

# ***AEROMONAS HYDROPHILA* AND MOTILE AEROMONAD SEPTICEMIAS OF FISH**

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## **INTRODUCTION**

*Aeromonas hydrophila* and other motile aeromonads are among the most common bacteria in freshwater habitats throughout the world, and these bacteria frequently cause disease among cultured and feral fishes. From descriptions of fish diseases in the early scientific literature, Otte (1963) speculated that septicemic infections in fish caused by motile aeromonads were common throughout Europe during the Middle Ages. Although the bacterial etiology of these early reports was inconclusive, the pathology was similar to that observed with red leg disease in frogs, in which *A. hydrophila* was identified as the causal organism. Because many bacteria isolated from fish with hemorrhagic septicemias in fish were often misidentified, it is now recognized that certain isolations of bacteria ascribed to the genera *Pseudomonas*, *Proteus*, *Bacillus*, *Aerobacter*, and *Achromobacter* actually belonged to the genus *Aeromonas*. The exact etiology of disease involving aeromonads is complicated by the diverse genetic, biochemical, and antigenic heterogeneity that exists among members of this group. Consequently, motile aeromonads are often referred to as a complex of disease organisms that are associated with bacterial hemorrhagic septicemias and other ulcerative conditions in fishes.

Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose part of the normal intestinal microflora of healthy fish (Trust et al. 1974). Therefore, the presence of these bacteria, by itself, is not indicative of disease and, consequently, stress is often considered to be a contributing factor in outbreaks of disease caused by these bacteria. Such stressors are most commonly associated with environmental and physiological parameters that adversely affect fish under intensive culture. As

one such example, Eisa et al. (1994) have shown that the prevalence of motile aeromonad septicemia in cultured and wild Nile tilapia (*Oreochromis niloticus*) was 10.0% and 2.5% respectively; it was 18.75% and 6.25% in cultured and wild Karmout catfish, respectively. Ventura and Grizzle (1987) produced systemic infections more readily among channel catfish (*Ictalurus punctatus*) by abrading their skin prior to exposing the fish to the bacterium. These researchers further showed that *A. hydrophila* infected internal organs through the digestive tract or through uninjured skin under conditions of crowding (13.1 g of fish/L) and high temperature (24°C). Such infections did not occur when catfish were held at a lower density (5.2 g of fish/L) and temperature (18°C). In another interesting study, Peters et al. (1988) subjected subordinate rainbow trout (*Oncorhynchus mykiss*) to social stresses of cohabitation with dominant cohorts and then exposed these fish to infection by *A. hydrophila*. By comparison to their dominant counterparts, the subordinate trout showed physical evidence of stress based on elevated plasma glucose concentrations and increased leukocyte volumes. Following exposure to the pathogen, the bacterium was also recovered from more organs and with greater prevalence among the subordinate fish than from their dominant cohorts. In feral fish, other factors may be important stressors that trigger motile aeromonad septicemias. Toranzo et al. (1989), for example, described the occurrence of such an epizootic associated with spawning stress among gizzard shad (*Dorosoma cepedianum*) in the Potomac River (Maryland, U.S.A.).

In the United States, motile aeromonads primarily cause disease in cultured warm water fishes: minnows, bait fishes, carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), striped bass (*Morone saxatilis*), largemouth bass (*Micropterus salmoides*) and tilapia. The pathogen may also affect a variety of cool and cold-water species, but is not necessarily restricted to fresh water environments. Rahim et al. (1985) have isolated *A. hydrophila* from wounds of five species of brackish water fish including species; *Plotosus anguillaris*, *Lates calcarifer*, *Epinephelus megachir*, *Labeo rohita* and *Serotherodon nilotica*. Thampuran et al. (1995) have not only isolated motile aeromonads from raw and processed products of marine fish, but also from marine fishing grounds, as well.

## TAXONOMY AND CLASSIFICATION

Because the biochemistry, genetics, and serology of the motile *Aeromonas* taxon are heterogeneous, the taxonomic position of this genus has been unstable. Kluyver and van Niel (1936) transferred many organisms that were associated with hemorrhagic septicemias in fish within the genera *Bacillus*, *Pseudomonas*, *Proteus*, and *Aerobacter* into the new genus *Aeromonas*. These aeromonads were short, gram-negative, motile bacilli with a single flagellum that fermented glucose with or without the production of gas. Snieszko (1957) later divided the genus into three species: *A. hydrophila*, *A. punctata*, and *A. liquefaciens*. In Snieszko's scheme, *Aeromonas liquefaciens* contained most of the fish pathogens. Schubert (1967) confirmed that there was enough biochemical similarity to establish the genus *Aeromonas*, but invalidated species-specific distinctions. Later, Popoff and Vernon (1976) demonstrated that the motile aeromonads could be classified into two distinct species: *A. hydrophila* (composed of the organisms previously described as *A. punctata* and *A. liquefaciens*), and a new species that they named *A. sobria*. Biochemically, *A. hydrophila* hydrolyzes esculin and ferments both salicin and arabinose, whereas *A. sobria* does not utilize these compounds (Lallier et al. 1981). Motile

