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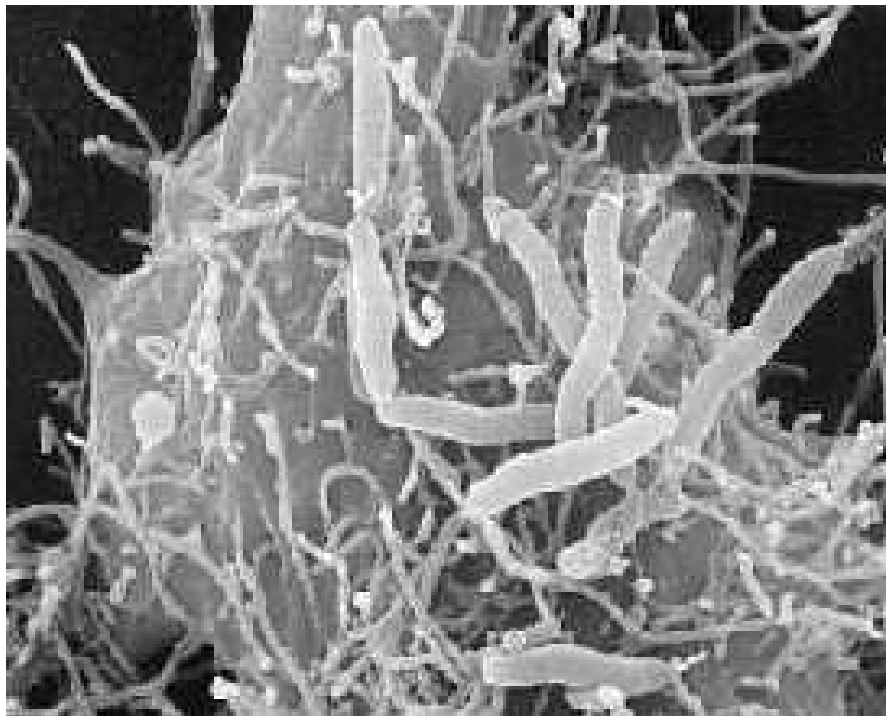
Aeromonas:
Human Health Criteria Document



Electron Micrograph of *Aeromonas*, courtesy of Northwest Fisheries Science Center

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Human Healh Criteria Document

Prepared for

Office of Ground Water and Drinking Water
Office of Water

Prepared by

Health and Ecological Criteria Division
Office of Science and Technology
Office of Water
U. S. Environmental Protection Agency
Washington, DC 20460

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The Cadmus Group, Inc
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FOREWORD

The mission of the United States Environmental Protection Agency's (EPA) Office of Water (OW) is to protect public health and the environment from adverse effects of pollutants (e.g., toxic chemicals and microbial pathogens) in ambient water, drinking water, wastewater, sewage sludge and sediments. The Safe Drinking Water Act (SDWA) requires the US EPA to regulate disease-causing organisms (pathogens) and toxic chemicals in drinking water.

SDWA, as amended in 1996, requires the US EPA to publish a list of unregulated microbiological and chemical contaminants that are known or anticipated to occur in public water systems and that may need regulation with a national primary drinking water regulation (NPDWR). SDWA also requires that the Agency make regulatory determinations on at least five contaminants on the list every five years. EPA published this list of unregulated contaminants, referred to as the Contaminant Candidate List (CCL), in March 1998 (63 FR 10273). SDWA requires a new CCL to be published every five years thereafter. The Agency published the second CCL (CCL 2) in February 2005 (70 FR 9071). Both 1st and 2nd CCLs (CCL 1 and CCL 2) contain *Aeromonas hydrophila (A. hydrophila)*, a member of the genus *Aeromonas*, as a potential waterborne pathogen. For each contaminant on the CCL, the Agency will need to obtain sufficient data to conduct analyses on the extent of occurrence and the risk posed to populations via drinking water. This information will, ultimately, assist the Agency in determining the appropriate course of action (e.g., develop a regulation, develop guidance, or make a decision not to regulate the contaminant in drinking water).

This *Aeromonas* Human Health Criteria Document constitutes a comprehensive review of published literature on the genus *Aeromonas* providing all relevant information on the *Aeromonas* group of organisms, including but not limited to, the general characteristics of *Aeromonas*, its occurrence in human and animal populations and in water, the health effects associated with *Aeromonas* infection, outbreak data, and an assessment of risk. This document also includes information about analytical methods used to detect and quantify *Aeromonas* in water and the effectiveness of removal for various water treatment practices. This document will be useful as the Agency evaluates *Aeromonas* through its CCL and regulatory determinations efforts.

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EPA also thanks the External Peer Reviewers (see External Peer Review Workgroup) for their excellent review and valuable comments on the draft document.

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The External Peer Review was managed by the Office of Science and Technology, Office of Water, US EPA under EPA Contract No. 68-C-02-091 to Versar Inc. The following professionals were part of the External Peer Review Workgroup that provided excellent technical and scientific review on the May 2005 Draft regarding the content and technical approach in response to EPA Charge to the Peer Reviewers.

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Their comments were reviewed and incorporated where appropriate to develop this final human health criteria document on *Aeromonas*.

Potential areas for conflict of interest were investigated with the Peer Reviewers and review of their current affiliations. No conflicts of interest were identified.

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1.0 Executive Summary

The Safe Drinking Water Act requires the U.S. Environmental Protection Agency (EPA) to publish regulations to control disease-causing organisms (pathogens) and hazardous chemicals in drinking water. *Aeromonas hydrophila (A. hydrophila)*, a member of the genus *Aeromonas*, is listed on the first and second Contaminant Candidate List (CCL 1 and CCL 2) of potential waterborne pathogens (USEPA 1998; 2005). This document constitutes a comprehensive review of published literature on the genus *Aeromonas*, with emphasis on the biology of the organisms, their occurrence in the environment, their effects on human health, the methods by which they may be detected, and their response to water treatment technologies.

1.1 General Information

Members of the genus *Aeromonas* are facultatively anaerobic, oxidase positive, gram-negative bacteria whose natural habitat is in the aquatic environment. Some species are pathogenic for animals and humans (Martin-Carnahan and Joseph 2005). *A. hydrophila* is listed on the CCL, and EPA Method 1605 (USEPA 2001) has been validated for detection and enumeration of *A. hydrophila* in drinking water.

The taxonomy of the genus *Aeromonas* has been confusing because of lack of congruity between phenotypic and genotypic characteristics of species, and multiple methods are required for accurate classification. Currently, 17 DNA-DNA hybridization groups (HG) have been described and 14 phenospecies are described in the 2005 edition of Bergey's Manual (Martin-Carnahan and Joseph 2005). Several additional *Aeromonas* species have been described recently, including *A. simiae, A. molluscorum*, and *A. culicicola*, though the latter may be a synonym of *A. veronii*. Other species that may be synonyms of recognized species include *A. ichthiosmia* (*A. veronii* biovar sobria) and *A. enteropelogenes* (*A. trota*). Continued use of hybridization groups is controversial, as all named phenospecies can be phenotypically identified. The controversies involving *Aeromonas* taxonomy are delineated on the LPSN web page at http://www.bacterio.cict.fr/a/aeromonas.html.

1.2 Occurrence

Aeromonas spp. are found worldwide in aquatic environments, including ground water, surface waters, estuarine and marine waters, drinking water, and wastewater (Holmes et al., 1996).

Aeromonads are found in foods, including fresh grocery produce, seafood, raw meats, packaged ready-to-eat meats, cheese, and milk (Palumbo 1996).

While *Aeromonas* spp. are not considered fecal bacteria, they are present in the feces of healthy animals and humans, presumably as the result of ingestion of food and water containing these organisms (Holmes et al., 1996; Demarta et al., 2000). They are present in high numbers in sewage before and after treatment (Monfort and Baleux 1991), thus they have been proposed as an indicator of sewage-contaminated surface water.

Aeromonas spp. may colonize drinking water distribution systems and produce biofilms that resist disinfection (Holmes et al., 1996; Bomo et al., 2004). Despite the presence of aeromonads in drinking water distribution systems, no point source outbreaks attributable to *Aeromonas* spp. have been reported from ingestion, inhalation, or dermal contact with drinking water.

1.3 Health Effects in Humans

von Graevenitz and Mensch (1968) reviewed 30 cases of infection and established *Aeromonas* spp. as significant human pathogens causing a variety of extra-intestinal infections in various organ systems. *Aeromonas* infections typically follow trauma in an aquatic environment, or dissemination from the gastrointestinal tract as the result of underlying diseases such as cancer. Both extra-intestinal and gastrointestinal infections are now known to occur in previously healthy hosts as well as immunocompromised or otherwise susceptible populations (Janda and Abbott 1996; Joseph 1996; Janda and Abbott 1998). Besides diarrheal illness, *Aeromonas* spp. cause wound infections, septicemia, meningitis, ophthalmitis, endocarditis, aspiration

pneumonia, and biliary tract infections. *A. hydrophila* (HG-1), *A. veronii* biovar sobria (HG-8), *A. caviae* (HG-4), *A. jandaei* (HG-9), *A. veronii* biovar veronii (HG-10), *A. schubertii* (HG-12), and *A. trota* (HG-14) are isolated from human infections (Janda and Abbottt 1998). Considerable strain variation occurs among *Aeromonas* species, and the proportion of strains within these phenospecies that cause human infection is unknown.

Virulence of *Aeromonas* spp. is multifactorial and incompletely understood. Factors contributing to virulence include toxins, proteases, hemolysins, lipases, adhesins, agglutinins, and various hydrolytic enzymes (Janda and Abbott 1996). Virulence factors are present in two forms, cell-associated structures, and extracellular products. Among the cell-associated structures are pili, flagella, outer membrane proteins, lipopolysaccharide, and capsules. The major extracellular products include cytotoxic, cytolytic, hemolytic, and enterotoxic proteins.

Aeromonads produce an array of filamentous structures, including short rigid, and long wavy pili, and polar and lateral flagella. Short rigid pili are similar to those of *E. coli* Type I and Pap pili (Ho et al., 1992), while long wavy pili belong to a class of Type IV bundle-forming pili (Bfp) (Kirov and Sanderson 1996). Removal of pili or neutralization of attachment sites by homologous antibody treatment limits or defeats adherence properties in cell culture systems (Iwanaga and Hokama 1992; Kirov et al., 1999). The Type IV pili are controlled by the *tapABCD* gene cluster (Pepe et al., 1996). *TapD* encodes a Type IV peptidase/methyltransferase which is responsible for extracellular secretion of aerolysin and other enzymes via a Type II secretion system (Strom and Pepe 1999), in addition to its *tap* pilin-related function. Barnett et al. (1997) cloned the *tap* cluster and demonstrated conclusively that Bfp and Tap pili were distinct families. Tap pili have a molecular mass of 17 kilodaltons (kDA), while Bfp pili have a molecular mass of 19-23 kDA. Tap pili are thought to facilitate attachment to enterocytes and enhance colonization. Bfp pili facilitate adherence to erythrocytes (Kirov et al., 2004). Polar flagella and lateral flagella were described by Rabaan et al. (2001) and Kirov et al. (2002). Polar flagellins function as adhesions, while lateral flagellins are thought to serve as colonization factors (Kirov et al., 2004).

Capsule production has been reported for *A. salmonicida* and *A. hydrophila* serogroups (Martinez et al., 1995), but the function of capsule material is vague. It is presumed to resist complement activity and perhaps enhance adherence (Merino et al., 1996).

S-layers (originally termed A-layer in *A. salmonicida*) are paracrystalline structures made up of identical protein subunits that are translocated across the cell membrane and assembled on the cell wall surface via an interaction with O-polysaccharide side chains of lipopolysaccharide. Stains producing S-layers are more pathogenic for fish, but the role of S-layer in human infection is not clear. Studies suggest that strains containing S-layers autoagglutinate (Kokka et al., 1991a).

The hemolysin produced by some *Aeromonas* species is termed ‘aerolysin’, and it posesses both hemolytic and enterotoxic activity. This hemolytic enterotoxin (aerolysin), has been shown to share significant homology with the cytotoxic enterotoxin (Act), and two cytotonic toxins (Alt and Ast) as reviewed by Xu et al. (1998). Early investigation of *Aeromonas* toxins was responsible for recognition of ‘aerolysin’ in several species of aeromonads; however, it appears that ‘aerolysin’ is really a family of toxins whose mechanisms of action are gradually being understood. The toxins reported by various investigators have fundamental differences in properties, making comparisons difficult (Chakraborty et al., 1987a; Buckley et al., 1981). Act increased levels of tumor necrosis factor " (TNF-") and interleukin (IL-1 $) in macrophage cell lines and other inflammatory cytokines that result in tissue damage. Prostaglandin activity (PG_1 or PG_2) is increased. Aerolysin is released from cells as proaerolysin that is activated by proteolytic cleavage of a C-terminal peptide fragment of approximately 40 amino acids. Another ‘aerolysin’ has been described that is released by a Type II secretion pathway under control of *exeAB* and *exeC-N* genes. The gene products produce a pore in the outer membrane protein for toxin secretion (Howard et al., 1996). This ‘aerolysin’ is a dimer with hemolytic activity for mouse erythrocytes. This toxin is involved in channel formation in target cells in a similar manner to the way it forms pores in bacterial outer membrane protein, by producing a heptamer that inserts into the cell membrane and forms a 1-2 nanometer (nm) channel which results in loss of cell permeability, cell leakage, and eventual cell destruction.

Aeromonas spp. produce a lipase, glycerophospholipid cholesterol acyltransferase (GCAT), that results in production of cholesteryl esters and phospholipase activity that digests plasma membranes of host cells (Buckley 1983). Another class of enzymes, the metallo- and serine proteases are involved in toxin activation and have a protective role in inimical environments (Rodriguez et al., 1992). Other enzymes that contribute to virulence include amylase, chitinase, elastase, lecithinase, and nucleases (Gosling 1996a).

A. salmonicida strains produce siderophores that facilitate iron acquisition (Chart and Trust 1983). Some species of mesophilic aeromonads produce a enterobactin-like siderophore under iron-limiting conditions.

1.4 Health Effects in Animals

Aeromonads have been reported as pathogens of fish, amphibians, and reptiles (Gosling 1996b). *Aeromonas* spp. cause hemorrhagic disease, ulcerative disease, furunculosis, and septicemia in fish (Austin and Adams 1996). Aeromonads cause pneumonia, peritonitis, abortion and other diseases in birds and domestic animals (Gray 1984; Gosling 1996b).

1.5 Risk Assessment

Exposure to *Aeromonas* spp. through ingestion of food and water is continuous, however most environmental strains do not produce gastrointestinal disease in normal humans. No point source waterborne disease outbreaks have been attributable to aeromonads through exposure to treated drinking water. Case reports suggest that susceptible individuals may acquire gastrointestinal illness from chronic exposure to high numbers of aeromonads in untreated water, and foodborne disease has been reported. The infectious dose by ingestion is remarkably high, based upon bacterial counts in foods implicated in disease. A human feeding study failed to induce illness in a significant percentage of volunteers (Morgan et al., 1985), however, this study is considered inconclusive since the strain use was selected for the presence of only one virulence factor (enterotoxin) and the subjects were healthy adults. In the only risk assessment study for exposure via drinking water, Rusin et al. (1997) reported that the oral infectious dose

exceeded 10 log_{10} colony-forming units per milliliter (CFU/mL) and the risk of infections attributable to drinking water was 7.3 per billion exposures.

The use of water purification devices installed at the tap has become popular, yet devices employing activated carbon filtration may actually degrade microbial quality of drinking water. Chaidez and Gerba (2004) demonstrated that counts of *Pseudomonas* and *Aeromonas* were higher in water samples taken after filtration though point-of-use devices. Despite these increases in total bacterial load in drinking water, the levels remained substantially below the number required to cause gastrointestinal disease.

Risk assessment studies have demonstrated an increased rate of transmission in daycare centers in Ecuador (Sempertegui et al., 1995), nursing homes in the U.S. (Bloom and Bottone 1990; Sims et al., 1995), and intensive care units in Italy (Torre et al., 1996). Poor personal hygiene and reuse of water have been shown to increase the risk of transmission.

The risk of *Aeromonas* infections is significant for animals in aquaculture, where crowding promotes transmission (Austin and Adams 1996).

1.6 Methods

The reconciliation of phenotypic and genotypic methods presented by Martin-Carnahan and Joseph (2005) in the Second Edition of Bergey's Manual of Systematic Bacteriology enables identification of most clinical and environmental aeromonads to group and many to species, leading Janda and Abbott (1998) to suggest that taxonomic advances make continued use of DNA-DNA hybridization groups unnecessary. The myriad of methods for detection, isolation, and enumeration of aeromonads from various sources was reviewed by Moyer (1996). Since then, the membrane filtration method using Ampicillin Dextrin Agar (ADA) has been published in Standard Methods for Examination of Water and Wastewater (APHA 1998), and EPA has validated Method 1605: *Aeromonas* in Finished Water by Membrane Filtration using Ampicillin-Dextrin Agar with Vancomycin (ADA-V) (USEPA 2001). Ampicillin has been incorporated into ADA in various concentrations from 10-30 mg/L by different investigators, but use of ampicillin

will inhibit the growth ampicillin sensitive aeromonads, including *A. trota* and some strains of *A. caviae* and *A. schubertii*.

Many investigators have published molecular detection methods for one or more species of *Aeromonas* in clinical or environmental samples. Some of these detection systems target genes controlling virulence, thereby seeking to differentiate pathogenic from nonpathogenic strains. Unless molecular detection methods are preceded by culture enrichment, the detection limit is typically greater than 3-4 $\log_{10}$ CFU/mL, rendering these methods too insensitive for direct detection of aeromonads in environmental samples.

1.7 Water Treatment

Aeromonas spp. are effectively removed by sand filtration and inactivated by disinfectants used in water treatment at concentrations commonly employed (Gerba et al., 2003). Aeromonads sequestered in biofilms may resist disinfection and persist for long periods of time. Use of granulated activated carbon (GAC) in water treatment may provide nutrient sources for aeromonads in the form of assimilable organic carbon, contributing to the presence and survival of aeromonads in distribution system water. The ability to detect aeromonads in drinking water distribution systems is related to temperature, where samples typically test positive only when water temperature exceeds 15° C and where chlorine residuals are below 0.2 mg/L. Because grab samples may contain cells suspended in the water column, particle-adsorbed cells from filter break-though, or microcolonies sloughed from biofilms accumulations, the true number and source of aeromonads present in drinking water distribution systems cannot be reliably determined.

1.8 Indicators of Occurrence and Treatability

Epidemiological evidence does not support the presence of aeromonads in drinking water as a threat to public health. A statewide case reporting system in California was discontinued after a short time because the number of cases reported was deemed insignificant (King et al., 1992).

The level of risk aeromonads comprise for immunocompromised hosts from exposure to drinking water is unclear, since their primary environmental exposure is from surface water and foods (Altwegg and Geiss 1989; Kirov 1993a; Janda and Abbott 1999). Enhanced treatment of drinking water using improvements in filtration and disinfection is unlikely to completely remove aeromonads from drinking water, since they gain access to water distribution systems during construction or maintenance, and maintain populations under favorable conditions of temperature and nutrient levels in the absence of disinfection residuals. The number of aeromonads typically found in surface source water does not approach an infectious dose for humans via ingestion.

Aeromonas spp. do not correlate with fecal indicators used for monitoring treated drinking water. Conventional water treatment processes are effective in removing or inactivating aeromonads, however aeromonads may persist in distribution system biofilms when disinfectant levels are low (< 0.2 mg/L free chlorine residual).

2.0 General Information

The order *Aeromonadales* comprises a single family of bacteria, *Aeromonadaceae*, where the genus *Aeromonas* resides (Martin-Carnahan and Joseph 2005). *Aeromonas hydrophila* is designated as the type species. Aeromonads are naturally occurring inhabitants of aquatic environments, namely fresh waters, marine waters, and estuarine waters. Some species of aeromonads cause diseases of aquatic animals and opportunistic infections in humans.

2.1 History

Bacteria resembling motile *Aeromonas* species were first isolated from water and diseased animals over 100 years ago. The history of their isolation and taxonomy was reviewed by Carnahan and Altwegg (1996) and Martin-Carnahan and Joseph (2005).

Associations of aeromonads with human disease were reported by von Graevenitz and Mensch (1968) in a review of 30 cases of *Aeromonas* infection or colonization, providing

evidence for their recognition as human pathogens and suggesting that some aeromonads may be associated with gastrointestinal disease. *Aeromonas* spp. are isolated most frequently from fecal specimens from children under five years of age, while isolation of aeromonads from other body sites typically occurred in adult populations. Aeromonads cause acute diarrheal disease of short duration or chronic loose stools in children, the elderly, or the immunocompromised, and they have been implicated as a cause of travelers' diarrhea. *Aeromonas* spp. cause cellulitis or wound infections following traumatic injury in an aqueous environment. They also cause septicemia associated with underlying disease such as cirrhosis, leukemia, cancer, and various infections associated with hospitalization such as rare urinary tract infections, surgical wound infections, meningitis, peritonitis, endocarditis, or other serious infections. Predisposing conditions for *Aeromonas* infection include cirrhosis or other hepatic disease, hematologic malignancies, hepatobiliary disease, diabetes, and renal disease. Use of medicinal leaches has resulted in infections in patients undergoing reconstructive breast surgery. The species distribution of *Aeromonas* infections as *A. hydrophila* (48%), with *A. sobria,* and *A. caviae* equally distributed (25-27%).

An environmental source of *Aeromonas* implicated in gastrointestinal infection was first proposed by Holmberg et al. (1986), who presented epidemiological evidence to support untreated well water as the source of infection in patients with diarrheal disease. *A. caviae* was present in 18 of 34 cases. The role of *A. caviae* as an agent of gastrointestinal disease remained controversial for the next decade until an overwhelming number of case reports overcame the last objections. Today, seven species of *Aeromonas* are recognized to cause a variety of intestinal and extra-intestinal infections in humans (Janda and Abbott 1998). *Aeromonas* spp. have been identified as the cause of community acquired infection, nosocomial infection, and travelers' diarrhea (Rautelin et al., 1995).

The intense interest in *Aeromonas* spp. in the 1980s lead to the search for improved culture methods for their recovery from water, food, clinical specimens, and especially feces, since the role of aeromonads as agents of gastrointestinal disease was controversial (Joseph 1996). Isolation and enumeration methods for all sample types were reviewed by Moyer (1996), and phenotypic identification methods were reviewed by Millership (1996). Current

identification methods for the recognized phenospecies are contained in the Second Edition of Bergey's Manual of Systematic Bacteriology (Martin-Carnahan and Joseph 2005). Methods for identification of the newly recognized phenospecies *A. hydrophila* ssp. *dhakensis, A. hydrophila* ssp. *ranae, A. simiae*, *A. molluscorum*, and *A. culicicola* are not published in this edition of Bergey's Manual.

An understanding of the mechanisms by which aeromonads cause gastrointestinal disease has been frustrated by an inconclusive human feeding study (Morgan et al., 1985) and the lack of suitable animal model systems for demonstrating the Koch-Henle postulates (Kelleher and Kirov 2000). Numerous virulence factors and toxins (Gosling 1996a; Howard et al., 1996; Chopra and Houston 1999) have been proposed and examined, but a complete understanding of the multi-functional mechanisms of pathogenesis continues to elude investigators. The first evidence supporting a mouse model for *Aeromonas* virulence studies was published by Sha et al. (2002). Other models include the medicinal leech model (Graf 1999) and the blue gourami model (Yu et al., 2005). None of these models reproduce gastrointestinal disease in humans.

Aeromonads are ubiquitous in the aquatic environment (Holmes et al., 1996). *Aeromonas* spp. cause disease in poikilothermic animals, and occasionally in mammals. Diseases caused by aeromonads represent a significant source of loss to the aquaculture industry. *Aeromonas* spp. have been found in foods (Palumbo 1996), and they have been shown to cause foodborne outbreaks of gastrointestinal disease (Zeng Shan et al., 1988). They have been isolated from treated drinking water (LeChavallier et al., 1983; Burke et al., 1984a; van der Kooij 1988; Fernandez et al., 2000). Drinking water standards for *Aeromonas* are in effect in The Netherlands (van der Kooij 1988), a bottled water standard is in effect in Canada (Warburton et al., 1998; Warburton 2000), and *A. hydrophila* is listed by EPA on the CCL in the U.S. (USEPA 1998). No disease outbreaks associated with treated drinking water have been reported, and the role of drinking water as a source of aeromonads causing gastrointestinal disease in not clearly and convincingly established. Burke et al. (1984a) demonstrated a seasonal association between the presence of aeromonads in a public drinking water supply and the presence of aeromonads in human fecal specimens collected from patients with gastroenteritis; however, the causal relationship of aeromonads to gastroenteritis was not shown.

2.2 Taxonomy

Until the late 1970s, aeromonads were divided into two groups, based upon physiological properties and host range. Motile aeromonads that grew at 35-37º C and were recognized to cause human infections were called *A. hydrophila*. Non-motile aeromonads that grew at 22-28º C and infected fish were called *A. salmonicida*. Besides optimum growth temperature and motility, production of indole, and elaboration of a melanin-like pigment on tyrosine agar provided reliable phenotypic markers for their differentiation.

The taxonomy of aeromonads between 1980 and 1990 was complicated by the fact that few phenotypic markers were available to reliably differentiate species for the newly recognized hybridization groups. Further complication arose from reports of genetically identical strains published under different names by different investigators. DNA hybridization groups (HGs) were recognized for which there were no reliable phenotypic characteristics, resulting in confusion among microbiologists and physicians. Some of the newly recognized aeromonads were described from a handful of strains, making it difficult to describe the species phenotypically. For example, *A. allosacchrophila* was described from three isolates (Martinez-Murcia et al., 1992), *A. encheleia* was described from four isolates (Esteve et al., 1995), and *A. popoffii* was described from eight isolates (Huys et al., 1997).

The current taxonomy of the genus *Aeromonas* is based upon DNA-DNA hybridization and 16S ribosomal DNA relatedness studies. The genera of the family *Aeromonadaceae* now include *Aeromonas*, *Oceanimonas*, *Oceanisphaera*, and *Tolumonas* (*incertae sedis*) (Martin-Carnahan and Joseph 2005). The current genomospecies and phenospecies within the genus *Aeromonas* are shown in Exhibit 2.1. *A. hydrophila* ssp. *dhakensis* (subsp. nov.) (Huys et al., 2002a), *A. hydrophila* ssp. *ranae* (subsp. nov.) (Huys et al., 2003), *A. culicicola* (sp. nov.) (Pidiyar et al., 2002), *A. simiae* (sp. nov.) (Harf-Monteil et al., 2004), and *A. molluscorum* (sp. nov.) (Minana-Galbis et al 2004a) have been proposed as new species and subspecies of *Aeromonas*, and more will undoubtedly be described.

The first attempts to identify aeromonads to genotype relied upon differences in 16S ribosomal DNA sequences (Martinez-Murcia et al., 1992), and several investigators developed probes for detection of various *Aeromonas* spp. (Demarta et al., 1999; Khan et al., 1999). *Aeromonas* taxonomy based upon 16S ribosomal DNA is complex and investigators have examined alternative means to sort out the genotypic maze. Borrell et al. (1997) were able to identify ten species using endonucleases *Alu*I and *Mbo*I, but needed to add *Nar*I and *Hae*III to differentiate *A. salmonicida* from *A. encheleia*, and their system did not distinguish between *A. salmonicida* and *A. bestiarum*, nor identify *A. popoffii*. Figueras et al. (2000) added two additional endonucleases *Nlw*NI and *Pst*I to this restriction fragment length polymorphism (RFLP) method to differentiate *A. salmonicida* and *A. bestiarum* and for recognition of *A. popoffii*. The genetic heterogeneity of aeromonads as the result of cross-over in ribosomal sequences makes it unlikely that 16S ribosomal DNA will be a practical means of differentiation of species, since additional endonucleases must be added as new species are recognized, resulting in an unwieldily and overly complex typing system (Morandi et al., 2005). Genetic crossover or recombination may obscure phylogenetic relationships among aeromonads.

Various other genetic methods are under investigation for species identification. Pidiyar et al. (2003a) sequenced the 16S-23S intergenic spacer regions and found that the resulting phylogeny did not agree with the results of 16S ribosomal DNA and DNA-DNA hybridization studies. The significance of intergenic spacer region analysis is under investigation. The sequence of the *gyrB* gene was used to construct a phylogenetic tree of all 17 hybridization groups (Pidiyar et al., 2003b). *A. culicicola* grouped with *A. veronii*, based upon *gyrB*, but it grouped with *A. jandaei* based upon 16S ribosomal gene sequence. From sequence analysis of the polymerase chain reaction (PCR) amplicon of *gyrB* and the *gyrB* gene, the *gyrB* gene sequence was viewed as a better phylogenetic chronometer than the 16S ribosomal gene. Yanez et al. (2003) reported that the *gyrB* gene agree with the 16S ribosomal data which lead to placement of the genus *Aeromonas* in the family *Aeromonadaceae*, and *gyrB* gene sequences were useful in resolving discrepancies between 16S ribosomal gene sequences and DNA-DNA hybridization results.

Several other methods have been used to characterize *Aeromonas* species. Minana-Galbis

et al. (2004b) examined the genetic diversity between *A. hydrophila, A. bestiarum, A. salmonicida* and *A. popoffii* by multilocus enzyme electrophoresis (MLEE). By MLEE, *A. popoffii* and *A. bestiarum* were closely related. MLEE has been used in genomospecies determination since 1991 (Altwegg et al., 1991b; Figueras et al., 2000). While some authors are enthusiastic about MLEE, few studies have applied the method and none of them have looked at the entire population structure of the genus *Aeromonas*. Multilocus sequence typing (MLST) using the four gene loci of 16S rDNA, *recA*, *chiA* and *gyrB* has revealed the taxonomic limitations of 16S rDNA alone (Carnahan 2001).

The current classification of some species is questionable (Martin-Carnahan and Joseph 2005). *A. ichthiosmia* is a later synomym of *A. veronii*, and the taxonomic status of *A. allosaccharophila* is unclear and deserves further investigation, though a later study supports the status of *A. allosaccharophila* as a separate species based upon *gyrB* gene sequences (Yanez et al., 2003). *A. allosccharophila* and *A. ichthiosmia* belong to *A. veronii* complex. The taxonomic status of *A. enteropelogenes* and *A. trota* is questionable as they share 81-99% relatedness and identical APE E20 and API 50CHE biochemical profiles. While *A. enteropelogenes* has taxonomic status, *A. trota* has been cited more frequently and it should be used preferentially in future publication to avoid ambiguity among ampicillin-susceptible aeromonads (Carnahan et al. 1991a; Huys et al. 2002b).

A. salmonicida contains four subspecies: *salmonicida*, *achromogenes*, *masoucida*, and *smithia*. However, the lack of congruence between phenotypic and genetic data result in great difficulty in accurately assigning isolates to the correct subspecies (Martin-Carnahan and Joseph 2005). *A. salmonicida* has not been shown to cause disease in humans, however *A. hydrophila*-like HG-3 strains that are motile and grow at 37° C have been isolated from clinical specimens on rare occassions (Carnahan and Joseph 1993), and its importance relates to animal diseases in the marine environment and in aquaculture (Austin and Adams, 1996).

Other species of *Aeromonas* have been described, but they have not yet received taxonomic recognition. These species include *A. arequipensis*, *A. dechromatica*, *A. guangheii*, and *A. pastoria* (Martin-Carnahan and Joseph 2005). *Vibrio proteolytica* is improperly published

in the chemical literature as *Aeromonas proteolytica* by investigators unfamiliar with the taxonomy of the *Aeromonadaceae* (Holz 2002).

Exhibit 2.1. Current Genomospecies and Phenospecies of the Genus *Aeromonas*

DNA Hybridization Group (HG)	Type Strain	Genomospecies	Phenospecies	Remarks
1	ATCC 7966	*A. hydrophila*	*A. hydrophila*	Isolated from clinical specimens
1	BCCM/LMG 19562	*A. hydrophila* subsp. *dhakensis*	*A. hydrophila* subsp. *dhakensis*	Isolated from clinical specimens
1	BCCM/LMG 19707	*A. hydrophila* subsp. *ranae*	*A. hydrophila* subsp. *ranae*	Pathogenic for frogs
2	ATCC 14715	*A. bestiarum*	*A. hydrophila*-like	Isolated from clinical specimens
3	ATCC 33658	*A. salmonicida*	*A. salmonicida* subsp. *salmonicida*	Nonmotile fish pathogen
3	ATCC 33659	*A. salmonicida*	*A. salmonicida* subsp. *achromogenes*	Nonmotile fish pathogen
3	ATCC 27013	*A. salmonicida*	*A. salmonicida* subsp. *masoucida*	Nonmotile fish pathogen
3	ATCC 49393	*A. salmonicida*	*A. salmonicida* subsp. *smithia*	Nonmotile fish pathogen
3	CDC 0434-84, Popoff C316	unnamed	*A. hydrophila*-like	Isolated from clinical specimens
4	ATCC 15468	*A. caviae*	*A. caviae*	Isolated from clinical specimens
5A	CDC 0862-83	*A. media*	*A. caviae*-like	Isolated from clinical specimens
5B	CDC 0435-84	*A. media*	*A. media*	
6	ATCC 23309	*A. eucrenophila*	*A. eucrenophila*	
7	CIP 7433, NCMB 12065	*A. sobria*	*A. sobria*	
8X	CDC 0437-84	*A. veronii*	*A. sobria*	
8Y	ATCC 9071	*A. veronii*	*A. veronii* biovar sobria	Isolated from clinical specimens
DNA Hybridization Group (HG)	**Type Strain**	**Genomospecies**	**Phenospecies**	**Remarks**
9	ATCC 49568	*A. jandaei*	*A. jandaei*	Isolated from clinical specimens
10	ATCC 35624	*A. veronii* biovar *veronii*	*A. veronii* biovar veronii	Isolated from clinical specimens, ornatine decarboxylase positive
11	ATCC 35941	unnamed	*Aeromonas* spp. (ornithine positive)	
12	ATCC 43700	*A. schubertii*	*A. schubertii*	Isolated from clinical specimens
13	ATCC 43946	*Aeromonas* Group 501	*A. schubertii*-like	Isolated from clinical specimens

Exhibit 2.1. Current Genomospecies and Phenospecies of the Genus *Aeromonas,* continued

DNA Hybridization Group (HG)	Type Strain	Genomospecies	Phenospecies	Remarks
14	ATCC 49657	*A. trota*	*A. trota*	Isolated from clinical specimens, ampicillin susceptible
15	ATCC 51208, CECT 4199	*A. allosaccharophila*	*A. allosaccharophila*	
16	ATCC 51020, CECT 4342	*A. encheleia*	*A. encheleia*	Pathogenic for eels
17	BCCM/LMG 1754	*A. popoffii*	*A. popoffii*	
Unassigned	MTCC 3249, NCIM 5147	*A. culicicola*	*A. culicicola*	Isolated from mosquitoes

ATCC - American Type Culture Collection, Rockford, MD; BCCM/LGM - Bacteria Collection, Ghent University, Belgium; CDC- Centers for Disease Control and Prevention; CIP - Collection de l'Institut Pasteur, Paris, France; NCMB - National Collection of Marine Bacteria, Aberdeen, Scotland; CECT - Coleccion Espanola de Cultivos Tipo, University of Valencia, Valencia, Spain; CCUG - Culture Collection, University of Gotenborg, Gotenborg, Sweden; MTCC - Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India; NCIM - National Chemical Laboratory, Pune, India
From Martin-Carnahan and Joseph 2005

2.3 Description of the Genus

Aeromonas species are autochthonous (natural inhabitants) to the aqueous environment where they may be pathogenic for poikilotherms (cold-blooded animals). They are found in soil, sediments, and water in freshwater, estuarine, and marine environments. They are present in foods, sewage, and biofilms of drinking water distribution systems. Aeromonads are found in the intestinal tract of animals and humans, with and without evidence of gastrointestinal disease. Some aeromonads are pathogenic for humans, and most human clinical isolates belong to HG-1, HG-4, HG-8, HG-9, HG-10, HG-12 or HG-14 (Janda and Abbott 1998). The proportion of strains within these hybridization groups that are capable of causing human disease is not known. HG-2, HG-3, HG-5, HG-6, HG-7, HG-11, HG-15, HG-16 and HG-17 are isolated from the environment or diseased animals, and they are not considered human pathogens. The aeromonads causing human diseases are associated with a variety of infections including septicemia, wound infections, meningitis, peritonitis, and hepatobilary infections. Some strains of *Aeromonas* produce enterotoxins responsible for causing gastroenteritis in humans; however, isolation of aeromonads from feces does not prove pathogenicity, since these bacteria are widely distributed throughout the environment in water and foods, especially during summer months (Janda and Abbott 1998).

