 and 0.001% KCl solutions. The survival of *A. hydrophila* in 0.1, 0.01, and 0.001 KCl solutions were almost similar until 120 h. In case of CaCl₂·2H₂O solutions, the highest survival was observed in 0.2% CaCl₂·2H₂O (Fig. 2C). The bacteria decreased until 24 h and then increased and the survival was static through 120 h. The survival in 0.02, 0.002 and 0.0002% CaCl₂·2H₂O solutions were similar. Among the MgCl₂·6H₂O solutions the survival was the highest in 0.4% solution (Fig. 2D). The bacteria

decreased at 3 h and 24 h and then the number was increased through 120 h. The survival at 0.040, 0.0040 and 0.0004% MgCl₂·6H₂O solutions were similar to each other through 120 h. Out of 4 different formulated waters the highest survival of *A. hydrophila* was observed in FW₂ (Fig. 3). The survival was lowest in FW₁ and almost similar in FW₃ and FW₄.

Pathogenicity of starved and cultured bacteria

Starved bacteria in FW₂ and 0.85% NaCl solution were used from the result in the previous experiment of

Fig. 3. Survival of cultured *Aeromonas hydrophila* strain A-3500 in various formulated waters. Formulated water 1 (▲), formulated water 2 (●), formulated water 3 (○) and formulated water 4 (△). , detection limit.

survival. The pathogenicity of starved and cultured bacteria determined after intraperitoneal injection to three kinds of fish is summarized in Table 1. The LD₅₀ of starved bacteria were 10^{6.75} (FW₂) and 10^{6.57} (PS) in ougon carp, 10^{6.13} (FW₂) and 10^{6.38} (PS) in kouhako carp and 10^{6.15} (FW₂) and 10^{6.05} (PS) in goldfish. The LD₅₀ of cultured bacteria was 10^{7.90} in ougon carp, 10^{7.69} in kouhaku carp and 10^{7.39} in goldfish. Pathogenicity of starved bacteria is obviously higher than that of the cultured bacteria in all kinds of fishes.

Discussion

The survivability study demonstrated the high survival rate or some proliferation of *A. hydrophila* in the solution with 0.35% and 0.85% NaCl, 1% KCl, 0.20% CaCl₂·2H₂O, 0.40% MgCl₂·6H₂O, FW₂, river and tap water preparations. In case of NaCl and FW₂, the bacteria could not survive long period in high concentrations of salts. There was a decline in the viable count between 0 and 3 h in all the preparations of water. There are reports on the long time survival of *A. hydrophila* in filtered lake water (Lowcock and Edwards, 1994) and in low nutrient waters (Van der Kooij and Hijnen, 1984). One of the reason for the ecologically broad distribution of *A. hydrophila* may be attributed to its survivability as described by Kersters *et al.*, (1996). Morita (1982)

suggested that 1% or less of bacterial cells could survive for a long period of time under starvation in the absence of an exogenous carbon source.

From etiological and epizootiological view points of pathogenic microorganisms, it is important to establish whether any of their physiological characteristics, either singly or in combination, can be related to virulence (Santos *et al.*, 1987). Starvation survival is a mechanism by which the species can control the time of existence analogous to bacterial spore (Dow *et al.*, 1983). Drastic changes take place in the lipid content of *Vibrio cholerae* in the membrane when cells are starved since phospholipids located in the membrane are metabolized (Hood *et al.*, 1986). During the period of starvation, the cis-monoenoic fatty acids are preferentially utilized in *V. cholerae* (Guckert *et al.*, 1986). Amy and Morita (1983) demonstrated that starved cells of *Vibrio* sp. lose many proteins, however they synthesize new proteins. These newly synthesized proteins were termed starvation protein. Carbon starvation results in an increase in the degree of cross linking in the murein layer in Gram negative bacteria (Pisabarro *et al.*, 1985). An increase in the thickness of the peptidoglycan layer has also been demonstrated by shifting from 2 to 3 layers of peptidoglycan to 4 to 5 layers in *Escherichia coli* (Leduc *et al.*, 1989). Starved *Vibrio* strains possess a large number of exoenzymes that allow efficient degradation of a variety of polymeric substances in the environment even in the host surface tissue, which will enable the bacterium to infect human and marine vertebrates and invertebrates (Davis, 1992). It is assumed that similar physiological changes reported in *Vibrio* sp. occurred in starved *A. hydrophila*. Based on the results of this study it may be concluded that the starved *A. hydrophila* is more pathogenic than the cultured *A. hydrophila*.

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Table 1. Virulence of starved and cultured *Aeromonas hydrophila* strain A-3500 after intraperitoneal injection to three kinds of fish

Name of fish	Bacterium	CFU/fish	Dead fish/injected fish	Mortality (%)	LD ₅₀
Ougon carp	Starved in FW ₂	1.2 × 10 ⁸	4/5	80	10 ^{6.75}
		1.2 × 10 ⁷	3/5	60	
		1.2 × 10 ⁶	2/5	40	
		1.2 × 10 ⁵	0/5	0	
		1.2 × 10 ⁴	0/5	0	
	Starved in PS	1.3 × 10 ⁸	4/5	80	10 ^{6.57}
		1.3 × 10 ⁷	4/5	80	
		1.3 × 10 ⁶	2/5	40	
		1.3 × 10 ⁵	0/5	0	
		1.3 × 10 ⁴	0/5	0	
	Cultured	2.3 × 10 ⁸	3/5	60	10 ^{7.90}
		2.3 × 10 ⁷	1/5	20	
		2.3 × 10 ⁶	1/5	20	
		2.3 × 10 ⁵	0/5	0	
		2.3 × 10 ⁴	0/5	0	
Kouhaku carp	Starved in FW ₂	2.4 × 10 ⁸	8/8	100	10 ^{6.13}
		2.4 × 10 ⁷	6/8	75	
		2.4 × 10 ⁶	4/8	50	
		2.4 × 10 ⁵	4/8	50	
		2.4 × 10 ⁴	0/8	0	
	Starved in PS	2.4 × 10 ⁸	7/8	87.5	10 ^{6.38}
		2.4 × 10 ⁷	5/8	62.5	
		2.4 × 10 ⁶	5/8	62.5	
		2.4 × 10 ⁵	2/8	25	
		2.4 × 10 ⁴	0/8	0	
	Cultured	2.2 × 10 ⁸	5/8	62.5	10 ^{7.69}
		2.2 × 10 ⁷	2/8	25	
		2.2 × 10 ⁶	2/8	25	
		2.2 × 10 ⁵	1/8	12.5	
		2.2 × 10 ⁴	0/8	0	
Goldfish	Starved in FW ₂	3.0 × 10 ⁸	6/6	100	10 ^{6.15}
		3.0 × 10 ⁷	5/6	83.3	
		3.0 × 10 ⁶	3/6	50	
		3.0 × 10 ⁵	3/6	50	
		3.0 × 10 ⁴	0/6	0	
	Starved in PS	2.9 × 10 ⁸	5/6	83.3	10 ^{6.05}
		2.9 × 10 ⁷	5/6	83.3	
		2.9 × 10 ⁶	4/6	66.7	
		2.9 × 10 ⁵	3/6	50	
		2.9 × 10 ⁴	0/6	0	
	Cultured	3.4 × 10 ⁸	4/6	66.7	10 ^{7.39}
		3.4 × 10 ⁷	3/6	50	
		3.4 × 10 ⁶	1/6	16.7	
		3.4 × 10 ⁵	2/6	33.3	
		3.4 × 10 ⁴	0/6	0	

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