aeromonads may be pleomorphic but generally produce circular, smooth, raised colonies on agar. Upon microscopic examination, the bacteria appear as short (0.5 X 1.0 ~m), gram-negative bacilli. Phenotypically, motile aeromonads are cytochrome oxidase positive, ferment glucose with or without the production of gas, and are insensitive to the vibriostatic agent O/129 (2,4-diamino,6,7-di-isopropyl pteridine). In addition, the bacteria produce 2,3-butanediol and reduce nitrate to nitrite. Hsu et al. (1984) noted that all (n = 164) of the isolates of motile aeromonads that they studied produced acid from fructose, galactose, maltose, mannitol, trehalose, dextrin, and glycogen; 99.4% of the strains produced acid from glucose, 98.8% from mannose, and 98.2% from glycerol. Acid production from other carbohydrates (arabinose, salicin, cellobiose, sucrose, and lactose) varied. Shotts et al. (1985) also found that all *A. hydrophila* complex strains hydrolysed albumin, casein, and fibrinogen; most strains also digested gelatin (99.9%), hemoglobin (94.3%), and elastin (73.2%), but none of the strains hydrolyzed collagen. Further phenotypic differentiation of the seven most commonly isolated motile aeromonads obtained from clinical isolates may be accomplished using the criteria of Carnahan et al. (1991) and modified by Joseph and Carnahan (1994) as described in Table 1. However, Austin and Austin (1989) have shown that *A. hydrophila*, *A. sobria*, and *A. caviae* comprise the most predominant clinical isolates that are typically associated with fish.

**Table 1:** Differentiation of common motile aeromonads isolated from clinical specimens as described by Carnahan et al. (1991) as modified by Joseph and Carnahan (1994). All bacterial isolates to this point would be short gram negative, oxidase-positive bacilli that ferment glucose and are resistant to the Vibrostatic agent O129.

Characteristic <sup>a</sup>	<i>A. hydrophila</i>	<i>A. veronii</i> bv. <i>sobria</i>	<i>A. veronii</i> bv. <i>veronii</i>	<i>A. caviae</i>	<i>A. scubertii</i>	<i>A. janddaei</i>	<i>A. trota</i>
Esculin hydrolysis	+	-	+	+	-	-	-
Voges-Proskauer reaction	+	+	+	-	V	+	-
Pyrazinamidase activity	+	-	-	+	-	-	-
CAMP-like factor (aerobic only)	+	+	+	-	-	V	-
Arabinose fermentation	V	-	-	+	-	-	-
Mannitol fermentation	+	+	+	+	-	+	+
Sucrose fermentation	+	+	+	+	-	-	-
Ampicillin susceptibility	R	R	R	R	R	R	S
Carbenicillin susceptibility	R	R	R	R	R	R	S
Cephalothin susceptibility	R	S	S	R	S	R	R

Colistin susceptibility <sup>b</sup>	V	S	S	S	S	R	S
Lysine decarboxylase	+	+	+	-	+	+	+
Ornithine decarboxylase	-	-	+	-	-	-	-
Arbutin hydrolysis	+	-	+	+	-	-	V
Indole production	+	+		+	-	+	+
H <sub>2</sub> S production <sup>c</sup>	+	+	+	-	-	+	+
Gas from glucose	+	+	+	-	-	+	+
Hemolysis (TSA with 5% sheep erythrocytes)	+	+	+	V	+	+	V

<sup>a</sup> +, positive for >70% of isolates; -, negative, i.e. positive for <30% of isolates; V, variable; R, resistant; S, susceptible.

<sup>b</sup>MIC (single dilution), 4 µg/mL.

## PATHOLOGY

Motile aeromonads cause diverse pathologic conditions that include acute, chronic, and covert infections. Severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiologic condition of the host, and the degree of genetic resistance inherent within specific populations of fishes. Motile aeromonads differ interspecifically and intraspecifically in their relative pathogenicity or their ability to cause disease. Under controlled laboratory conditions, De Figueredo and Plumb (1977) found that strains of motile aeromonads isolated from diseased fish were more virulent to channel catfish than were those isolated from pond water. Lallier et al. (1981) performed studies on rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) to compare the relative virulence of *A. hydrophila* and *A. sobria*, as taxonomically described by Popoff and Vernon (1976). Their results indicated that strains of *A. hydrophila* isolated from either healthy or diseased fish were more virulent than strains of *A. sobria*. Additionally, *A. sobria* was not isolated from fish with clinical signs of motile aeromonad septicemia (Boulanger et al. 1977). Paniagua et al. (1990), for example, collected aeromonad isolates along the River Porma, Leon Province (Spain) and found that their isolates grouped within three species; *A. hydrophila* (n=74 strains), *A. sobria* (n =11 strains), and *A. caviae* (n = 12 strains). The authors additionally observed that 72.02% of *A. hydrophila* isolates and 63% of *A. sobria* isolates were virulent for fish by intramuscular challenge, but all of the strains of *A. caviae* were avirulent. Pathologic conditions attributed to members of the motile aeromonad complex may include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, hemorrhagic septicemia, red sore disease, red rot disease, and scale protrusion disease.



