Relation of Water Temperature to Infection of *Salmo gairdneri* with Motile *Aeromonas*

Teresa P. NIETO*¹, M. J. R. CORCOBADO*¹, Alicia E. TORANZO*²,
and Juan L. BARJA*²

*¹Departamento de Microbiología, Colegio Universitario de Orense,
Universidad de Santiago de Compostela, Spain

*²Departamento de Microbiología, Facultad de Biología,
Universidad de Santiago de Compostela, Spain

We have studied two epizootic outbreaks occurred in March, 1983 and April, 1984, in fingerling rainbow trout of about 4 cm length reared in freshwater at North-West of Spain. A total of 84 strains were isolated from peritoneal cavity, liver, kidney and gills of diseased fish and were assigned to five main bacterial groups: *Aeromonas*, *Flavobacterium-Cytophaga*, *Pseudomonas-Xanthomonas*, Gram positive Cocci and Enterobacteria. All these bacterial groups were present in the peritoneal cavity, kidney and gills, whereas *Pseudomonas-Xanthomonas* and Gram (+) Cocci were not recovered from liver. *Aeromonas* (50%) and *Flavobacterium-Cytophaga* (15.6%) were the prevalent groups in the peritoneal cavity, being also detected in high percentage in kidney and gills. The majority of the *Aeromonas* strains belonged to the motile *Aeromonas* group, and were further classified to the species level.

Most of the *Aeromonas* strains resulted to be pathogenic for rainbow trout. Four representative strains were tested for LD₅₀ values and included in the "virulent" (LD₅₀ ≤ 10^{4.5}) or "weakly virulent" (LD₅₀ = 10^{5.5}) categories. These strains produced gas from glucose, were VP positive and fermented salicine, being assigned to the *A. hydrophila* species, and three of them shared the characteristics of the ideal phenotype of *A. hydrophila* biovar *hydrophila* displaying a strong elastase and staphylytic activities.

The analysis of physico-chemical and bacteriological parameters of the water, revealed that both epizootics appeared to be "stress" mediated since the mortalities occurred when the temperature of water suddenly increased from 5.5–8°C to more than 11°C. Then, our data confirm that motile *Aeromonas* septicemia is usually enhanced by elevations of water temperature.

Introduction

Motile *Aeromonas* septicemia is a common bacterial disease syndrome in cultured and reservoir fish populations (BOULANGER *et al.*, 1977; De FIGUEIREDO and PLUMB, 1977; LARSEN and JENSEN, 1977). *Aeromonas* species are widely distributed in nature, being considered autochthonous inhabitants of aquatic environments (HAZEN *et al.*, 1978; SEIDLER *et al.*, 1980; LARSEN and WILLEBERG, 1984) and they comprise a portion of the normal microbial flora of apparently healthy fishes (TRUST and SPARROW, 1974; SAKATA *et al.*, 1980; NIETO *et al.*, 1984; TORANZO *et al.*, 1985).

Aeromonas hydrophila is one of the main opportunistic fish pathogens which under environmental or physiological stress can produce epizootic outbreaks (HALEY *et al.*, 1967; FLIERMANS *et al.*, 1977;

GROBERG *et al.*, 1978). In addition, it has long been also considered as a pathogen in Amphibians, reptiles, snails (SHOTTS *et al.*, 1972; RIGNEY *et al.*, 1978), and more recently has received particular attention because its association with human disease (DAVIS *et al.*, 1978; JOSEPH *et al.*, 1979).

At present, the taxonomy of *Aeromonas* spp. is confused, and it has been demonstrated that a great variation in virulence exists within the motile *Aeromonas* isolated from fishes (De FIGUEIREDO and PLUMB, 1977; MITTAL *et al.*, 1980; WAKABAYASHI *et al.*, 1981). Then, it is apparently not clear at this time whether *A. hydrophila* or *A. sobria* has more virulent characteristics (OLIVIER *et al.*, 1981; WAKABAYASHI *et al.*, 1981; HSU *et al.*, 1981).