The genus *Aeromonas* consists of straight, coccobacillary to bacillary gram-negative bacteria with rounded ends measuring 0.3-1.0 x 1.0-3.5 Fm (Martin-Carnahan and Joseph 2005). They occur singly, in pairs, and rarely as short chains. Motile strains produce a single polar flagellum, though peritrichous or lateral flagella may be formed on solid media by some species. *Aeromonas* spp. are facultatively anaerobic, catalase positive, oxidase positive, chemoorganotrophic bacteria that exhibit both oxidative and fermentative metabolism on carbohydrates. *Aeromonas* spp. produce a wide variety of extracellular hydrolytic enzymes such as arylamidases, amylase, deoxyribonuclease, esterases, peptidases, elastase, chitinase, and lipase (Carnahan et al., 1988). *Aeromonas* spp. grow optimally within a temperature range between 22-35º C, but growth occurs in a temperature range from 0-45º for some species (Mateos et al., 1993). Some species, including most nonmotile *A. salmonicida* strains, do not grow at 35º C (Martin-Carnahan and Joseph 2005). They tolerate a pH range from 4.5 to 9.0, but the optimum pH range is from 5.5 to 9.0 (Isonhood and Drake 2002), and optimum sodium chloride concentration range is from 0 to 4%.

Serotyping is based upon somatic (O) antigen determinants as described by Sakazaki and Shimada (1984). Several typing schema have been proposed (Fricker 1987; Cheasty et al., 1988; Thomas et al., 1990), but only one comparison study of two of these schema has been published (Shimada and Kosako 1991). The schema of Sakazaki and Shimada recognizes 44 serogroups, with an additional 52 provisional serogroups proposed by Albert et al. (1995). *Aeromonas* spp. are found to be serologically heterogeneous, with individual serogroups found in more than one species (Janda et al., 1996). Most type and reference strains were not serologically representative of a genomospecies. Three serotypes predominate in clinical specimens, O:11 (24%), O:16 (14%), and O:34 (10%). Korbsrisate et al. (2002) characterized the distribution of *A. hydrophila* serogroups in clinical specimens and developed polyclonal antibodies for rapid identification of clinical isolates by direct agglutination. Only 50% of strains fell into the common serogroups O:11, O:16, O:18, O:34, or O:83. Rough strains (15.2%) and untypable strains (2.3%) reduced the effectiveness of serotyping for identification of clinical strains. A polyvalent antiserum was produced that resulted in positive agglutination of 102 or 105 strains, for a calculated sensitivity of 97.1% and specificity of 90.7%. This test could be useful in rapid identification of aeromonads to genus where they are isolated from samples that may also contain vibrios.

Motile aeromonads are generally resistant to penicillin, ampicillin, carbenicillin and ticarcillin. They are typically susceptible to second and third generation cephalosporins, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, trimethoprim-sulfamethoxazole, and quinolones (Koehler and Ashdown 1993; Janda and Abbott 1998). Most aeromonads produce an inducible chromosomal β-lactamase (Walsh et al., 1997). *A. trota* and up to 30% of *A. caviae* are susceptible to ampicillin. Antibiotic resistance to streptomycin, chloramphenicol, tetracycline, cephalexin, cefoxitin, erythromycin, furazolidone, and sulfathiazole is mediated by plasmids (Chaudhury et al., 1996).

The genus *Aeromonas* is differentiated from *Plesiomonas* and *Vibrio* by its resistance to O/129 (150 Fg) and variable presence of ornithine decarboxylase (Martin-Carnahan and Joseph 2005). Other key differential characteristics include its inability to grow in the presence of 6.5% sodium chloride, gelatin liquefaction, inability to ferment i-inositol, and a negative String Test. Additional useful but variable phenotypic characteristics include an inability to grow on thiosulfate citrate bile salts sucrose agar (TCBS), and ability of most but not all *Aeromonas* species to ferment D-mannitol, and sucrose.

Differentiation of species is possible for most species, however the congruity between DNA-DNA hybridization group and phenotype is not complete and some strains remain unnamed. The biochemical identification of *Aeromonas* spp. is shown in Exhibits 2.2 to 2.4.

Exhibit 2.2. Identification of Genomospecies of the *Aeromonas hydrophila* Group

Characteristic	*A. hydrophila* (HG-1)		*A. bestiarum* (HG-2)		Motile, mesophilic *A. salmonicida* subspecies (HG-3)	
	25º C	35º C	25º C	35º C	25º C	35º C
D-rhamnose	-	-	+	+	-	-
D-sorbitol	-	-	-	-	+	+
Lactose	-	d	-	D	d	+
DL-Lactate	d	+	-	-	-	-
Urocanic acid	-	-	+	+	+	+
Elastase	+	d	+	-	+	d
Gluconate oxidation	nd	d	-	-	nd	-
Lysine decarboxylation	+	+	+	-	+	d
Maximum growth temperature	41º C		38-39º C		38-39º C	

HG, hybridization group; +, > 75% of strains positive; d, 26-74% of strains positive; -, < 25% of strains are positive; nd, not determined
From Martin-Carnahan and Joseph 2005

Exhibit 2.3. Identification of Genomospecies of the *Aeromonas caviae* Group

Characteristic	*A. caviae* (HG-4)	Unnamed (HG-5A)	*A. media* (HG-5B)
Lactose	d	+	+
Cellobiose	d	+	+
DL-lactate	+	-	+
Citrate	+	-	-

HG, hybridization group; +, > 75% of strains positive; d, 26-74% of strains positive; -, < 25% of strains are positive
From Martin-Carnahan and Joseph 2005

Exhibit 2.4. Identification of Genomospecies of the Motile *Aeromonas*

Characteristic	*A. hydrophila*	*A. bestarium*	*A. caviae*	*A. media*	*A. eucrenophila*	*A. sobria*	*A. veronii biovar sobria*	*A. jandaei*	*A. veroni biovar veroni*	*A. schubertii*	*A. trota*	*A. allosaccharophila*	*A. encheleia*	*A. popoffii*
Hybridization Group	HG-1	HG-2	HG-4	HG-5	HG-6	HG-7	HG-8	HG-9	HG-10	HG-12	HG-14	HG-15	HG-16	HG-17
Esculin hydrolysis	+	+	+	+	+	-	-	-	+	-	-	d	+	-
Gas from Glucose	+	+	-	-	+	+	+	+	+	-	+	+	+	+
Voges-Proskauer	+	+	-	-	-	+, weak	+	+	+	d	-	-	-	+
Indol	+	+	+	d	+	+	+	+	+	-	+	+	+	d
Pyrazinamidase	+	-	+	d	+	nd	-	-	-	-	-	nd	nd	nd
L-arabinose	d	+	+	+	+	-	-	-	-	-	-	d	-	nd
D-mannitol	+	+	+	+	+	+	+	+	+	-	+	+	+	+
sucrose	+	+	+	+	d	+	+	-	+	-	-	+	+	-
lysine decarboxylase	+	-	-	-	-	+, weak	+	+	+	+	+	+	-	-
ornithine decarboxylase	-	-	-	-	-	-	-	-	+	-	-	d	-	-
arginine dihydrolase	+	+	+	+	+	-	+	+	-	+	+	d	d	+
arbutin hydrolysis	+	+	+	+	+	nd	-	-	+	-	-	nd	-	nd
H_2S production	+	+	-	-	+	nd	+	+	+	-	+	nd	nd	nd
hemolysis	+	+	d	-	-	-	+	+	+	+	+	nd	nd	-
ampicillin, 10 : g	R	R	R	S	S	R	R	R	R	R	S	R	R	R
carbenicillin, 30 : g	R	R	R	nd	nd	S	R	R	R	R	S	nd	R	nd
cephalothin, 30: g	R	R	R	d	S	S	S	S	d	S	R	nd	nd	nd
colistin, 4 : g/mL	d	d	S	S	S	nd	S	R	S	S	S	nd	nd	nd

HG, hybridization group; +, > 75% of strains positive; d, 26-74% of strains positive; -, < 25% of strains are positive; nd, not determined; R, resistant; S, sensitive
From Martin-Carnahan and Joseph 2005

2.4 Methods

Isolation and enumeration of *Aeromonas* spp. from various sources was reviewed by Moyer (1996). Clinical specimens are collected and transported according to recommended clinical laboratory practice (Altwegg 1996). Environmental samples are collected and handled according to the principles outlined in Standard Methods for Examination of Water and Wastewater, 20th edition (APHA 1998). Food samples are collected and handled in accordance

with the procedures contained in the FDA Bacteriological Analytical Manual (USFDA 2001). Clinical specimens and environmental sample processing may require preparation of serial dilutions, and samples may be cultured directly, in dilution, or by membrane filtration, followed by incubation of the filter membrane on culture media.

Broth enrichment methods are frequently used to recover aeromonads from samples where they may be present in low numbers together with larger numbers of other bacteria. von Graevenitz and Bucher (1983) found that use of APW enrichment increased recovery of aeromonads from clinical specimens. APW with or without ampicillin (10 or 30 mg/L) may be used for qualitative detection of aeromonads when using the membrane filtration method for sample processing (Moyer 1996). Use of ampicillin at the higher concentration may inhibit some strains, and *A. trota* is sensitive to both concentrations of ampicillin. Sachan and Agarwal (2000) incorporated cephalothin at 10 mg/L into buffered dextrin broth (BCDB-10) and compared it to alkaline peptone water with cephalothin at 10 mg/L (CAPW-10) for selective enrichment of aeromonads from chicken samples. BCDB-10 performed slightly better than CAPW-10 for naturally infected chickens, while the isolation rate from spiked samples was best for CAPW-10 followed by BCDB-10, and APW. Optimal recovery may require use of more than one enrichment broth, with and without antibiotic to recover strains that may be sensitive to antibiotics.

Aeromonads grow readily on laboratory culture media, and a large number of selective and differential culture media have been developed for recovery of *Aeromonas* spp. from the environment, foods, and clinical specimens. Comparative studies suggest that no single medium results in optimum recovery of aeromonads, and combinations of media and methods are frequently employed by direct plating, membrane filtration, or multiple tube tests for determining most probable number. The membrane filtration method, EPA Method 1605, has been validated for isolation of *Aeromonas hydrophila* from drinking water samples (USEPA 2001). This method employs ampicillin dextrin agar (ADA) which uses 10 mg/L ampicillin and 2 mg/L vancomycin as selective agents. While this medium is capable of isolating aeromonads in addition to *A. hydrophila*, *A. trota* and some strains of *A. caviae* are inhibited by ampicillin and will not be detected by this method.

Several new or modified culture media have been introduced since the review of Moyer (1996). Neyts et al. (2000a) modified bile salts irgasan brilliant green agar for enumeration of *Aeromonas* spp. in foods by increasing pH to 8.7, replacing xylose with soluble starch as the carbon source and decreasing the concentration of bile salts. The resulting mBIBG medium suppressed growth of the *Enterobacteriaceae* except for *Citrobacter freundii*. While *Pseudomonas* spp. grew, they were easily differentiated from *Aeromonas* spp by differences in colony morphology. Quantitative recovery of aeromonads from foods with densities from 2-7 $\log_{10}$ CFU/g was possible in the presence of 5-6 $\log_{10}$ CFU/g of background flora.

Aeromonads have been shown to interfere with methods for detecting and enumerating coliform bacteria. Geissler et al. (2000) used LMX® broth (Merck), Chromocult Coliform® (CC) agar and Chromocult Coliform® agar plus cefsulodin (CC-CFS) with standard methods for multiple-tube fermentation (MTF) enumeration of coliforms and *E. coli* in marine waters. β-galactosidase positive non-coliform bacteria (*Aeromonas* spp. and *Vibrio* spp.) contributed to the numbers obtained for coliform tests when methods are used that include chromogenic and fluorogenic media. False positive tests due to *Aeromonas* spp. occurred with LMX® and CC, since most *Aeromonas* spp. are β-galactosidase positive. Holler et al. (1995) found a high percentage of *Aeromonas* spp. in laurylsulfate-tryptose broth with 4-methylumbelliferyl-β-D-glucuronide (LSTB-MUG) and Ley et al. (1993) observed that *Aeromonas* spp. comprised 76% of β- galactosidase positive colonies isolated on X-Gal. Fricker and Fricker (1996) reported that most of the false positive result for coliforms on LMX® were due to *Aeromonas* spp. Of the media evaluated, CC-CFS was most effective in inhibiting aeromonads that may interfere with coliform tests.

Methods for identification of aeromonads based upon phenotypic characterization of strains using biochemicals resulted in confusion due to extreme phenotypic diversity between and within genomospecies. The schema of Abbott et al. (2003) and Martin-Carnahan and Joseph (2005) make it possible to identify most isolates to named species. Atypical strains are frequently isolated from environmental samples, and accurate identification of these strains is problematic.

While there are more than 17 genomospecies of *Aeromonas,* only 7 are recognized as human pathogens (Carnahan et al., 1991b; Janda and Abbott 1998). Aeromonads associated with human diarrhea include *A. hydrophila, A. caviae, A. veronii* biovar sobria, *A. veronii* biovar veronii, *A. schubertii*, *A. jandaei*, and *A. trota* (Carnahan 1993). To assist clinical laboratories with identification of *Aeromonas* spp., Carnahan et al. (1991b) devised Aerokey II, which provided a reliable and accurate system for identification of most of the currently recognized strains of clinical significance. In a two year evaluation of Aerokey II, it successfully identified 95.3% of clinical strains isolated in 2000 and 98.2% of clinical strains isolated in 2001 (Sinha et al., 2004). *A. caviae* was the predominant species, followed by *A. hydrophila, A. veronii* biovar sobria, *A. trota, A. veronii* biovar veronii, *A. schubertii* and *A. jandaei*. Four strains could not be identified.

In an alternative approach, Carson et al. (2001) developed a phenotypic identification system miniaturized for use in 96 well microtiter plates. A computer assisted identification program was developed based upon this identification system which produced a matrix of substrate reaction probabilities for known aeromonads. Carnahan and Joseph (1991) found that colistin resistance could be used as an additional phenotypic marker for identification of aeromonads. The *A. veronii* group shows 2.5% resistant to colistin with the exception of *A. jandaei*, which is 100% colistin resistant. *A. hydrophila* group is 85.8% resistant, while *A. caviae* group is 2.1% resistant. When colistin was included in a 14 panel test format, 96.2% of strains could be identified to phenospecies and 93.6% of strains could be identified to genomospecies (Fosse et al. 2003a).

Huys et al. (1995) used whole cell fatty acid analysis (FAME) by gas-liquid chromatography to correctly classify 73.5% of water isolates to the correct DNA hybridization group. While this method offers the advantages of an instrumental method with autosampling for unattended operation, the reliability of identifications does not compare to newer genomic methods.

Antigen-antibody methods for identification of *Aeromonas* spp. have not been widely used. Shin et al. (2000) developed a latex agglutination test for the rapid identification of

Aeromonas hydrophila isolates from various sources. Delamare et al. (2002a) developed a monoclonal antibody ELISA test for rapid detection of *Aeromonas* spp. in human feces. The limited response of the monoclonal antibody to other aeromonads causing diarrhea in humans limits the utility of the test in clinical diagnosis of *Aeromonas* spp. associated gastrointestinal disease.

2.4.1 Isolation and Enumeration from Environmental Samples

Isolation of *Aeromonas* spp. from environmental samples provides a challenge because of the presence of competing bacteria and the possibility of sample matrix interference with sample preparation and culture methods. The use of dilution schemes and enrichment media facilitate isolation of aeromonads heavily contaminated samples such as sewage sludge and sewage effluents. Kersters et al. (1996a) compared several culture media for isolation and enumeration of aeromonads from water samples and concluded that ampicillin dextrin agar (ADA) produced the best overall results. Water and food samples are usually processed using a multiple tube technique such as the multiple tube fermentation test (MFT) used for coliform analyses but employing alkaline peptone water for determining the most probable number (MPN). Non-turbid water samples may be processed using the membrane filtration (MF) method, where samples are filtered through a membrane with a pore size of 0.45 Fm, and the membrane is either placed into APW enrichment broth or placed onto the surface of selective and differential culture media such as ampicillin dextrin agar (ADA) and incubated aerobically at 35° C for 24-48 hr. EPA Method 1605 employing MF and ADA-V has been validated for detection and enumeration of *A. hydrophila* from drinking water (USEPA 2001).

The use of a presence-absence screening test is proposed and PA Broth, Nutrient Broth and Buffered Peptone Water were compared for recovery of coliforms, aeromonads, pseudomonads, and *S. aureus* (Warburton 2000). Several plating media were evaluated for detection of target bacteria. A combination of PA broth and buffered peptone water provided best recovery of target organisms when used in conjunction with plating on differential/selective media. Use of the double enrichment method reduces costs associated with individual tests for each regulated indicator organism.

Several culture enrichment and culture media have been evaluated for isolation of aeromomads from foods (Moyer, 1996; Palumbo, 1996). Starch ampicillin agar (SAA) and bile salts inositol brilliant green agar (BIBG) with prior enrichment in APW or tryptose broth containing ampicillin (TSB-30) (ampicillin 30 mg/L) are recommended, together with commercially available media such as *Aeromonas* Medium (Ryan's Medium). Starch glutamate ampicillin penicillin (SGAP-10) medium was used to isolate aeromonads from sewage sludge. This medium is highly selective, and it has been used to isolate aeromonads from foods and other challenging matrices. Samples are prepared in dilution, inoculated into culture media with or without enrichment, and incubated aerobically at 35º C for 24-48 hr. Colonies are screened by performing a spot oxidase test and identified using biochemical methods or commercially-available bacterial identification kits.

Merino et al. (1993c) developed an antibody capture method using microtiter plates and enzyme-linked immunosorbent assay for detection of *A. hydrophila* O:11 in food samples. This assay was highly specific for the target antigen, and other species or serotypes of *A. hydrophila* were not detected.

2.4.2 Isolation from Clinical Specimens

Aeromonas species grow readily in blood culture media and on 5% sheep blood agar used in clinical laboratories for isolation of human pathogens for normally sterile body sites. Isolation of aeromonads from contaminated specimens such as feces require the use of selective and differential plating media such as McConkey agar, cefsulodin irgasan novobiocin (CIN) agar, or blood ampicillin agar (10 mg/L ampicillin). To facilitate recovery of aeromonads from heavily contaminated specimens such as feces, enrichment broths such as alkaline peptone water are incubated overnight and subcultured to blood ampicillin agar and CIN agar. Culture plates are incubated aerobically at 35º C for 24-48 hr. *Aeromonas* spp. produce characteristic colonies, with or without hemolysis on blood agar, and colonies may be quickly screened using the spot oxidase test. Oxidase positive colonies are further screened using tube biochemicals or by inoculation of a cell suspension into one of the commercially available bacterial identification kits.

2.4.3 Culture Identification Methods

Commercial systems for bacterial identification are notoriously inaccurate for identification of *Aeromonas* spp., since they do not incorporate the key substrates necessary for correct identification (Abbott et al., 1998; Vivas et al., 2000; Soler et al., 2003a; Park et al., 2003). Bascomb et al. (1997) found the MicroScan system to be more reliable than its competitors because it relies upon more substrates for bacterial identifications. Contrary to these findings, Soler et al. (2003a) reported that the MicroScan/WalkAway system correctly identified only 14.8% of isolates, while the BBL Crystal Enteric/Nonfermenter system correctly identified 20.3% of isolates originally assigned species by using 16S rRNA gene restriction fragment length polymorphism analysis. Most misidentifications placed *Aeromonas* spp. in the genus *Vibrio*, though one strain of *A. veronii* was identified as *Burkholderia cepacia* by the BBL Crystal system. MicroScan was able to identify *Aeromonas* spp. to group, but was not able to differentiate species within groups. BBL Crystal frequently assigned an incorrect species within the genus *Aeromonas*.

Vivas et al. (2000) compared MicroScan Walk/Away in conjunction with the MicroScan Combo Negative type 1S panels with conventional biochemical methods for identification of 85 environmental, clinical and reference strains of *Aeromonas* spp. Using the MicroScan Combo Negative type1S substrate panel, 67 of 88 (78.8%) of strains were correctly identified, 4 of 88 (4.7%) of strains were incorrectly identified, and 10 of 88 (11.8%) of strains represented rare biotypes that could not be identified.

Differences in the reliability of identifications between Soler et al. (2003a) and Vivas et al. (2000) result from the use of different reference methods, conventional biochemicals in the case of Vivas et al. (2000) and 16S RFLP in the case of Soler et al. (2003b). Neither reference method is 100% accurate in assigning the correct species identification. Incubation temperature appears to alter the physiological activity of aeromonads, complicating the use of phenotypic characteristics for identification and classification of isolates (Merino et al., 1995).

Commercial kits have difficulty in correct identification of *Aeromonas* species because they do not include some of the key substrates required for differentiation of species. Even more problematic is the inaccurate identification of *Aeromonas* species as members of other genera. For example, *Aeromonas veronii* biovar sobria is identified as *Vibrio algiolyticus* by the Vitek system (Park et al., 2003). The API 20NE kit correctly identified this isolate as *A. sobria* (later recognized as *A. veronii* biovar sobria). Other examples of misidentification are shown in Exhibit 2.5.

Exhibit 2.5. Misidentification of Aeromonads by Commercial Kits

Correct Identification	Misidentified As	Commercial Kit
A. caviae	*V. fluvialis*	Not specified
A. hydrophila	*V. alginolyticus*	API 24; Vitek GNI+
A. schubertii	*V. damsela*	Vitek GNI
A. veronii biovar sobria	*V. alginolyticus*	Vitek GNI+
A. veronii biovar sobria	*V. cholerae*	API 20E
A. veronii	*Burkholderia cepacia*	BBL Crystal
A. caviae *A. veronii* *A. media* *A. jandaei* *A. bestiarum* *A. salmonicida*	*V. fluvialis* *V. fluvialis* *A. hydrophila* *V. fluvialis* *A. hydrophila* *V. fluvialis* *A. hydrophila* *A. hydrophila* *V. fluvialis*	MicroScan

From Park et al., 2003; Soler et al., 2003a

Because of the difficulty and expense of identifying *Aeromonas* spp. in clinical laboratories, Janda and Abbott (1998) suggest that it may be sufficient to continue to report aeromonads by group, i.e. *A. hydrophila* group, *A. caviae* group, and *A. veronii* biovar sobria group. This approach would result in misidentification of less than 15% of aeromonads important in clinical medicine and have little impact on patient treatment. By adding a few additional biochemical tests to a basic system, differentiation of *A. jandaei* and *A. schubertii* would be possible, thus covering the clinically relevant species. Epidemiological studies, taxonomic studies, and published reports require identification of isolates to genomospecies.

Abbott et al. (2003) characterized a culture collection of 193 strains representing the 15 species recognized at the time of the study for 63 phenotypic traits. The results of this study were used to design a schema for identification of aeromonads, first to group, then to species within each group. Tests recommended for performance in clinical and reference laboratories are shown in Exhibit 2.6.

Exhibit 2.6. Recommended Tests for Identification of *Aeromonas*

Laboratory Type	Carbohydrate Tests	Tube/Slant Tests	Plate Tests
Clinical Laboratories	L-arabinose D-glucose (gas) glycerol D-mannitol sucrose	esculin citrate gelatin indole Voges-Proskauer	ampicillin susceptibility cephalothin susceptibility
Reference Laboratories (additional tests to those performed in clinical laboratories)	lactose sucrose cellobiose L-rhamnose D-sorbitol D-mannose lactulose glucose-1-phosphate glucose-6-phosphate	gluconate potassium cyanide (KCN) DL-lactate urocanic acid pyrazinamidase (PZA)	elastase stapholysin lipase

From Abbott et al., 2003

By using selected substrates recommended by Abbott et al. (2003), it is possible to identify clinical isolates of *Aeromonas* spp. to one of the three major complexes of significance in human disease, as shown in Exhibit 2.7.

Exhibit 2.7. Biochemical Tests for *Aeromonas* spp. Group Identification (% Positive)

Test	***A. hydrophila* group (*A. hydrophila, A. bestiarum, A. salmonicida*)**	***A. caviae* group (*A. caviae, A. media, A. eucrenophila*)**	***A. sobria* group (*A. veronii, A. jandaei, A. schubertii, A. trota*)**
Esculin	87 (92, 81, 85)	71 (76, 55, 78)	0
Voges-Proskauer	74 (88, 63, 62)	0	54 (88, 87 17, 0)
Glucose (gas)	81 (92, 69, 77)	16 (0, 0, 78)	87 (92, 100, 0, 69)
L-arabinose	93 (84, 100, 100)	96 (100, 100, 78)	4 (12, 0, 0, 0)

From Abbott et al., 2003

The biochemical reactions necessary to separate individual species within complexes are shown in Exhibits 2.8 through 2.10.

Exhibit 2.8. Biochemical Tests for Separation of *A. hydrophila* Group (% Positive)

Test	*A. hydrophila*	*A. bestiarum*	*A. salmonicida*
citrate	92	38	85
DL-lactate	84	0	0
urocanic acid	16	94	100
gluconate	64	13	0
cellobiose	4	38	69
lactose	64	13	92
L-rhamnose	24	69	0
D-sorbitol	0	0	85

From Abbott et al., 2003

Exhibit 2.9. Biochemical Tests for Separation of *A. caviae* Group (% Positive)

Test	*A. caviae*	*A. media*	*A. eucrenophila*
citrate	88	82	0
DL-lactate	96	56	0
urocanic acid	100	100	0
glucose (gas)	0	0	78
pyrazinamidase	88	18	100
glucose-1-phosphate	4	100	100
glucose-6-phosphate	4	100	100
lactulose	68	55	0
D-mannose	32	100	100

From Abbott et al., 2003

Exhibit 2.10. Biochemical Tests for Separation of *A. sobria* Group (% Positive)

Test	*A. veronii*	*A. jandaei*	*A. schubertii*	*A. trota*
indole	100	100	17	100
Voges-Proskauer	92	87	17	0
lipase (corn oil)	92	100	100	0
D-glucose (gas)	92	100	0	69
cellobiose	20	20	0	100
glycerol	100	100	0	94
D-mannitol	100	100	0	69
sucrose	100	0	0	19
ampicillin resistence (10 : g/mL)	100	93	92	6

From Abbott et al., 2003

2.4.4 Molecular Detection Methods

Direct detection of aeromonads by polymerase chain reaction (PCR) or use of genetic probes has advanced rapidly in the past decade. PCR methods have been developed for detection of *Aeromonas* spp. in a variety of matrices as shown in Exhibit 2.11.

Exhibit 2.11. PCR Methods for Detection of *Aeromonas* species

Organism	Matrix	Type PCR	Target Gene	Detection Method	Detection Limit	Reference
A. hydrophila	raw milk	semi-nested multiplex	*aerA*	gel	2 $\log_{10}$ CFU/mL	Ozbas et al., 2000
A. hydrophila	marine water	multiplex	*aerA*	gel	2 $\log_{10}$ CFU	Kong et al., 2002
A. hydrophila	water	immuno-capture	16S ribosome	gel	10 CFU	Peng et al., 2002
A. trota					10-15 CFU	Khan et al., 1999
A. caviae	flies	culture enrichment	16S ribosome	gel	ND	Nayduch et al., 2001
A. hydrophila	fish	culture enrichment	*aerA* and *hlyA*	gel	1-10 CFU/g	Gonzalez-Rodriguez et al., 2002

Exhibit 2.11. PCR Methods for Detection of *Aeromonas* species, continued

Organism	Matrix	Type PCR	Target Gene	Detection Method	Detection Limit	Reference
A. hydrophila	feces	duplex real-time	aerA	SYBR green	4-5 log10 without enrichment	Fukushima et al., 2003
A. hydrophila	various	universal primer	16S ribosome	denaturing gradient gel electro-phoresis	ND	Ji et al., 2004

PCR methods may be more sensitive than culture for complex matrices with dense background flora that interferes with culture detection, and for organisms for which culture methods are inadequate or unavailable. Ozbas et al. (2000) developed a PCR method for detection of *A. hydrophila* in raw milk. The detection limit was 2 $\log_{10}$ CFU/g and the detection rate was 23% for PCR and 14% for culture.

The use of selective agents, particularly antibiotics, in culture media may inhibit some strains of the target bacteria. Kannan et al. (2001) developed a PCR method for direct detection in feces targeting the aerolysin toxin gene (*aer-1* and *aer-2* primer pairs). The sensitivity was 100% and specificity was 98%, but the detection limit not given, though it compared favorably with culture. This method detected *A. trota* strains that are missed using culture media containing ampicillin.

Nucleic acid amplification methods targeting virulence genes are used for detection of pathogenic bacteria and to differentiate pathogenic from non-pathogenic strains (Kingombe et al., 1999; Chacon et al., 2003; Sen and Rodgers 2004). Wang et al. (2003) developed a multiplex PCR method for detection of hemolysin and aerolysin genes in *A. hydrophila* and *A. sobria*, and used it to screen 121 clinical and 7 reference strains for *ahh1*, *asa1*, and *aerA* genes. Five genotypes were identified, Type 1 contained only the *ahh1* gene, Type 2 contained only the *asa1* gene, Type 3 contained both *ahh1* and *asa1* genes, Type 4 contained *ahh1* and *aerA* gene, and Type 5 containing no hemolysin genes. Phenotypic expression of toxins was evaluated in Vero cell culture cytotoxicity assay and the presence and number of hemolysin genes corresponded

with cytotoxin titers. The range of virulence of aeromonads is thought to result from the variety of genotypes present in the environment. Both phenotypic and genotypic heterogeneity are common among aeromonads.

Stine et al. (2003) constructed microarrays of DNA probes to study the population dynamics of microbial communities, and used one such microarray to study population interactions of marine bacteria in coastal waters where aeromonads make up a significant percentage of the microbial flora. Microarray technology provides a powerful tool for studying bacterial populations in biofilms. In a novel application of microarray technology, Galindo et al. (2003) and Galindo et al. (2004a) used microarrays to identify *A. hydrophila* cytotoxic enterotoxin-inducing genes in macrophages, thus demonstrating the potential of microarrays in elucidating intracellular mechanisms of pathogenesis. Microarrays and proteomics have also been used to examine the effects of cytotoxic enterotoxin on human epithelial cells (Galindo et al. 2005).

In another novel application of probe technology, Asfie et al. (2003) used fluorescent *in situ* hybridization (FISH) to characterize the fecal microflora of goldfish. Tetramethylrhodamine labeled rRNA targeted oligonucleotides were used as group specific probes (AER66 for *Aeromonas*). *Aeromonas* spp. made up 18.9% to 34.5% of total cell density in fecal samples, at counts from $2.5x10^{8}$ to $2.3x10^{10}$ cells/g.

No single phenotypic or genotypic identification system has been found that will reliably classify all strains of *Aeromonas* spp. Ciapini et al. (2002) used phenotyping by Biolog system and amplified 16S rDNA restriction analysis (ARDRA) to identify aeromonads. Discrepancies were noted between biolog results and ARDRA. Chromosomal DNA restriction analysis results did not agree with ARDRA profiles. These data suggest the need for a polyphasic approach to defining the taxonomic status of species in the genus *Aeromonas*. Phenotypic (biochemicals), phylogenic (16S rDNA sequencing) and genomic DNA-DNA hybridization) data are required for accurate classification. New identification systems such as MLST (Stackebrandt et al. 2002) and matrix-assisted laser desorption/ionization mass spectormetry (MALDI-MS) (Donohue et al. 2005) offer promise of rapid and specific species identification.

2.4.5 Typing Methods

Typing methods are used in a variety of ways:

- Taxonomic studies of genetic relatedness
- Analysis of microbial populations in complex matrices
- Analysis of genetic relationships between clinical and environmental strains of the same species

Attempts to epidemiologically relate clinical and environmental isolates required typing methods such as isoenzyme analysis or molecular genetic methods such as rDNA restriction patterns (ribotyping) (Moyer et al., 1992a), restriction fragment length polymorphism analysis (RFLP) (Borrell et al., 1997), pulsed-field gel electrophoresis (PFGE) (Borchardt et al., 2003), or polymerase chain reaction (PCR) (Khan and Cerniglia 1997).