Figure 1: Scale protrusion on a carp (*Cyprinus carpio*) caused by *Aeromonas hydrophila*

In the acute form of disease, a fatal septicemia may occur so rapidly that fish die before they have time to develop anything but a few gross signs of disease. When clinical signs of infection are present, affected fish may show exophthalmia, reddening of the skin, and an accumulation of

fluid in the scale pockets (Faktorovich 1969). The abdomen may become distended as a result of an edema and the scales may bristle out from the skin to give a “washboard” appearance (Figures 1 and 2). The gills may hemorrhage and ulcers may develop on the dermis. Ogara et al. (1998) noted severe eye pathology and heavy mortality among yearling and older rainbow trout accompanying a severe outbreak of motile aeromonad septicemia. The condition at first affected one eye, progressed into the other eye, after which the orbits ruptured causing blindness and death. Similarly, Yambot and Inglis (1994) described an acute mortality among Nile tilapia in which the most apparent clinical signs included an opaqueness in one or both eyes, accompanied by exophthalmia and eventual bursting of the orbit. Motile aeromonads were isolated from the eyes, liver and kidneys of affected fish. Histopathologically, fish may exhibit epithelial hyperplasia in the foregut; leptomenigeal congestion in the brain, as well as a thrombosis and inflammation in the perisclerotic region and corneal epithelium of the eye (Fuentes and Perez 1998). There may also be a severe branchitis, as indicated by leukocytic infiltration and dilation of the central venous sinus (Grizzle and Kiryu 1993). These authors also noted that catfish with septicemic or latent infections had enlarged nuclei in the branchial epithelium and that there was a significant correlation between the presence of these gill lesions and the severity of hepatic and pancreatic lesions. Rodriguez et al. (1993) further noted that there was an increase in bacterial acetylcholinesterase activity in the brain tissue of moribund fish.



Figure 2: Severe distention and accumulation of ascites in the abdomen of a goldfish (*Carassius auratus*) caused by *Aeromonas hydrophila*. Also note the “washboard” effect of the dermis caused by the protrusion of scales from the body surface.

Systemic infections were characterized by diffuse necrosis in several internal organs and the presence of melanin-containing macrophages in the blood (Ventura and Grizzle 1988). Internally, the liver and kidneys are target organs of an acute septicemia. The liver may become pale or have a greenish coloration while the kidney may become swollen and friable. These organs are apparently attacked by bacterial toxins and lose their structural integrity (Huizinga et al. 1979). Even when tissue damage in the liver and kidneys is extensive, the heart and spleen are not necessarily damaged. However, splenic ellipsoids are often centers of intense phagocytic activity

by macrophages. Bach et al. 1978 observed pathological changes in the spleens of fish injected with virulent *A. hydrophila*, whereas fish infected orally showed little or no splenic involvement. Bacteria were present within the reticular sheaths of the ellipsoids, where intense phagocytic activity by macrophages occurred. Phagocytized bacteria divided intracellularly and extracellularly and destroyed the endothelial and reticular cells of the ellipsoids. . The lower intestine and vent, which sometimes protrude from the body, are often swollen, inflamed, and hemorrhagic. Additionally, the intestine is devoid of food and may be filled with a yellow mucus-like material.

Chronic motile aeromonad infections manifest themselves primarily as ulcerous forms of disease, in which dermal lesions with focal hemorrhage and inflammation are apparent (Figures 3 and 4). Both the dermis and epidermis are eroded and the underlying musculature becomes severely necrotic (Huizinga et al. 1979). Inflammatory cells are usually lacking in the necrotic musculature, whereas the adjacent epidermis undergoes a hyperplasia that results in a raised margin. At this stage, the infection has usually become systemic and pinpoint hemorrhages (petechiae) may occur throughout the peritoneum and musculature. Fish with only cutaneous infections may have several types of concealed lesions including increased amounts of lipofuscin and haemosiderin in the liver and spleen; however, most visceral organs were not necrotic (Ventura and Grizzle 1988).

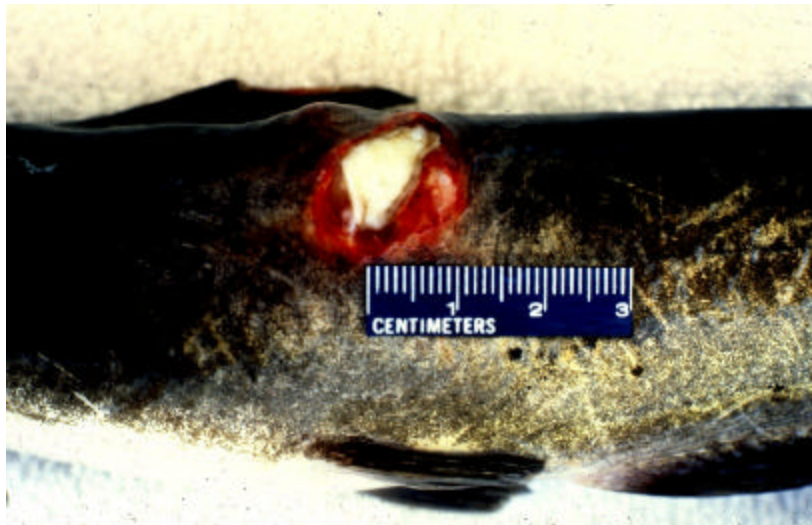


Figure 3: Lesion produced by *Aeromonas hydrophila* in the dermis of a channel catfish (*Ictalurus punctatus*).



Figure 4: Hemorrhage and ulcerations caused on the dermis of American shad

(*Alosa sapidissima*) by *Aeromonas hydrophila*.