A number of extracellular virulence factors, including proteases, enterotoxins, cytotoxins and hemolysins, have been associated with the patho-

Table 1. Total numbers of fish sampled during the epizootics

Date	Total fish sampled	Moribund fish (with clinical signs)	Dead fish	Apparently healthy fish
March, 1983	32	10	8	14
April, 1984	31	8	8	15

genesis of *Aeromonas*, but their role in the fish disease requires further assessment (ALLAN and STEVENSON, 1981; THUNE *et al.*, 1982; LALLIER *et al.*, 1984). The present study was conducted to provide more information about the role of the temperature in the origin of epizootic outbreaks in cultured rainbow trout (*Salmo gairdneri*) carrying motile *Aeromonas*.

Materials and Methods

Collection of Samples and Ambient Conditions

We have followed two epizootic outbreaks that occurred during March, 1983 and April, 1984, in fingerling rainbow trout reared in a freshwater hatchery located at North-West of Spain.

Mortalities reached a maximum of 20% mainly in fingerlings of about 4 cm length. There was not noticeable mortalities in adult fish.

The clinical signs observed in some diseased rainbow trout involved swelling of the body cavity and hemorrhaging in the internal organs, which sporadically showed petechiae. However, mortalities occurred most frequently without external symptoms.

We sampled moribund or recently dead fish taken from different tanks, which presented the highest rate of mortality. For comparative purposes we also analyzed apparently healthy rainbow trout. Total numbers of fish examined during each epizootic are shown in Table. 1. During these studies, a group of healthy fish were maintained in the laboratory with water of the hatchery, to observe their posterior evolution.

Samples of the water supply were collected before it entered into the fish tanks, to study the physico-chemical and bacteriological parameters that could adversely affect to the fish, being a cause of "stress".

Temperature (degrees Celsius), dissolved Oxygen (mg/l) and pH, were monitored along this study using a portable electrode (YSU-57, Yellow Spring Inst. USA).

The bacteriological quality of the river water was assessed by determining the Total Viable Count (TVC) of heterothrophic mesophilic bacteria on TSA at 20 °C for 48 hr, as well as the total Coliforms (TC) and Faecal Streptococci (FS) by the Most Probable Number (MPN) method following the procedures of The American Public Health Association (APHA, 1980).

Bacterial Isolation and Biochemical Characteristics

Samples from liver, kidney, peritoneal cavity and gills were taken under aseptic conditions, and streaked directly onto plates of Trypticase-Soy-Agar (TSA) (Difco). After 48 hr incubation at 25°C, a representative number of the most numerous colony types from all samples were picked off from the inoculated plates, subcultured repeatedly until pure and stored on TSA slants under mineral oil.

All the bacterial isolates were subjected to taxonomic analysis by examination of their morphological, physiological and biochemical characteristics as previously described (NIETO *et al.*, 1984), and final identification was based in the schemes proposed by McDANIEL (1979) and POPOFF (1984).

The biochemical profiles of the presumptive *Aeromonas* strains were confirmed using the API 20-E system (Analytab Products), and results were compared with those obtained in the conventional tube tests. The multitest medium devised by KAPER *et al.* (1979) for identification of *A. hydrophila* (AH medium) was also utilized.

Additional tests as fermentation of salicine, production of gas from glucose and SH₂ from cysteine, hemolysis, elastase and staphylolysis, were also performed as important differential traits within the motile *Aeromonas* group. Elastase activity was determined according to the modified medium of HSU *et al.* (1981) which allow to reduce the incubation time to 72 hr. The staphylolysis test was carried out in TSA plates using *Staphylococcus aureus* strain ATCC 25923.

A. hydrophila Reference strain ATCC 7966 and strain No. 67-P-24 (isolated from *Plecoglossus altivelis* in Japan), were used as controls in the biochemical tests conducted.

Virulence Tests

To define levels of virulence among the bacterial isolates, pathogenicity assays were basically conducted as previously described (TORANZO *et al.*, 1983) using fingerling rainbow trout with an average weight of 10 g.