The marked heterogeneity and incongruity between phenotypic and genomic identification of aeromonads lead to the application of various typing methods for epidemiological investigations. Altwegg (1996) reviewed typing systems and concluded that phenotypic methods such as biotyping, phage typing, serotyping, antibiograms, and protein typing did not provide sufficient discrimination among strains to be useful. Among the genotyping methods, plasmid analysis was equally useless. Restriction enzyme analysis methods such as ribotyping and pulsed-field gel electrophoresis provided discrimination at the strain level, and ribotyping results had both epidemiological significance and sufficient taxonomic value to permit assignment of strains to DNA-DNA hybridization groups. The recent widespread application of PFGE in microbiology laboratories lead to recognition that genomic DNA of aeromonads may be characterized by PFGE (Garcia et al., 2000), and PFGE is replacing ribotyping as the preferred method for genomic typing of aeromonads (Bonadonna et al., 2002).

Considerable progress in typing methods has been made since the review of Altwegg (1996). Minana-Galbis et al. (2002) was able to identify 91% of clinical and environmental isolates from Spain in a numerical taxonomy schema using 16 phenotypic tests. By introducing

non-traditional substrates, Borrell et al. (1998) reported that phenotypic identification was successful to the genomospecies level for 93% of 983 environmental and clinical strains of *Aeromonas*. Citrate and sorbitol enabled separation of the *A. hydrophila* complex. Of human isolates, *A. veronii* biovar sobria and *A. caviae* were most common intestinal aeromonads, while *A. hydrophila* isolates were from extraintestinal sources. *A. veronii* biovar sobria was found in lakes, reservoirs, and treated drinking water, and *A. caviae* was common in seawater and milk products. *A. hydrophila* was second most common freshwater isolate. The tests used were based upon publications by Altwegg et al. (1990) and Abbott et al. (1992). All substrates were incubated at 36º " 1º C except sorbitol and citrate, which were incubated at 30º C according to Altwegg et al. (1990). Some strains (7% in this study) cannot be identified using phenotypic characteristics. These findings are similar to those reported by Hanninen and Siitonen (1995). Molecular methods are still necessary for clarification of the taxonomic standing of unknown strains.

Several investigators are using restriction fragment length polymorphism of the intergenic spacer region between the 16S and 23S rRNA gene for genotyping aeromonads in investigations of water treatment plant colonization and foodborne outbreaks (Martinez-Murcia et al., 2000; Solar et al., 2003b). A comparison of three genomic typing methods, RFLP of the 16S-23S intergenic spacer region (ISR), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR), and repetitive extragenic palindromic (REP-PCR) on 26 isolates of *A. popoffii* from different geographical regions suggested that all three methods were required to obtain strong geographical discrimination among aeromonads (Solar et al., 2003b).

Szczuka and Kaznowksi (2004) analyzed 120 isolated from water and human stools from patients with gastroenteritis by random amplified polymorphic DNA PCR (RAPD), REP-PCR, and ERIC-PCR. No determinant clone was associated with human diarrhea. There was no genetic similarity between clinical and environmental strains isolated from the same or different geographical areas. Some clones colonized a specific ecosystem, e.g. drinking water. RAPD and ERIC-PCR had the same discriminatory power and provided the same utility for epidemiological investigations and population genetic analysis, while REP-PCR was less effective for differentiating aeromonads. These techniques were used to show that the hospital environment was not the source of aeromonads in hospitalized children with diarrhea since no clonal

relationship could be demonstrated between stool and environmental isolates. Aeromonads exhibit extreme genetic diversity, and it is difficult to link clinical and environmental isolates epidemiologically using typing methods. Borchardt et al. (2003) compared clinical and well water isolates by PFGE and found them to be genetically unrelated, suggesting that gastrointestinal infection were not linked with groundwater exposure.

Alavandi et al. (2001) used RAPD-PCR and SDS-PAGE whole cell profiles to examine the relationship between *Aeromonas* isolates from children with diarrhea and their water sources. Water isolates clustered in two major groups while clinical isolates were scattered across the dendogram. RAPD-PCR could distinguish environmental from clinical isolates but it could not distinguish phenospecies or genomospecies of aeromonads. Whole cell protein fingerprinting and cluster analysis could neither differentiate isolated from clinical or environmental sources or differentiate phenospecies of the genus *Aeromonas*.

Delamare et al. (2002b) used SDS-PAGE showed promise for species differentiation but lacked discrimination to differentiate strains within a species, while RAPD offered strain differentiation but was not useful in species identification. These results differ from those of Alavandi et al. (2001) where SDS-PAGE analysis could not differentiate phenospecies of *Aeromonas*.

Another promising method was described by Donohue et al. (2005), consisting of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). This method is based upon protein fingerprinting using whole cell preparations.

2.5 Summary

Aeromonas spp. are oxidase-positive, gram-negative bacteria that are widely distributed in the aquatic environment. Aeromonads have been isolated from diseased animals and fish for over 100 years, but they have been recognized as human pathogens only in past 50 years, and there is still controversy concerning their relationship to enterotoxin production and resulting gastrointestinal disease (Albert et al 2000). Aeromonads may produce a variety of extracellular

products that confer virulence in some strains. The taxonomy of the genus *Aeromonas* was codified with publication of the Second Edition of Bergey's Manual of Systematic Bacteriology (Martin-Carnahan and Joseph 2005), however some recognized species have not been named and new species continue to be described. No universal selective/differential medium has been developed for isolation of aeromonads (Merino et al., 1995). The available media used for isolation of aeromonads suffer from insufficient differentiation and selectivity (allowing overgrowth and differential ambiguity) or over-selectivity (suppression of ampicillin-sensitive strains). EPA Method 1605 was validated recently for detection and enumeration of *A. hydrophila* from drinking water. Molecular systems such as PCR have been developed for detection of aeromonads, however unless culture enrichment methods precede PCR amplification, the detection limit is too low for practical use with environmental samples. While ribotyping, PFGE, or other typing methods are useful in epidemiological investigations, the results must be interpreted cautiously because of the extreme biological diversity inherent among aeromonads.

3.0 Occurrence

Mesophilic, motile aeromonads are ubiquitous (everywhere) and autochthonous (naturally occurring) in the aquatic environment. They inhabit freshwater, esturine waters, and wastewater, and have been found in chlorinated and unchlorinated drinking water (Havelaar et al., 1992). They are also found in soil and the feces of aquatic and terrestrial animals and humans. They occur in fish and other seafoods, vegetables, raw milk, and processed food products. *Aeromonas* spp. occur in the human gastrointestinal tract both in the presence and absence of disease, but the presence of *Aeromonas* spp. in other body sites is usually associated with infection and disease. Aeromonads cause disease in poikilothermophiles (cold blooded animals) such as frogs, eels, and fish, where they are an economic liability to the aquaculture industry. Aeromonads have been found in association with marine copepods and plankton, where they are present at cell densities from 4 CFU/mL to 1.3×10^{3} CFU/100mL in seawater and from 1.5×10^{1} CFU/100mL to 6×10^{2} CFU/100mL on plankton.

3.1 Worldwide Occurrence

Aeromonas spp. are found worldwide in surface water, ground water, non-chlorinated drinking water, chlorinated drinking water, and bottled mineral water (Holmes et al., 1996). Aeromonads are found in a wide variety of foods (Palumbo, 1996). They are found in the intestinal tract of humans and animals, raw sewage, sewage effluents, activated sludge, and sewage-contaminated waters (Holmes et al., 1996).

Aeromonads reach population densities of 10^6-10^8 CFU/mL in raw sewage and 10^3-10^5 CFU/mL remain in sewage effluents after treatment (Holmes et al., 1996). Their occurrence in the environment is not dependent upon fecal pollution; however, they reach higher numbers in nutrient-rich waters contaminated by sewage. They may reach 3-5 $\log_{10}$ CFU/mL in surface waters during summer months. They are not common in groundwater, though they may colonize poorly constructed wells.

3.1.1 Occurrence in Human Population

Humans carry *Aeromonas* spp. in their gastrointestinal tract both in the presence and absence of disease. The rates of fecal carriage in asymptomatic persons in developed countries range from 0% to 4.0% (Millership et al., 1983; Agger et al., 1985; Quan et al., 1986; Svenungsson et al., 2000), while the isolation rate from persons with diarrheal illness ranges from 0.8 to 7.4% (Agger et al., 1985; Moyer 1987; Albert et al. 2000). In Southeast Asia, asymptomatic carriage rates as high as 27.5% and recovery rates from patients with diarrhea as high as 34% have been reported (Pazzaglia et al., 1990). Among western Peace Corp workers in Thailand, aeromonads were recovered from 8.5% of healthy persons and 30.8% of persons with diarrhea (Echeverria et al., 1981).

Recovery rates among children with diarrhea vary geographically: 0.62 to 4% in Malaysia (Lee and Puthucheary 2001; Lee and Puthucheary 2002), 0.75% in Nigeria (Kehinde et al., 2001), 2% in Sweden (Svenungsson et al., 2000), 2.3% in Taiwan (Juan et al., 2000), 4.8% in Switzerland (Essers et al., 2000), and 6.8% in Greece (Maltezou et al., 2001). Sinha et al. (2004)

reported aeromonads in 6.5% of all patients in India, and Chan et al. (2003) reported aeromonads in 6.9% of adult patients with acute diarrhea in Hong Kong. Other investigators report isolation rates from symptomatic patients from 0.04% to 21% (Kuijper and Peeters 1991; Dumontet et al., 2003; Maraki et al., 2003). Pazzaglia et al. (1990) reported that 23.1% of newborns in Peru demonstrated transitory gastrointestinal colonization with *Aeromonas* spp. during the first days of life. The isolation rates for human fecal specimens vary widely, as geographical areas, patient populations, food habits, level of sanitation, and culture methods influence the recovery rates (Dumontet et al., 2003).

Saad et al. (1995) observed that the frequency of recovery of *Aeromonas* spp. from stools corresponded to the warm summer months when *Aeromonas* growth reached their maximum and postulated that fresh vegetables may be a source. No corresponding increase in the number of aeromonads in water was evident, suggesting food as the primary contributor to human carriage of *Aeromonas* spp. The relationship between presence of *Aeromonas* spp. in human fecal specimens and clinical manifestations of disease continues to challenge epidemiologists.

Kannan et al. (2001) reported that *Aeromonas* spp. isolated from clinical specimens in India include *A. hydrophila* (59.3%), *A. caviae* (18.7%), *A. veronii* (10.9%), *A. schubertii* (4.6%), *A. jandaei* (3.1%), and *A. trota* (3.1%). Fecal carriage rates of 6.6% of symptomatic 10-year olds have been reported (Komathi et al., 1998). Seventeen of 2,565 stool samples (0.66%) were positive for *Aeromonas* spp. (Borchardt et al., 2003). Agger et al. (1985) reported *Aeromonas* spp. in 1.1% of stools in Wisconsin and Moyer (1987) reported a fecal isolation rate of 7.1% in Iowa. The choice of culture methods especially the use of alkaline peptone water or other enrichment methods prior to plating fecal samples markedly affects the detection rate both within and between patient populations.

The clinical significance of isolates in these surveys is not clear, even when all patients in the surveyed population had diarrhea. Enteropathogenicity is influenced by growth temperature, where strains of O:34 grown at 20º exhibit enhanced virulence over strains grown at 37º C (Merino et al., 1992). Strains isolated at 35-37º C, the typical incubation temperature used in clinical laboratories may produce false negative tests for virulence factors, making the

retrospective assessment of clinical significance impossible.

3.1.2 Occurrence in Animal Population

Aeromonas spp. have been recognized as animal pathogens since they were first isolated from diseased frogs and fish (Gosling 1996b). They are now recognized to cause disease in birds and domestic animals (Gray 1984; Shane and Gifford 1985).

Fish

A. salmonicida and *A. hydrophila* cause hemorrhagic disease, ulcerative disease, furunculosis, red sore disease and septicemia in fish (Austin and Adams 1996). Lehane and Rawlin (2000) reviewed zoonoses acquired from fish and reported that aeromonads caused cellulitis, myositis, and septicemia following injuries from handling fish, working in aquaculture, or keeping fish as pets.

Reptiles and Amphibians

A. hydrophila has been isolated from diseased turtles, alligators, snakes, and frogs (Gosling 1996b).

Birds

Aeromonads have been isolated from feces of wild and pet birds. Aeromonads may cause septicemia in poultry (Saif and Busch 1974). Okrend et al., 1987 demonstrated that chicken meat is contaminated with *Aeromonas* spp., and Hudson and De Lacy (1991) showed that 3 of 36 ready to eat products involving chicken meat contained *Aeromonas* spp.

Domestic Livestock

Gray (1984) isolated *A. hydrophila* from feces of normal horses (7 of 110, 6.4%), pigs (11

of 115, 9.6%), sheep (10 of 111, 9.0%), and cows (26 of 123, 21.1%). Most positive cultures were recovered only after enrichment in alkaline peptone water. The total fecal carriage rate in animals is slightly higher than the fecal carriage rate of normal humans, which is < 1 to 7% for most studies, although some studies report higher rates (Pitaragnsi et al., 1982). Populations in animals probably reflect the presence of aeromonads in their feed and water.

In their study of *Aeromonas* spp. in the feces of domestic animals, Figura and Marri (1985) isolated *A. hydrophila* more frequently than *A. caviae*. They did not detect *A. sobria* in tongue swabs or cecal contents of pigs. Stern et al. (1987) isolated aeromonads from 1 of 32 cows and 3 of 21 turkeys, but none were isolated from 22 pigs or 24 sheep. Gray and Stickler (1989) reported finding predominantly *A. hydrophila* in cow feces and *A. caviae* in pig feces. Diet and water sources influenced recovery of aeromonads from feces of domestic animals. *Aeromonas* spp. have been isolated from feces, bedding, and drinking water of health cows and pigs (Hathcock et al., 1999). They survive in soil for months (Brandi et al., 1996). Both healthy and diseased animals shed aeromonads in feces (Hathcock et al., 1999).

Household Pets

Ghenghesh et al. (1999a) isolated *Aeromonas* spp. from rectal swabs of 13 of 120 dogs and 1 of 15 cats in Libya by using alkaline peptone water (pH 8.6) enrichment and blood agar containing ampicillin at a concentration of 15 mg/L. Nine of 14 isolates were examined for hemolytic activity using human erythrocytes, and 6 strains were found positive. These qualitative studies provide no estimate of the number of aeromonads shed in the feces of dogs and cats, and therefore the public health significance of these findings is unknown.

Feral Animals

Apart from fish and amphibians, little is known of the occurrence of *Aeromonas* disease in feral animals. *Aeromonas* may be an opportunistic pathogen of stressed animals (Gosling, 1996b).

Invertebrates

A. caviae has been cultured from common houseflies captured on two farms and in a restaurant in South Carolina (Nayduch et al., 2001). APW enrichment was followed by PCR detection (APW-PCR). *A. caviae* was found in 272 of 349 (78%) of dairy farm flies, 54 of 99 (55%) of pig farm flies, and 77 of 200 (39%) restaurant flies. Feces from cows (58%) and pigs (100%) on the farms were positive for *A. caviae*. Nayduch et al. (2002) proposed that houseflies could serve as vectors for transmission of *Aeromonas* spp. since the bacteria multiplied in the gut and persisted for several days. Fly to fly transmission was demonstrated and transmission of *A. caviae* from fly to food was observed. Though *A. culicicola* has been isolated from mosquitoes, there is no epidemiological evidence of transmission to humans (Pidiyar et al. 2002).

Gu and Mitchell (2002) isolated *A. media, A. veronii, A. salmonicida*, and other bacteria from zebra mussels. *A. media* and *A. salmonicida* were shown to be pathogenic for zebra mussels, with high morbidity in 5 days when challenged with an inoculum of 7 $\log_{10}$ CFU per mussel. Pathogenicity of *A. media* was not temperature dependent, though longer incubation times were required to produce a lethal effect. Mussels harbor bacterial populations as high as 5 $\log_{10}$ CFU per mussel and pseudomonads are the predominate flora of healthy mussels. The microflora of mussels may pose a public health concern, especially when aeromonads are the predominant component of the microflora of mussels fouling drinking water intakes and pipes (Austin and Adams 1996; Gosling 1996b; Palumbo 1996). The use of aeromonads for biological control of zebra mussels warrants further investigation.

Aeromonads have been found in association with marine copepods and plankton, where they are present at cell densities from 4 to 1.3×10^{3} CFU/100mL in seawater and from 1.5×10^{1} CFU/100mL to 6×10^{2} CFU/100mL in plankton (Dumontet et al 1996; 2000).

The use of medicinal leeches to treat vascular infiltration in surgical wounds has been recognized as a risk factor for *Aeromonas* infections since 1983, and there are numerous reports of cellulitis and septicemia resulting from leech therapy (Mercer et al. 1987; Snower et al. 1987). The symbiotic association between *A. veronii* biovar sobria and medicinal leeches has been

reported by Graf (1999), who proposed medicinal leeches as a model for studying pathogenesis of *Aeromonas* infection.

3.1.3 Occurrence in Water

Surface Waters

Maalej et al. (2002) studied the seasonal occurrence of aeromonads in urban effluents and the costal marine environment. In urban sewage effluents, presence of aeromonads exhibited a seasonal cyclic distribution similar to fecal coliforms, with the highest numbers (29×10^6 CFU/100mL) in winter months and the lowest levels in summer months. In coastal waters, aeromonads reached highest levels (56 CFU/100mL) in summer months. Lowest levels of aeromonads occurred under conditions of maximal solar irradiation and minimum turbidity. The lack of correlation between fecal indicator bacteria and aeromonads suggests that the former group of organisms is not predictive of the presence of aeromonads in ambient waters.

Bonadonna et al. (2002) studied the occurrence of bacteria of anthropomorphic (human) origin and those of autochthonous (natural) origin using model systems for prediction of public health risk to marine bathers. The resulting model used salinity, total coliforms, fecal coliforms, *E. coli*, and location as predictive variables for presence of aeromonads. Presence of *E. coli* and fecal coliforms were associated with lower *Aeromonas* counts, while predominance of total coliform was associated with higher *Aeromonas* counts. Fecal coliforms and increased salinity was associated with higher *Aeromonas* counts. *Aeromonas* counts ranged from < 100 CFU/100mL to $> 10^5$ CFU/100mL. The complexity of the association between anthropomorphic and autochthonous bacteria confounds development of a predictive model for estimating public health risk of recreational exposure to marine waters.

Maalej et al. (2003) studied seasonal dynamics on *Aeromonas* populations in urban effluent and natural seawater. Effluents contained 1.48×10^5 CFU/100mL to 2.2×10^8 CFU/100mL, while seawater densities were from below the detection limit to 7.9×10^3 CFU/100mL. The seasonal density of aeromonas was inversely related to the seasonal density of fecal coliforms.

During cold months, aeromonads increased in effluent and decreased in seawater, while during warm months, aeromonads decreased in effluent and increased in seawater. Inactivation of aeromonads in seawater is thought to result from lower water turbidity with increased solar penetration and lower levels of available organic nutrients compared to urban effluents.

Ivanova et al. (2001) isolated *A. veronii* biovar sobria and *A. popoffii* from surface drinking water reservoir in Russia in the absence of apparent sources of fecal pollution. *A. popoffii* was also described by Huys et al. (1997) and it is common in freshwater and seawater (Soler et al., 2002). *A. popoffii* has been isolated from humans only once, though strains may produce virulence factors such as aerolysin, serine protease, DNAse, and lipase (Hua et al 2004).

Ground Waters

Anoxic groundwater may support growth of aeromonads, and aeration has proven successful in reducing the organic nutrients that supported growth.

Massa et al. (2001) studied occurrence of aeromonads in natural mineral water and well water in Italy. Aeromonads were not detected in the 60 natural mineral waters examined. Aeromonads were found in 5 of 20 well samples with counts ranging from 26-1,609 CFU/250 mL. Presence of aeromonads did not correlate with fecal indicators in 2 of 5 wells where they were detected. Putative virulence factors were demonstrated in well water isolates. The clinical and epidemiological significance of these findings is unknown.

Villari et al. (2003) examined 103 isolates obtained over 3 years from natural mineral water and surface streams within the watershed of the wells from which the mineral water samples were collected. Evidence of clonal identity was found in the *A. caviae* isolates and among *A. hydrophila* strains in the mineral water samples using PFGE. Aeromonads from surface waters did not show clonal identity. Biofilm was thought to be responsible for the clonal nature of well water isolates.

Drinking Water

Aeromonas spp. have been isolated from chlorinated drinking water supplies in several countries (Hazen et al., 1978; Burke et al., 1984a; van der Kooij 1988; Fernandez et al., 2000; Figueras et al., 2005). Aeromonads are typically below 10 CFU/mL in drinking water distribution systems. Aeromonads grow in distribution systems (Havelaar et al., 1990; van der Kooij 1991). They occur in distribution system biofilms where they may be protected from disinfection (van der Kooij et al., 1995; Holmes and Nicolls 1995). A drinking water standard of 200 CFU/100 mL at 25° C has been established in the Netherlands (Havelaar et al 1990). Multiple strains are frequently found in water sources (Kuhn et al., 1997; Sen and Rodgers 2004).

Gavriel et al. (1998) studied a drinking water distribution system in Scotland for the presence of *Aeromonas* spp. and their relationship to chlorine concentration, pH, temperature, and rainfall. They isolated aeromonads from chlorinated reservoirs and suggested a relationship between rainfall and increased recovery, probably due to increased organic load caused by formation of chloramines.

The presence of aeromonads in distribution system water indicates neither fecal pollution nor treatment failure; however, a large number of aeromonads present in distribution water suggests that water conditions support growth. Aeromonads in biofilm may resist disinfection.

Aeromonas spp. have been found at a frequency of 1-27% of drinking water supplies examined (Rusin et al., 1997). They are found in all aqueous environments except thermal springs, hypersaline lakes, and extremely polluted waters (Janda and Abbott 1999**.** Aeromonads have been isolated from ground pork, chicken, beef, milk, produce, shellfish, fish, and fish eggs. Most of the foodborne outbreaks attributed to *Aeromonas* spp. resulted from ingestion of fish or shellfish. Because of the prevalence of aeromonads in foods, water appears to be an incidental source of colonization of the human gastrointestinal tract. The only incidence data reported for drinking water exposures show a predicted lifetime exposure case rate of 0.07 cases per 100,000 population (Rusin et al., 1997).

A. popoffii has been isolated from drinking water (Huys et al., 1997) and *A. culicicola* was isolated from drinking water by Figueras et al. (2005). The longer survival rate of *A. caviae* compared to *A. hydrophila* may explain its frequent isolation from drinking water (Hanninen and Siitonen 1995) and its high concentrations in treated sewage (Holmes et al., 1996).

Simmons et al. (2001) studied aeromonads in 125 roof-collected rainwater systems in New Zealand and found that 22 of them exceeded regulatory levels of indicator bacteria. *Aeromonas* spp. were isolated from 20 of 125 (16%) supplies and there was a positive association between presence of aeromonads and presence of fecal indicators. Households reporting gastrointestinal illness were more likely to have aeromonads in their water.

Aeromonas spp. were isolated form 11.4% of water samples supplied to a slaughterhouse and 25.7% of water samples drained from carcasses (Bizani and Brandelli 2001). Water was shown to contaminate meats and may be a factor in food processing.

Bottled Water

Aeromonas spp. have been cultured from mineral water by Gonzales et al. (1987), Quevedo-Sarmiento et al. (1986), Slade et al. (1986), Manaia et al. (1990), Havelaar et al. (1990), Warburton et al. (1998), and Tsai and Yu (1997). Isolations rates as high as 35.5% and cell concentrations greater than 3 $\log_{10}$ CFU/mL have been reported.

Croci et al. (2001) studied the behavior of aeromonads in bottled mineral waters under various conditions of temperature and nutrient levels. They demonstrated growth in polyethylene bottles at 10º C reaching peak cell densities of 4.47 $\log_{10}$ CFU/100mL in 28 days. It took 60 days to reach this cell density at 20º C.

Bottled water standards in Canada require testing for heterotrophic plate count (HPC), most probable number (MPN) for coliforms and *E. coli,* and membrane filtration (MF) for *Aeromonas* and *Pseudomonas*. Warburton (2000) proposed using enrichment methods to reduce the cost associated with testing bottled water. In the U.S., bottled water is classified as a food and

therefore it is under FDA jurisdiction. No regulatory standards exist, although industry guidelines have been proposed. Of 3,460 samples tested, 0.6% contained aeromonads, 1.2% contained pseudomonads, 3.7% contained total coliforms, and 2.1% contained fecal coliforms.

Wastewaters

Aeromonads are widespread in wastewater treatment processes (Poffe and Op de Beeck 1991; Kampfer et al., 1996). *Aeromonas* spp. were not detected in aerosols generated by wastewater treatment plants (Brandi et al., 2000). Nsabimana et al. (2000) studied microbial populations in activated sludge over time using phenotyping and ribotyping. The initial variety of hybridization groups was reduced and replaced by dominant hybridization groups. Over the course of a year, *Aeromonas* populations shifted as shown in Exhibit 3.1.

Exhibit 3.1. *Aeromonas* Population Shift in Activated Sludge

Initial Populations		Altered Populations	
A. caviae group	54.5%	*A. enucrenophila*	47.1%
A. hydrophila group	22.7%	*A. caviae* group	41.2%
A. sobria group	18.2%	*A. hydrophila* group	5.4%
A. eucrenophila	4.5%	*A. sobria* group	5.8%

From Nsabimana et al., 2000

Albrechtsen (2002) evaluated microbial quality of rainwater and graywater systems used for flushing toilets in Denmark. Rainwater systems introduced bacteria not found in toilets flushed with treated water. Graywater was associated with odor and high coliform counts. *Aeromonas* counts in stored rainwater were < 10 to 30 CFU/mL. Toilet bowls flushed with rainwater contained < 10 to 4,400 CFU/mL, while toilet bowls flushed with treated water contained 10 to 8,800 CFU/mL.

Irrigation Waters

Irrigation water contains aeromonads at concentrations between 2 to 4 $\log_{10}$ CFU/mL (Pianetti et al., 2004). Irrigation waters may contaminate fruits and vegetables.

Marine and Estuarine Waters

Several investigators have reported the presence of *A. hydrophila* in seawater (Echeverria et al., 1983; Brandi et al., 1996). Additionally, *A. caviae* has been cultured from seawater at densities of 2 $\log_{10}$ CFU/mL, whereas in fecally contaminated water, wastewater, and sewage, *A. caviae* may reach densities of 7-8 $\log_{10}$ CFU/mL.

3.1.4 Occurrence in Food

Isonhood and Drake (2002) reviewed *Aeromonas* spp. in foods. While aeromonads have been isolated from fish, shellfish, meats, dairy products, and fresh vegetables, few foodborne outbreaks have been reported. A growing body of epidemiological evidence supports the possibility of aeromonads causing foodborne gastroenteritis. While a plethora of putative virulence factors has been postulated and demonstrated in food isolates, the exact role and mechanism of aeromonads in causing diarrheal illness has not been elucidated. Evidence suggests that a high infective dose is necessary to produce gastrointestinal disease in a susceptible host, and the fact that aeromonads may survive and grow at refrigerator temperatures provides a reservoir of bacteria that may achieve an infective dose when foods are mishandled.

A variety of foods have been shown to harbor aeromonads (Yadav and Verma 1998). Ibrahim and MacRae (1991) reported aeromonads present in beef (60%), lamb (58%), pork (74%), and milk (26%) samples. Krovacek et al. (1992) found aeromonads in 43% of random samples from retail food outlets in Sweden. Walker and Brooks (1993) reported aeromonads in fish and fresh salads, and aeromonds have been isolated from lamb (Sierra et al., 1995), oysters (Tsai and Chen 1996), cheese and raw milk (Melas et al., 1999), and fish and seafood (Hanninen et al., 1997a). Szabo et al. (2000) isolated *Aeromonas* spp. from 70 of 120 samples of lettuce in Australia. Aeromonads are found in ready to eat foods, including seafoods (Hudson and Avery 1994; Tsai and Chen 1996). Studies published before 1990 relied upon phenotypic identification, while several studies published after that time identified isolates to hybridization group. While hybridization groups containing virulence factors are found in environmental samples and foods, aeromonads only cause gastroenteritis when their presence exceeds an infective dose for a

susceptible host (Neyts et al., 2000b). Strain variability and undetermined host susceptibility factors have made it impossible to determine a nominal infective dose, however anecdotal evidence suggests that the infective dose is highly strain and host dependent, and probably exceeds 6 log_{10} CFU/g. Such high doses are unlikely to be ingested in drinking water, since ambient water concentrations rarely exceed 4 log_{10} CFU/mL except in sewage-polluted waters.

Exhibit 3.2. Occurrence of *Aeromonas* spp. in Foods

Food	Cell Density (log_{10} CFU/g)	Reference
vegetables	< 2 to > 6	McMahon and Wilson 2001
fish	2-4	Pin et al., 1995
seafood	< 2 to > 5	Hanninen et al., 1997a
luncheon meats	3-4	Gobat and Jemmi 1993
cheese	3-5	Pin et al., 1995
meats (beef, pork, lamb)	2-4	Pin et al., 1995
poultry	2-4	Pin et al., 1995
milk (pasteurized)	4-5	Pin et al., 1995

Agarwal et al. (2000) isolated aeromonads from fish (22%), snails (6.25%), and quail eggs (18%), buffalo milk (2.8%), and goat meat 8.9% – all foods of animal origin in India.
These findings are consistent with those of Tsai and Chen (1996), who found 22.2% of fish samples contained aeromonads, and Glunder and Siegmann (1993), who reported finding aeromonads in birds and poultry eggs. Abbey and Etang (1988) reported finding aeromonads in 28-29% of snails in Nigeria.

Neyts et al. (2000b) cultured 68 food samples quantitatively to determine the presence of mesophilic *Aeromonas* spp. Aeromonads were found in 26% of vegetable samples, 70% of meat and poultry samples, and 72% of fish and shrimp samples at numbers from < 2 log_{10} CFU/g to > 5 log_{10} CFU/g.

3.1.5 Occurrence in Other Media

Soil

There are few studies of *Aeromonas* spp. in soil apart from the contribution of water. Gray and Stickler (1989) reported the presence of aeromonads in pasture soil, probably as a contribution from manure.

Air

Aerosolization of waters occurs in wastewater treatment. Fannin et al. (1985) studied airborne counts of aeromonads in an activated sludge wastewater treatment plant, finding them in airborne particles before and after treatment. Aeromonads were insignificant compared to the numbers of the *Enterobacteriaceae* that were present.

Surfaces

Aeromonads forms biofilms on surfaces and may pose a threat of contamination in food processing. Bal'a et al. (1998) found that heat and chlorine were effective against biofilm on stainless steel surfaces, however older biofilm was more resistant to heat and less established biofilm. Eight-day old biofilm was destroyed by heating to 60º C and by exposure to 75 mg/L chlorine for 1 min.

3.2 Environmental Factors Affecting Survival

Environmental survival of bacteria is dependent upon many physical and biological factors. Temperature, pH, ionic strength, sunlight (UV irradiation), moisture, available nutrient, presence of suspended solids, cell-specific protection mechanisms, and the presence of toxic substances and predators all interact to determine survival times.

3.2.1 Survival in Water

Aeromonas spp. have their natural habitat in water and grow over a wide temperature range. Because *Aeromonas* spp. grow between 0º C and 45º C, with a temperature optimum of 22º C to 32º C, there are few environmental habitats where they are not found. Both high (Tsai and Yu 1997; Warburton 2000; Croci et al., 2001) and low (Kersters et al., 1996b) survival rates have been reported. Nutrient availability, temperature, and water activity most affected growth rates. Growth was optimal at 30º C at pH 7 and a water activity of 0.99 (Sautour et al., 2003). Imbert and Gancel (2004) studied the effect of temperature downshift on protein synthesis of *A. hydrophila*. While a few proteins were under-expressed, two-dimensional electrophoresis revealed that numerous new proteins appeared with a decrease in temperature and some others were over-expressed. Cold shock proteins distinct from those produced by *E. coli* were recognized. Additional studies are required to elucidate the nature of heat and cold shock proteins produced by aeromonads.

Aeromonads grew in filtered autoclaved tapwater but were inhibited by the presence of heterotrophic bacteria, suggesting that competition for nutrients is a selective factor limiting the growth of aeromonads (Mary et al., 2001). Environmental strains had no competitive advantage over clinical isolates. Survival rates vary by strain, with HG-4 > HG-5B > HG-17 > HG-1. Cell density increased from 1-2 $\log_{10}$ CFU/mL to 6 $\log_{10}$ CFU/mL in 3-5 days in both filtered-autoclaved and unfiltered-autoclaved tapwater. Cells suspended in unfiltered water that had not been autoclaved declined from initial concentrations of 4 $\log_{10}$ CFU/mL to 1 $\log_{10}$ CFU/mL within 14 days. Cells suspended in filtered water that had not been autoclaved showed strain-specific differences in survival with decay rates from 4 to 1 $\log_{10}$ CFU/mL on day 1 *(A. popoffii*), day 3 (*A. media*), day 7 (*A. hydrophila*) and day 14 (*A. caviae*).

The upper growth limit is approximately 6×10^5 CFU/mL in nutrient-poor water (Miettinen et al., 1997; Tsai and Yu 1997; Kersters et al., 1996b; Mary et al., 2001). Phosphorous (Miettinen et al., 1997) and assimilable organic carbon (AOC) (van der Kooij 1992) are considered to be the primary factors regulating growth. *A. hydrophila* (HG-1) grows less well than other *Aeromonas* spp. in nutrient poor water (van der Kooij and Hijnen 1988; Mary et al., 2001).

Studies in low nutrient waters such as bottled mineral water show that *Aeromonas* spp. remain viable for extended periods of time. *A. hydrophila* survives in distilled bottled water between 30-60 days, and for > 223 days in spring water (Warburton 2000). *A. hydrophila* increased by 1 $\log_{10}$ during the first 24 hr. then declined 3 $\log_{10}$ CFU in 90 days and 6 $\log_{10}$ CFU in 150 days (Messi et al., 2002). Experiments using mixed cultures reduced the survival time of aeromonads. van der Kooij and Hijnen (1988) have shown that microgram per liter concentrations of amino acids and long-chain fatty acids promote growth of aeromonads in water distribution systems. Aeromonads can also metabolize a wide variety of biopolymers, including proteins, carbohydrates and lipids, which are attacked by aeromonads wide array of extracellular enzymes.