*Aeromonas hydrophila* was generally considered to be a secondary invader in red sore disease, in which the primary etiological agent was believed to be the protozoan ciliate *Epistylis* (Rogers 1971). Recently Hazen et al. (1978b) reexamined the etiology of red sore disease and found that *A. hydrophila* was present in 96% of the initial lesions on fish, whereas *Epistylis* was present in only 35% of such lesions. Furthermore, electron microscopy showed that *Epistylis* lacked structures that produced lytic enzymes and, therefore, could not initiate the development of lesions. This study strongly suggested that *A. hydrophila* is indeed the primary etiological agent of red sore disease and that *Epistylis* is a secondary pathogen that rapidly colonizes the dermal lesions initiated by bacterial proteolytic enzymes.

In frogs and other amphibians, *A. hydrophila* infections cause distention of capillaries on the ventral surface of the legs and abdomen, giving them the red coloration that is the source of the name of the disease; - red leg. Outbreaks of aeromonad septicemias in frogs and warm water fishes usually occur in the spring and coincide with an increase in water temperature. Resistance to disease is lowered at this time because aquatic organisms are often anemic and have a substantial decrease in serum proteins resulting from periods of dormancy and starvation that occurred during the winter. Huizinga et al. (1979) also indicated that rising water temperatures increased metabolism, decreased overall condition, and stressed the fish. Stressed fish increased production of corticosteroids, which in turn increased their susceptibility to infection.

Motile aeromonads can also cause disease in warm-blooded vertebrates. In immunocompromised human hosts, for example, *A. hydrophila* may cause septic arthritis, diarrhea, corneal ulcers, skin and wound infections, meningitis, and fulminating septicemias (von Gravenitz and Mensch 1968; Davis 1978). Clinical isolates of *A. hydrophila* have been obtained from retail foods (fish, seafood, raw milk, poultry and red meats) and all isolates had biotypes identical to those of enterotoxin-positive strains (Palumbo et al. 1989). The ability of these bacteria to grow competitively at 5°C may be indicative of their potential as a public health hazard. However, most *A. hydrophila* were reduced to non-detectable levels on catfish filets cooked to 70°C (Huang et al. 1993).

## **VIRULENCE FACTORS**

The ability of a pathogen to locate, attach to, and subsequently infect a susceptible host is a primary step in the development of disease. Consequently, factors produced by motile aeromonads, which can facilitate contagion, are important elements of bacterial virulence. In fact, motile aeromonads that have been taken from lesions on diseased fish have been shown to have a greater chemotactic response to skin mucus than isolates that were obtained as free-living organisms from pond water (Razen et al. 1982). Ascencio et al. (1998) have shown that *A.*



*hydrophila*, *A. caviae*, and *A. sobria* can actually adhere to animal cell lines that have mucous receptors. These workers also determined that the proportion of *A. hydrophila* strains which bound various mucins was significantly greater than the proportions of *A. caviae* and *A. sobria* that acted similarly. Trust et al. (1980) also indicated that *A. hydrophila* had adhesive agglutination characteristics which facilitated attachment to eukaryotic cells. Electron microscopy further demonstrated that motile aeromonads produce fimbriae (pili) that facilitate adhesion but these structures were common to cells regardless of their virulence (delCorral 1990). The results of the latter study, therefore, suggest that attachment may facilitate contagion but prevented correlation between haemagglutination, yeast cell co-agglutination and virulence. In addition to adhesins, Dooley and Trust (1988) characterized a tetragonal surface protein array consisting of a 52 kD protein from virulent isolates of *A. hydrophila*. This S-layer was also reported by Ford and Thune (1991) among motile aeromonads that they isolated from clinically diseased catfish. The existence of such layers structurally permeate bacterial cell membranes which generally increases cellular hydrophobicity. The increased surface tension enhances resistance of the bacterium to complement-mediated serum lysis and phagocytosis by leukocytes. Indeed, Gado (1998) noted that virulent aeromonad isolates from Nile tilapia share a common resistance to the killing effect of tilapia serum. Byers et al. (1986) have also shown that *A. hydrophila* can produce siderophores that confer resistance against the ability of serum transferrin to inhibit bacterial growth.

Many studies have attempted to further delineate the virulence mechanisms of motile aeromonads. Kou (1973) found that many of the virulent, avirulent, and attenuated aeromonads that he studied possessed hemorrhagic factors and lethal toxins. The virulent bacteria had quantitatively more toxic potential than did their avirulent or attenuated counterparts. Olivier et al. (1981) indicated that both *A. hydrophila* and *A. sobria* produced enterotoxins, dermonecrotic factors, and hemolysins. Although both species produced hemolysis on blood agar plates at 30°C, only *A. hydrophila* did so at 10°C. Because these researchers were working with salmonid fish, they suggested that the hemolysis of red blood cells by *A. hydrophila* at temperatures comparable to those of the water in which fish live may at least partially account for the difference in virulence between *A. hydrophila* and *A. sobria*. Enterotoxins, haemolysins, proteases, haemagglutinins, and endotoxins produced by this complex of bacterial organisms have been the subject of much research (Cahill 1990). However, the composite nature of virulence observed both inter- and intra-specifically among the motile aeromonads may be best defined by looking at synergistic relationships between virulence factors. For example, Rigney et al. (1978) found that individual injections of either endotoxin or hemolysin alone did not produce clinical pathology in frogs. However, when both the endotoxin and hemolysin were injected in the same inoculum, frogs exhibited pathology that mimicked clinical signs of red leg disease. Thune et al. (1982a) also found that channel catfish were tolerant to injections of endotoxin alone at concentrations up to 400 µg endotoxin per 7.2 g of fish. However, an extracellular cell-free extract of a spent culture medium had an LD<sub>50</sub> value of 15.7 µg protein within 48 h after its injection into 7.2 g fish. Thune et al. (1982b) later showed that this extract contained was proteolytic but not hemolytic. Two proteases were further refined from this extract; one was heat labile and had an LC<sub>50</sub> concentration of 18 µg of protein per gram of fish, and the other was heat stable and had an LC<sub>50</sub> concentration of 3 µg of protein per gram of fish. Allan and Stevenson (1981) also found hemolytic and proteolytic activities in crude extracellular preparations from *A. hydrophila*. These researchers indicated that aeration increased growth