Pure cultures of a representative number of strains from each bacterial group, were grown overnight in TSB at 20 °C. Logarithmic-phase cultures were centrifuged, and the cells resuspended in Ringer solution. Bacterial concentrations in the inocula were adjusted to contain 10^8 cells/ml by measure of the optical density at 540 nm, and viable bacteria numbers were estimated by the standard plate count method on TSA. Fish were inoculated intraperitoneally with 0.1 ml of the corresponding suspension of each bacterial strain (15 fish were inoculated per strain), and were maintained in freshwater aquariums with a temperature range of 16 to 20 °C. Mortalities were recorded daily for a

two weeks period, and were considered to be due to the inoculated strain only if bacteria could be recovered as pure cultures from the internal organs of dead fish.

A group of *Aeromonas* strains of high virulence according to the criteria of WAKABAYASHI *et al.* (1981) (100% mortality in seven days), were further tested in order to determine the degree of virulence expressed as LD₅₀ (50% Lethal Dose). Fingerling rainbow trout were inoculated with 0.1 ml of ten-fold dilution of each bacterial suspension, ranging from 10^2 to 10^7 cells/ml (5 to 8 fish were used per dose). The mean LD₅₀ values were determined after seven days by the REED and MUENCH method (1938).

Results

The physico-chemical and bacteriological parameters of the water supply at the start of each epizootic outbreak are shown in Table 2. The pH was slightly acid and the dissolved oxygen was near saturation. Only the water temperature suffered in both epizootics a sudden elevation. So, during the winter the temperature ranged between 5.5 and 8 °C, but in March, 1983 (first epizootic) and in

Table 2. Physico-chemical and bacteriological parameters of the water supply at the start of each epizootic outbreak

Date	Temperature (°C)	pH	Dissolved oxygen (mg/ml)	TVC/ml	TC	FS
March, 1983	11	6.6	9.0	300	15	23
April, 1984	12	6.4	8.5	350	23	9

TVC, Total Viable Count on tryptic-soy-agar.

TC, Total Coliforms/100 ml.

FS, Faecal Streptococci/100 ml.

Table 3. Percentage of strains isolated from diseased rainbow trout in the epizootic outbreaks

Bacterial groups	Peritoneal cavity	Liver	Kidney	Gills
<i>Aeromonas</i>	50	44	23	21
<i>Flavobacterium-Cytophaga</i>	15.6	44	15.4	28
<i>Pseudomonas-Xanthomonas</i>	9.3	—	23	21
Gram (+) Cocci	12.5	—	30.7	7.1
Enterobacteria	12.5	12	7.7	14.2
Total number of isolates	32	25	13	14

April, 1984 (second epizootic), reached values of 11°C and 12°C, respectively. After these rapid shifts, the temperature increased progressively until a maximum in August. The bacteriological param-

eters of water quality were within the normal values for a clean river.

A total of 84 strains were isolated from either moribund or recently dead fish, and were assigned

Table 4. Biochemical characteristics and virulence degree of selected *Aeromonas* strains isolated from diseased rainbow trout