Photooxidation from visible and ultraviolet light has an inimical effect on aeromonads. *A. salmonicida* populations were reduced by 99.9% after 2 hrs of exposure to sunlight (Liltved and Landfald 2000). The presence of humic acids enhanced the effectiveness of photoinactivation, while the presence of sodium chloride at 0.9% increased survivability. The efficacy of UV irradiation in conjunction with photoinactivation was used to characterize the parameters needed for inlet and effluent water in aquaculture. Benchokroun et al. (2003) examined the effects of solar radiation on *Aeromonas* spp. in an algal pond. Inactivation was enhanced by increasing oxygenation or pH, while addition of catalase or sodium pyruvate prevented die-off, suggesting that damage to cells was through photooxidation. *A. sobria* was slightly more resistant to photooxidation which may explain its relative presence at high density in waste stabilization ponds.

Aeromonads grow best between pH 7-9 (Vivekanandhan et al., 2003). Sautour et al. (2003) reported that variation in pH had little effect upon survival over a range of pH 5-9, and this is consistent with the growth range reported by Popoff and Lallier (1984). *Aeromonas* spp. are sensitive to acid conditions below pH 3.5; however, they exhibit an acid stress response in that when they are acclimated at pH 5, the kill time at pH 3.5 is extended. Treatment with protein-inhibiting antibiotics prior to exposure to low pH eliminated the acid stress response, suggesting that protein synthesis is an important part of the acid stress response.

Delamare et al. (2000) examined salt tolerance among aeromonads. All strains challenged were able to grow in the presence of 0.34 M sodium chloride, and all but *A. veronii* biovar sobria (referred to as *A. ichthiosmia*), *A. sobria*, and *A. salmonicida* subspecies *salmonicida* were able to grow in the presence of 0.51 M sodium chloride. *A. hydrophila, A. caviae, A. encheleia, A. trota, A. euchrenophila*, and *A. media* tolerated 0.68 molar salt concentration; *A. caviae* and *A. trota* grew at 0.85 M salt concentration, and *A. trota* tolerated 1.02 molar salt concentration.

3.2.2 Survival in Food

Aeromonads have been shown to grow in foods held at refrigerator temperatures (Palumbo and Buchanan 1988; Majeed and MacRae 1991; McMahon 2000). *A. hydrophila* counts increased from 3-5 $\log_{10}$ CFU to 6-7 $\log_{10}$ CFU in ground meat at 7º C over 7 days (Vaid and Garg 2002). Counts reached 8 $\log_{10}$ CFU in one day at 25º C. Background microflora were not inhibitory for aeromonads. While members of the *Enterobacteriaceae* are inhibited, growth of *A. caviae* was stimulated from 4 $\log_{10}$/g to 8 $\log_{10}$ CFU/g at ambient temperatures and from 5 $\log_{10}$ CFU/g to 7 $\log_{10}$ CFU/g at refrigerator temperature in salad materials packaged under superatmospheric oxygen (Allende et al., 2002).

The Food Micromodel (UK) and the Pathogen Modeling Program (US) were developed to model growth of bacteria in foods using variables of temperature, sodium chloride concentration, sodium nitrate (US model only), and pH.

Growth temperature is an important feature in differentiation of clinical and environmental strains. Approximately half of clinical isolates show some growth at 4-5º C, all food isolates grow at this temperature (Knochel 1990). While most clinical strains grow at 42º C, only a few isolates from vegetables stored at 5º C grew at elevated temperature. Knochel (1990) found that some aeromonads isolated from cold water did not grow at 37º C. The growth temperature range for aeromonads is from 4 to 44º C, but individual strains typically have a restricted growth range according to their ecological niche, and growth of a strain at both extremes of the range are rare (Kirov et al., 1993b). Aeromonads are considered heat sensitive with respect to other foodborne pathogens.

Aeromonads tolerate high pH well and this feature has been exploited by using alkaline peptone water at pH 8.6 for sample enrichment. Aeromonads grow at pH 5.8 or higher, and may survive at pH 4.6 or higher according to computer modeling using Food Micromodel. Species-specific acid tolerance is know to occur, since *A. caviae* grown on glucose or other simple sugars produces sufficient acetic acid to auto-sterilize a broth culture within 48 hr. in weakly buffered systems. This metabolic activity has been called the suicide phenomenon (Namdari and Cabelli 1989).

Aeromonads do not tolerate high salt concentrations. Knochel (1990) reported that a few strains tolerated 6% NaCl, but generally aeromonads do not tolerate concentrations above 5% NaCl. Growth in a competitive environment may not occur at salt concentrations between 3-4%, and a few isolates are sensitive to 2% NaCl. Vivekanandhan et al. (2003) examined the effects of salt concentration on *A. hydrophila*. Salt concentrations above 2% inhibited growth somewhat. Some growth occurred at 4% NaCl concentration; however, no growth occurred at 5% though cells remained viable. These results may explain the distribution of aeromonads in seawater and have implication in food preservation.

Modified atmospheres are increasingly being used in food packaging. Pin et al. (2004) studied the response of *A. hydrophila* to various combinations of pH, temperature, and CO_2 and O_2 concentrations. The results were used to develop and validate a predictive model for growth and death estimates under modified atmospheres at refrigerator temperature. Reduced oxygen levels do not exert a detrimental effect on survival and growth of aeromonads, and they may be isolated from vacuum packed foods (Berrang et al., 1989). Expression of proteases and hemolysins was inhibited, particularly at low temperature (McMahon 2000). High amounts of carbon dioxide (94-99%) markedly affected growth and viability (Golden et al., 1989). Devlieghere et al. (2000) reported that the use of carbon dioxide in the package atmosphere in combination with reduced water activity effectively inhibited growth. Gas-packed cured cooked meat products did not support growth of *Aeromonas* spp stored at < 7º C.

Irradiation is effective at doses of 125 to 150 kilorads, which is sufficient to eliminate

viable cells from foods. Polyphosphates together with sodium chloride, sorbates, and smoke are inhibitory to aeromonads (Gram 1991; Palumbo et al., 1995). Velazquez et al. (2001) studied the antibacterial effects of phosphates, common food additives in meat products. *A. hydrophila* was totally inhibited at concentrations between 0.5 and 3%. 0.5% sodium acid pyrophosphate exhibited the greatest inhibition as it possesses both bactericidal and bacteriolytic properties.

Mary et al. (2003) studied the tolerance of *A. hydrophila* to ethanol, sodium chloride, drying, and temperature. Starved cells developed increased tolerance to salt and ethanol challenge but not to heat. These findings suggest that less rigorous food processing and preservation methods may not eradicate aeromonads from foods. Uyttendaele et al. (2004) examined the effect of lactic acid, chlorinated water, and thyme essential oil solution for control of *Aeromonas* spp. in fresh vegetables. Lactic acid at 1% or 2%, and 0.5% or 1% thyme essential oil solutions were able to control growth of aeromonads, while waters with chlorine concentrations of 0.1 to 0.5 mg/L were not.

Usual food preservative processes (pH < 5, sodium chloride > 3.5%,) are sufficient to suppress growth of aeromonads with the exception of refrigeration, as refrigeration alone does not suppress growth of aeromonads (Palumbo et al., 1995).

3.2.3 Viable but Non-culturable (VNC) State

Starvation (nutrient deprivation) of bacteria has been reported to induce physiological changes that reduce the ability to detect them using culture methods. Nutritional depletion is correlated with detachment of *Aeromonas* cells adsorbed to surfaces (Sawyer and Hermanowicz 2000).

Maalej et al. (2004) studied survival of *A. hydrophila* in natural filtered seawater. Populations declined below the detection limit at both 5º C and 23º C in 3-5 weeks. Cells grown at 5º C were more resistant to stress than cells grown at higher temperature. A temperature shift from 5º C to 23º C did not result in cell resuscitation. Cells lost respiratory activity before they lost membrane integrity. The shift to VNC state is associated with formation of hydrogen

peroxide sensitive cells populations. Catalase or sodium pyruvate may permit cell damaged by photooxidation to be recovered, but this was not true for *A. hydrophila* in seawater experiments. *A. hydrophila* has been shown by exposure to hydrogen peroxide to resist loss of viability due to oxidative stress by exposure to sub-lethal levels of oxidants which induce polypeptide synthesis in a stress response similar to other bacteria (Landre et al., 2000).

Sun et al. (2000) claims to have induced *A. hydrophila* into a VNC state by incubation at 4º C for 45 days. Cells were resuscitated using liquid media and solid media containing catalase or sodium pyruvate. Wai et al. (2000) also reported induction of VNC aeromonads with recovery on media through addition of catalase or sodium pyruvate. Contrary to these reports, Rahman et al. (2001) reported that induction of the VNC state was not reversible in aeromonads. Mary et al. (2002) reported that *A. hydrophila* declined to non-detectable levels in nutrient-poor filter sterilized distilled water at 4º C within 7 weeks, while the number of cells with intact membranes by the Live/Dead® method decreased by 1 $\log_{10}$ CFU. Cells could not be resuscitated by an increase in temperature to 25º C, and neither catalase or sodium pyruvate improved recovery. Whether or not aeromonads exist in a reversible VNC state remains to be determined.

3.2.4 Preservation in Stock Cultures

While freezing at -70º C is common for culture preservation, survival after freezing has not been systematically studied with foods. Palumbo and Buchanan (1988) reported poor survival of stock cultures lyophilized in skim milk, while Marino et al. (1995) reported excellent survival rates for up to two years for cultures lyophilized in a mixture of glycerol-skim milk-tryptone soy broth. The recovery rate of aeromonads lyophilized in serum-inositol cryopreservative has been variable, with good recovery for 2-3 years, but poor recovery after 10 years or more.

3.3 Summary

Aeromonas spp. are found worldwide in surface water (Hazen et al., 1978; Kersters et al., 1995), ground water (Kersters et al., 1995), non-chlorinated drinking water (Gavriel et al., 1998), chlorinated drinking water (LeChevallier et al., 1983; Gavriel et al., 1998), and bottled mineral

water (Tsai and Yu 1997). Aeromonads are found in a wide variety of foods (Kirov 1993a). They are found in the intestinal tract of humans and animals (Janda and Abbott 1998), raw sewage (Holmes et al., 1996), sewage effluents (Holmes et al., 1996), activated sludge (Kampfer et al., 1996), and sewage-contaminated waters (Boussaid et al., 1991). Aeromonads have been shown to grow in foods held at refrigerator temperatures (Palumbo and Buchanan 1988; Majeed and MacRae 1991; McMahon 2000). However, while aeromonads have been isolated from fish, shellfish, meats, dairy products, and fresh vegetables, few foodborne outbreaks have been reported.

4.0 Health Effect in Humans

Some *Aeromonas* spp. are opportunistic pathogens of humans, causing a wide variety of extra-intestinal infections and occasionally associated with gastrointestinal disease. *Aeromonas* infections occur in four broad groups of patients:

a. Persons with impared immune function or serious underlying disease, especially cirrhosis or hematologic malignancy.
b. Persons with hospital-acquired postoperative infections or infections associated with health care.
c. Previously healthy persons with community-acquired infection following trauma and/or exposure to contaminated water.
d. Previously healthy persons, especially children, who ingest contaminated foods and subsequently develop gastrointestinal illness.

The frequency of *Aeromonas* infections remained stable over the past 15 years, so that *Aeromonas* infection should not be considered an "emerging" problem.

The first comprehensive review of human infections caused by *Aeromonas* spp. was undertaken by von Graevenitz and Mensch (1968). Many comprehensive reviews have followed (Janda and Abbott 1996; Chopra and Houston 1999; Figueras 2005). *Aeromonas* spp. reportedly cause cellulitis, abscess, wound infection, necrotizing fasciitis, myonecrosis, pneumonia,

empyema, septicemia, septic arthritis, osteomyelitis, endocarditis, meningitis, gastroenteritis, appendicitis, peritonitis, acute suppurative cholangitis, and corneal ulcer.

4.1 Clinical Symptoms

Clinical symptoms of *Aeromonas* infection depend upon the site and severity of infection. Wound infections frequently result in cellulitis and rarely necrotizing fasciitis. Septicemia may follow wound infection, or may be secondary to systemic diseases such as cancer, cirrhosis, diabetes, biliary disease, or diseases resulting in gastrointestinal perforation. Dissemination may result in meningitis or endocarditis. Pneumonia is rare and it is usually associated with aspiration, such as in near drowning. Gastroenteritis symptoms range from mild self-limiting to dysentery or cholera-like illness.

Patients present with a spectrum of disease symptoms from mild self-limiting diarrhea to acute, severe diarrhea with abdominal cramps, vomiting, and fever. Bloody stools occur with some strains. Adults have chronic diarrhea and abdominal cramps, whereas children 12 years or younger are likely to have more acute and severe illness. *A. caviae* and *A. hydrophila* have been associated with chronic diarrhea lasting up to one year (del Val et al., 1990). A syndrome resembling ulcerative colitis has been observed by endoscopy, and segmented colitis has also been reported (Farraye et al., 1989). Colonoscopy reveals exudates, superficial ulcerations, erythema, and friability of the mucosa, as well as loss of vascular pattern and overlying mucus.

4.1.1 Mechanism of Virulence

Virulence of aeromonads is multifactorial and incompletely understood despite decades of intense investigation (Trower et al., 2000). Many putative virulence factors have been described, including toxins, enterotoxins, proteases, hemolysins, lipases, adhesins, agglutinins, hydrolytic enzymes, outer membrane proteins, S-layer, flagella, and pili. Janda (2002) reviewed the many virulence factors produced by *Aeromonas* spp., and readers are referred to this review for details of virulence mehanisms that are beyond the scope of this document.

Only one virulence factor, A-layer of *A. salmonicida*, had unequivocally been linked to disease causation in 1981, but much progress has been made in understanding virulence mechanisms since then (Kay et al., 1981). No animal model has been found for testing other aeromonads, though streptomycin-treated mouse, clindamycin-treated rat, and suckling mouse models have been used to study virulence factors (Sanderson et al., 1996). Pazzaglia et al. (1990) reported that the removable intestinal tie adult rabbit diarrhea (RITARD) model was the preferred animal model for studying diarrheal disease. Sanyal et al. (1975) demonstrated multiplication of aeromonads within the gut of experimental animals; however, these experiments did not result in identification of an animal model. Several in vitro cell lines have been used to study attachment, invasion and toxic effects of *Aeromonas* virulence factors. Vero, HEp-2, INT 407, mouse Y1 adrenal cells, Caco-2 have been used with varying success (Thornley et al., 1997). Studies using purified ileal and colonic enterocytes and human mucosal slices are currently being evaluated.

Structural factors of bacteria promote attachment (pili, flagella) colonization (adhesins, outer membrane proteins) and protect cells from host response (S-layer, lipopolysaccharide (LPS), capsule). Long wavy flexible fimbriae and afimbrial adhesions are associated with colonization of *A. hydrophila*. Kirov et al. (1995a) and Kirov et al. (1996) demonstrated that removal of surface structures reduces adherence in HEp-2 cells by up to 80%. *A. caviae* and *A. veronii* biovar sobria were found to adhere better than *A. hydrophila*. Most adherence studies were done with clinical isolates and little is known about adherence of environmental strains. Carrello et al. (1988) reported differences in pili of clinical and environmental strains. Adhesion to HEp-2 cells correlated with clinical strains possessing low numbers of thin flexible long L/W type pili, while environmental strains expressed a larger number of short rigid pili termed the S/R type.

Nonfilamentous adhesins include S-layer, LPS, and outer membrane proteins. Collagen-binding protein is found extracelluarly and in loose association with cells, and it is thought to have adherence properties. Surface array proteins are thought to have antiphagocytic properties (Sara and Sleytr 2000). The role of LPS is not clear, but it is thought to be associated with colonization. Tso and Dooley (1995) suggested that it might confer resistance to complement-mediated lethal effects. Martinez et al. (1995) found capsules in pathogenic *A. hydrophila*

serotypes O:11 and O:34. Gavin et al. (2003a) reviewed molecular mechanisms of *Aeromonas* pathogenesis. Aeromonads possess all of the requirements of pathogenic bacteria. Attachment and entry into host cells is facilitated through production of flagella, pili, and adhesins. Multiplication in host tissue is aided by production of siderophores and outer membrane proteins, while resistance to host defenses is conferred by production of capsule, S-layer, lipopolysaccharide and porins. Enterotoxins, proteases, phospholipases, and hemolysins effect damage to host cells leading to cell death.

Several exotoxins and enzymes are elaborated by *Aeromonas* spp. with putative virulence properties (Chopra et al 1986). The cytotoxic group of extracellular products includes hemolysin, aerolysin, and phospholipase. The enterotoxin group is comprised of heat-labile enterotoxin, heat-stable enterotoxin, non-cholera toxin cross reactive, cholera toxin cross reactive, aerolysin (Asao-toxin, β hemolysin), and non-channel forming hemolysin (HlyA). The protease group contains thermostable metalloprotease (38 kDa), thermolabile serine protease (68kDa), thermostable serine protease (22 kDa), and zinc protease (19 kDa). The hydrolase group includes DNAse, gelatinase, acetylcholinesterase, amylase, lipase, and chitinase. Other non-enzyme proteins such as histone-like protein, multidrug-resistance protein, and collagen-binding protein are thought to play a role in virulence.

Thornley et al. (1997) reviewed the virulence genes of *Aeromonas* spp. A summary of virulence factors is shown in Exhibit 4.1.

Exhibit 4.1. Virulence Factors of *Aeromonas* species

Cell-Associated Virulence Factors	Extracellular Virulence Factors
Pili (fimbriae)	Hemolysin
Flagella	Enterotoxin
Outer membrane proteins	Cytotoxin
A or S layer	Protease
Lipopolysaccharide	Glycerophospholipid cholesterol acetyltransferase (GCAT)
Capsule	Other hydrolytic enzymes

From Thornley et al., 1997

4.1.1.1 Cell-Associated Virulence Factors

Invasins

Several studies have shown invasive ability of *Aeromonas* to equal that of *Campylobacter* (Nishikawa et al., 1994; Shaw et al., 1995). While intracellular bacteria have been demonstrated by electron microscopy, no gene or product has been identified specifically with invasion.

Adhesins

Cell properties promoting adhesion of aeromonads to host cells were recognized early in the studies of the pathogenesis (Gosling 1996a). Ascencio et al. (1998) reported cell surface extracts containing active mucin-binding components from 22-95 kDa from *Aeromonas* spp. Adhesions to HEp-2, Caco-2 and INT407 cells has been reported by several investigators (Nishikawa et al., 1994; Bartkova and Ciznar 1994; Kirov et al., 1995b). A correlation between high level HEp-2 cell adherence and enteropathogenicity has been reported (Kirov and Sanderson 1995). Some investigators have reported weak adherence of *A. caviae* to HEp-2 cells (Carrello et al., 1988).

Outer Membrane Proteins

The literature contains several conflicting reports concerning attachment mechanisms, but it is generally accepted that outer membrane proteins (OMP) mediate bacterial adherence to host cells. Recent studies suggest that non-pilar adhesins play a major role in adhesion of *A. hydrophila* and *A. veronii* biovar sobria. Nishikawa et al. (1994) suggested the role of an outer membrane protein in binding of aeromonads to Caco-2 cells. The role of OMPs has been proposed for attachment to HEp-2, HeLa, Chineses hamster ovary (CHO) and Vero cells (Bartkova and Ciznar 1994). Some OMP have hemagglutination activity, while other OMPs are thought to have pore-forming capability.

Rocha de Souza et al. (2001) identified a 43 kDa OMP from *A. caviae* that promoted adhesion to HEp-2 cells, and demonstrated that expression of the 43 kDa OMP is greater in cells

grown at 22° C than those grown at 37° C. This characteristic has been observed with *A. veronii* biovar sobria (Kirov et al., 1995a). All *A. caviae* strains of Rocha de Souza et al. (2001) exhibited the aggregative adherence pattern and the aggregates were able to adhere to HEp-2 cells, forming a stacked-brick appearance. Microbeads coated with the protein adhered to HEp-2 cells and antibody to the 43 kDa protein blocked adherence of the protein-coated beads. Rocha de Souza et al. (2003) studied interaction of adherence and invasion properties of *A. caviae* using Caco-2 cells and observed that the 43 kDa OMP facilitated cell binding. TEM did not demonstrate fimbrial structures on cell surfaces of highly-adherent *A. caviae* strains. These data suggest that OMPs mediate adherence in *A. caviae* instead of pili. Merino et al. (1992) suggested that LPS and OMP might be other important adhesins of *Aeromonas* strains to HEp-2 cells and the findings of Rocha de Souza et al. (2001) support the role of OMPs in adherence. Fang et al. (2004) cloned and characterized the major 43 kDa adhesin (Aha1) of *A. hydrophila*. The amino acid sequences showed a high degree of similarity to OMPs of *A. hydrophila.*

A 43 kDa OMP exhibiting hemagglutinin activity produced by *A. hydrophila* was inhibited by L-fucose (Atkinson et al., 1987). This OMP was shown to have pore-forming activity using an artificial planar bilayer membrane (Quinn et al., 1993). None of the *A. caviae* strains of Rocha de Souza et al. (2001) demonstrated hemagglutinin activity to human erythrocytes, nor did they show L-fucose sensitive adherence to HEp-2 cells.

Guzman-Murillo et al. (2000) showed that Omp48 is associated with adherence properties of *A. veronii*. Vazquez-Juarez et al. (2003) cloned and sequenced the gene encoding Omp48, a major outer membrane protein of *A. veronii*. BLAST analysis revealed over 50% identity with the LamB porin of other pathogenic gram-negative bacteria. Vazquez-Juarez et al. (2004) identified mucin and lactoferrin binding proteins in *A. veronii*. The 48 kDa protein was identified as Omp48 which is similar to *E. coli* OMP LamB, a porin mediating maltose transport across the outer membrane. Omp48 binds collagen and fibronectin in addition to having mucin and lactoferrin binding activity. OMPs exhibit immunoprotective attributes, and specific antibody inhibited adhesion to HeLa cells Vazquez-Juarez et al. (2004).

Contrasting results were obtained by Kirov et al. (1998), who reported that Type IV pili

were present in *A. caviae* strains isolated from clinical sources in Australia, and they suggested that pili were the principal adhesins.

S-Layer Proteins

S-layer (also termed A-layer) has been extensively studied and it has been linked to virulence by providing protection against bactericidal effects of the immune response, protecting cells from proteolysis, facilitating association with macrophages, binds collagen, and plays a role in colonization (Gavin et al., 2003b). The S-layer has a paracrystalline array made up of protein units measuring 49-51 kDa, and made up of two domains. Virulence is markedly reduced when the S-layer is lost. S-layer caries binding sites for fibronectin, laminin and other extracellular proteins, and provides protection against proteases and phagocytic cells (Kay and Trust 1991). The protein is produced under control of the *ashA* gene (Thomas and Trust 1995). S-layers of *A. hydrophila* and *A. veronii* biovar sobria are morphologically similar but antigenically diverse (Kostrzynska et al., 1992). S-layer producing *Aeromonas* spp. are associated with extraintestinal infection of humans (Kokka et al., 1992; Janda et al., 1994). Generally, the S-layers of *A. veronii* biovar sobria are less associated with virulence than the S-layer of *A. salmonicida*.

Sara and Sleytr (2000) reviewed the structural and functional nature of S-layers, which are formed of monomolecular crystalline arrays of protein subunits. The ahs gene encodes S-layer of *A. hydrophila* and the vapA gene encodes S-layer in *A. salmonicida*. S-layers are thought to protect against bacterial parasites such as *Bdellovibrio bacteriovorus*, but they are ineffective against protozoa. S-layer of *A. salmonicida* is thought to contribute to virulence by rendering cells resistant to bactericidal activity of complement in immune and nonimmune sera. A-layer plays a role in uptake of porphyrins and shows unique immunoglobulin and extracellular matrix protein-binding capacity.

Lipopolysaccharide

Lipopolysaccharide plays a role in adhesion to epithelial cells (Merino et al., 1996), resistance to nonimmune serum (Merino et al., 1991), and virulence (Aguilar et al., 1997). It also

comprises the major structural element of the gram-negative cell wall that is responsible for somatic antigenic specificity (O-antigen). Serotyping of aeromonads is based upon O-antigen of the LPS. LPS is thought to be a colonization factor, as its presence facilitates adherence to HEp-2 cells (Francki and Chang 1994). Loss of LPS reduced adhesion by 61%. Tso and Dooley (1995) suggested that O-polysaccharides were not found in a high percentage of clinical isolates and were not required for virulence. They also suggested that LPS was required for colonization of cells by non-invasive or enterotoxigenic strains, but would be required by invasive strains. The structure and function of O-polysaccharides of *A. hydrophila* O:34 were determined by Knirel et al. (2002). Knirel et al. (2004) elucidated the structure of R-type lipopolysaccharide of *A. hydrophila* in an effort to understand the nature of rough strains compared to strains with intact O-antigen determinants. Recently published work on the LPS of *A. hydrophila* serotype O:34 suggests that the UDP N-acetylgalactosamine-4-epimerase gene is essential for mesophilic *A.hydrophila* serotype O:34 virululence (Canals et al 2006).

Capsule

Few investigations have examined the role of capsule polysaccharide as a virulence factor since most motile strains are not encapsulated. Capsules have been shown in *A. hydrophila* serotypes O:11 and O:34 when they are grown in glucose-rich media (Martinez et al., 1995). Preliminary work suggests that capsule may play a role in septicemia, as non-encapsulated strains are less virulent. Zhang et al. (2002) report that *Aeromonas* capsule material has the capability of protecting cells from complement-mediated serum killing activity. Aguilar et al. (1999) also reports serum resistance properties of capsule polysaccharide.

Flagella

Flagellation in *Aeromonas* is usually monotrichous and polar. However, lateral flagella occur in some strains and some strains are nonmotile. Peritrichous flagella are unsheathed and they are associated with swarming movement across solid media surfaces (Kirov et al., 2002). Flagella allow *Aeromonas* to reach target cells where they colonize (Barnett et al., 1997). Flagellar production is under the control of over 40 genes (Macnab 1996). Lateral flagella

facilitate biofilm formation (Gavin et al., 2002) and persistence during infection (Costerton et al., 1987). Polar sheathed flagella are expressed constitutively in liquid media, and peritrichous lateral flagella are produced in matrices which do not permit motility by single polar flagella such as growth on solid culture media (Gavin et al., 2003b). Merino et al. (2001a) suggest that the mgtE gene encodes a Mg^{2+} and Co^{2+} transport system that have a role in swarming ability and consequently, adherence and biofilm formation.

Gryllos et al. (2001) studied the *flm* gene locus of *Aeromonas* spp. relative to adherence and concluded that flagella and the LPS O-antigen are involved in adherence to human epithelial cells. *FlmA* and *flmB* are widely distributed among aeromonads and they are involved in flagellar assembly. In *A. caviae*, *neuA*, *flmD* and *neuB* are located on the same operon with *flmA* and *flmB* and they are involved in O-antigen biosynthesis and flagellar assembly. Nine lateral flagella genes have been identified in *A. hydrophila.* Five polar flagellar genes have been found in *A. caviae* and polar flagella are required for optimal adherence of *A. caviae* to HEp-2 cells (Rabaan et al., 2001). Merino et al. (2003) described nine polar flagellar genes in *A. salmonicida* but found no lateral flagella. Production of lateral flagella would confer no advantage to *A. salmonicida*, since it is adapted to fish. Genes controlling polar flagella were required for expression of lateral flagella (Altarriba et al., 2003).

Kirov (2003) reviewed expression of lateral flagella and their multi-functional role in pathogenesis. Lateral flagella enable swarming motility on surfaces, and function as adhesins which contribute to microcolony formation and biofilm formation on surfaces. Polar flagellar genes *flaA, flaB, flaG, flaH*, and *flaJ* have been identified. Lateral flagellar genes identified are *lafA1* and *lafA2*, which code for flagellin subunits, plus *lafB, lafC, laf,E, lafF, lafS, lafT, lafU, lafX*, and *fliU*, most of which have known gene function. *FlaA* and *flaB*, *lafA1*, and *lafA2* genes have been sequenced. Only *laf*-positive strain swarm. Lateral flagella promote auto-agglutination and function as adhesions. The role of lateral flagella in intestinal colonization is not fully understood; however, swarming has been associated with urinary tract colonization in *Proteus* spp.

Kirov et al. (2004) reported that both polar and lateral flagella are enterocyte adhesins that

contribute to biofilm formation on surfaces. Approximately 60% of mesophilic species produce lateral flagella. Strains that loose polar flagella are virtually nonadherent to cell lines, while strains lacking lateral flagella have a reduced capacity for cell binding. Flagellar mutants were shown to have decreased binding capacity by more than 80%.

Gavin et al. (2002) reported that lateral flagella are essential for adherence to epithelial cells and biofilm formation. Mutations in lateral flagellar genes did not affect polar flagellation; however, mutation of genes controlling polar flagella inhibit lateral flagellar expression. Gavin et al. (2003b) report that lateral flagella are required for increased cell adherence, invasion and biofilm formation. Aeromonads expend considerable resources of cell energy in flagellar regulation, and the overlapping mechanisms controlling polar and lateral flagella is not fully understood.

Pili (Fimbriae)

Attachment to host cells is mediated by extracellular appendages of bacteria such as pili, and these filamentous structures were described as potential colonization factors in *A. hydrophila* and *A. veronii* biovar sobria (Hokama and Iwanaga 1991). Kirov (1993b) reported that pili were important adhesive factors for mucosal surface attachment and described filamentous and nonfilamentous adhesins. Two morphotypes of pili have been observed in *Aeromonas* spp., short rigid pili (S/R type) and long wavy flexible pili (L/W type).

S/R pili are present in 95% of strains regardless of source (Kirov et al., 1995a). S/R pili facilitate autoagglutination but not hemagglutination. Contrary to Kirov et al. (1995a), Ho et al. (1990) found they were more common in environmental strains, and were not thought to be colonizing factors. S/R pili have diameters of 7-10 nm. They have been purified from *A. hydrophila* and the 17kDa pillin protein shows homology in 28 of 40 residues with *E. coli* Type I and the Pap-pyelonephritis-associated pilin.

L/W pili cause hemagglutination, are associated with outer membrane proteins, and are found on clinical isolates Kirov et al., 1995b). L/W pili, bundle-forming pili and filamentous

networks showed maximal expression when grown at 22º C. (Kirov et al., 1995a; Kirov et al., 1995b). The L/W pili have a diameter of 4-7 nm and can form bundles or filamentous networks (Kirov et al., 1995a). Removal of these pili blocks adherence and reaction of removed pili with cell receptors competitively block the adhesion of the parent bacterial strain (Iwanaga and Hokkama 1992). *Aeromonas* pili share sequence similarities with Type IV pilins but they are immunologically and electrophoretically distinct.

Kirov and Sanderson (1996) reported the first purification of bundle-forming pili from *A. veronii* biovar sobria. The N-terminus sequence of 21 kD *A. sobria* flexible pilus was most similar to the mannose-sensitive hemagglutinin of *V. cholerae* with 22 of 26 amino acids being identical (Kirov and Sanderson 1996). Bundle-forming pili were most closely related to Type IV-A class. The *fxp* gene of *A. hydrophila* has been sequenced. It is found on a plasmid and encodes the 4 kDa mini pilin of Ho et al. (1990). The fxp gene was not found in other *A. hydrophila* strains so its role in pathogenesis is doubtful.

Aeromonas spp. from cases of gastroenteritis may exhibit Type IV pili (Tap) (Barnett and Kirov 1999) or bundle-forming pili (Bfp) (Kirov and Sanderson 1996; Kirov et al., 1999). Barnett et al. (1997) also reported the presence of two distinct families of Type IV bundle-forming pili and Tap pili in *Aeromonas* strains from patients with gastroenteritis.

Kirov et al. (2000) investigated the role of Type IV pili (Tap) in the pathogenesis of gastrointestinal disease. While Type IV bundle-forming pili (Bfp) have been linked to intestinal colonization (Kirov et al., 1999), less is known about Tap pili, though the presence of Tap pili is thought to promote biofilm formation (Bechet and Blondeau 2003). Mutations in genes controlling Tap pili did not affect adhesion to Henle 407 intestinal cells or HEp-2 cells. Tap mutants produced disease in rabbits (ileal loop model) and suckling mice (oral challenge model). Tap and Bpf differ in amino acid sequence and molecular weight. Bpf are significant colonization factors. The role of Tap pili requires further study.

4.1.1.2 Extracellular Virulence Factors

Enzymes

Aeromonas spp. produce a wide variety of extracellular enzymes, some of which are thought to contribute to pathogenesis. Pemberton et al. (1997) reviewed the secreted enzymes of *Aeromonas* spp. and their genetic control and biological function. Examples of some of these enzymes and their activity are shown in Exhibit 4.2.

Exhibit 4.2. Extracellular Enzymes Secreted by *Aeromonas* spp.