rates, cell yield, and the amount of proteolytic activity. Proteolytic activity was reduced, however, when cultures were incubated at 37°C. Furthermore, extracts from a protease deficient mutant were more toxic to fish than were similar extracts from isogenic wild strains. Because the extract from the protease deficient mutant had a fivefold increase in hemolytic activity, Allan and Stevenson concluded that hemolysin, not protease, was the principal virulence factor of *A. hydrophila*. The results of other studies, however, might caution against such conclusions. Although Rogulska et al. (1994) found that haemolytic activity was high in 93% of very pathogenic strains of *A. hydrophila* and the activity was also high in 87.5% of pathogenic *A. sobria*. Measurement of hemolytic activity alone was not the decisive characteristic in terms of isolate virulence because even some (15%) avirulent *A. hydrophila* isolates were hemolytic. Additional measurements showed that a combination of high hemolytic and high proteolytic activity, which was detected in 90% of virulent *A. hydrophila* strains and 87.5% of the *A. sobria* strains, may be a better measure of total virulence. Only one non-pathogenic strain of *A. hydrophila* had high levels of both activities. Rodriguez et al. (1992) have purified a metalloprotease, a serine protease and a haemolysin from culture supernatants of *A. hydrophila*. Each of these factors have lethal activity for rainbow trout. The metalloprotease had a molecular weight of 38 kd, was stable at 56°C for 10 min, had no cytotoxic activity but produced an LC<sub>50</sub> value of 150 ng/g of trout. The serine protease had a molecular weight of 22 kD, was also stable at 56°C for 10 min, possessed cytotoxic activity and also had an LC<sub>50</sub> value of 150 ng/g of trout. The haemolysin was alpha -hemolytic and had a molecular weight of 68 kD and produced an LC<sub>50</sub> value of 2 µg/g of trout. The haemolysin was stable at 56°C deg for 20 min and at 60°C for 10 min. Because the haemolysin possessed esterase activity on beta -naphthyl acetate, Rodriguez et al. suggested that the compound may be distinct from either alpha - or beta - haemolysin. Cascon et al. (2000) have cloned a gene encoding elastolytic activity, ahyB, from *Aeromonas hydrophila*. AhyB is synthesized as a pre-proprotein which is further processed into a mature protease and a C-terminal propeptide. The protease hydrolyzed casein and elastin and showed a high sequence similarity to other metalloproteases. Results of a recent molecular analysis, which was conducted by Zhang et al. (2000) to underscore genetic differences between virulent and avirulent isolates, confirms that virulence among the motile aeromonads depends upon a multiplicity of factors. Their results showed that 22 DNA fragments were present in most of the virulent strains and that these genes encoded for five known virulence factors of *A. hydrophila* including haemolysin (hlyA), protease (oligopeptidase A), outer-membrane protein (Omp), multidrug-resistance protein and histone-like protein (HU-2). These same fragments were mostly absent in the avirulent isolates that were examined.

## IDENTIFICATION AND DIAGNOSIS

Presumptive diagnosis of *A. hydrophila* may be based on the species of fish affected, the past disease status of those fish, and the presence of clinical signs of disease. However, bacteria must be isolated and identified biochemically to provide a definitive diagnosis. Either tryptic soy agar (TSA) or brain-heart infusion agar (BRIA) is a suitable medium for the primary isolation of motile aeromonads from diseased fish. Because mixed bacterial infections are common in fish affected with hemorrhagic septicemias, it is often difficult to isolate pure cultures of a single species of motile aeromonads from clinically diseased tissues. In order to facilitate the recovery of motile aeromonads upon primary isolation, Shotts and Rimler (1973) designed a differential

medium for selective isolation of motile aeromonads. This medium, termed R-S agar, is prepared by dissolving the following ingredients (grams) in distilled water to a volume of 1 liter: L-lysine hydrochloride (5.0), L-ornithine hydrochloride (6.5), L-cystine hydrochloride (0.3), maltose (3.5), sodium thiosulfate (6.8), bromothymol blue (0.03), ferric ammonium citrate (0.8), sodium deoxycholate (1.0), novobiocin (0.005), yeast extract (3.0), sodium chloride (5.0), and agar (13.5). The mixture is constantly stirred, heated to boiling for 1 min, and brought to pH 7.0. The medium is cooled to 45°C, dispensed into sterile petri dishes, and can be refrigerated in plastic sleeves until used.



Figure 5: Growth of yellow colonies that are characteristic of *Aeromonas hydrophila* after incubation on Rimler-Shotts agar.