Characteristics	<i>Aeromonas</i> strains					
	Collection strains		Present study			
	ATCC 7966	67-P-24	T-12	T-16	T-80	T-2
Gram stain	—	—	—	—	—	—
Motility	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
Growth on TCBS-agar	(+) ^a	—	—	—	—	—
Growth on McConKey-agar	+	+	+	+	+	+
Growth on SS-agar	+	+	+	+	+	+
Sensitivity to 0/129 (150 µg)	R ^b	R	R	R	R	R
β-Galactosidase	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+
Lysine decarboxylase	—	(+)	—	(+)	—	(+)
Ornithine decarboxylase	—	—	—	—	—	—
Citrate	—	+	+	+	+	+
H ₂ S from tiosulphate	—	—	—	—	—	—
Urease	—	—	—	—	—	—
Tryptophane deaminase	—	—	—	—	—	—
Indole	+	+	+	+	+	+
Voges-Proskauer	+	+	+	+	+	+
Gelatinase	+	+	+	+	+	+
Fermentation of glucose	+	+	+	+	+	+
Fermentation of mannitol	+	+	+	+	+	+
Fermentation of inositol	—	—	—	—	—	—
Fermentation of sorbitol	—	—	—	+	—	—
Fermentation of rhamnose	—	—	+	+	—	—
Fermentation of sucrose	+	+	+	+	+	+
Fermentation of melibiose	—	—	—	—	—	—
Fermentation of amygdalin	—	+	+	+	+	+
Fermentation of arabinose	+	+	+	+	+	—
Fermentation of salicine	+	+	+	+	+	+
Gas from glucose (25°C)	+	+	+	+	+	+
H ₂ S from cysteine	(+)	+	+	+	+	+
Elastase production (72 h)	—	+	+	+	+	—
Staphylolysis	—	+	+	+	+	—
Hemolysis (sheep erythrocytes)	+	+	+	+	+	+
Virulence (LD ₅₀) ^d	ND ^c	ND	10 ^{5.5}	10 ^{4.5}	10 ^{4.3}	10 ^{5.6}

^a (+), weak or delayed positive reactions.

^b R, resistant strain.

^c ND, not determined.

^d LD₅₀=Number of bacteria needed to kill 50% of the inoculated fish in a 7-day period.

to five bacterial groups: *Aeromonas*, *Flavobacterium-Cytophaga*, *Pseudomonas-Xanthomonas*, Gram positive Cocci and Enterobacteria. The percentage of strains belonging to each bacterial group within the fish organs studied, are shown in Table 3. All the bacterial groups were present in the peritoneal cavity, being *Aeromonas* (50%) and *Flavobacterium-Cytophaga* (15.6%) the prevalent groups. These genera predominated also in the liver, whereas *Pseudomonas-Xanthomonas* and Gram (+) Cocci were not recovered from this organ. Although we have detected in high percentage four of the five bacterial groups present in the kidney, the number of isolates from this organ was shorter than in the other organs sampled. We can not rule out that the presence of Gram (+) Cocci in peritoneal cavity, kidney and gills was due to the handling or contaminated fish food.

We focused our further studies on the characterization of *Aeromonas* strains, since was the only group found in high percentage in all organs from all the fish examined. The majority of the isolates were identified as motile *Aeromonas* based on the following characteristics: Gram-negative rods, motile, oxidase and catalase positive, glucose fermentative, β -galactosidase positive, nitrate positive, indole positive, arginine dihydrolase positive, ornithine decarboxylase negative, mannitol positive, inositol negative, resistant to the vibriostatic agent 0/129 and Novobiocin.

The results of virulence tests demonstrated that most of the *Aeromonas* strains fell in the categories of "high virulence" or "low virulence", as defined by WAKABAYASHI *et al.*, (1981), because they produced in a preliminary screening, mortalities between 70% and 100% of the inoculated fish with a bacterial dose of 10^7 cells. Some strains of high virulence were further tested for LD₅₀ values. Table 4 shows the biochemical characteristics as well as the virulence degree of four representative pathogenic motile *Aeromonas*, in comparison with control *A. hydrophila* strains.

Our *Aeromonas* strains were classified to the species level according to the POPOFF and VERON schemes (1976), taken as important differential traits production of gas from glucose, fermentation of salicine and arabinose, Voges-Proskauer reaction and elastase production. Three strains (T-12 and T-16 isolated from gills and T-80 from liver) share the

characteristics of the ideal phenotype of *A. hydrophila* biovar *hydrophila*, similarly to the control strain 67-P-24. The strain T-2 isolated from peritoneal cavity, although did not ferment arabinose and was elastase negative, was also assigned to the *A. hydrophila* species (as defined by SCHUBERT, 1974).

According to the degrees of virulence described by LALLIER *et al.* (1981), two strains were included in the "virulent category" because displayed a mean Lethal Dose $\leq 10^{4.5}$, whereas the other two *Aeromonas* tested (Table 4) belonged to the level of "weakly virulent" (LD₅₀ $\approx 10^{5.5}$).