Enzyme	Activity	Abbreviation	Size (kDa)	Source
Aminopeptidase	prolyl	Pap	48	*A. veronii* biovar sobria
Amylase	"-amylase	AmyA	49	*A. hydrophila*
	"-amylase	AmyA	48	*A. hydrophila*
	"-amylase	AmyB	72	*A. hydrophila*
$-lactamase	metallo-$-lactamase	CphA	28	*A. hydrophila*
	metallo-$-lactamase	CphA2	28	*A. hydrophila*
	metallo-$-lactamase	ImiS	28	*A. veronii* biovar sobria
	cephalosporinase	CepS	38	*A. veronii* biovar sobria
	penicillinase	AmpS	27	*A. veronii* biovar sobria
Cell elongating toxin			70	*A. hydrophila*
Chitinase	Group A	ChiA	94	*A. caviae*
	Group B	ChiIII	53	*A. hydrophila*
	Group B	ORF-3	57	10S-24
	Group C	ChiIII	5	10S-24
Enterotoxin	cytolytic	Ahh1	52	*A. hydrophila*
	cytotonic		35	*A. hydrophila*
Hemolysin	"-hemolysin	Ahh5	54	*A. hydrophila*
	"-hemolysin	Asa1	54	*A. veronii* biovar sobria
	aerolysin	AerA	57	*A. hydrophila*
	aerolysin	Ahh3	54	*A. hydrophila*
	aerolysin		54	*A. salmonicida*
	aerolysin		63	*A. salmonicida*

Exhibit 4.2. Extracellular Enzymes Secreted by *Aeromonas* spp., continued

Enzyme	Activity	Abbreviation	Size (kDa)	Source
Hemolysin	aerolysin		65	*A. salmonicida*
Lipase	acetlycholine esterase		15-5	*A. hydrophila*
	glycerophospho-lipid-chloesterol acyltransferase	GCAT	31	*A. hydrophila*
	glycerophospho-lipid-chloesterol acyltransferase	GCAT	26	*A. salmonicida*
	lipase	H3	72	*A. hydrophila*
	phospholipase	Apl1	73	*A. hydrophila*
Nuclease	DNAse	Dns	25	*A. hydrophila*
	DNAse	NucH	110	*A. hydrophila*
	RNase	RNaseA	24	*A. hydrophila*
Porin	carbohydrate reactive	CROMP	40	*A. hydrophila*
	carbohydrate reactive	CROMP	43	*A. hydrophila*
	maltoporin	LamB	49	*A. salmonicida*
	porin		28	*A. salmonicida*
	porin	OmpA1	36	*A. salmonicida*
	porin	OmpA2	35	*A. salmonicida*
	protein I		47	*A. hydrophila*
	protein II		39	*A. hydrophila*
Porin	protein III		36	*A. hydrophila*
	protein IV		27	*A. hydrophila*
Protease	fibrinolytic protease		87.5	*A. salmonicida*
	metallo-caseinase	AsaP1	20	*A. salmonicida*
	metallo-protease	P2	20	*A. salmonicida*
	serine protease	P1	70	*A. salmonicida*
	serine protease	AspA	67	*A. salmonicida*
	zinc protease	Ahp	19	*A. hydrophila*
Siderophore receptor	ferric siderophore	FstA	86	*A. salmonicida*
Superoxide dismutase	iron		50	*A. salmonicida*
	manganese		46	*A. salmonicida*
Xylanase	xylanase I	XynA	23	*A. caviae*
	xylanase IV		41	*A. caviae*

Amended from Pemberton et al., 1997

Proteases

The role of proteases as virulence factors has not been fully elucidated (Gonzalez-Serrano et al., 2002). Differences in protease elaboration have been attributed to differences in strain origin, incubation temperature, and culture media (Mateos et al., 1993). Proteases of *A. hydrophila* and *A. veronii* biovar sobria are broadly shown to consist of two distinct types, a thermostable metalloprotease (TSMP) that is sensitive to EDTA, and a thermolabile serine protease (TLSP) that is sensitive to phenylmethylsulphonyl fluoride (PMSF) (Leung and Stevenson 1988). Both types of proteases are produced by most strains, though a small number of strains produce only TSMP. Very few strains produce only TLSP. *A. caviae* protease has been investigated by Karunakaran and Devi (1995), who found evidence for production of a serine protease, while most work with *A. caviae* supports production of thermostable metalloproteases. Serine protease has been shown to activate proaerolysin (Howard and Buckley 1985; Abrami et al., 1998a) and glycerophospholipid-cholesterol acyl-transferase (GCAT) (Vipond et al., 1998); however, Gonzalez-Serrano et al. (2002) found no relationship between protease activity and expression of hemolysins. McMahon (2000) reported that proteases and hemolysins of *A. hydrophila* has lower activity when cells were grown at sub-optimal temperature.

Elastase

Elastase was described in aeromonads and pseudomonads in 1972 (Scharmann 1972), and Nord et al. (1975) evaluated elastase production for characterization of aeromonads and pseudomonads. Elastase production has been recommended as a phenotypic marker for characterization of *Aeromonas* spp. (Carnahan et al., 1991b). Hasan et al. (1992) developed a sensitive method for elastase detection using a bilayer plate assay. Recently, elastase has attracted interest as a potential virulence factor (Cascon et al., 2000a; 2000b). Kawakami et al. (2000) cloned the *apk* gene encoding the extracellular metallo-protease of *A. caviae* which exhibits elastase activity, and Keller et al. (2004) demonstrated that a metallo-protease of *A. hydrophila* possessed elastase activity. The role of elastase in pathogenesis has not been fully explored.

Lipases

Lipase genes such as *plc*, *lipH3*, *pla* and *lip* have been identified as potential virulence factors, and mutations in these genes reduce lethal effects in mice and fish (Merino et al., 1999).

Nucleases

Nucleases have been described, but their role in pathogenesis has not been verified (Pemberton et al., 1997).

Glycerophospholipid Cholesterol Acyl-Transferase (GCAT)

A. salmonicida produces glycerophospholipid cholesterol acyl-transferase (GCAT) as a major virulence factor resulting in furunculosis in fish. *A. hydrophila* secretes a similar enzyme that hydrolyzes both neutral and phospholipids, however the role of GCAT in human pathogenicity is unknown (Brumlik and Buckley 1996). GCAT has lipase or phospholipase activity and could cause erythrocyte lysis by digesting their plasma membrane (Pemberton et al., 1997).

Superoxide dismutase

Leclere et al. (2004) found that the manganese superoxide dismutase of *A. hydrophila* has the equivalent function as the copper/zinc (Cu/Zn) superoxide dismutase of *E. coli*. It is located in the periplasmic space and may confer a virulence advantage on the bacteria.

Toxins

β-*hemolysin*

Two hemolytic toxins have been described in aeromonads, hemolysin encoded by the *hlyA* gene and aerolysin controlled by the *aerA* gene (Heuzenroeder et al., 1999). AerA is a channel

forming β-hemolysin promoting channel formation leading to cell death. HylA is a non-channel forming hemolysin similar to the *V. cholerae* hemolysin.

Epple et al. (2004) investigated the effect of aeromonads and their secretory products on ion secretion and barrier function of human intestinal cells HT-29/B6. Chloride ion secretion activity followed exposure of cells with an aerolysin-like β-hemolysin, probably as the result of channel insertion into the apical membrane and activation of protein kinase C. Xia et al. (2004) cloned the β-hemolysin gene from *A. hydrophila* isolated from freshwater fish in China. The cloned β-hemolysin sequences were used in a PCR assay to survey environmental isolates to detect potential pathogenic *A. hydrophila* strains. Such screens have economic importance in aquaculture.

Cytotoxic Toxins

The major hemolysin produced by aeromonads is called aerolysin, though it is know by several other names (cytotoxic enterotoxin, Asao toxin, and cholera toxin cross-reactive cytolytic enterotoxin). Aerolysin is produced by some strains of *A. hydrophila, A. veronii* biovar sobria and *A. caviae* (Singh and Sanyal 1992). The *aerA* gene that encodes the structural protein is present in all *Aeromonas* strains and the conditions that result in expression are being elucidated. Singh and Sanyal (1992) reported that environmental strains could be induced to produce aerolysin with repeated passage (1-3 times) in rabbit ileal loops. The implication is that all environmental strains are potentially enterotoxigenic when passed from host to host, but environmentally adapted strains are not pathogenic when acquired directly from the environment. Toxin is expressed in the latter stages of logarithmic growth. Production of aerolysin in *A. caviae* is enhanced by iron deficiency and repressed by glucose (Singh and Sanyal 1992).

Aerolysin is a 50-52 kDa heat labile protein that causes fluid accumulation in rabbit ileal loops and lyses a wide range of cells including CHO and rabbit erythrocytes (Chopra et al., 1993). It belongs to a class of pore-forming cytotoxins that disrupt cell membranes and is highly lethal for rats and mice. Aerolysin from *A. hydrophila* and *A. veronii* biovar sobria share properties with the cardiotoxic thermostable hemolysin of *V. parahaemolyticus*. The structure has been

determined by X-ray crystalography and the monomer protein is divided into four domains (Parker et al., 1994). Antibodies specific for cholera toxin react with aerolysin, but there is no sequence homology and cholera antisera do not neutralize aerolysin activity. Aerolysin crosses the bacterial cell membrane as an inactive proaerolysin, which binds to the receptor glycophorin on the membrane of erythrocytes. The toxin is then activated by proteolysis, either by bacterial or host proteases, and forms a transmembrane channel. The mechanism of action has been elucidated using erythrocytes and little is know about the toxins effect on intestinal cells, though antisera to aerolysin neutralized the toxin effects in rabbit ileal loops (Ferguson et al. 1997). The role of aerolysin in gastroenteritis is controversial.

Cytotonic toxins

At least four non-cytoxic but cytotonic factors have been described in *Aeromonas* spp. (Gosling et al., 1993). They elongate CHO cells, round Y1 adrenal cells and increase the intracellular levels of cAMP (McCardell et al., 1995). A 15 kDa heat labile protein from *A. hydrophila* causes fluid accumulation in rabbit ileal loops and increases the intracellular concentration of cAMP in Y-1 adrenal cells. A toxin of similar size and characteristics has been purified from *A. veronii* biovar sobria (Gosling et al., 1993). A 44 kDa toxin was isolated from *A. hydrophila* by Chopra et al. (1993) with similar properties, and the ability to cause an increase in prostaglandin E_2 (PGE_2) levels in CHO cells. Another 70 kDa toxin has been described in *A. hydrophila* that caused elongation of CHO cells but does not increase intracellular levels of PGE_2 or cAMP. None of these toxins cross-react with antisera specific for cholera toxin. A heat-stable toxin that cross-reacts with cholera toxin has been reported in *A. hydrophila*, *A. veronii* biovar sobria, and *A. caviae*. Several cytotonic enterotoxins have been cloned, including a heat-stable and a heat-labile cAMP-stimulating toxin from *A. hydrophila* (Chopra et al., 1993). Further characterization of heat-labile and heat-stable enterotoxins of aeromonads was published by Chopra et al. (1996) and Sha et al. (2002). Chakraborty et al. (1984) cloned a heat-stable toxin of undetermined size from *A. hydrophila*. The relationship between these cloned products is not known.

Few *A. caviae* strains have been reported to produce cytotonic enterotoxins (Namdari and Bottone 1990a). The mechanism of pathogenicity is obscure, though cholera toxin has been shown to cross-react with some variants of aerolysin (Honda et al., 1985). The role of *A. caviae* as a cause of gastroenteritis is becoming established as investigators have correlated non-cytotonic enterotoxin producing strains of *A. caviae* with gastroenteritis. Aeromonads may represent a paradox. Although they may be capable of producing enterotoxins, the enterotoxins may play little role in pathogenicity of some strains (Wilcox et al., 1994).

Aerolysin

Fivaz et al. (2001a) reviewed aerolysin of *A. hydrophila*. Aerolysin was identified by Bernheimer et al. (1975) and later purified by Buckley et al. (1981). Chakraborty et al. (1987b) confirmed that aerolysin was a characteristic virulence factor of *Aeromonas* spp. Aerolysin is secreted as a 52 kDa protein called proaerolysin because it is inactive until it is proteolytically activated. The active toxin binds to the target host cell and oligomerizes into a ring-like structure that is able to insert into the cell membrane and form aqueous trans-membrane pores. Membrane perforation triggers a number of intracellular events leading to cell death.

Aerolysin, a pore-forming toxin of *Aeromonas* spp., is a known virulence factor (Krause et al., 1998). The toxin is secreted in an inactive precursor form (Parker et al., 1996) which becomes active with cleavage of a C-terminal peptide. The toxin is thought to bind to specific receptors located on host target cells (Nelson et al., 1997; Abrami et al., 1998b). Binding is thought to concentrate the toxin and facilitate polymerization into heptameric complexes that penetrate the cell membrane and form water-filled channels leading to cell lysis (Abrami et al., 1998b; Krause et al., 1998; Rossjohn et al., 1998).

Krause et al. (1998) reported that aerolysin-induced chemotaxis and release of calcium ions (Ca^{2+}) from intracellular stores was mediated via the activation of a toxin-sensitive G-protein. Proaerolysin and aerolysin were found to increase cytosolic Ca^{2+} in a dose-dependent manner, which suggested that protoxin and toxin bind to receptors on the target cell which express proteases that convert the protoxin to active toxin, that is then inserted through the host

cell membrane through the heptamerization pores. Abrami et al. (2000a; 2000b) showed that heptimer formation occurred following attachment of GPI-anchoring proteins to permit aerolysin to bond to its receptor. These pores modify potassium ion (K^+) and sodium ion (Na^+) gradients, membrane potential, and cytosolic Ca^{2+} levels. Abrami et al. (2003) studied the interaction of aerolysin and GPI-anchoring proteins to form pores in the plasma membrane of host cells. Fujii et al. (2003) studied the mechanism of action of aerolysin-like hemolysins of *A. sobria* for stimulating T84 cells to product cAMP. Song et al. (2004) showed that aerolysin is activated by a metalloprotease in *A. veronii* biovar sobria.

Barry et al. (2001) found that proaerolysin, the pro-form of the channel-forming protein aerolysin, is secreted as a dimer. There is controversy about whether the protein is active in its dimer or monomer form, as suggested by Fivaz et al. (1999). Barry et al. (2001) showed by several means that the protein remains in dimeric form at low concentration of the protein and that the dimeric protoxin is capable of receptor binding.

Diep et al. (1999) reported that aerolysin contains two receptor-binding sites. One is located on the large lobe for glycosylphosphatidylinositol-anchored proteins; and the other is on a site in the small lobe that binds surface carbohydrate determinants. The different binding properties of Act compared with aerolysins of some strains suggests that separate proteins are involved and several isomers of aerolysin may exist.

Fivaz et al. (2001b) concluded that the mechanism of cell death is far more complicated that simple disruption of the cytoplasmic membrane with resulting cell lysis. Laohachai et al. (2003) reviewed the role of bacterial toxins that induce changes in membrane transport leading to diarrheal disease. While progress is being made in understanding the mechanism of aerolysin activity, the actual mechanism is complex and incompletely understood.

Entertoxins

Aeromonas spp. may produce a variety of virulence factors including enterotoxin (Krovacek et al., 1991; Majeed and MacRae 1991). Three cytotoxic and cytotonic enterotoxins

have been described in *Aeromonas* spp., *Aeromonas* cytotoxic enterotoxin (Act), *Aeromonas* heat-labile (56º C) cytotonic enterotoxin (Alt), and *Aeromonas* heat-stable cytotonic enterotoxin (Ast). Ast and Alt are not related to cholera toxin (Chopra and Houston 1999; Chopra et al. 2000).

Asao et al. (1984) purified a hemolysin from *A. hydrophila* with cytotoxic and enterotoxin activities. Rose et al. (1989) and Ferguson et al. (1997) purified a 52 kDa single peptide chain with cytotoxic properties that is now recognized as Act.

Act is a single-chain polypeptide 52 kDa in length and is related to aerolysin, with hemolytic, cytotoxic and enterotoxic activity. Act activity has been demonstrated in LD_{50} studies and by the inability of Act isogenic mutants to induce a fluid-secretory response in mouse ligated ileal loops (Xu et al., 1998).

Act is a cytotoxic enterotoxin that has hemolytic, cytotoxin, and enterotoxic properties. Act causes fluid secretion and tissue damage in mouse ileal loops. Act has no homology with CT coding gene ctx, however it cross-reacts with antibody to CT. Potomski et al. (1987) isolated a similar 63 kDa toxin from *A. sobria*, but it did not cross-react with CT. The aerolysin gene from *A. trota* and *A bestiarum* (originally identified as *A. hydrophila*), was cloned, and these gene products are related to the hemolysin of Asao (Chakraborty et al., 1986; Howard and Buckley 1986). Sha et al. (2004) showed that the *gidA* gene regulates act production. Presence of the *gidA* gene markedly increased the median lethal dose (LD_{50}) for mice inoculated by interperitoneal injection. Addition of iron or glucose repressed gene expression.

The *act* gene is known to stimulate proinflammatory cytokine and eicosanoid cascades in macrophages in the rat intestinal epithelial cell line ICE-6, leading to tissue damage and fluid secretory response (Chopra et al., 2000). There is good correlation between the cytotonic enterotoxins Alt and Ast and elongation of Chinese hamster ovary cells and production of c-AMP, which is typical enterotoxic activity (Chopra et al., 1994). Previous data show that Act is the major enterotoxin contributing to fluid secretory response, followed by alt and ast in *A. hydrophila* (Sha et al., 2002). Sinha et al., 2004 found that the majority of the *A. veronii* biovar sobria strains contained act. Presence of all three genes is rare (Albert et al., 2000; Sinha et al.,

2004). The presence of these three enterotoxin genes in various combinations may increase or decrease expression of specific enterotoxin genes and mediate the severity of diarrhea (Sha et al., 2002).

Act has multiple biological functions such as lysis of red blood cells, and destruction of cell culture lines, which evokes a fluid secretory response in ligated intestinal loop models and lethality for mice. Chopra et al. (2000) demonstrated that Act increased cAMP production in macrophages along with PGE2 and promoted fluid secretion in animal models. Act upregulates the production of proinflammatory cytokines and antiapoptotic protein Bcl-2 and activates arachidonic acid (AA) metabolism in macrophages and monocytes.

Alt is a cytotoxic enterotoxin which is heat labile at 56º C. It consists of a single 44 kDa polypeptide chain that is unrelated to cholera toxin (Albert et al., 2000). Alt elevates cyclic AMP and prostaglandin levels in Chinese hamster ovary and intestinal epithelial cells and caused fluid accumulation in rat ileal loops.

Ast is a cytotoxic enterotoxin consisting of a single 44 kDa polypeptide chain that is heat stable at 56º C. Chopra et al. (1994) identified *ast* gene products of 32 kDa and 67 kDa which evoked fluid secretion in rat small intestine and cAMP levels in mucosal cells. McCardell et al. (1995) purified an Ast-like toxin, is not related to cholera toxin, and does not increase cAMP, CGMP or PGE2 levels in CHO cells, but it does cause fluid accumulation in infant mice.

Falcon et al. (2001) reported that cytotoxic enterotoxin of *A. hydrophila* induced apoptosis in HT29 human intestinal cells. Toxin exposure resulted in cell detachment, cytoplasmic disorganization, and blebbing. The cytotonic enterotoxins Ast and Alt cause fluid accumulation in ligated ileal loops in animal models and probably have an undescribed role in causing diarrhea in humans (Sha et al., 2002). Galindo et al. (2004b) described apoptosis as an effect of cytotoxic enterotoxin activity in murine macrophages and human epithelial cells

Chopra and Houston (1999) reviewed enterotoxins of *Aeromonas* spp. associated with production of gastrointestinal disease. Since publication of this review, TagA, a lipoprotein

controlled by the *toxR* gene, has been identified in *Aeromonas.* This gene is also found in *E.coli* O157:H7 were it mediates serum resistance. The regulation of genes associated with cytotoxic enterotoxins is under continuing investigation (Sha et al., 2002).

4.1.1.3 Type II Secretion Systems

Aerolysin is released from *A. hydrophila* and *A. salmonicida* by a Type II secretion system as a soluble precursor referred to as pro-aerolysin (Abrami et al., 2000b; Burr et al., 2001). It undergoes a monomer to dimer transition at high concentrations. Aerolysin shares high sequence similarity with the alpha toxin of *Clostridium septicum* and has a similar mode of action. Proaerolysin binds to GPI-anchored proteins expressed on cell surfaces. Heptemerization results in a hole in the plasma membrane of receptor cells with disruption of membrane polarization and chloride ion leakage. Transit across the outer membrane may be rate limiting and there is evidence for periplasmic accumulation of the protein. Secrete of toxins via the Type II pathway is under the control of at least two genes, *exeAB* (*gspAB*) and *exeC-N* (*gspC-N*) (Ast et al., 2002).

4.1.1.4 Type III Secretion Systems

Burr et al. (2002) found genes in *A. salmonicida* which are associated with Type III secretion systems in other bacteria, suggesting that Type III secretion systems contribute to virulence of aeromonads. Burr et al. (2003) presented evidence that *A. salmonicida* exoenzyme T (AexT) is translocated via a Type III secretion system. AexT plays a role in cytotoxicity to fish cells.

Chacon et al. (2004) identified genes in 50% of clinical strains of *Aeromonas* that encode for a putative Type III secretion system. The genes were found more frequently in *A. hydrophila* and *A. veronii* strains than in *A. caviae*. Type III secretion systems play an essential role in pathogenicity as they facilitate the delivery of toxins directly into host cells. The presence of Type III secretion systems suggests that pathogenicity islands may be present in *Aeromonas* spp. A *cagA*-like gene of *H. pylori* was found in *Aeromonas* spp. isolated from water samples in India

(Datta et al., 2003). Nucleotide sequencing demonstrated 97-98% relatedness, lending support for the presence of pathogenicity islands in *Aeromonas* spp.

Yu et al. (2004) located the gene cluster responsible for a Type III secretion system and presented evidence that it is required for *A. hydrophila* pathogenesis. Several proteins associated with Type III secretion systems in aeromonds have homologs in *Pseudomonas* spp. and *Yersinia* spp. Characterization of these proteins has value in development of vaccines and for enhanced understanding of the pathogenesis of disease caused by aeromonads.

Sha et al. (2005) used isogenic mutants to demonstate reduced Act production and associated virulence for mice. Production of DNA adenine methyltransferase encoded by the dam_{AhSSU} gene is necessary for viability and over-production of Dam is associated with reduced virulence for mice (Erova et al. 2006). These recent studies on the relationships between Type III secretion systems, cytotoxic enterotoxin and Dam are providing new information on the complex inter-relationship among virulence factors of *Aeromonas* spp.

4.1.1.5 Siderophores

During infection, microbes must acquire iron from the host, and this is accomplished by production of siderophores; thus, siderophores are considered to be virulence factors. Siderophores are iron-specific ligands of low molecular mass. The ferric siderophore gene *fstA* of *A. salmonicida* has significant sequence similarity with the *fstA* gene of several known pathogens (Pemberton et al., 1997).

Motile aeromonads produce either of the phenolate siderophores, enterobactin or amonabactin. Amonabactin is unique to *Aeromonas* spp.; whereas, enterobactin is produced by many enteric bacteria. *A. hydrophila* expresses primarily amonabactin; while *A. veronii* biovar sobria expresses mostly enterobactin. Amonabactin is thought to drive an iron-delivery system that is able to trap Fe-transferrin in serum. Enterobactin is believed to be inhibited by albumin so it is inactive in serum. Most isolates have a siderophore-independent chelation system that can

acquire iron from haem-containing molecules. Both amonabactin and enterobactin genes have been cloned and they are genetically distinct from those of *E. coli* (Byers et al., 1991).

4.1.1.6 Quorum Sensing

Quorum sensing is a mechanism for controlling gene expression in response to an expanding bacterial population. It has been reported in *Aeromonas* spp., and it is a subject of intensive investigation in many bacteria (Swift et al., 1997). The quorum sensing signal generator and response regulator were cloned from *A. hydrophila* and *A. salmonicida* and termed *ahyRI* and *asaRI*, respectively. The *iciA* gene is downstream and a similar gene in *E. coli* is known to inhibit chromosome replication, which suggests that cell division in *Aeromonas* is linked to quorum sensing. Expression of the LuxR protein of *Aeromonas* may function to regulate production of virulence factors in addition to regulate cell division as a manifestation of quorum sensing.

Sha et al. (2005) observed that lactone production was decreased in *act* and *aopB* mutants of A. hydrophila, suggesting a relationship between reduction in virulence and a reduction in quorum sensing ability. The role of quorum sensing in regulation of Type III secretion systems is under current investigation.

4.1.1.7 Opsonins

Merino et al. (2001b) characterized genes responsible for opsonophagocytosis resistance in *A. hydrophila* O:34. Expression of a filamentous phenotype is associated with cell aggregation and it is hypothesized that filaments may interfere with phagocytosis directly or by encouraging cell aggregation.

4.1.1.8 Glycosylation

Power and Jennings (2003) reviewed the genetics of glycosylation in gram-negative bacteria, including *A. caviae*, where the virulence role of glycosylation of proteins associated with virulence such as pili, adhesins, and flagella is unresolved.

4.1.1.9 Suicide Phenomenon

A. caviae, and some strains of *A. hydrophila* and *A. veronii* biovar sobria, grown in broth cultures in media containing 0.5% glucose are known to undergo auto-sterilization after 24 hr. This effect has been referred to as the "suicide phenomenon" (Namdari and Cabelli 1989). Studies have shown that acetate accumulation rapidly lowers pH resulting in cell death. Ballal et al. (2001) observed that non-suicidal strains were lethal for mice while suicidal strains were not; thus, suggesting a relationship between the suicide phenomenon and virulence.

4.1.1.10 Vacuolation

Guimaraes et al. (2002) reported that a strain of *A. hydrophila* isolated from a patient with diarrhea did not produce cytotonic enterotoxin in mice and only small quantities of aerolysin; however, it produced vacuolating activity in Caco-2 cells. Other effects on cells besides enterotoxins, such as vacuolation, may be operative in mild infections.

4.1.1.11 Pathogenicity Islands

A *cagA*-like gene of *H. pylori* was found in *Aeromonas* spp. isolated from water samples in India (Datta et al., 2003). Nucleotide sequencing demonstrated 97-98% relatedness. The gene was unstable and tended to be lost with repeated subculture in the laboratory. The mechanism of acquiring this gene and the reason for its instability on subculture is unknown.

The distribution of some of the recognized virulence genes in *Aeromonas* spp. is shown in Exhibit 4.3.

Exhibit 4.3. Distribution of Virulence Genes in *Aeromonas* spp. Isolated from Patients with Watery Diarrhea

Species	No. Strains	Toxin gene (%)				
		alt	***act***	***ast***	***hly*A**	***aer***
A. caviae	74	72 (97.3)	1 (1.4)	1 (1.4)	3 (4.1)	3 (4.1)
A. veronii biovar sobria	25	12 (48.0)	21 (84.0)	2 (8.0)	6 (24.0)	1 (4.0))
A. hydrophila	35	31(88.6)	14 (40.0)	0	31 (88.6)	10 (28.6)
A. veronii biovar veronii	9	7 (77.7)	7 (77.7)	0	0	0
A. trota	10	5 (50.0)	0	1 (10.0)	1 (10.0)	0
A. schubertii	4	3 (75.0)	2 (50.0)	0	1 (25.0)	0
A. jandaei	1	1 (100%)	0	0	0	0
Unidentified	6	5 (83.3)	3 (50.0)	0	4 (66.7)	0

From Sinha et al., 2004

4.1.2 Gastrointestinal Infection

The role of aeromonads as causes of diarrheal disease is problematic and frustrating. While some stains of *Aeromonas* undoubtedly cause diarrhea, the ability to unequivocally demonstrate cause and effects continues to elude investigators, who must rely upon clinical and epidemiological associations rather than conclusive evidence. This dilemma results from the inability to find an animal model system that replicates the pathogenesis of gastroenteritis in humans.

While the production of extra-intestinal disease in humans is incontrovertible, the role of mesophilic *Aeromonas* spp. as agents causing gastroenteritis is controversial. Much of the controversy results from the inconclusive human volunteer feeding study of Morgan et al. (1985),

where ingestion of *Aeromonas* strains in concentrations as high as 9 $\log_{10}$ CFU/mL failed to produce disease. Because no animal model has been identified to fulfill Koch's postulates for gastrointestinal disease in humans, the role of aeromonads as agents of gastroenteritis has been extrapolated from anecdotal case reports, case-control studies, and a handful of outbreaks epidemiologically associated with food or water ingestion (Kirov 2001).

Aeromonads produce multiple virulence factors typically associated with gastrointestinal disease in other bacteria, but the direct relationship between most of these virulence factors and gastrointestinal disease has not been proven. No epidemiological studies have indisputably linked aeromonads with outbreaks of diarrheal disease. The strongest evidence to support the thesis that some strains of *Aeromonas* cause diarrhea is found in individual case reports (Moyer and Larew 1988; Bloom and Bottone 1990).

Though the role of aeromonads as agents of gastroenteritis remains controversial, several microbiological, epidemiological, and clinical/immunological investigations indicate that some strains of *Aeromonas* are enteric pathogens (Altwegg and Geiss 1989; Joseph, 1996). Gastroenteritis has been linked with but not necessarily caused by *Aeromonas* spp. worldwide (Altwegg and Geiss 1989). The association is strongest in children under the age of 2 years, adults over 50 years of age, and the immunocompromised (Agger 1986; Moyer 1987; San Joaquin and Pickett 1988). A summer peak for isolation of aeromonads from stools corresponds with their increased presence in the environment (Burke et al., 1984a; Agger et al., 1985; Nishikawa and Kishi 1988). While strains possess virulence properties such as the ability to produce enterotoxin, cytoxtoxin, hemolysins, adhesins, invasins, and an array of hydrolytic enzymes, not all strains possessing these properties cause disease in humans, and the host factors predisposing to colonization and disease are unknown (Kirov 1993b). While *A. hydrophila* and *A. veronii* biovar sobria are generally recognized as agents of gastroenteritis, the causal role of *A. caviae* was considered controversial despite the fact that a causal role was first proposed by Fritsch et al. (1975) thirty years ago. Today, most investigators acknowledge *A. caviae* as the cause of gastrointestinal disease (Rautelin et al., 1995). *Aeromonas* spp. have been established as an enteric pathogen (Albert et al., 2000), but the mechanisms of pathogenicity remain elusive. *A. caviae* adheres to mucosal epithelial cells (Krzyminska et al., 2001). *A. hydrophila* and *A. sobria*

produce a number of virulence factors that result in diarrhea, including enterotoxins, cytotoxins, hemolysins, aerolysins, proteases and hemagglutinatins (Albert et al., 2000).

Aeromonads may be present in the gastrointestinal tract of humans, and most epidemiological studies show higher numbers in stools of patients with gastroenteritis than in asymptomatic individuals. Acute self-limiting diarrhea occurs in children, and chronic gastroenteritis or enterocolitis may occur in children and the elderly. The presentation of gastroenteritis caused by aeromonads includes various combinations of fever, vomiting, and increased fecal leucocytes or erythrocytes (Janda and Abbott 1996).

Exhibit 4.4 lists representative case reports where *Aeromonas* spp. are strongly associated with gastrointestinal disease.

Exhibit 4.4. Documented Cases of Gastroenteritis Caused by *Aeromonas*

Patient (age/gender)	Symptoms	Serological Titer	Biopsy	Organism
10/F	typhoid fever	yes	not done	*A. hydrophila*
67/F	cholera	yes	not done	*A. hydrophila*
35/M	dysentery	not done	yes	*A. hydrophila*
67/F	cholera	yes	not done	*A. veronii* biovar sobria
67/M	enteritis	yes (convalescent only)	not done	*A. hydrophila*
30/F	dysentery	yes (convalescent only)	not done	*A. media*
80/M	enteritis	yes (convalescent only)	not done	*A. veronii*
24/F	ulcerative colitis	yes (acute only)	yes	*A. veronii*

Janda and Abbott 1998

The few epidemiological studies that link *Aeromonas* spp. to gastroenteritis are shown in Exhibit 4.5.

Exhibit 4.5. Cases of Gastroenteritis Epidemiologically Linked to *Aeromonas*

Patient (age/gender)	Symptoms	Typing Method	Source	Organism	Reference
3 mo./M	chronic diarrhea	ribotyping	drinking water, rice cereal	*A. caviae, A. veronia* biovar sobria	Moyer and Larew 1988
38 yr/M	gastroenteritis	ribotyping	shrimp	*A. hydrophila*	Altwegg et al., 1991a
2 yr/M	gastroenteritis	ribotyping	well water	*A. caviae*	Moyer 2002

Khan and Cerniglia (1997) consider *Aeromonas* species to be enteric pathogens. Certain strains of aeromonads ingested at a high inoculum levels (probably > 8 $\log_{10}$ CFU) may produce diarrheal disease in susceptible hosts (Janda and Abbott 1998). Buchanan (1984) suggested that aeromonads may be associated with up to 13% of gastroenteritis cases in the U.S. O:11 and O:34 serotypes are common in gastroenteritis (Thomas et al., 1990; Kokka et al., 1991b, Merino et al., 1993a; Merino et al., 1993b). According to Kirov (2001), the majority of aeromonads associated with gastroenteritis are *A. veronii* biovar sobria (HG-8/10), *A. hydrophila* (HG-1), and *A. caviae* (HG-4), though *A. veronii* biovar veronii (HG-8/10), *A. trota* (HG-13), and *A. jandaei* (HG-9) occur occasionally. Gastroenteritis attributed to *A. sobria* was characterized by acute watery diarrhea, vomiting abdominal pain, and fever (Taher et al., 2000). A fatal case of gastroenteritis resulted from eating contaminated mozzarella cheese (Montagna et al., 1998). Goldsweig and Pacheco (2001) reviewed infection colitis caused by *Aeromonas* spp. Picard et al. (1984) suggested the intestinal tract as the source of invasive infections, but Outin et al. (1984) observed that the source of gastrointestinal infection with *Aeromonas* is rarely proven.

While some investigators refuse to accept aeromonads as a cause of diarrheal disease, the available evidence supports the conclusion that some strains do cause either acute or chronic gastrointestinal illness in susceptible hosts.

4.1.3 Skin and Soft Tissue Infection

Skin and soft tissue infection caused by *Aeromonas* spp. resulting in cellulitis and bacteremia were reviewed by Gold and Salit (1993). Skin and soft tissue infections may follow

traumatic injury in environments where soil and water may contaminate the wound. Voss et al. (1992) reported that 82% of *Aeromonas* wound infections resulted from penetrating injury and 43% of these infections resulted from environmental exposures to *Aeromonas* spp. Swimming, diving, boating, and fishing are all aquatic recreational activities placing persons at risk of *Aeromonas* infection. A review of 32 foot injuries revealed that at least one-third of cases resulted from introduction of aeromonads in soil-contaminated glass, nails, or sticks (Wakabongo 1995). Infections from severe trauma associated with automobile accidents or other accidents resulting in crushing injury, compound fractures, or severe burns may lead to osteomylitis, myonecrosis, or gangrene. Campbell (2001) reviewed infectious complications of lawn mower injuries and identified *Aeromonas* spp. as a cause of resulting wound infections, and Larka et al. (2003) observed that *Aeromonas* infection following foot trauma may not always be associated with an aquatic environment.

Necrotizing fasciitis is a rapidly advancing form of cellulitis characterized by muscle necrosis. Minnaganti et al. (2000) reported necrotizing fasciitis caused by *A. hydrophila* in patients with suppressed immune systems, burns, and trauma in aquatic settings. The case of an 85-year old man with no history of trauma suggests that sepsis from an intestinal source resulted in soft tissue infection and subsequent necrotizing fasciitis. Angel et al. (2002) reported a case of necrotizing fasciitis caused by *A. hydrophila* following a water moccasin bite. Cheng et al., (2004) reported a fatal case of nosocomial necrotizing fasciitis caused by *A. hydrophila* in a 44-year old man who underwent valve replacement surgery.