After inoculation, R-S agar should be incubated at 37°C for 24 to 48 h, to ensure optimal differentiation of bacteria. Colonies of motile aeromonads are yellow; those of *Pseudomonas*, *Escherichia*, and *Enterobacter* are green; and those of *Edwardsiella* are green with black centers. Although *Proteus vulgaris* and some *Citrobacter* are also yellow on R-S agar, these colonies have black centers that indicate the bacteria are producing hydrogen sulfide gas.

Although R-S agar has facilitated primary isolation of motile aeromonads, yellow colonies should not be accepted as a basis for definitive diagnosis. *Aeromonas salmonicida* will also produce yellow colonies on R-S agar, but unlike the motile aeromonads growth of this bacterium is inhibited at 37°C. Davis and Sizemore (1981) also indicated that R-S agar supported the growth of a limited number of yellow colonies whose DNA homology ratios were inconsistent for the genus *Aeromonas*. Many of these isolates, especially those obtained from low-salinity sites, were also identified by the API 20E system as *A. hydrophila*. Therefore, additional biochemical tests should be performed on isolated colonies that have been cloned from purified colonies on either TSA or BHIA. It is important to note that reactions inconsistent for the species can occur if one uses colonies picked directly from the differential R-S agar medium. For example, Overman et al. (1979) found that 8% of the isolates they examined were cytochrome oxidase negative when taken from differential media.

Glucose fermentation is a critical reaction that differentiates the motile aeromonads from species of *Pseudomonas* (Bullock 1961). Bacteria are inoculated into two tubes of oxidation fermentation (OF) basal medium supplemented with 1% glucose. The medium in one tube is overlaid with a plug of sterile petrolatum and both tubes are incubated at 25°C for 24 to 48 h.

Results are interpreted as follows: yellow coloration in both tubes indicates acidic fermentation of glucose typical of *Aeromonas*, whereas yellow coloration only in the tube without petrolatum indicates oxidation of glucose characteristic of *Pseudomonas*. A single-tube modification of this test was described by Walters and Plumb (1978), in which a tube is prepared with twice the amount of glucose medium contained in the two-tube test. After incubation, a yellow coloration throughout the tube indicates fermentation, whereas a yellowish coloration only at the top of the medium indicates oxidation. Motility can also be determined by examination for diffused bacterial growth away from the origin of inoculation. Production of gas is evidenced in either test by the formation of bubbles in the medium. Although most strains of *A. hydrophila* produce gas during the fermentation of glucose, some motile aeromonads isolated from diseased fish are anaerogenic--that is, they do not generate gas (Ross 1962).

Because motile aeromonads are ubiquitous and have considerable antigenic diversity, serodiagnostic identification is not reliable. Agglutination procedures, fluorescent antibody tests (Eurell et al. 1978, Soltani and Rabani Khorasgani 1999), and immunoenzyme procedures (Lewis 1981, Mishra 1998) have been adapted for use within homologous antigen/antibody systems. These assays may accurately detect strains of motile aeromonads against which homologous detection antibodies were developed, but detection of heterologous strains makes serodiagnostic identification impractical. Fliermans and Hazen (1980) found that a total of three different antisera to *A. hydrophila* gave a positive fluorescent antibody reaction with only 27.5% of the *A. hydrophila* isolates tested. Therefore, the lack of an effective polyvalent antiserum specific for *A. hydrophila* still limits reliable serodiagnostic detection of this pathogen. Calabrez and Lintermans (1993) have also developed a polymerase chain reaction (PCR) technique which has been used to detect strains of *A. sobria* in water, sediments, and fish. Genetic analysis suggests that there are presently 14 genomospecies recognized within the aeromonads (Joseph and Carnahan 1994) and, consequently a number of species-specific PCR tests would need to be developed and run on individual clinical samples in order to achieve accurate diagnosis.

## SEROLOGY AND VACCINATION

Motile aeromonads are one of the most taxonomically and antigenically diverse groups of bacteria pathogenic to fish. The amount of antigenic diversity inherent within this group is especially expressed within H and O somatic antigens. Ewing et al. (1961) described 12 O-antigen groups and 9 H-antigen groups. Each group was further divided into a number of additional serotypes. Chodynieski (1965) also found a high degree of antigenic diversity among strains of motile aeromonads obtained from the same population of fish and even from different organs of the same fish. Monovalent bacterins were initially prepared against *A. hydrophila*, but these vaccines only provided acceptable levels of protection against challenge with a homologous bacterium. Fish were not immune to infection by heterologous strains of *A. hydrophila* (Post 1966; Schaperclaus 1967). Although some strains of motile aeromonads have common somatic antigens (Rao and Foster 1977; Lallier et al. 1981), it has been consistently demonstrated that a monovalent antiserum could agglutinate only a small percentage of the total isolates examined. Kingma (1978) produced seven rabbit antisera to heat stable antigens of seven isolates. Collectively, these antisera agglutinated only 19.5% of the total number of motile

aeromonad isolates studied. Despite the tremendous degree of serological diversity that exists within this taxon, Mittal et al. (1980) found that strains of *A. hydrophila*, which were highly virulent for rainbow trout, shared a common O-antigen. Furthermore, these bacteria did not agglutinate in acriflavine, settled after boiling, and resisted the bactericidal activity of normal mammalian sera. In contrast, low-virulence strains of *A. sobria* did not share the common O-antigen; these bacteria settled after boiling and were lysed by normal mammalian sera.