Discussion

Until recently, the few attempts made to correlate the biochemical characteristics of *A. hydrophila* with production of extracellular virulence factors have been practically conducted with clinical or environmental strains. So, BURKE *et al.* (1982 and 1984) studying the biochemical properties of motile *Aeromonas* isolated from different environmental sources, found that most of the strains displaying enterotoxic and hemolytic activities produced gas from glucose, were Voges-Proskauer positive, and did not ferment arabinose which, as defined by SCHUBERT (1974), should correspond to *A. hydrophila* subsp. *hydrophila*. However, if we consider the simplified classification of POPOFF and VÉRON (1976) and POPOFF (1984), these strains could be included either in *A. hydrophila* or *A. sobria* species.

A correlation between cytotoxin production and the presence of a positive VP phenotype was also reported by CUMBERBACH *et al.* (1979) in clinical isolates of *A. hydrophila*.

Similarly to the findings of these authors, in our study the four characterized pathogenic *Aeromonas* strains (Table 4), shared together with the control strains, the characteristics of to produce gas from glucose and to be VP positive.

There are several reports that show the *A. hydrophila* described by POPOFF and VÉRON (1976) to be more virulent than *A. sobria* (BOULANGER *et al.*, 1977; MITTAL *et al.*, 1980). However, both of these proposed species are potentially virulent with regard to enterotoxigenicity (SEIDLER *et al.*, 1980). By the other hand, the results of WAKABAYASHI *et al.* (1981) and HSU *et al.* (1981), show that if motile *Aeromonas* isolated from fishes possess a high elastase or staphylolytic activity, they will usually be

virulent strains.

In the present study, three pathogenic *Aeromonas* (T-12, T-16 and T-80) displayed a strong elastase and staphylolytic activities, similarly to the Japanese *A. hydrophila* 67-P-24. In addition, our *Aeromonas* as well as the control strains produced hemolysins (Table 4). Recent studies of BURKE *et al.* (1982 and 1984) and RAHIM *et al.* (1984), demonstrated that hemolysin production correlates closely with enterotoxigenicity in *A. hydrophila* isolated from water and fish skin. Then, these strains can represent a potential public health hazard.

The mechanism of pathogenicity of *Aeromonas* for fish has not been clearly identified. Whereas THUNE *et al.* (1982), obtained a toxic fraction for fish with proteolytic but no hemolytic activity, ALLAN and STEVENSON (1981), considered the hemolytic activity as a significant toxic factor for fish. Unfortunately, until now, the biological activities of *Aeromonas* products have been defined in terms of their effects on mammalian cells and systems, even when the disease process occurs in fish or other poikilotherm animals.

The analysis of the physico-chemical and bacteriological parameters of the water during the two epizootic outbreaks, seems to indicate that the disease was "stress" mediated, since in both cases the mortalities of rainbow trout occurred in late winter and spring when the water temperature suddenly increased to more than 11°C (Table 2). The relationship of water temperature with numbers of fish-infections induced by *A. hydrophila* has also been reported (FLIERMANS *et al.*, 1977; GROBERG *et al.*, 1978). That is, GROBERG *et al.* (1978), found that fatal experimental infections with *A. hydrophila* in juvenile steelhead trout, chinook salmon and coho salmon, were progressively enhanced as the temperature was increased from 12°C, and were suppressed at 9°C and below. In addition, temperatures of 15–20°C, which gave the highest "*in vitro*" growth rates of *A. hydrophila*, were also those that displayed the greatest fish mortalities in the shortest time period.

Interestingly, during these epizootics we have isolated certain *Pseudomonas* and Enterobacteria which also proved to be virulent strains. Then, we can not rule out the possibility that some fish had a mixed bacterial infection involving mainly motile *Aeromonas*.

Actually, we are conducting more extensive

studies (using both homeotherm and poikilotherm cell-systems) in order to a better understanding of the relationships between the biotype and virulence characteristics of *Aeromonas* species isolated from diseased and healthy fishes, which will allow us a more adequate classification of these strains in relation to their role in the production of disease.

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