Bite wounds are associated with *Aeromonas* infection, but it is not known whether the organism originated from the oral flora of the animal, or was introduced into the puncture wound from the environment. Wound infections have been reported following bear, alligators (Raynor et al., 1983), piranha (Revord et al., 1988), and snake (Angel et al., 2002) bites.

Burn infections caused by aeromonads are rare events – only 29 cases have been reported in English language literature (Kienzle et al., 2000). These authors reported 5 cases of *A. hydrophila* or *A. caviae* infections from burns associated with explosions (4 of 5) and a campfire accident (1 of 5). In 4 of 5 cases, water was used to quench the fire or as a first aid treatment.

Wilcox et al. (2000) reported *A. hydrophila* infection in 73-year old victim of an electrical burn on the hand.

Trauma is most closely associated with *Aeromonas* wound infections. While the typical presentation in persons with normal immune status is cellulitis, more serious infections and sepsis may occur as shown in Exhibit 4.6. Patients developing myonecrosis have a mortality rate exceeding 90%.

Exhibit 4.6. Circumstances Promoting *Aeromonas* Wound Infections

Injury	Event	Location	Source	Immune Status	Presentation
laceration, abrasion	water sports, seafood shucking	community	water, soil	normal	cellulitis, abscess, ulceration, septicemia
puncture	occupational exposure	community	soil, water	normal	cellulitis, abscess, ulceration
crush	motor vehicle accidents	community	soil, water	normal	pyomyositis, myonecrosis, osteomyelitis, gangrene
burn	occupational	community	water, soil	normal	cellulitis, septicemia
invasive medical procedure	intraabdominal surgery, catheterization	nosocomial	feces, fomites	impaired	purulent exudate, abscess

Modified from Janda and Abbott 1998

The significance of aeromonads as a cause of skin and soft-tissue infections was made abundantly clear as a result of the tsunami that devastate Southeast Asia in December 2004 (Hiransuthikul et al. 2005; Lim 2005). Among 777 patients hospitalized for injuries suffered as a result of the tsunami, 515 had skin and soft-tissue infections and 145 isolates for 305 patients were either *A. hydrophila* or *A. veronii* biovar sobria. Many of these infections developed because traumatic wounds were not cleaned properly or because of delay in obtaining medical care.

4.1.4 Respiratory Tract Infections

Pneumonia and lung abscess in adults has been reported (Goncalves et al., 1992). Pneumonia may also occur in children (Sirinavin et al., 1984), and Kao et al. (2003) reported a case of bacteremic pneumonia caused by *A. hydrophila* in a previously healthy 5-year old child. Predisposing conditions were present in 11 of 15 (73%) of cases. Reported predisposing conditions include alcohol abuse (20%), neurologic disease (20%), cardiovascular disease (27%), chronic renal failure (7%), chronic obstructive lung disease (20%), traffic accidents (7%), and malignancy (7%). Murata et al., 2001 reported fulminant pneumonia caused by *A. hydrophila* in a patient undergoing hemodialysis with chronic renal failure and cirrhosis. The source of infection was not determined.

Respiratory infections occur in the immunocompetent persons who involuntarily aspirate surface water while swimming or as the result of an accident (Mukhopadhyay et al., 2003). Miyake et al. (2000) reported *Aeromonas* pneumonia from near-drowning experiences. Respiratory infections may also occur in persons with underlying diseases placing them at risk for *Aeromonas* bacteremia originating from an intestinal source as shown in Exhibit 4.7.

Exhibit 4.7. Respiratory Tract Infections Caused by *Aeromonas*

Patient (age/gender)	Underlying Disease	Infection	Specimen	Source
38/?	None	Lung abscess	Bronchial lavage	Fresh water
24/M	None	pulmonary edema, ARDS	Endotracheal aspirate, blood	Water
41/F	Chronic renal failure	Parapharyngeal soft-tissue infection	oral secreations, blood	Denture cup water
24/M	None	Pneumonia	blood, lung	Seawater
8/F	β-thalessemia	Epiglottitis	Epiglottis	Unknown
59/M	None	Lung abscess	Sputum, bronchial wash	Unknown
69/M	Diabetes melitus, cirrhosis, ethanol	Pneumonia, sepsis	Blood, Bronchial wash	GI tract
43/M	Ethanol abuse, cocaine abuse	Pneumonia	Blood, sputum	River water
50/M	Cirrhosis	Empyema	Blood, plural fluid	GI tract

From Janda and Abbott 1998

Isolation of *Aeromonas* spp from respiratory specimens must be interpreted together with clinical findings, since the upper respiratory and nasopharyngeal tracts may be transitorily colonized.

4.1.5 Meningitis

Meningitis is a rare complication of extraintestinal infections with *Aeromonas* spp. Of eight cases reviewed by Parras et al. (1993), four cases occurred in children and two cases had underlying hematologic disorders (sickle cell anemia and β-thalessemia).

4.1.6 Endocarditis

Brouqui and Raoult (2001) reviewed endocarditis and found only two cases caused by *Aeromonas*, both in patients with cirrhosis.

4.1.7 Osteomyelitis

Osteomyelitis has been reported following compound fractures or crushing trauma where wounds were contaminated by soil or water (Janda and Abbott 1998).

4.1.8 Hepatobiliary Infections

Liver disease is a recognized predisposing factor leading to *Aeromonas* infection resulting from septicemia (Clark and Chenoweth 2003). Underlying hepatitis B infection and cirrhosis are predisposing factors for liver disease. Liver abscess, supperative cholangitis, and empyema may occur following septicemia in patients with underlying hepatobiliary disease. *A. veronii* biovar sobria and *A. veronii* biovar veronii are typically involved in these infections; while *A. caviae* has been reported as the cause of cholecystitis (Kumar et al., 2000a).

Chan et al. (2000) reviewed 30 cases of acute suppurative chlolangitis caused by *Aeromonas* spp. Septicemia occurred in 4 of 30 patients with *Aeromonas* infection. Most patients (83%) had bile duct stones, 13% had chloangiocarcinoma and 7% had pancreatic cancer. Previous

instrumentation, e.g. catheterization, or cystoscopy, predisposed patients (83%) to *Aeromonas* infection.

4.1.9 Bacteremia/Septicemia

Bacteremia resulting from *Aeromonas* infection was reviewed by Ko and Chuang (1995), and Ko et al. (2000). *A. hydrophila* sepsis is associated with gastrointestinal disease, liver cirrhosis, diabetes, malignancy, pancreatitis, trauma, cardiac anomalies, and respiratory disease. Sepsis is accompanied by fever, hypotension, jaundice, and chills, and complications of *Aeromonas* infection may include intravascular coagulation, purpura fulminans, and ecthyma gangrenosum (Ko et al., 2000). Disseminated infection progresses rapidly and has a high fatality rate (Shiina et al., 2004).

Llopis et al. (2004) reviewed *Aeromonas* bacteremia in 75 elderly patients to determine the clinical and epidemiological characteristics. Bacteremias were mostly in males (72%) suffering from chronic liver disease (36%) or neoplasm (33%). The origin of infection was from the abdomen in 52% of cases. Nosocomial infections constituted 29% cases, and 48% of cases occurred in the elderly, and 28% were polymicrobic infections. *Aeromonas* spp. were isolated from blood, ascitic fluid, bile, urine, surgical wound infection, and liver abscess. *A. hydrophila* was most frequently isolated (68%), followed by *A. veronii* biovar sobria, and *A. caviae*.

4.1.10 Peritonitis

Peritonitis sometimes occurs as a secondary infection following colonization of the intestinal tract, and is also associated with peritoneal dialysis or intestinal perforation (Ramos et al., 1996). Most infections occur in patients with chronic liver disease, where the case-fatality rate approaches 60%. Tsoufa et al. (2000) described a case of peritonitis caused by *A. veronii* biovar sobria that occurred following intestinal perforation resulting from cancer surgery.

4.1.11 Hemorrhagic Uremic Syndrome

Bogdanovic et al. (1991) reported a case of hemolytic uremic syndrome (HUS) caused by *A. hydrophila* in a 23-month old child that occurred six days following an episode of bloody diarrhea. In a follow-up report, Robson et al. (1992) reported that 2 of 82 cases of HUS were due to *A. hydrophila*. This report provides additional evidence that this organism produces a cytolytic hemolysin (aerolysin) and/or cytotoxic enterotoxin (Act), which behaves in a similar fashion to the Shiga-like toxin of *E. coli* O157:H7, while remaining genetically and antigenically distinct.

4.1.12 Ocular Infections

Ocular infections caused by *Aeromonas* spp. are rare, and they usually result from water-related trauma (Carta et al., 1994). *A. hydrophila, A. sobria* and *A. caviae* have all been implicated as the cause of ocular infections. Most ocular infections follow eye trauma and water or soil contamination (Carta et al., 1994). Puri et al. (2003) reported a case of corneal ulcer in a 58-year-old patient caused by *A. sobria* where neither prior trauma nor water/soil exposure could be identified. Pinna et al. (2004) report a case of keratitis caused by *A. caviae* in a patient who wore soft contact lenses. Contamination of the contact lens case probably resulted in infection.

The causal role of *Aeromonas* spp. in ocular disease must be evaluated in conjunction with clinical presentation, since aeromonads have been cultured from eye swabs of persons without eye disease (Smith 1980).

Exhibit 4.8. Ocular Infections Caused by *Aeromonas*

Disease	Exposure	Reference
Keratitis	contact lenses trauma	Carta et al., 1994 Pinna et al., 2004 Carta et al., 1994
Endophthalmitis	trauma	Carta et al., 1994
Conjunctivitis	unknown	Smith 1980
Corneal ulcer	unknown	Puri et al., 2003

4.1.13 Septic Arthritis

Septic arthritis caused by *Aeromonas* spp. is relatively rare (Chmel and Armstrong 1976; Steinfeld et al. 1998; Roux et al., 2000).

4.2 Predisposing Factors

Predisposing factors identified with *Aeromonas* infection of the gastrointestinal tract include hospitalization, antimicrobial therapy, neutralization or underproduction of gastric acid, hepatic disease, and underlying enteric conditions such as gastric or colon cancer, colon surgery, gastrointestinal bleeding, or idiopathic inflammatory bowel disease (Joseph 1996). Formula-fed infants and children with disruption of normal bowel flora for antibiotic therapy may become colonized with aeromonads resulting in chronic diarrheal illness (Moyer 1987; Moyer and Larew 1988; Namdari and Bottone 1991). *Aeromonas* spp. have been isolated from chlorinated and non-chlorinated drinking water supplies (LeChevallier et al., 1983; Burke et al., 1984a, 1984b; Havelaar et al., 1992), and drinking untreated ground water has been identified as a risk factor (Moyer 1987). Selective infection among persons with common exposure have been attributed to differences in host susceptibility, especially among children, thought the nature of these host determinants has not been elucidated (Demarta et al., 2000).

Predisposing factors for extra-intestinal *Aeromonas* infection include trauma, cirrhosis, diabetes, alcoholism, severe malnutrition, renal failure, and severe peripheral vascular disease (Altwegg and Geiss 1989; Furusu et al., 1997). The pathogenicity of aeromonads differs based upon the genospecies and phenospecies involved and strain differrences (Janda and Kokka 1991). Based upon intraperitoneal infection of mice, the relative order of virulence for highest to lowest was *A. jandaei, A. hydrophila, A. schubertii, A. veronii* biovar veronii, *A. veronii* biovar sobria, HG-11 (unnamed), *A. bestiarum*, HG-3 (*A. hydrophila* -like), *A. caviae, A. eucrenophila, A. media*, and *A. sobria*.

4.3 Chronic Conditions/Sequelae

Underlying diseases associated with *Aeromonas* infection include liver dysfunction

(cirrhosis), blood dyscrasias (leukemias, lymphomas, myelomas), biliary disease, and diabetes (Janda and Duffy 1988). Complications associated with infections caused by *Aeromonas* include hemolytic uremic syndrome, septicemia, meningitis, peritonitis, wound infections, respiratory tract infections, and ocular infections.

4.4 Dose Response

The single human feeding study failed to determine the infectious dose (Morgan et al., 1985). Empirical evidence from case reports and food borne outbreaks suggests that the infectious dose is very large (> 6-8 $\log_{10}$ CFU/g) and that only select strains have the ability to produce gastrointestinal disease; however, it is possible that some strains may have a lower infective dose in sensitive sub-populations.

4.5 Immunity/Vaccines

Jiang et al. (1991) first demonstrated a secretory immunoglobulin A (sIgA) response following *Aeromonas* infection, and *Aeromonas* exotoxins induce both IgM and IgG response. Crivelli et al. (2001) characterized the sIgA response to *Aeromonas* exoproteins in patients with naturally acquired infection. Fecal antibodies (sIgA response) could be used to assess the progression of intestinal infection for some strains of *Aeromonas* where IgM and IgG response was delayed or nonexistent. While no reference strains tested induced sIgA, 11 of 14 patient isolates of *A. bestiarum* induced sIgA production, and 2 of 8 strains of *A. caviae* failed to induce sIgA. *Ahh1* and *aerA* gene products were shown to induce antibodies, which supports the conclusion that at least some strains of *Aeromonas* are enteropathogenic.

4.6 Therapeutic Measures

Zong et al., 2002 reviewed clinical aspects of *Aeromonas* infection and therapeutic options. Aggressive antibiotic therapy is indicated for disseminated infections. Gastrointestinal infections are typically self-limiting, and antibiotic therapy is required only in prolonged cases in immunocompromised hosts. Rehydration therapy may be required in infants with watery diarrhea.

Surgical intervention may be necessary in cases of nectotizing fasciitis. In addition, cellutis may require debridment, and abscesses may require draining.

4.6.1 Antibiotic Treatment

The role of antibiotics in treatment of gastrointestinal *Aeromonas* infections is controversial, since most patients regain health without treatment. Antimicrobials are indicated for only severe and unresponsive cases of *Aeromonas* gastroenteritis (Phavichitr and Catto-Smith 2003). Antimicrobials should be considered for chronic gastrointestinal disease or extra-intestinal infection (Ghenghesh et al., 1999b). For adult patients with severe gastrointestinal illness such as colitis, quinolones, chloramphenical, trimethoprim-sulfamethoxazole, and tetracyclines are effective (Farraye et al., 1989). However, Ko et al. (1996) reported resistance in strains from Taiwan to tetracycline, trimethoprim-sulfamethoxazole, ceftriaxone, cefotaxime, cefixime and tobramycin. Prophylactic antimicrobial therapy is recommended for patients undergoing leech therapy (Snower et al., 1989).

In addition to selection of antibiotic therapy in the clinical setting, antibiotic sensitivity patterns are sometimes useful as phenotypic characteristics for species identification, especially for clinical isolates (Overman and Janda 1999). *Aeromonas* spp. are characteristically resistant to ampicillin (94.9%), with variable resistance to cephalexin (76.3%), trimethoprim (37.3%), tetracycline (11.9%), cefuroxime (5.1%), and ceftazidime (1.7%). All strains tested were susceptible to gentamicin, chloramphenicol, and ciprofloxacin (Murphy et al., 1995). In another study, antibiotic susceptibility tests were performed on 164 strains, and resistance to ciprofloxacin (12-22%), nalidixic acid (54-62%), and norfloxacin (14-19%) were recorded (Sinha et al., 2004). Wide annual fluctuations in resistance during two consecutive years occurred with furazolidone (7% vs. 74%) and streptomycin (45% vs. 72%). These data support antibiotic susceptibility testing of isolates from extra-intestinal source.

Susceptibility results show discrepancies depending upon methods used (Overman 1980). Disc diffusion and broth dilution tests for amoxicillin-clavulanic acid, sulfonamide, trimethoprim, and trimethoprim-sulfamethoxazole are unreliable (Koehler and Ashdown 1993). Overman and

Janda (1999) published antimicrobial susceptibility patterns for *A. jandaei, A. trota, A. schubertii*, and *A. veronii* biovar veronii, using the Microscan system and found that the antibiograms were useful in establishing the identification of these four species when isolates originated from clinical specimens. In the U.S., more than 90% of strains are susceptible to third-generation celphalosporins (cefotaxime, ceftriaxone, ceftazidime and cefoperazone) and aminoglycosides (gentamicin, tobramycin, amikacin, sisomicin, netilmicin, kanamycin, and neomycin). Nearly all aeromonads are susceptible to quinolones (ciprofloxacin, norfloxacin, ofloxacin, levofloxacin, sparfloxacin, moxifloxacin and gatifloxacin (Zong et al., 2002). Most U.S. strains are susceptible to chloramphenicol, tetracycline, minocycline, doxycycline, and nitrofurantoine, but resistant to clindamycin, vancomycin, and erythromycin. Imipenem is effective for treatment of *Aeromonas* infection (Lupiola-Gomez et al., 2003).

Aeromonas spp. are typically sensitive to tetracycline, aminoglycosides, trimethoprim-sulfamethoxazole, third-generation cephalosporins, and quinolones (Koehler and Ashdown 1993; Janda and Abbott 1996). Ko et al. (1996) reported increasing resistance to tetracyclines, trimethoprim-sulfamethoxazole, some extended-spectrum cephalosporins (ceftriaxone, cefotaxime, and cefixime), and tobramycin in strains from Taiwan. Huys et al. (2001) found oxytetracycline resistant strains in water from fish farms and in hospital sewage.

Aeromonas species exhibit differences in their susceptibility to antibiotics. Clinical isolates of *A. caviae* were more susceptible to ticarcillin than *A. veronii* and *A. hydrophila* (Vila et al., 2002). Use of clavulanic acid with amoxicillin enhanced antibacterial activity but tazobactam did not enhance the effect of piperacillin. *A. veronii* (79%) were more susceptible to cefazolin than *A. caviae* (53%) or *A. hydrophila* (40%). Gentamicin and amikacin were more active than tobramycin. Tobramycin resistance and gentamicin susceptibility has been observed in Australia, Taiwan and the U.S. Fluoroquinolones have been the first choice treatment for *Aeromonas* infections. Strains resistance to nalidixic acid and susceptibility to ciprofloxacin are known to have a mutation in the *gyr*A gene; thus, naladixic acid resistant strains should not be treated with fluoroquinolones. Resistance to nalidixic acid is a function of a mutation of the *gyrA, gyrB, parC*, and *parE* genes which make up the quinolone resistance-determining regions (Goni-Urriza et al., 2002). Gyrase and topoisomerase IV are the primary and secondary targets for quinolones,

respectively.

Ko et al. (2001) demonstrated that minocycline and cefotaxime administered together produced a synergistic effect against *A. hydrophila* using a murine model. Ciprofloxacin was as effective as cefotaxime-minocycline in vitro and in a murine model, suggesting that clinical studies are warranted (Ko et al., 2003).

Two metal ion binding sites are conserved in the metallo-β-lactamase produced by *A. hydrophila* (Valladares et al., 2000). Class B metalloproteins require bivalent transition-metal ions for activity, and Zn^{2+} is required for activation. Quiroga et al. (2000) found that cefotetan was efficacious against *Aeromonas* spp. because it behaved as a transitory inactivator of metallo-β-lactamase activity. Walsh et al. (2002) used Etest strips for detection of metallo-β-lactamases in clinical isolates. The test is based upon reduction of imipenem or ceftazidime in the presence of EDTA or 2-mercaptopropionic acid. Fosse et al. (2003b) demonstrated the β-lactamase classes produced by *Aeromonas* spp., and proposed they had taxonomic value as an adjunct in species identification.

Edwards et al. (2001) reported antibiotic resistance in *Aeromonas* spp. isolated from a eutrophic lake in England. Goni-Urriza et al. (2000a; 2000b) reported antibiotic resistance to ampicillin (99%), ticarcillin (87%), cephalothin (93%), cefoxitin (56%), streptomycin (65%), sulfamethoxazole (90%), trimethoprim (42%), pipemidic acid (67%), oxolinic acid (67%), naladixic acid (59%), and tetracycline (14%) in *Aeromonas* spp. isolated from European rivers. Susceptibility to fluoroquinolones varied from 54 to 98%. Most strains were susceptible to ciprofloxacin, colistin, fosfomycin, cotrimoxazole, chloramphenicol, gentamicin, tobramycin, cefotaxime, and imipenem (Goni-Urriza et al., 2000b). Urban wastewater effluents are thought to contribute to the increasing rate of antibiotic resistance in environmental aeromonads (Goni-Urriza et al., 2000a).

Oxytetracycline is used in aquaculture and the development of resistant strains of *Aeromonas* spp. has both commercial and public health implications (Adams et al., 1998). Because resistance is plasmid mediated, it is easily transferred among strains, and has been

demonstrated in isolates from hospital sewage and a German hospital patient, in addition to fish farming environments. Dissemination of resistant strains has occurred in Norway, Scotland, England, and Germany (Rhodes et al., 2000). Bruun et al. (2003) demonstrated transfer of large plasmids conferring oxytetracycline resistance among environmental aeromonads and between *Aeromonas* spp. and *E. coli*.

A. popoffii has been isolated from freshwater and seawater, but not from humans, though they are known to produce virulence factors and exhibit antibiotic resistance (Soler et al., 2002). Third-generation cephalosporins and quinolones are most active against *A. popoffii* in laboratory studies.

4.6.2 Other Therapeutic Strategies

Rehydration therapy is sufficient intervention in most pediatric cases of gastroenteritis caused by *Aeromonas* spp. (San Joaquin and Pickett 1988). Draining obstructions and antibiotic therapy cleared infection in patients with acute suppurative cholangitis (Chan et al., 2000). Debridment or more intensive surgical intervention may be necessary to arrest progression of soft tissue infections.

4.7 Sensitive Sub-populations

Aeromonads cause disease in elderly and immunocompromised patients (San Joacquin and Pickett 1988; Merino et al., 1995), as well as in previously healthy hosts (Goncalves et al., 1992). Dumontet et al. (2003) recommends including *Aeromonas* in routine stool culture for patients under 10 years of age, the elderly and immunocompromised.

4.7.1 Elderly

Echeverria et al. (1981) reported a strong association between *Aeromonas* and gastroenteritis in adults older than 60 years and in travelers. Cryan et al. (1990) reported a case of diarrhea in a 70-year-old man caused by *A. sobria* presumably acquired from drinking water. An

organism with the same biotype and antibiogram was isolated from the group water system that supplied his house. Because no other persons exposed to the contaminated water source became ill, age is thought to be a predisposing factor resulting in illness. Champsaur et al. (1982) reported the case of a 67-year-old Thai woman with cholera-like illness caused by *A. sobria*. The source of infection was not determined, but either food or water could have been the source.

Persons with underlying diseases are susceptible to *Aeromonas* infections, and elderly patients are at increased risk of acquiring *Aeromonas* bacteremia (Ko and Chuang 1995). Thomsen and Kristiansen (2001) reported 3 cases of bacteremia caused by *A. sobria* where 2 of 3 patients were 75-years old or older. Leukemia, HIV infection, and colorectal cancer were underlying factors. Gelbart et al. (1985) reported a case of cholera-like diarrhea in a 62-year-old male caused by *A. sobria*. The patient has a history of chronic alcoholism with organic brain syndrome, but no underlying immunocompromising disease. The nature of exposure leading to illness was not determined.

Elderly patients with diabetes are subject to opportunistic infections caused by *Aeromonas* spp. Furusu et al. (1997) reported necrotizing fasciitis and gas gangrene in a diabetic patient on hemodialysis. Grobusch et al. (2001) reported a case of cellulitis and septicemia caused by *A. hydrophila* in a 68-year-old patient with multiple underlying medical conditions including diabetes. The patient soaked his legs in a bucket of tapwater and infrequently changed the water. Moawad and Zeiderman (2002) reported a case of surgical wound infection in an 83-year-old diabetic patient cause by *A. hydrophila*. Sarma (2002) reported a case of *A. jandaei* cellusitis and bacteremia in a 75-year old patient with diabetes following a snake bite. Roux et al. (2000) reported a case of septic arthritis in an elderly diabetic.

4.7.2 Children

A strong association between gastroenteritis and *Aeromonas* species has been shown in children (Agger et al., 1985; Moyer 1987; San Joaquin and Pickett 1988; Namdari and Bottone 1990b). Diarrheal illness seems to be more common in children under the age of three years (Sack et al., 1988), while children under the age of two years with multiple underlying medical

complications are at risk of sepsis from aeromonads in their intestinal tract (Janda and Abbott 1998). Acute gastrointestinal disease in children usually resolves within 7 days, and it is characterized by watery diarrhea (100%), fever (70%) and vomiting (30%). Bloody stools occur in up to 30% of cases for *A. hydrophila and A. sobria*, but not with *A. caviae* (Lee and Puthucheary 2001). Chronic disease with soft stools persisting for months to years have been reported (Moyer et al., 1992a).

de la Morena et al. (1993) reported an outbreak of diarrhea associated with *Aeromonas* spp. in daycare centers. In this outbreak, 6 of 25 children had *Aeromonas caviae* and/or *A. hydrophila* as the only pathogen, and 5 of 6 of these children had diarrhea. The source of infection was not determined; however, the authors discounted person-to-person transmission. Person-to-person transmission of *A. caviae* from an infant to an adult was shown by ribotyping (Moyer and Larew 1988).

Kafetzis et al. (2001a) isolated *Aeromonas* spp. from 7 of 294 (2.5%) of children (median age 1 year) with acute diarrhea, and observed a seasonal peak incidence in summer months in Greece.

Saxena et al. (2002) cultured stools of healthy and diseased children and adults and found higher counts of *Aeromonas* spp. in healthy subjects than in those with disease. The only culture method for aeromonads was MacConkey agar, which may have affected the results.

Sirinavin et al. (1984) reviewed septicemia in infants and children, where disease manifestations resemble those found in adults. Septicemia, gastroenteritis, peritonitis, skin and wound infections, septic arthritis, osteomyelitis, ocular infections, myositis, urinary tract infections, pneumonia, meningitis, and hemolytic uremic syndrome have been reported in children (Janda and Abbott 1998).

4.7.3 Immunocompromised

Persons with compromised immune systems associated with malignant hematologic

disease, mainly leukemia, other malignancies, hepatobiliary disease such as cirrhosis, hepatitis, hepatic abscess, and biliary obstruction are at risk of opportunistic infections caused by aeromonads (Altwegg and Geiss 1989; Furusu et al., 1997). *A. hydrophila* and *A. veronii* biovar sobria are more pathogenic than other aeromonads for immunocompromised populations (Janda et al., 1994).

Wolff et al. (1980) reviewed bacteremic *Aeromonas* infections in ambulatory immunocompromised hosts. Patients with malignancy, hepatobiliary disease, diabetes, pancreatitis, trauma, cardiac anomalies, gastrointestinal disorders, anemia, and respiratory problems are subject to developing septicemia caused by aeromonads (Janda and Abbott 1996). Kafarakis et al. (2001b) reported septicemia in an immunosuppressed female patient. The source of infection in these patients is thought to be endogenous resulting from gastrointestinal colonization. Mortality rates among these groups ranges from 25-50%.

Lau et al. (2000) reviewed underlying factors predisposing patients to bacteremia and reported *Aeromonas* infections in 14 of 42 patients with neoplasm, 11 of 42 patients with cirrhosis, 10 of 42 patients with biliary tract disease, and 7 of 42 patients with other illnesses. In this study, 33 of 42 (79%) of cases were community acquired. Alcoholism and chronic hepatitis B infections were frequent predisposing factors for *Aeromonas* infection (Kim et al., 2001; Lau et al., 2000). Neutropenic patients (Orlando et al., 2001) and patients with underlying conditions such as E-β thalassemia (Cigni et al., 2003), ovarian cancer (Kafarakis et al., 2001b), and hematological malignancies or solid tumors (Martino et al., 2000) are at increased risk of infection. Flynn and Knepp (1987) reported a case of septicemia in an immunocompromised patient who apparently acquired his infection while shucking seafood.

The mortality rate for immunocompromised patients with soft tissue infection and septicemia ranges from 56 to 75% despite aggressive surgical intervention and antibiotic therapy (Altwegg and Geiss 1989; Gold and Salit 1993).

Reports of gastroenteritis in HIV patients have been limited (Dumontet et al., 2003). Interestingly, Suthienkul et al. (2001) reported that *Aeromonas* diarrhea was present in 4.6% of

AIDS patients and 8.3% of non-AIDS patients in Thailand.

4.8 Summary

Aeromonas spp. cause a variety of extraintestinal infections such as wound infection, memingitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, eye and urinary tract infections. *A. hydrophila, A. caviae, A. veronii* biovar sobria, *A. veronii* biovar veronii, *A. jandaei*, *A. trota*, and *A. schubertii* are known to be pathogenic to humans. While all of these species have been isolated from fecal specimens, *A. hydrophila, A. veronii* biovar sobria, and *A. caviae* are most frequently associated with gastrointestinal disease. A high number of asymptomatic persons carry aeromonads in their stools (Schiavano et al., 1998). Expression of virulence factors is multifactorial and host susceptibility dependent (Kuijper and Peeters 1991).

Kuhn et al. (1997) suggests that only certain strains of aeromonads are associated with gastroenteritis in humans, though humans continually ingest aeromonads in food and water. Despite demonstration of virulence factors such as enterotoxins, cytotoxins, hemolysins, aerolysins, proteases, hemagglutinins, and invasins, it has not been possible to predict pathogenicity for the human gastrointestinal tract based upon presence and production of recognized virulence factors alone (Thornley et al., 1997). The difficulty in unequivocally determining the pathogenesis of aeromonads results from their extreme strain heterogeneity and the lack of a suitable animal model system (Albert et al., 2000).

Aeromonas cause gastroenteritis in children, the elderly, and in immunocompromised individuals. Several putative virulence factors have been identified, and food isolates frequently produce one of more of these factors. Presence of aerolysin (cytotoxic hemolytic exotoxin), heat-labile or heat-stable cytotoxins, phospholipases and various extracellular enzymes (amylase, proteinase, deoxyribonuclease, elastase, chitinase, etc.) does not consistently correspond to virulence in humans (Dumontet et al., 2003). Strains containing the *ast* gene (heat-stable cytotonic enterotoxin) were more common in environmental isolates than in clinical isolates from children with diarrhea (Albert et al., 2000). Apparently, both the alt (heat-labile cytotonic enterotoxin) and *ast* genes must be present for a strain to have the ability to cause watery diarrhea,

while strains containing only the *alt* gene are associated with loose stools.

The relationship of aeromonads to waterborne and foodborne disease is controversial (Joseph and Carnahan 2000). Pathogenic strains, primarily HG-1, HG-4 and HG-8Y, are not frequently isolated from drinking water (Havelaar et al., 1992; Hanninen and Siitonen 1995; Hanninen et al., 1997b), while others report the presence of pathogenic strains to be frequent in the aquatic environment, including drinking water (Goni-Urriza et al., 1999).

Virulence factors associated with aeromonads include straight (Sato et al., 1989) and flexible (Ho et al., 1990) type IV pili. Two distinct class IV pili (Bundle forming and Tap) are produced (Kirov et al., 1999; Kirov et al., 2000), in addition to collagen-binding protein (Gullberg et al., 1989), hemagglutinin (Majeed and MacRae 1994), and S-layers (Kokka et al., 1991a). Several extracellular products are elaborated, including cytotoxic and cytotonic enterotoxins, hemolysins, and various hydrolytic enzymes. The presence of both Type II and Type III secretions systems has been demonstrated (Sha et al. 2005; Yu et al. 2005) and several new virulence factors such as enolase (Sha et al. 2003), UDP N-acetylgalactosamine 4-epimerase (Canals et al. 2006), and DNA adenine methyltransferase (Erova et al. 2006) are being investigated. Despite the plethora of putative and proven virulence factors produced by aeromonads, the precise combination of virulence factors that invariably confer virulence on a particular strain has not been determined. Presence and expression of virulence genes are not unequivocally associated with the ability of a strain to cause disease in a susceptible host.

Extra-intestinal infections are usually serious and potentially life-threatening, and aggressive antibiotic therapy is indicated. Because multi-drug resistance is commom among aeromnads, antimicrobial susceptibility testing is a necessary prerequisite to successful therapy. Gastrointestinal infections are usually self-limiting; however, antibiotic therapy may be required for infants or immunocompromised patients with prolonged infection.

5.0 Health Effect in Animals

Aeromonas infection of aquatic animals has been recognized for over 100 years, but they

are less commonly recognized in other vertebrates (Gosling 1996b; Austin and Adams 1996). While many animals shed aeromonads from their gastrointestinal tract, there is no evidence that they suffer from gastrointestinal disease. *Aeromonas* spp. cause outbreaks of disease and represent an economic threat to the aquaculture industry. The role of aeromonads in aquaculture was reviewed by Nielsen et al. (2001).

5.1 Clinical Symptoms

Aeromonas hydrophila has been reported to cause septicemia in snakes (Esterabadi et al., 1973), turtles (Pasquale et al., 1994), and rabbits (Paniagua et al., 1998). Forga-Martel et al. (2000) reported a case of infectious abortion caused by *A. hydrophila* in a mare. Contamination from an adjacent dairy farm was suspected as the source of infection for the mare and transplacental infection was thought to result in fetal sepsis and abortion. Kumar et al. (2001) reported a case of mastitis in a dairy cow and isolated *A. sobria* from milk. The organism was resistant to ampicillin, colistin, cotrimoxazole, gentamicin, nitrofurantion, streptomycin, cefotaxime, cephalexin, chloramphenicol, nalidixic acid, furazolidone, norfloxacin and oxytetracycline. Ciprofloxicin was administered therapeutically with success.

Disease in aquatic animals is characterized by hemorrhagic lesions, ulcers, furunculosis, and septicemia in frogs and fish (Gosling 1996b; Austin and Adams 1996).

5.1.1 Mechanism of Action/Disease

The mechanisms of disease action in animals (Gosling 1996b) and fish (Austin and Adams 1996) were reviewed recently. The virulence factors operative in producing animal disease are essentially the same ones that produce disease in humans.

5.1.2 Chronic Conditions/Sequelae

Mastitis (Kumar et al., 2001), septic arthritis (Traubdargatz et al., 1994), and abortion (Forga-Martel et al., 2000) have been reported in animals. A chronic form of furunculosis has

been reported in fish (McCarthy 1980).