Schachte (1978) also found that the delivery of vaccine by injection or immersion or *per os* stimulated differential kinetics of antibody production. However, no significant protection against natural exposure to heterologous *A. hydrophila* was afforded to channel catfish vaccinated by any of the methods. Anbarasu et al. (1998) found that formalin inactivated vaccines were superior to heat killed preparations, especially when the bacterins were injected with adjuvants. Thune and Plumb (1982) found that both sac fry and swim-up fry vaccinated by immersion in sonicated polyvalent bacterin were protected against challenge with homologous bacteria, indicating an early onset of immunocompetence in channel catfish. This study was important because *A. hydrophila* causes a severe problem in channel catfish in spring and early summer, when fry are abundant. The early onset of immunocompetence among channel catfish fry indicates that immunization, if effective, could be used to reduce outbreaks of *A. hydrophila* infection. Regardless of whether whole cells, freeze-thawed cells, or cell sonicates were used, Thune and Plumb (1982) further indicated that injection was superior to either immersion or spray vaccination for developing humoral antibodies. However, cell sonicates evoked the best antibody response. Sonication may disrupt the cell and allow better processing of certain somatic antigens (e.g. – bacterial lipopolysaccharide). Consequently, Baba et al. (1988) were able to evoke better protection against *A. hydrophila* among carp that were vaccinated with crude lipopolysaccharide (LPS) rather than whole-cell, formalin killed vaccine. Immersion of fish in the LPS vaccine for 2 h at 25°C was more efficacious and less stressful than injection, but vaccination with crude LPS did not invoke an observed humoral immune response as measured by bacterial agglutination, passive haemagglutination and agar gel diffusion tests.

Because of the antigenic diversity that exists among this complex group of organisms, additional vaccination strategies included research on development of polyvalent vaccines, immunization against inactivated extracellular toxins (toxoids), and the development of vaccines consisting of cellular antigens plus toxoid. Liu (1961) noted that the biological activity of extracellular toxins from motile aeromonads was neutralized by a single antiserum prepared against an isolate of *A. liquefaciens* (synonym for *A. hydrophila*). He therefore concluded that the motile aeromonads shared extracellular antigens. Bullock et al. (1972) also indicated that aerogenic and anaerogenic strains of *A. hydrophila* possessed common extracellular antigens. Schaperclaus (1970) recognized that common carp (*Cyprinus carpio*) vaccinated against *A. hydrophila* developed both agglutinating serum antibodies against cellular antigens and antitoxic activity against extracellular antigens. In a later study, he found that common carp vaccinated by intraperitoneal injection of bacteria produced more circulating antibodies than did carp vaccinated by the oral route (Schaperclaus 1972). Furthermore, vaccination with soluble extracellular antigens was more efficacious and provided a wider base of protection against heterologous serotypes than did vaccination with whole-cell antigen. Shieh (1987) found that Atlantic salmon immunized by intramuscular injections of extracellular protease from *A. hydrophila* were protected from challenge with the homologous and some heterologous isolates

of *A. hydrophila*. Shieh (1990) repeated these studies and produced similar results when *A. sobria* extracellular protease was used as the immunogen and challenges were conducted with homologous and heterologous strains of the same bacterium. However, no report was made on the ability of extracellular protease derived from one of these species to cross protect against the other motile aeromonad species. Despite the great amount of research that has been expended to develop a safe and efficacious vaccine, there is still no product that has been licensed for use against the motile aeromonads within the United States.

## OCCURRENCE AND RANGE

Motile aeromonads cause diseases wherever bait fishes or warm water or ornamental fishes are propagated. To a lesser extent, these bacteria also initiate disease in cold-water species. Although diseases associated with motile aeromonads are most severe among fish that are propagated under conditions of intensive culture, these bacteria may also affect feral fish and are common in the intestinal flora of apparently healthy fish (Trust et al. 1974). The bacterium is ubiquitous and occurs in most fresh water environments. It can be found both in the water column and in the top centimeter of sediment (Hazen 1979). Motile aeromonads are adapted to environments that have a wide range of conductivity, turbidity, pH, salinity, and temperature (Hazen et al. 1978a). Temperature optimums may depend upon the particular strain under investigation, but generally range from 25°C to 35°C. Consequently, most epizootics among warm water fishes in the southeastern United States are generally reported in spring and early summer (Meyer 1970).

Pond water, diseased fish, and diseased frogs, as well as convalescent frogs and fishes, may become reservoirs of infection. Certain algae (Kawakami and Hashimoto 1978) and other protozoa (Chang and Huang 1981) that are grazed upon by fish, can harbor motile aeromonads. In the latter study, *Tetrahymena pyriformis* was experimentally shown to graze on populations of *Aeromonas hydrophila*. The bacterium, at concentrations of  $1 \times 10^6$  cells/mL co-existed with the protozoan. If a second bacterial species was introduced, predation by the protozoan increased. In reservoir studies, Hazen (1979) found high densities of *Aeromonas hydrophila* in mats of decomposing *Myriophyllum spicatum* and, enterically, within largemouth bass, several other species of fish, turtles, alligators, and snails. The densities of *A. hydrophila* were highest in the water from March to June, although a second peak occurred in October. Mean monthly densities of *A. hydrophila* were positively correlated with incidence of incubation in largemouth bass. Largemouth bass from thermally altered parts of the reservoir had a significantly higher incidence of infection. Kawakami and Hashimoto (1978) found greater quantities of *A. hydrophila* (as many as  $10^3$  cells) in algae fed upon by ayu (*Plecoglossus altivelis*) than were present in the water itself where the density of *A. hydrophila* was generally less than 10 cells/mL. Osborne et al. (1989) found high densities of motile aeromonads within the environment during midsummer when sedimentary chlorophyll sub(a) and water temperature were highest. This also correlated temporally with the highest prevalence of dermally ulcerated striped mullet (*Mugil cephalus*) that also contain large concentrations of the bacteria within their stomachs and on their skin. The authors suggest that mullet graze on bacteria-laden sediment for algae and consequently bioaccumulate the pathogen within their guts and on their skin which, in turn, enhances disease. Thus, the intestinal tract or epidermal abrasions are likely portals of

bacterial entry. Under conditions of stress, it is even likely that some strains of motile aeromonads that are ordinarily part of the normal gut flora become pathogenic. It is believed that infection occurs in winter, when fish are relatively inactive, and that the disease breaks out in spring. Aquarium fish, which are usually maintained at constant water temperature, can develop this disease at any time. Rainbow trout appear to be among the most susceptible of the salmonids that develop motile aeromonad septicemias.

## METHODS OF CONTROL

### *Prevention*

Effective hatchery management is the best approach to avoid infections and subsequent epizootics caused by these bacteria. In water reuse hatcheries, both ozonation (Colberg and Lingg 1978) and filtration combined with ultraviolet irradiation (Bullock and Stuckey 1977) effectively eliminate the threat of *A. hydrophila*. Colberg and Lingg (1978) showed that a specific microbial oxygen demand was exerted during batch ozonation that caused greater than a 99% mortality of bacteria within a 60-second contact during continuous flow exposure at 0.1-1.0 mg ozone per liter. Calesante et al. (1981) eliminated recurrent motile aeromonad mortality among muskellunge (*Esox masquinongy*) fry by ultraviolet irradiation of lake and well water supplies during egg incubation and yolk absorption.

Motile aeromonad septicemias are generally mediated by stress. Elevated water temperature (Esch and Hazen 1980), a decrease in dissolved oxygen concentration, or increases in ammonia and carbon dioxide concentrations have been shown to promote stress in fish and trigger motile aeromonad infections (Walters and Plumb 1980). The monitoring of environmental variables can therefore enable one to forecast stressful situations and possibly avoid problems before they arise. Wherever this disease occurs frequently, pond fish should not be handled but transferred only after water temperature is high enough for fish to be active and feeding normally (Rychlicki and Zarnecki 1957). Mortalities were reduced dramatically (80-90%) when fish, at the time of spring transfer, were injected intraperitoneally with 10-20 mg of chloromycetin or by dissolving 10-20 mg chloromycetin in water per kilogram of fish (5-10 mg/lb). In the United States, however, this drug is not registered for use on food fish nor is it legal to treat fish prophylactically (in the absence of disease).

It is prudent that managers should avoid introducing fish into their hatcheries that have recently undergone infection. Shipments of new eggs should be surface disinfected to prevent contamination of facilities and stocks. Wright and Snow (1975) found that either acriflavine (500-700 mg/L for 15 min) or iodine as Betadine (100-150 mg/L iodine for 15 min) successfully disinfected eggs of largemouth bass (*Micropterus salmoides*), but neither Roccal nor formalin was effective. When warm water fishes are held in tanks or hauled in trucks or plastic bags, the value of adding disinfectants or antibiotics should be examined. The most promising compounds include chloramphenicol, oxytetracycline, chlortetracycline, and a mixture of penicillin and streptomycin added to water at a rate of 10-15 mg/L. Again, it is important to note that the use and administration of such prophylaxis must be in compliance with local regulations.

## ***Treatment***

Oxytetracycline (Terramycin) has been the drug of choice for treating motile aeromonad septicemias in fishes. The drug is approved for use with pond fishes, channel catfish, and salmonids. It is administered in feed at a daily rate of 50 to 75 mg/kg of fish for 10 days. Fish must be withdrawn from treatment for 21 days before they are stocked or eaten. This treatment sometimes produces dramatic results when it is administered for even 2 or 3 days, and is particularly effective when fish become infected after they have been handled, crowded, or held under stress for short periods of time (Meyer 1964; Meyer and Collar 1964).

Furanace, though not registered for use in the United States, is extremely effective against motile aeromonads if the affected fish are immersed for 5 to 10 minutes in water containing 1-2 mg/L furanace, or by maintaining fish for 1 week in water containing 0.1 mg/L drug. However, furanace can be toxic to fishes if used improperly (Mitchell and Plumb 1980).

Chloramphenicol (chloromycetin) was successfully used to treat frogs with red leg disease by gastric intubation of 3 to 5 mg per 100g of frog for 5 days, twice daily. Chloromycetin, like oxytetracycline, was effective in treating fish when it was administered orally. However, its use is prohibited in food fishes and discouraged in other fishes because it is the drug of last resort in certain human diseases--e.g., typhoid fever. Indiscriminant use of chloramphenicol can result in drug resistance and thus reduce the value of the antibiotic in human medicine. Against strains of motile aeromonads that show multiple drug resistance, piromidic acid administered orally has been experimentally shown to be more effective than either chloromycetin or oxytetracycline (Katae et al. 1979). However, this drug is not registered for use on food fishes in the United States.

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