5.2 Dose Response

Paniagua et al. (1990) determined that doses of 7 $\log_{10}$ CFU of *A. hydrophila* (72% of strains) and *A. sobria* (63% of strains) infected intramuscularly produced disease in trout, while no *A. caviae* strains did. The dose response for animals is unknown.

5.3 Immunity/Vaccines

Research is underway to characterize immunity in fish and to produce effective vaccines to prevent economic loss in aquaculture. Live attenuated cells (Vaughan et al., 1993), outer membrane proteins (Hirst and Ellis, 1994), recombinant serine protease (Bennett et al., 1992), and lipopolysacccharide (Rodgers 1990) have been investigated with varying degrees of success.

5.4 Therapeutic Measures

Apart from aquaculture and veterinary medicine, little information is available on treatment of *Aeromonas* infections. Generally, the same antibiotics and therapeutic management techniques are used in human and veterinary medicine.

5.4.1 Antibiotic Treatment

Specific antibiotic therapy is used to treat domestic animals and to reduce losses in the aquaculture industry. Sulfonamides, oxytetracycline, and quinolones have been used successfully (Martinsen et al., 1991).

5.4.2 Other Therapeutic Measures

Management consists of removal of diseased animals and water treatment to control the density of aeromonads in aquaculture. Maintaining high standards of water quality, disinfection of equipment, temperature control, and stress reduction reduce the prevalence of disease (Austin and

Adams, 1996).

5.5 Sensitive Subpopulations

Animals in aquaculture are crowded and vulnerable to disease caused by *Aeromonas* spp.

5.6 Summary

Aeromonas spp. cause considerable economic loss in aquaculture, and methods are being developed to manage disease and increase production. Aeromonads are a minor veterinary pathogen, and the therapeutic measures resemble those practiced in human medicine.

6.0 Epidemiology and Disease Outbreaks

6.1 Transmission Routes and Mechanisms

Aeromonas spp. are ubiquitous in the environment and there are multiple opportunities for transmission to humans through food, water, animal contact, and direct human contact. Extra-intestinal infections are typically acquired following trauma in an aquatic environment, and intestinal infections are acquired by ingestion of contaminated food or water. Intestinal infections in immunocompromised patients may disseminate resulting in septicemia with multiple organ involvement. Inhalation of surface water in near drowning incidents has been reported to cause pneumonia.

6.1.1 Foodborne Transmission

Aeromonas spp. have been recognized as potential foodborne pathogens since 1984 (Buchanan 1984). Kirov (1993a) reviewed the public health significance of *Aeromonas* spp. in foods, and Merino et al. (1995) reviewed aeromonads as emerging pathogens present in foods. Aeromonads are common on foods, especially green vegetables, and they are found in raw milk, ice cream, meats, and seafood.

The presence of aeromonads in fish and seafoods lead the U.S. Food and Drug Administration to designate them as a "new" foodborne pathogen (Tsai and Chen 1996). Butt et al. (2004) reviewed foodborne diseases associated with seafood consumption. Foodborne illnesses cause an estimated 76 million illnesses in the U.S. each year, and seafood is implicated in 10-19% of these illnesses (Butt et al., 2004). The causative agent is identified in approximately 44% of illnesses, with viruses accounting for approximately 50% of known illnesses, and 9% were caused by bacteria. The prevalence of seafood-related illnesses is dependent upon cultural dietary habits, and 20% of foodborne illnesses in Australia and 70% of illnesses in Japan are associated with consumption of raw or under-cooked seafood. In the U.S., shellfish cause about 64% and finfish cause 31% of outbreaks. *A. hydrophila, A. caviae*, and *A. veronii* biovar sobria are commonly found in seafoods (Tsai and Chen 1996). *Aeromonas* spp. have been recovered from 93% of fish, 100% of fish eggs, and 16% of shrimp (Hanninen et al., 1997a). Wang and Silva (1999) reported that 36% of channel catfish samples from 3 processing plants contained *A. hydrophila* and 35.7% contained *A. veronii* biovar sobria. The rates of contamination were higher in summer months when water temperatures are warm (Nishikawa and Kishi 1988). Cockle ingestion resulted in *A. hydrophila* gastroenteritis (Bernardeschi et al., 1988) and shellfish shucking has caused septicemia from superficial abrasions on the hands of a worker (Flynn and Knepp 1987).

6.1.2 Waterborne Transmission

Diarrheal disease was associated with drinking untreated well water (Holmberg et al., 1986). The use of ribotyping to link environmental and clinical strains of aeromonads has been widely applied; however, strain diversity has confounded success in all but a handful of studies. Altwegg et al. (1991a) used ribotyping to demonstrate that shrimp ingestion resulted in gastroenteritis in the first report of foodborne illness attributed to *Aeromonas* spp. Subsequently, others have shown the same ribotype in well water and stools of patients with gastroenteritis. Ribotyping was used to demonstrate that a patient with chronic diarrhea carried the same strain for years (Moyer et al., 1992a), and ribotyping was used to demonstrate person-to-person transmission of *Aeromonas* spp. between a foster child and a foster parent (Moyer and Larew 1988). Aquarium water has been suggested as the source of aeromonads resulting in

gastrointestinal infection (San Joaquin et al., 1989).

Filler et al. (2000) reported a case of acute renal failure in a 6-month old infant caused by *A. sobria* acquired from aquarium water. The infection presented with water diarrhea that progressed to bloody diarrhea and hemolytic uremic syndrome. Renal function was restored by kidney transplant at 16 months of age.

There is no evidence of association of strains of *Aeromonas* with enteric infection through the waterborne route among the general population (Leclerc 2003). Implementation of a general water safety plan by health care facilities affords sufficient protection of compromised populations.

6.1.3 Person-to-person Transmission

Ashdown and Koehler (1993) did not observe person-to-person transmission to secondary household contacts in 45 cases of *Aeromonas* gastroenteritis in Australia. Person-to-person transmission was shown by ribotyping in the case of a foster child with *A. sobria* and *A. caviae* infection, where the foster mother developed diarrhea and *A. caviae* matching the child's isolate was isolated from her stool (Moyer and Larew 1988). Transmission among children in daycare centers (Sempertegui et al., 1995), nursing homes (Bloom and Bottone 1990), and patients in intensive care (Torre et al., 1996) have been reported.

6.1.4 Animal-to-person Transmission

Animal-to-person transmission may occur through direct contact, or by ingestion of contaminated food products of animal origin.

6.1.5 Environmental Transmission

Extra-intestinal infections originate from environmental sources directly from soil or water contact, or indirectly by ingestion and bacteremic dissemination of aeromonads from the

gastrointestinal tract.

6.2 Disease Outbreaks

Aeromonas spp. have been reported to be the cause of the disease outbreaks shown in Exhibit 6.1. Foodborne outbreaks are discussed below in section 6.2.4. The source of infection is difficult to determine and is frequently unknown.

Exhibit 6.1. Cases or Outbreaks Caused by *Aeromonas* spp.

Location	Number Ill	Symptoms	Source	Organism	Reference
Florida, community acquired	1	septicemia	seafood shucking (crabs)	*A. hydrophila*	Flynn and Knipp 1987
Italy, community acquired	1	gastroenteritis, septicemia	Cockles	*A. hydrophila*	Bernardeschi et al., 1988
U.S., nursing home	4 or 11	gastroenteritis	unknown	*A. hydrophila*	Bloom and Bottone 1990
Canada, industrial camp					Glover et al., 1992
Mexico, day care center	6 or 25; 5 of 24	gastroenteritis	unknown	*A. caviae*	de la Morena et al., 1993
U.S., nursing home	1	gastroenteritis	unknown	*Aeromonas* spp.	Sims et al., 1995
Libya, community acquired	28	gastroenteritis	unknown	*A. sobria*	Taher et al., 2000
U. S., community acquired	1	gastroenteritis. acute renal failure	aquarium water	*A. sobria*	Filler et al., 2000
Australia, community acquired	26	cellulitis	mud football	*A. hydrophila*	Vally et al., 2004

Modified from Joseph, S. W. 1996

6.2.1 Outbreaks Associated with Drinking Water

Aeromonads are frequently isolated from drinking water (Havelaar et al., 1990; Holmes and Nicholls 1995; Holmes et al., 1996), and temporal and seasonal associations between presence of aeromonads in drinking water and their presence in the stools of patients with

gastroenteritis have been reported (Burke et al., 1984b). While some investigators claim that drinking water is responsible for outbreaks of *Aeromonas* gastroenteritis (Ghanem et al., 1993), epidemiological evidence linking water ingestion to gastrointestinal illness has been limited to untreated drinking water supplies, and no waterborne disease outbreaks associated with treated drinking water supplies have been reported. Molecular typing studies have shown that the strains most frequently found in feces belong to HG-1 and HG-4, while HG-2, HG-3 and HG-5A are more commonly found in drinking water and the environment (Moyer et al., 1992b; Hanninen and Siitonen 1995), suggesting that environmental strains are fundamentally different from clinical strains. The high infectious dose, the differences in temperature optima, and the variation in expression of putative virulence factors between clinical and environmental strains suggests that outbreaks of gastrointestinal illness resulting from water ingestion are unlikely to occur.

6.2.2 Outbreaks Associated with Recreational Water

While superficial infections resulting from environmental exposure are relatively common, no outbreaks attributable to recreational water exposure have been reported.

6.2.3 Outbreaks Associated with Other Water Sources

One report linked exposure to aquarium water to a case of gastroenteritis caused by *A. sobria* with fatal disseminated disease in a 6-month old child (Filler et al., 2000).

6.2.4 Food

Aeromonas spp. have been reported as the cause of individual cases and point source outbreaks of foodborne disease as shown in Exhibit 6.2. Raw or under cooked seafood and meat are most frequently implicated, but snails, soups and egg salad have resulted in illness or outbreaks.

Exhibit 6.2. Foodborne Gastroenteritis Associated with *Aeromonas* spp.

Location	Attack Rate	Incubation Period (h)	*Aeromonas* spp. Isolated		Reference
			Food	Feces	
School, Russia	"mass" poisoning	ND	Fish	ND	Kalina 1977
Restaurant, England	3 of 3	24-36	oysters	ND	McSwiggan and Jones 1978*
Hungary	"several" cases	20	Soups, starchy broths	ND	Janossy and Tarjan 1980
Pub, Scotland	> 20	< 24	prawns	no	Small 1980*
Dinner party, Nigeria	1 of 2	20	snails	yes	Agbonlahor et al., 1982
Florida	7 of 7	22-34	oysters	yes	Abeyta et al., 1986
China	115	ND	pork	yes	Zeng Shan et al., 1988
School, Japan	29 of 37	ND	ND	yes	Kobayashi and Ohnaka 1989
Household, Japan	4 of 5	ND	sashimi (fish balls)	yes	Kobayashi and Ohnaka 1989
Restaurant, England	14	< 24	prawns	ND	Hospital report*
Restaurant, England	2	6-8	prawns	no	Hospital report*
Specialty store, Switzerland	1	48	shrimp cocktail	yes	Altwegg et al., 1991a
Japan	2 of 8	ND	seafoods	yes	Tanaka et al., 1992
Hospital cafeteria, U.S.	4	< 24	egg salad sandwich	yes	Bottone 1993
Smorgasbord, Sweden	22	20-34	meats	yes	Krovacek et al., 1995
Norway	3 of 4	ND	raw fish	yes	Granum et al., 1998

Modified from Kirov 1993a
*Cited by Todd et al., 1989
ND - no data

6.2.5 Travelers

Echeverria et al. (1981) noted that travelers were at risk of acquiring diarrheal disease caused by *Aeromonas* spp. Hanninen et al. (1995) reported travelers' diarrhea in Finnish tourists who visited Morocco, and Yamade et al. (1997) reported *Aeromonas* infections in Japanese travelers to developing countries. Both Sierra et al. (2001) and Vila et al. (2003) reported *Aeromonas* spp. as the cause of diarrhea in 2% of travelers to Africa, Latin America, and Asia.

Rautelin et al. (1995) reported a case of *A. caviae* diarrhea in a 45-year old male who traveled to Turkey. *A. caviae* was isolated from his stool on 14 occasions over 17 months. Treatment with three courses of ciprofloxacin were required to eradicate the infection. Ribotyping revealed that all isolates were the same strain.

6.2.6 Other Sources

Lehane and Rawlin (2000) reviewed zoonoses acquired from fish and reported that aeromonads caused cellulitis, myositosis, and septicemia following injuries from handling fish, working in aquaculture, or keeping fish as pets.

Several investigators have reported *Aeromonas* infections concurrently with use of therapeutic leeches. Indergand and Graff (2000) and Braschler et al. (2003) both reported symbiotic relationships between *Aeromonas veronii* biovar sobria and medicinal leeches. Cellulitis is the most common infection following application of medicinal leeches (Fenollar et al., 1999), and up to 20% of patients undergoing leech therapy experience infections (Mercer et al., 1987). Ouderkirk et al. (2004) reported a case of meningitis following leech therapy. Use of tap water to fill aquariums used for maintaining leeches is discouraged (Sartor et al., 2002).

6.3 Summary

Aeromonas spp. are ubiquitous in the environment and there are multiple opportunities for transmission to humans through food, water, animal contact, and direct human contact.

Aeromonads are common on foods, especially green vegetables, and they are found in raw milk, ice cream, meats, and seafood. The presence of aeromonads in fish and seafoods lead the U.S. Food and Drug Administration to designate them as a "new" foodborne pathogen (Tsai and Chen 1996). While aeromonads are frequently isolated from drinking water (Havelaar et al., 1990; Holmes and Nicholls 1995; Holmes et al., 1996), and claims that drinking water is responsible for *Aeromonas* gastroenteritis (Ghanem et al., 1993), epidemiological evidence supporting water ingestion to illness has been limited to untreated drinking water supplies, and no waterborne disease outbreaks associated with treated drinking water supplies have been reported. There is no evidence of association of strains of *Aeromonas* with enteric infection through the waterborne route among the general population (Leclerc 2003). Implementation of a general water safety plan by health care facilities affords sufficient protection of compromised populations (CDC 2001). Lehane and Rawlin (2000) reviewed zoonoses acquired from fish and reported that aeromonads caused cellulitis, myositosis and septicemia following injuries from handling fish, working in aquaculture, or keeping fish as pets.

7.0 Risk Assessment

The health implication of heterotrophic bacteria, including *Aeromonas* spp. is controversial (Edberg et al., 1996; Grabow 1996; Rusin et al., 1997). Payment et al. (1991) found that 35% of gastrointestinal disease was attributed to consumption of water meeting current treatment standards. This finding has not been confirmed in recent epidemiological studies (Colford et al., 2005). Pavlov et al. (2004) studied virulence factors in heterotrophic bacteria isolated from treated and untreated drinking water and concluded that while the presence of potential pathogens in drinking water is of epidemiological concern, the extent of health risk cannot be defined and further studies are needed. *Aeromonas* was isolated from 18.1% of samples; however, Rusin et al. (1997) report the risk of infection to be less than 6.8 cases per 10,000 population exposed and 95% of the time would be less than 1/1000 for a single day exposure.

One means of assessing the risk of infection associated with *Aeromonas* spp. is to characterize isolates from various sources for presence of putative virulence factors. Several

investigators have demonstrated production of multiple virulence factors and multidrug resistance in *Aeromonas* spp. isolated from surface waters (Gibotti et al., 2000;). While putative virulence factors have been demonstrated in water isolates, Havelaar et al. (1992) showed that aeromonads from human fecal specimens were not very similar to strains isolated from drinking water. The *ast* gene was significantly more common in environmental isolates than in isolates from children with diarrhea, while isolates possessing both the *alt* and *ast* genes were significantly more numerous for children with diarrhea than from other sources (Isonhood and Drake 2002).

Biscardi et al. (2002) isolated cytotoxic *A. hydrophila* from Italian bottled mineral and thermal waters. Overall, 61 bottled waters yielded 4 positives (6 strains) and 23 thermal waters yielded 6 positives (12 strains). All 6 isolates from mineral water were cytotoxic and contained the aerolysin gene, while 7 of 12 environmental strains were cytotoxic and 11 of 12 strains contained the aerolysin gene.

Murphy et al. (1995) reviewed hospitalized cases of *Aeromonas* infection over a 15-year period and reported that 52.5% of extra-intestinal *Aeromonas* infections were hospital-acquired and 55.9% of infections occurred in patients with serious underlying disease. Only 13.6% of isolates were from community-acquired cases in previously health individuals. Polymicrobial infections occurred in 40.7% of patients. Post-operative infection accounted for 27.1% of cases. Clinically significant infections occurred in 79.7% of cases. The mortality rate was 5.1%. *Aeromonas* spp. were isolated from patients with bacteremia, skin and soft tissue infections, respiratory tract infections, genitourinary tract infections, biliary tract infections, and peritonitis. Malignant disease was a common underlying illness.

Immunocompromised, infants less than 2 years of age, and individuals with severe wounds associated with trauma are the primary risk groups for *Aeromonas* infection. The mortality of disseminated cases is 25-75% (Janda and Abbott 1998).

Drinking water standards have not provided absolute public health protection, though they have been responsible for reducing the number of waterborne disease outbreaks associated with treated public drinking water supplies (Padiglione et al., 1997). Any deviation from usual plant

performance may signal a problem that potentially increases the risk of contaminants entering the distribution system, and such events require immediate intervention (Padiglione et al., 1997).

A. hydrophila was placed on the U.S. EPA Candidate Contaminant List in 1998 (USEPA 1998). This list is published every five years. It identifies chemicals and potential waterborne pathogens for further evaluation, research and regulatory consideration, which includes risk assessment.

7.1 Risk Assessment Paradigms/Models

The current framework for microbial risk assessment is based upon the report, *A Conceptual Framework for Assessing the Risks of Human Disease Following Exposure to Waterborne Pathogens*, published in condensed form in 1996 (ILSI 1996). This framework was further revised during the Workshop on Water and Foodborne Pathogen Risk Assessment (May 1999), cosponsored by the ILSI Risk Science Institute and EPA Office of Ground Water and Drinking Water (ILSI 2000). Microbial risk assessments involve analyses of data related to characterization of potential exposure and characterization of associated human health effects. The characterization of pathogen virulence factors is thought to be of paramount importance in determining the potential for a particular strain to cause disease in an exposed host. This concept has lead to exhaustive characterization of virulence factors in collections of isolates from clinical and environmental sources.

7.1.1 Assessment of Virulence Factors as an Indication of Risk

Figueroa et al. (1988) proposed that all strains containing virulence factors be considered pathogenic, but Schiavano et al. (1998) isolated strains of *A. veronii* biovar sobria capable of producing cytotoxin and showing invasive properties from asymptomatic hosts. While our knowledge of virulence factors of aeromonads is incomplete, carrying the genes responsible for production of known virulence factors, and demonstration of structural properties suggestive of virulence is not sufficient evidence to classify aeromonads as human pathogens. Unrecognized host factors undoubtedly play the pivotal role in determining the condition under which virulence

factors are expressed and disease occurs.

7.1.1.1 Studies Characterizing Virulence Markers in Clinical and Environmental Isolates

Gonzalez-Serrano et al. (2002) used the presence of the hemolysin genes *aerA* and *hlyA* genes to predict virulence in isolates from freshwater fish and a case of human diarrhea. The strain from a case of human diarrhea was positive for both hemolysin genes. Nine of 11 strains of *A. hydrophila* contained both hemolysin genes, while 2 of 11 *A. hydophila* strains lacked the *hlyA* gene. All three strains of *A. veronii* biovar sobria lacked both hemolysin genes.

Growth temperature may affect the expression of several virulence-associated factors (Gonzalez-Serrano et al., 2002). Clinical isolates (invariably isolated at 35° C) express virulence traits more frequently at 37° C; whereas, isolates from foods or the environment express virulence traits more efficiently at refrigeration temperatures (Knochel 1989; Kirov 1997). Curiously, the *A. veronii* biovar sobria strain that was *aerA* and *hlyA* negative showed enterotoxic activity in the suckling mouse assay. Wong et al. (1998) has suggested that *aerA* (aerolysin) and *hlyA* (CT-like hemolysin) must be produced together to contribute to virulence of *A. hydrophila*, PCR detection of *aerA* and *hlyA* genes (Pollard et al., 1990; Heuzenroeder et al., 1999) may not be sufficient to characterize a strain as potentially virulent, and other factors determine whether or not a strain is pathogenic (Gonzalez-Serrano et al., 2002). No criteria presently exist for classification of environmental strains as enteropathogenic.

Chacon et al. (2003) studied the distribution of virulence genes in clinical isolates from wounds (7), stools (92), abscesses (2), urine (3), sputum (1) and blood (3), and food, and environmental isolates from cake (2), fish (23), seawater (15), freshwater (83), and other sources (3). Markers included *aerA* gene, hemolysis on sheep and human blood, serine protease gene, GCAT gene, lipase gene, and DNAse gene.

Several genes have been identified that play a role in pathogenesis of aeromonads (Janda 2002). These genes encode for secreted enzymes such as lipase, hemolysin, enterotoxins, proteases, and nucleases (Pemberton et al., 1997; Heuzenroeder et al., 1999; Merino et al., 1999).

Detection of virulence genes is a crucial step in determining the potential pathogenicity of *Aeromonas* isolates (Wong et al., 1998; Heuzenroeder et al., 1999.

The aerolysin/hemolysin genes of *Aeromonas* spp. have frequently been targeted in molecular probe and PCR methods (Wang et al., 1996; Khan et al., 1998). Differences in PCR results for characterizing virulence genes results from choice of primers, though several investigators have used the primers developed by Kingombe et al. (1999) in an effort to standardize and compare results. The degree of similarity of sequences of *Aeromonas* spp. deposited in GenBank and the European Molecular Bacteriology Laboratory (EMBL) show marked variability with sequence similarity for aerolysin/hemolysin (50.2%), lipase (74.3%), DNAse (50.8%), serine protease (92.3%), and GCAT (91.9%) (Chacon et al., 2003).

Aerolysin/hemolysin genes were not detected in *A. media, A. allosaccharophila,* and *A. schubertii* (Chacon et al., 2003). Considerable discrepancies were found between presence of virulence genes and the phenotypic evidence of gene product expression (Chacon et al., 2003). Such discrepancies may result from differences in growth conditions that mediate expression of virulence genes under one set of conditions but not under other conditions. The absence of expression of one or more virulence factors may not indicate absence of virulence. No significant differences were found between presence of virulence factors among clinical and environmental strains for the two most clinically relevant species, *A. caviae*, and *A. veronii*. A statistically significant relationship between the presence of aerolysin/hemolysin and serine protease supports the role of protease in activation of aerolysin (Chacon et al., 2003).

Aerolysin/hemolysin, DNAse, and β-hemolysis were prevalent in clinical isolates (Chacon et al., 2003). DNAse was present more frequently in clinical than environmental strains. Lipases were present in both clinical and environmental strains. Aerolysin/hemolysin genes were more frequent in clinical isolates that in environmental strains. β-hemolysis was more common in clinical strains using sheep blood, but not human blood.

Sechi et al. (2002) characterized virulence factors from clinical and environmental strains of *Aeromonas* using slime, hemolysin, gelatinase, adhesin for epithelial cells, and the virulence

genes coding for the cytolytic enterotoxin, type IV pili (*tap*), bundle forming pili (*bfpA* and *bfpGH*). Patient strains had greater prevalence of hemolysin, protease, and gelatinase production, as well as greater adhesion.

7.1.1.2 Studies Characterizing Virulence Markers in Clinical Isolates

Schiavano et al. (1998) reported cytotoxic activity together with the presence of adhesins and invasins in 4 of 8 stool isolates for patients with diarrhea; however, one or more of these putative virulence factors were also found in isolates from normal patients. Hayashi et al. (2002) compared endoscopic findings in patients with enterocolitis caused by *Aeromonas* with *V. parahaemolyticus* and *Staphylococcus aureus*. *Aeromonas* infections were high in the sigmoid colon, but characteristic pathology was not observed.

Ghenghesh et al. (1999b) compared virulence factors and antibiotic susceptibility in aeromonads from children with diarrhea compared to matched normal controls in Lybia. They used hemolysis and hemagglutination as virulence traits, and isolated aeromonads from14.6% from diarrhea and 17.8% from controls. Only hemagglutinin showed a significant difference with greater prevalence in diarrheal patients.

Mokracka et al. (2001) characterized virulence factors of CT-like toxin, adhesive ability, and siderophores in clinical isolates of *A. caviae*. All isolates were adherent and produced siderophores, while 6 of 13 produced CT-like toxin. None of the isolates produced hemolysins. All isolates had high LD_{50} values in mice, which suggested that there is no relationship between pathogenicity in mice and the ability to produce gastroenteritis in humans.

Sinha et al. (2004) enriched fecal samples in APW, pH 9, incubated overnight, then inoculated onto 5% sheep blood agar with 30 mg/L ampicillin and xylose/deoxycholate citrate agar. Isolates were identified using Aerokey II (Carnahan et al., 1991b). PCR primers included *alt* (Granam et al., 1998), *ast* and *act* (Galindo et al 2005), *hlyA* (Heuzenroeder et al., 1999) and *aer* (Pollard et al., 1990). Ribotyping was performed using BglI restriction enzyme and a 7.5 kb BamHI fragment of plasmid pKK3535 as the probe. RFLP (RAPD-PCR) was used for DNA

fingerprinting. Among 78 patients with watery diarrhea, serotypes O:85, O:16: and O:83 were found most frequently, but 27 other serotypes were identified (Sinha et al., 2004). Four strains were rough, 5 were nontypable, and 1 was not available for serotyping. The presence of toxin genes in isolates from patients with diarrhea is shown in Exhibit 7.1. RFLP failed to show clonality among strains with one or more virulence factors.

Exhibit 7.1. Distribution of Toxin Genes in Clinical Isolates of *Aeromonas* spp.

Species	No. Strains	Number of Isolates with Toxin Genes (%)				
		alt	***act***	***ast***	***hlyA***	***aer***
A. caviae	74	72 (97.3)	1 (1.4)	1 (1.4)	3 (4.1)	3 (4.1)
A. veronii biovar sobria	25	12 (48.0)	21 (84.0)	2 (8.0)	6 (24.0)	1 (4.0)
A. hydrophila	35	31 (88.6)	14 (40.0)	0	31 (88.6)	10 (28.6)
A. veronii biovar veronii	9	7 (77.7)	7 (77.7)	0	0	0
A. trota	10	5 (50.0)	0	1 (10.0)	1 (10.0)	0
A. schubertii	4	3 (75.0)	2 (50.0)	0	1 (25.0)	0
A. jandaei	1	1 (100)	0	0	0	0
Unidentified	6	5 (83.3)	3 (50.0)	0	4 (66.7)	0

From Sinha et al., 2004.

7.1.1.3 Studies Characterizing Virulence Markers in Environmental Isolates

Gene disruption experiments have shown that enterotoxin Act and Alt (Chopra et al., 1994; Chopra et al. 1996; Xu et al., 1998; Sha et al., 2002), enterotoxin Ast (Chakraborty et al., 1984; Sha et al., 2002), elastase (Cascon et al., 2000a) and flagellin (Rabaan et al., 2001) are directly involved in pathogenesis in animal models and cell lines.

Ormen and Ostensvik (2001) demonstrated widespread occurrence of aerolysin-producing cytotoxic *Aeromonas* spp. in ambient water and drinking water in Norway. 79% of isolates carried the aerolysin gene and 83% were found to be cytotoxic to Vero cells.

Messi et al. (2003) screened 30 water isolates for bacteriocin-like substances. *A. hydrophila* showed antibacterial activity to one or more indicator bacteria or pathogens. These data support the empirical finding of inhibition of indicator bacteria in coliform tests.

Schubert (2000) examined 650 isolates from surface water and water treatment plants for adhesion and maximum growth temperature. INT-407 cells and 39º C were used to determine potential pathogenicity of environmental strains. Clinical strains grew at 39º C and adhered; while most environmental strains lacked the ability to grow at 39º C or adhere to cells. Strains encountered in drinking water are very unlikely to cause gastrointestinal disease.

Balaji et al. (2004) determined the cytotoxicity of 39 environmental isolates. All isolates produced cytotoxin in Vero cells.

Bondi et al. (2000) constructed virulence and antibiotic resistance profiles of 30 *A. hydrophila* environmental isolates. Hemolysin, cytotoxin, and adhesive capacity were use as virulence factors. Isolates were positive for adherence (70%), hemolysin (87%), and cytotoxin (100%).

Sen and Rodgers (2004) characterized the presence of six virulence factors in *Aeromonas* spp. isolated from U.S. drinking water supplies using PCR for detection of elastase (*ahyB*), lipase (*pla/lip/lipH3/alp*-1), flagella A and B (*flaA, flaB*), and enterotoxin (*act, alt, ast*) genes. Genes

were present in 88% (*ahyB*), 88% (*lip*), 59% (*fla*), 43% (*alt*), 70% (*act*), and 30% (*act*) of strains. Only 1 of 205 isolates tested had all virulence genes. In the work of Sen and Rodgers (2004), 67% of isolates had at least four virulence genes, and 100% of isolates had at least one gene. Only 1 strain of *A. hydrophila* HG-1 had all three toxin genes *act*, *alt* and *ast*, and this isolate had all virulence genes. The combination of virulence factors that would be required to predict infectivity remains to be determined because strains expressing multiple putative virulence factors have been isolated from asymptomatic individuals.

Hanninen and Siitonen (1995) used a combination of phenotypic methods and ribotyping to classify 79% of water isolates. Many environmental strains have not been assigned to a hybridization group and new species continue to be recognized.

Martinez-Murcia et al. (2000) used RFLP of PCR amplified intergenic spacer region between the 16S and 23S rRNA genes to demonstrate colonization of a public water supply with a particular strain of *A. veronii*. The method could be used to trace strains during epidemiological investigations of foodborne and waterborne outbreaks.

Rahim et al. (2004) characterized environmental isolates using an *act* gene probe to evaluate cytotoxic enterotoxin potential. 32 of 69 environmental isolates had the *act* gene. Isolates were examined for enterotoxigenicity by suckling mouse assay, hemolytic activity on blood plates, CAMP-like factor and cytotoxicity in Vero cells. The CAMP-hemolysis phenotype was significantly associated with rabbit ileal loop assay, vero cell assay, and hemolysin production suggesting that the CAMP-hemolysin assay can be used to evaluate enterotoxin production potential of aeromonads.

Mateos et al. (1993) reported that environmental strains of *Aeromonas* were inhibitied at 37° C and production of hemolytic and cytotoxic activity decreased at this temperature. All environmental isolates of *A. hydrophila* were pathogenic for trout while only 44% of human strains caused disease in fish.

Demarta et al. (2000) used ribotyping to show the same strain in ill and well family

members, suggesting host factors determined susceptibility and expression of disease. The same ribotype was isolated in the drinking water supply in the same region but not in samples from the household distribution system. Others (Moyer et al., 1992b; Hanninen and Siitonen 1995; Borchardt et al., 2003) have been unable to demonstrate strain similarity between patient and environmental isolates. Moyer reported the first known case where the same ribotype was isolated from a patient and the household water supply at the 7th International Symposium on *Aeromonas* and *Plesiomonas*, Orihuela, Spain in September 2002.

7.1.1.4 Studies Characterizing Virulence Markers in Food Isolates

Many investigators have characterized food isolates for their ability to produce putative virulence factors (Kumar et al., 2000b; Kingombe et al., 2004). *Aermonas* spp. exhibit a high degree of genetic heterogeneity. When PFGE was used to characterize a group of cultures isolated from ready-to-eat foods, 24 unique patterns were detected in 27 strains of *A. hydrophila* and 20 unique patterns were detected in 23 strains of *A. caviae*.

Tsai et al. (1997) evaluated hemolysin and cytotoxin production at various temperatures and determined that *A. hydrophila* produced both virulence factors at 5º, 28º and 37º C; however, the activity was greater when cultures were incubated at 28º C. Toxin production may occur in meats at refrigerator temperature (Majeed and MacRae 1991). Kirov et al. (1993a) studied virulence factors in strains isolated from milk, and concluded that aeromonads could grow to high numbers in refrigerated milk without spoilage. Most milk isolates were not enterotoxigenic; however, a strain of *A. sobria* from milk produced hemolysins, cytotoxins, and enterotoxin.

Strains grown at low temperature have enhanced adhesin production as the result of increased production of pili (Kirov et al., 1993b). Studies suggest that the presence of pre-formed enterotoxins in foods are of little concern, since toxin production is considerably reduced in food products compared to the amounts of toxins produced in laboratory media. This observation explains why so few foodborne outbreaks are attributed to *Aeromonas* spp. despite their presence at high numbers in foods.

Alcides et al. (2003) isolated *Aeromonas* spp. were isolated from parsley and watercress from street markets in Brazil. Strains expressed virulence factors and antibiotic resistance to trimethoprim (39%) and gentamicin (21%). Cell populations remained constant for 7 days at 5º C. *A. caviae, A. hydrophila*, and unidentified aeromonads were isolated. Vegetables may pose a risk factor to immunocompromised populations when they are eaten raw.

Of 141 food isolates examined for virulence factors, 66% produced cytolytic enterotoxin, as did 67% of clinical isolates; however, 58% of environmental isolates also produced enterotoxin (Kingombe et al., 1999). Wang and Silva (1999) reported that 86% of catfish isolates were hemolytic.

Okrend et al. (1987) demonstrated the capability of isolates from meats to produce toxins, and Tsai and Chen (1996) reported toxigenic strains isolated from seafood.

Phospholipase and proteases are thought to be responsible for invasive properties (Ishiguro et al., 1985). Hemolysins (Santos et al., 1999), enterotoxin and cytotoxin (Wong et al., 1998), and adherence factors (Neves et al., 1994) all have virulence-enhancing potential. The complex multifactorial pathogenic mechanisms in aeromonads are not completely understood.

Yadav and Kumar (2000) isolated 5 (5%) *Aeromonas* strains from 100 clinical specimens and 40 (14%) *Aeromonas* strains from 285 food samples (fish, milk and ice cream). *A. hydrophila* (9), *A. sobria* (6) and *A. caviae* (1) were identified from foods. *A. hydrophila* (2) and *A. sobria* (3) were isolated from stools. Enterotoxigenicity was shown in 48.8% to 57.5% of food isolates and 60% of stool isolates.

Kingombe et al. (2004) surveyed 78 meats and 123 processed, ready-to-eat foods for *Aeromonas* spp. containing virulence genes for cytotoxic enterotoxin and hemolysin by using conventional culture and a newly developed PCR method for direct detection of virulence genes in food samples. Using culture methods, 32.3% of samples contained aeromonads, while PCR detected target genes in 25.4% of samples. Fatty acid methyl ester (FAME) analysis and amplified fragment length polymorphism (AFLP) were used to determine the hybridization group

for 97% of isolates. HG-3 (50%) and HG-8/10 (38%) were most frequently isolated. The combination of PCR-RFLP and PCR-amplicon sequence analysis (PCR-ASA) allowed classification of strains to hybridization group, thereby providing a sensitive, specific system for assessment of aeromonads harboring virulence factors in food samples.

Nishikawa and Kishi (1988) isolated *Aeromonas* spp. from 11.1% of diarrheal stools compared to 2.2% of normal stools. Among food isolates, *A. hydrophila* and *A. sobria* predominated, while *A. caviae* was most common in seafood and vegetables. The common occurrence of aeromonads in foods suggested that the primary source of exposure may be foodborne rather than waterborne. Stool, food and water isolates all produced putative virulence factors, and differentiation of pathogenic from non-pathogenic strains was not possible from *in vitro* tests.

Hanninen et al. (1997a) isolated aeromonads from fish, fish eggs, shrimp, and water to determine the distribution of genomospecies in these samples. Hybridization groups, HG-2 and HG-3, were found most frequently in all samples; however, no strains of HG-2 were found in fish eggs. Twenty-six *Aeromonas* strains are suspected of being associated with foodborne illness. They include HG-1 (2), HG-2 (7), HG-3 (3), HG-7 (7), HG-8/10 (2), and 5 untyped strains. Strains of HG-1 were not found in market fish, fish eggs, shrimp, or fresh water samples. Strains isolated from suspected cases of diarrheal illness typically belong to HG-1 (*A. hydrophila*), HG-4 (*A. caviae*), or HG-8/10 (*A. veronii* biovar sobria). Because HG-2 and HG-3 are uncommon in diarrheal stools, their role in causing diarrhea is unclear.

Karabasil et al. (2002) examined *Aeromonas* isolated from fish and other seafood from retail markets in Hungary for their ability to produce toxins associated with virulence. Nine isolates were recovered from 78 samples, 6 *A. hydrophila* and 3 *A. sobria*. All 9 strains produced varying amounts of cytotoxin in Vero cell cultures. Because enrichment broth was used for primary isolation, it was not possible to quantitatively determine the number of organisms per gram of sample.

Quantitative culture revealed no significant differences between the number of

aeromonads in ambient water and on the skin, gills, or visera for fish at the time they are caught (Gonzalez et al., 2001). Aeromonads grow on fish skin and visera during storage on ice. Counts ranged from 2-5 $\log_{10}$ CFU/g with intestinal contents demonstrating the higher counts. Bacterial counts increased 2-3 $\log_{10}$ CFU/g during iced storage up to 14 days. *A. hydrophila*, *A. veronii* biovar sobria, *A. caviae*, *A. jandaei*, *A. schubertii*, *A. bestiarum*, and the unnamed hybridization group HG-11 were isolated. The diversity of strains was representative of the degree of water pollution. *Aeromonas* spp. contribute to spoilage of iced freshwater fish, and may pose a public health concern for fish handlers. Cooking is sufficient to protect persons ingesting fresh fish.

Martins et al. (2002) examined a series of clinical and food isolates for production of enterotoxin, hemolysin and cytotoxin, and the results are summarized in Exhibit 7.2. These results are consistent with those of other current investigators; however, others have reported higher correlation between hemolysin and cytotoxin production when hemolysin was detected using rabbit erythrocytes. Methods for determining hemolytic and cytotoxic activity of aeromonads has not been standardized, and cautious comparisons of between published reports are advised.

Exhibit 7.2. Putative Virulence Factors Produced by *Aeromonas* spp. Isolated from Clinical and Food Sources

Source	Presence of Virulence Factor		
	Hemolysin	Cytotoxin	Enterotoxin
Clinical Specimens	43.1%	89%	29.4%
Foods	17.1%	72.7%	18.2%

From Martins et al., 2002

A collection of 107 isolates from ready to eat meats and cheese in Italy were examined for cytotoxin (Montagna et al., 1998). Cytotoxin activity (Vero cells) was demonstrated in all isolates, but cytotoxin activity of *A. hydrophila/caviae* strains was temperature dependent, with 100% cytotoxity at 5º C but 98.2% cytotoxicity at 31º C.

Tsai and Chen (1996) cultured 66 seafood samples and reported that 17 (25.8%) were contaminated with *Aeromonas* spp. *Aeromonas hydrophila* was cultured from 50% of oysters (3.2-5.8 $\log_{10}$ CFU/g), 22.2% of fish fillets (1.9-3.9 $\log_{10}$ CFU/g), and 14.3% of shrimp (2.1-2.7 $\log_{10}$ CFU/g). No *Aeromonas* spp. were isolated from 12 fish ball samples. From this survey, 48 isolates were examined for hemolysin and cytotoxin. Hemolysin was found in 79.2% of isolates

(n = 38) and 91.7% of isolates (n = 44) produced cytotoxin. These isolates grew best and virulence factors were produced at higher titers at 28º C rather than at 37º C.

Scoglio et al. (2001) examined 18 strains of *Aeromonas* isolated from foods for presence of virulence factors including caseinase, lipase and lecithinase, DNAse mucinase, elastase, hemolysin, cytotoxin, and adherence. Most *A. hydrophila* (7 strains) and *A. caviae* (11 strains) produced caseinase, lecithinase, lipase, and DNAse, while all strains were negative for elastase and mucinase. All but 3 strains of *A caviae* were cytotoxic in either Vero or HEp-2 cells. Adherence was demonstrated in 11 strains. Strains with multiple virulence factors are found in seafoods that may have the potential to cause gastrointestinal disease.

7.1.1.5 Studies Characterizing Virulence Markers in Animal Isolates

Boynukara et al. (2004) characterized *A. sobria* strains from animal feces using SDS-PAGE. Turkish van cat, chicken, gull, and trout strains were included in the study.

7.2 Risk Assessment Case-Control Studies

Hellard et al. (2001) conducted a double-blinded epidemiological study to determine the contribution of drinking water to the incidence of gastrointestinal disease in Melbourne, Australia. They found no evidence of waterborne disease, even though the water supply was drawn from a forest catchment and the chlorination was the only treatment. A similar study conducted in the U.S. by Colford et al., (2005). The U.S. study showed no increased risk of acquiring gastrointestinal infections from exposures to treated drinking water.

7.2.1 Laboratory Studies

Nzeako and Okafor (2002) studied patients with diarrhea in Nigeria during the rainy season between April and October. Found *A. hydrophila* in 13% of patients with the highest recovery in children age 6-10 years (6%), followed by children age 11-15 years (3.6%). The asymptomatic carriage rate was 1%. Food and drinking untreated water were thought to be the

sources of *Aeromonas* spp.

The distribution of *Aeromonas* spp. isolated in Bangladesh are shown in Exhibit 7.2, and the presence of toxin genes in clinical and environmental isolates is shown in Exhibit 7.3.

Exhibit 7.2. Distribution of Aeromonas Strains from Various Sources in Bangladesh

Source	No. Isolates	Number of Isolates (% Positive)								
		A. hydrophila	*A. bestiarum*	*A. veronii biovar sobria*	*A. caviae*	*A. media*	*A. eucrenophila*	*A. trota*	*A. jandaei*	*A. species (unknown)*
Diarrheal children	115	11 (9.6)	3 (2.6)	21 (18.3)	39 (33.9)	14 (12.2)	1 (0.9)	25 (21.8)	0 (0.0)	1 (0.9)
Control children	27	1 (3.7)	0 (0.0)	4 (14.8)	11 (40.7)	1 (3.7)	1 (3.7)	6 (22.2)	1 (3.7)	2 (7.4)
Environment	120	18 (15.0)	0 (0.0)	16 (13.3)	7 (5.8)	2 (1.7)	0 (0.0)	72 (60.0)	4 (3.3)	1 (0.8)
Total	262	30 (11.5)	3 (1.2)	41 (15.7)	57 (21.8)	17 (6.5)	2 (0.8)	103 (39.3)	5 (1.9)	4 (1.5)

From Albert et al., 2000

Exhibit 7.3. Presence of Toxin Genes in Diarrheal Children, Control Children and the Environment

Source	No. Isolates	*alt*	*ast*	*alt + ast*
Diarrheal children	115	19 (16.5)	18 (15.7)	65 (55.7)
Normal children	27	9 (33.3)	7 (25.9)	6 (22.2)
Environment	120	20 (16.7)	36 (30.0)	40 (33.3)

From Albert et al., 2000

Isolates with both the *alt* and *ast* genes were from patients with watery diarrhea; while isolates with only the *alt* gene came from patients with loose stools (Albert et al., 2000). *A. caviae* was found most frequently; however, loose or watery stools also occurred in patients whose isolates were *A. veronii* biovar sobria, *A. media*, or *A. hydrophila*.

7.2.2 Studies in Younger Children

In a case-control study in Bangladesh in children under the age of five years, potential bacterial pathogens were isolated from 74.8% of symptomatic children and 43.9% of controls Albert et al., 1999). *Aermonas* infection did not show seasonality as in temperate climates. *Aeromonas* spp. were isolated from 99 (12.2%) sick individuals and 44 (5.4%) healthy individuals (P value 0.0001) (Albert et al., 1999). *Aeromonas* infections were more common during the first two years of life. These results differ from the findings in Thailand where no association between aeromonds and diarrhea was found (Echeverria et al., 1989).

Dallal and Moezardalan (2004) conducted a case-control study of diarrhea in children in Iran. Isolation rate was significantly higher from cases than controls. Aeromonads were isolated from 14 of 310 (4.5%) of cases of diarrhea in children.

7.2.3 Field Studies

Studies of military troops deployed in various regions of the world have not shown significance incidence of gastrointestinal disease caused by aeromonads related to their deployment (Haberberger et al., 1991; Oyofa et al., 1995). Joseph (1996) compiled the available data on gastrointestinal disease causation for *Aeromonas* spp. and found widely differing rates of asymptomatic carriage. He concluded that while some strains of aeromonads are undoubtedly capable of causing diarrhea in susceptible hosts, the factors contributing to their pathogenesis are not sufficiently characterized to permit reliable predication based upon presence and expression of known virulence factors.

7.2.4 Animal Studies

Pretreatment of experimental animals with antibiotics has been suggested to render them susceptible to gastrointestinal infection following intragastric inoculation with *Aeromonas* spp. (Haberberger et al., 1991). Kelleher and Kirov (2000) found that while intestinal colonization occurred, pathology characteristic of *Aeromonas* infection was absent in Norwegian rats. The clindamycin-treated rat model is not suitable for studies on the pathogenesis of aeromonads, and no other animal model has been identified.

Krzyminska et al. (2001) reported serial passage in mice-enhanced virulence of *A. caviae* isolated from diarrheal patients. Adhesion and siderophore properties increased, and the LD_{50} decreased with some strains. An increase of cross-reactive cholera-like toxin occurred with serial passage in mice. These studies confirm that *A. caviae* strains may be virulent, and demonstrate the adaptive nature of pathogenic aeromonads. The host factors responsible for pathogenic enhancement were not identified. These data suggest that human-to-human transmission of *A.*

caviae would likely result in more severe gastrointestinal infections that would occur when environmental starins are ingested.

7.2.5 Typing Studies

Demarta et al. (2000) showed that strains isolated from the household environment matched strains isolated from stools of children age 4 months to 6 years with gastroenteritis by using ribotyping. It was not determined whether the patients contaminated the environment or vice versa. The high rate of asymptomatic carriers and the wide distribution of potentially pathogenic strains in the environment suggest that host susceptibility factors determine the course of infection.

7.3 Risk Factors

Aeromonas spp. are infrequently cultured from treated drinking water. When detected, they are usually present at concentrations < 10 CFU/100 mL. Concentrations in groundwater are also low, typically < 1 CFU/100 mL; however, wells and associated distribution systems may become colonized, and well water may contain > 200 CFU/100L (Holmes et al., 1996).

Kilb et al. (2003) found that aeromonads colonizing rubber-coated valves in drinking water distribution systems were released into drinking water at a cell density of 17 CFU/cm^2. However, sloughing of biofilms of this cell density are unlikely to constitute a public health risk of infection, considering humans regularly ingest foods containing aeromonads at 2-5 $\log_{10}$ CFU/g without ill effects.

Ambient water has been shown pose a risk of food contamination. Pianetti et al. (2004) found *Aeromonas* spp. in 14 of 52 samples of irrigation water in Italy at concentrations from 2 to 4 $\log_{10}$ CFU/mL. This suggests that irrigation water may be a source of contamination for vegetables, and that because some strains produced virulence factors, they could serve as a source of infection to humans.

7.4 Potential for Human Exposure

Point-of-use (POU) devices on household taps increased the number of bacteria ingested in drinking water due to amplification in activated carbon (Chaidez and Gerba 2004). *Aeromonas* was rarely isolated and when found, it was present in low numbers (10 CFU/mL in tapwater; 29.5 CFU/mL in POU effluent). There is no evidence to implicate *Aeromonas* as a cause of illness from consumption of treated water (van der Kooij 2003).

Because aeromonads are capable of growing to large numbers in foods, it is theoretically possible that food-poisoning could result from colonization of the intestinal tract or from ingestion of pre-formed toxins. However, experience provides little evidence for the role of pre-formed toxins in causing gastroenteritis following ingestion of foods contaminated by aeromonads (Kirov 1993a). Many strains that grow at low temperatures and produce enterotoxin in culture media do not grow at 37° C, so they pose little threat to humans (Todd et al., 1989).

Since aeromonads are sensitive to heat and most food preservation methods, recontamination after cooking is suspected as the cause for outbreaks resulting from ingestion of cooked products (Marino et al., 1995). Ingestion of raw foods and untreated water remain the primary modes of transmission for aeromonads causing foodborne and waterborne illness.

Host factors seem to be related strongly to diarrheal disease caused by aeromonads (Schiavano et al., 1998). While humans are constantly exposed to aeromonads from various environmental sources, manifestations of disease occur infrequently and only in persons with predisposing conditions.

7.5 Risk Management

Occurrence and growth of aeromonads in drinking water distribution systems is dependent upon access from ambient waters, temperature, and nutritional parameters conducive to growth, and presence of a disinfectant residual to suppress microbial populations. *Aeromonas* spp. are rarely detected in distribution system water below 14° C and with free chlorine residuals above

0.2 mg/L. Limitation of available organic carbon restricts nutrient thereby limiting growth potential.

Factors for control of access and growth of aeromonads include:

- operating filter beds at optimal efficiency;
- maintaining a free chlorine residual of at least 0.2 mg/L in all parts of the distribution system;
- monitoring and maintaining the microbiological quality of GAC used in treatment processes;
- control of biofilms by preventive maintenance, e.g. hydrant flushing; and
- ensuring disinfection during maintenance and new construction.

These actions will ensure adequate treatment to minimize the opportunity for release of aeromonads into distribution water and to prevent colonization of biofilms.

7.6 Summary

Differentiation of pathogenic from non-pathogenic strains of *Aeromonas* spp. is difficult to impossible because the presence of traits typically associated with virulence are widespead among environmental strains (Thornley et al., 1997). Several authors have suggested that the presence of aeromonads capable of producing virulence factors in drinking water is a threat to public health (Bondi et al., 2000; Handfield 1996; Kuhn et al., 1997). However, the lack of correlation between presence of virulence factors and the ability of strains to cause gastroenteritis in humans suggests that the potential is not realized in any epidemiologically meaningful way. Demonstration of virulence genes is not sufficient evidence to ascribe pathogenicity since Pin et al. (1995) and Schiavano et al. (1998) have found virulence genes in isolates from healthy humans.

The presence of genes does not correlate with expression of gene products (Bondi et al., 2000). The presence of virulence factors does not correlate with manifestations of disease in

animals or humans (Bondi et al., 2000). There is no predictive value in genetic, molecular, or phenotypic characteristics for *Aeromonas* (Bondi et al., 2000). There is no significant correlation between hybridization group and virulence properties of individual strains (Marino et al., 1995).

The public health significance of finding virulence genes in isolates from drinking water must been interpreted carefully since the relationship between the presence of virulence factor genes and the ability of these strains to cause disease has not been firmly established (Sen and Rodgers 2004). Host factors may play an important role in determining susceptibility to infection (Sen and Rodgers 2004).

Various types of foods are considered possible sources of potentially pathogenic aeromonads (Abeyta et al., 1986; Altwegg et al., 1991a). Unfortunately, no reliable methods presently exists for differentiation of pathogenic and non-pathogenic strains that are predictive for the ability of a strain to produce gastrointestinal disease in humans (Kingombe et al., 2004). Because differences between clinical strains have not been demonstrated (Kingombe et al., 1999), the presence of aeromonads in foods carries potential public health risk to susceptible hosts.

The vast diversity among aeromonads suggests that predictive models based upon a few strains are not necessarily reliable (Merino et al., 1995). This was certainly the case in the human feeding study of Morgan et al. (1985), where high number of aeromonads failed to produce illness by ingestion.

Application of the ILSI model to risk assessment of *Aeromonas* spp. results in estimates of potential risk if infection from various sources that are unsupported by clinical and epidemiological data.

8.0 Water Treatment

Aeromonads have been reported in water distribution systems around the world (Havelaar et al., 1987; van der Kooij 1988; Hanninen and Siitonen 1995; Huys et al., 1997). Regrowth in distribution system water has been demonstrated to be a function of water temperature and

availability of assimilable nutrient. Aeromonads are known to colonize the surfaces in distribution system producing biofilms (Burke et al., 1984a). Most drinking water treatment process are capable of reducing the density of aeromonads from 3-4 $\log_{10}$ CFU to less than 1 CFU/100 mL (WHO 1996).

8.1 Conventional Water and Wastewater Treatment

In a survey of water treatment plants in Belgium, Meheus and Peeters (1989) found that the mean reductions for stages of treatment was 30-60% for flocculation/sedimentation, 70-90% for rapid sand filtration, 80-90% for GAC, and 98-100% for slow sand filtration. Hyperchlorination in conjunction with direct filtration removed 99-100% of aeromonads.

Aeromonas cell densities in surface water reach 3-4 $\log_{10}$ CFU/100mL in summer months in Indiana (Chauret et al., 2001). Counts from water filtered through GAC ranged from < 1 to 490 CFU/100 mL. Aeromonads were not detected in treated water plant effluent, however *A. hydrophila* was detected in 7.7% of biofilm samples from the distribution system. The issue of regrowth vs. distal contamination as the source of aeromonads in biofilms is difficult to assess.

El Taweel and Shaban (2001) sampled eight water treatment plants in Egypt for removal of indicator bacteria and *Aeromonas*. The number of aeromonads at plant intakes (raw water) ranged from 2.26-5.5 $\log_{10}$ CFU/100 mL. The number of aeromonads in finished water ranged from below detection limit to 47 CFU/100 mL in two or eight plants (mean 7-11 CFU/100 mL) and no aeromonads were detected in finished water produced by the other six plants. Because aeromonads exeeded coliforms in raw water contaminated with sewage, they are considered pollution indicators that are complementary to coliform monitoring (El Taweel and Shaban 2001).

8.1.1 Slow Sand Filtration

Slow sand filtration effectively removes 98-100% of aeromonads from raw water (Meheus and Peeters 1989).

8.1.2 Activated Sludge Plant/Trickling Filter Plant

Aeromonas spp. are present in raw sewage and the effluent of wastewater treatment plants (Stecchini et al., 1994). Overall reduction of aeromonads varies by treatment process, with 93% reduction in stabilization ponds (Monfort and Baleux 1990; Boussaid et al., 1991), and 99.9% reduction in activated sludge plants (Poffe and Op de Beeck 1991), and 98% in trickling filter plants (Poffe and Op de Beeck 1991). *A. caviae* is the dominant aeromonad in raw sewage and sewage effluents (Stecchini et al., 1994), but *A. hydrophila* has been found at 6 $\log_{10}$ CFU/g in partially dried sludge (Poffe and Op de Beek 1991). Ramteke et al. (1993) observed high levels of *A. caviae* in sewage pollution river water, and they proposed *A. caviae* as an indicator of fecal pollution.

8.1.3 Treatment Plants and Distribution Systems

Holmes and Nicolls (1995) and Chauret et al. (2001) reported the presence of *Aeromonas* in pipe biofilms in drinking water supplies. Biofilms are defined as a consortium of microorganisms within extensive polymer matrices (Costerton et al., 1987). Organisms forming biofilms typically produce extracellular polysaccharides and accumulate in hydrated structures on surfaces. It is estimated that 99% of all bacteria in natural environments exist in biofilms. The dynamic nature of biofilms suggested that a mechanism for cell-to-cell communication termed "quorum sensing" may exist to regulate attachment and maturation of biofilms (Williams and Stewart 1994). In many bacteria, N-acylhomoserine lactone (AHL) interacts with a sensor regulator of transcription. *A. hydrophila* possesses the AHL-dependent quorum-sensing system as an expression of the *ahyRI* locus (Lynch et al., 2002). Sha et al. (2005) showed that aeromonads used quorum sensing and that over-production of AHL was associated with reduced virulence.

Huys et al. (1995) showed that water plant isolates were dominated by *A. hydrophila* (38.8%), *A. caviae* complex (22.7%), and *A. sobria* complex (16.7%), with HG-3, HG-5A/B, and HG-8 as the representative genomospecies.

MacDonald and Brozel (2000) evaluated biofilm production in a simulated recirculating

cooling water system using fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for *Pseudomonas*, *Shewanella*, *Aeromonas*, *Proteobacteria*, and *Eubacteria*. Aeromonads were not detected in resulting biofilms. While presence of aeromonads in distribution system biofilms has been demonstrated, the conditions leading to their colonization are unknown.

Kilb et al. (2003) examined biofilm formation on rubber-coated valves in drinking water distribution systems. *Citrobacter* spp. were determined to be the predominant bacteria colonizing valves. However, in the 21 biofilms samples examined, 2 contained *Aeromonas* spp., 1 contained *A. hydrophila* at a cell density of 17 CFU/cm^2, and 1 had an unidentified *Aeromonas* spp.

Aeromonas spp. adhere to distribution system material such as stainless steel, copper, polybutylene after exposures as short as 1-4 hours (Assanta et al., 1998). Development of a mature biofilm is controlled through quorum sensing, a chemical signaling system by which bacteria poll cell density and regulate the expression of genes at high cell densities (Kirov 2003; Sha et al., 2005).

8.1.4 Rapid Gravity Filters

Rapid gravity filters remove 70-90% of aeromonads from raw water, while hyperchlorination in conjunction with filtration results in 99-100% reduction (Meheus and Peeters 1989).

8.2 Disinfection/Inactivation

Aeromonas spp. are susceptible to free chlorine at concentrations used in water treatment, though failure to maintain a chlorine residual in the distribution system may result in growth of aeromonads (Burke et al., 1984a; van der Kooij and Hijnen 1988).

Several studies on response to disinfection have been published (Cattabani 1986; Knochel 1991; Ozbas and Aytec 1994). Environmental strains resemble *E. coli* in their response to chlorine exposure at 0.5 mg/L; however, strain differences in chlorine response have been

demonstrated. Chlorine activity was higher at pH 6 than at pH 8. Some strains survive treatment at 0.3 mg/L (Cattabani 1986) and at 0.5 mg/L (Ozbas and Aytac 1994), while Ozbas and Aytac (1994) reported survivers at chlorine concentrations up to 2.5 mg/L after 30 min. exposure.

Chlorine concentrations of 0.1, 0.2 and 0.5 mg/L at pH 6,7, and 8 and temperature of 4, 21, and 32º C were used to determine disinfection response of *Aeromonas* spp. Strains isolated from the environment were inactivated within 1 min., but strains isolated from treated water distribution systems demonstrated less sensitivity to chlorine than the other strains.

Aeromonads are isolated from chlorinated waters, including distribution system water where colifoms counts are below the detection limit, leading to the suggestion that they are moderately resistant to chlorine (LeChevallier et al., 1983). Later studies do not support that aeromonads are any more resistant to chlorine than other waterborne bacteria, e.g. *E. coli*, *Pseudomonas* spp. *Acinetobacter* spp., etc. Knochel (1991) reported that aeromonads had a slightly higher susceptibility to combined chlorine compared to other water bacteria. Cattabiani (1986) reported that aeromonads were more resistant to chlorine that *Vibrio* spp. Three of four strain of aeromonas were sensitive to chlorine at 0.625 mg/L at pH 6.5 and temperature of 25º C. All aeromonads experienced >4 $\log_{10}$ reduction in 1 min. at a chlorine concentration of 5 mg/L (Palumbo and Buchanan 1988).

Because *Aeromonas* spp. are pathogenic for fish, their presence in aquaculture waters is of great economic concern (Bomo et al., 2003). These investigators used slow sand filtration to remove and inactivate *A. hydrophila* and other bacteria in fish pond effluents. A bacterial load of 8 $\log_{10}$ CFU/mL was applied to course and fine sand filters, and filter effluents were cultured to determine recovery. For course sand filters, a 4 $\log_{10}$ removal occurred on day 1, gradually decreasing to 3 $\log_{10}$ removal on day 3, followed by a progressive increase in removal to 5 $\log_{10}$ by day 7, and 6 $\log_{10}$ by day 15. For fine sand filters, a 4 log removal occurred between day 1-4, followed by a increase in removal to 6 $\log_{10}$ by day 7, and 7 $\log_{10}$ by day 15. Sand filtration appears to be an effective means of removing aeromonads from surface waters during water treatment.

Lee and Deininger (2000) used ozonation to reduce the heterotrophic plate count (HPC) of raw water from $1.33x10^5$ to 13 CFU/mL after treatment. The average reduction was 2.6 $\log_{10}$ following ozonation. When GAC was use after ozone treatment, the HPC increased to the levels of raw water. Gram-positive bacteria and mycobacteria survived ozone treatment better than gram-negative bacteria. Since aeromonads are gram-negative bacteria that comprise a significant proportion of the HPC of raw water, aeromonads would be susceptible to ozonation.

8.2.1 Disinfection

Gavriel et al. (1998) studied the response of *Aeromonas* to chlorine and chloramine disinfection and showed that *Aeromonas* spp. are more susceptible to chlorination than *E. coli*. The reported inactivation studies by various treatments are shown in Exhibits 8.1 to 8.3.

Exhibit 8.1. Inactivation of *Aeromonas hydrophila* by UV Irradiation

Strain	Water	UV dose (mWs/cm^2)	Reduction (%)
A. hydrophila	PBW	- 2.5	99
A. hydrophila	ND	3	90
A. hydrophila	ND	8	99

From Gerba et al., 2003
PBW, phosphate buffered water, ND, no data

Exhibit 8.2. Inactivation of *Aeromonas hydrophila* by Chlorine Dioxide

Strain	Water	ClO_2 (mg/L)	Temp (º C)	pH	Time (min)	Reduction (%)	CT_{99} (mg-min/L)
Ah M800	BDF	0.2	2 " 2	8	ND	ND	0.11-0.14
Ah M800	BDF	0.2	2 " 2	8	ND	ND	0.04-0.14

From Gerba et al., 2003
Ah, Aeromonas hydrophila; BDF, buffer demand free; ND, no data

Exhibit 8.3. Inactivation of *Aeromonas hydrophila* by Chlorine

Strain	Water	Free Chlorine (mg/L)	Temp (º C)	pH	Time (min)	Reduction (%)	CT_{99} (mg-min/L)
Ah TW11	CDF	0.2	21	6	7	99	1.4
Ah TW27	CDF	0.2	21	6	1	99	0.2
Ah 10693H	SDW	0.14	10	ND	5	99.999	ND
Ah 10693H	SDW	0.55	20	ND	5	99.999	ND
Ah 10693H	SDW	0.60	30	ND	5	99.999	ND
Ah 10693H	SDW	0.52	37	ND	5	99.999	ND
Ah clinical	Tap	0.05	5	7.8	20	90	1.0
Ah clinical	Tap	0.3	5	7.8	20	90	6.0

From Gerba et al., 2003
Ah, *Aeromonas hydrophila*; CDF, chlorine demand free; SDW, sterile distilled water; ND, no data

8.2.2 Inactivation

All treatments currently used by public drinking water supplies effectively control aeromonads in distribution water to a level that does not represent a threat to public health.

8.2.3 Distribution Systems

Municipal drinking water supplies may contain aeromonads in relative high levels, particularly in the distal ends of the system (Havelaar et al., 1990). Concentrations from <1 to 860 CFU/100 mL have been reported (Knochel and Jeppesen 1990; Havelaar et al., 1990). *Aeromonas* counts are higher in summer months and virtually undetectable with water temperatures drop below 14º C (Holmes et al., 1996). Control of aeromonads in distribution water may be achieved by controling biofilm production and maintaining a disinfectant residual.

Aeromonas hydrophila was placed on the EPA Contaminant Candidate List in 1998, establishing it as a priority for Agency drinking water research, including assessment of

occurrence in public drinking water supplies under the Unregulated Contaminant Monitoring Regulation (UCMR) (USEPA, 1999; USEPA, 2002). Under the UCMR Screening Survey, 293 distribution systems across the United States were monitored for aeromonads over a twelve month period using EPA Method 1605 for sample analysis (Best and Frebis, 2005). Other analyses conducted concurrently with detection and enumeration of aeromonads included pH, water temperature, turbidity, and free and total disinfectant residual. Aeromonads were detected in 14% of samples, with water systems serving more than 10,000 customers having a detection rate of 11% and water systems serving less than 10,000 customers having a detection rate of 17%, suggesting that aeromonads may be present more frequently in smaller water systems. Altogether, 32 of 42 (76%) water supplies observed 3 or fewer detections out of 18 total samples collected. In this study, 78% of positive samples contained 10 CFU/100 mL or less. No marked seasonal variation was observed. Aeromonads were more likely to occur in systems using ground water, rather than those using surface waters. No apparent differences were found in detection rates in high flow vs. low flow parts of the distribution systems, and there was no correlation between detection of aeromonads in areas of the distribution systems and low disinfectant residual. While aeromonads occur in drinking water distribution systems, there have been no reported outbreaks of waterborne disease associated with aeromonads.

8.2.4 Survival

Water temperature, available nutrient and disinfection residual affect the regrowth potential of *Aeromonas* in drinking water distribution systems (Burke, et al., 1984a; Holmes et al., 1996). *A. hydrophila* can survive and establish biofilm up to 0.6 mg/L of monochloramine (Mackerness et al., 1991). Holmes et al. (1996) reported that aeromonads persisted in 10% of pipe lengths after disinfection with 1 mg/chlorine. Freely suspended cells are susceptible to this level, but elevated levels are required to destroy *Aeromonas* in biofilm.

Bomo et al. (2004) investigated biofilm formation on stainless steel, polyvinyl chloride and glass surfaces using recycled water systems, and PVC an stainless steel using potable water systems. Biofilm formed on all materials at 1-2 $\log_{10}$ CFU/cm^2. Culture methods proved unreliable for detecting aeromonads in water samples, suggesting that molecular methods may be

required in addition to culture methods for characterization of *Aeromonas* populations in distribution systems. None of the aeromonads were identified as potential human pathogens. The public health implications of aeromonads in drinking water distribution systems are unknown.

8.3 Summary

Aeromonads have been reported in water distribution systems around the world (Havelaar et al., 1987; van der Kooij 1988; Hanninen and Siitonen 1995; Huys et al., 1997). Most drinking water treatment processes are capable of reducing the density of aeromonads from 3-4 log_{10} to less than 1 CFU/100 mL (WHO 1996). In a survey of water treatment plants in Belgium, Meheus and Peeters (1989) found that the mean reductions for stages of treatment was 30-60% for flocculation/sedimentation, 70-90% for rapid sand filtration, 80-90% for GAC, and 98-100% for slow sand filtration. Hyperchlorination in conjunction with direct filtration removed 99-100% of aeromonads. Aeromonads are isolated from chlorinated waters, including distribution system water where colifoms counts are below the detection limit, leading to the suggestion that they are moderately resistant to chlorine (LeChevallier et al., 1983). Later studies do not support that aeromonads are any more resistant to chlorine than other waterborne bacteria, e.g. *E. coli*, *Pseudomonas* spp., *Acinetobacter* spp., etc. Knochel (1991) reported that aeromonads had a slightly higher susceptibility to combined chlorine compared to other water bacteria. Water temperature, available nutrient and disinfection residual affect the regrowth potential of *Aeromonas* in drinking water distribution systems (Burke, et al., 1984a; Holmes et al., 1996).

9.0 Indicators for Occurrence and Treatability

A. hydrophila has been proposed as an indicator of tropical water quality (Miranda and Castillo 1996); however, some investigators contend that *Aeromonas* spp. are indigenous to the aquatic environment and do not correlate with fecal indicators.. Chao et al. (2003) conclude that the use of the commonly employed indicators for assessing subtropical water quality is questionable, especially for pristine waters such as springs and groundwater. The presence of *Aeromonas* spp. does not correlate with the presence of coliform bacteria in drinking water supplies (Fernandez et al., 2000). *Aeromonas* spp. did not correlate to chlorination levels in the

distribution system, but they proposed using *Aeromonas* spp. as an additional indicator of drinking water quality related to presence of biofilm

9.1 Relationship of Aeromonads to Other Microbial Indicators

Aeromonads comprise 36% of "atypical" colonies in coliform tests based upon membrane filtration and M-Endo broth (Brion and Mao 2000). The ratio of "atypical" colonies to coliphage was found to correlate with the level and type of fecal pollution in water; thus, this ratio may provide a supplemental indication of watershed quality (Brion et al., 2000).

Marcel et al. (2002) characterized *Aeromonas* populations in a tropical estuary in Africa. Overall, 63% of water samples contained aeromonads. Aeromonads were most abundant during the rainy season when salinity levels were lowest. The highest *Aeromonas* counts occurred with the *E. coli* counts were high. Counts ranged from < 1 to $> 10^4$ CFU/100 mL. Aeromonads are a good supplemental fecal pollution indicator.

9.2 Summary

A. hydrophila has been proposed as an indicator of tropical water quality (Miranda and Castillo 1996); however, some investigators contend that *Aeromonas* spp. are indigenous to the aquatic environment and do not correlate with fecal indicators. The ratio of "atypical" colonies to coliphage was found to correlate with the level and type of fecal pollution in water, thus this ratio may provide a supplemental indication of watershed quality (Brion et al., 2000).

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