ANTIBIOTIC- AND METAL-RESISTANT *Aeromonas* ISOLATED FROM ENVIRONMENTAL SOURCES

by

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ABSTRACT

Aeromonas is a ubiquitous aquatic bacterium that causes serious infections in both cold- and warm-blooded animals, including humans. Clinical isolates of the organism have shown an increasing incidence of antibiotic and antimicrobial drug resistances since the widespread use of antibiotics began. The genes for antibiotic resistance and metal resistance are frequently carried on the same plasmids, imparting both characteristics to the host bacterium. When there are either antibiotics or metals present in the environment, both markers are co-selected. Two hundred eighty-three *Aeromonas* isolates belonging to eleven different species were isolated from several streams and both urban and rural playa lakes in Lubbock, TX and New Mexico. The minimal inhibitory concentrations of seven metals, six antibiotics, and two synthetic drugs were determined. Low incidences of trimethoprim resistance, mercury resistance, and arsenite resistance were found. Antibiotic and metal resistances were not linked in almost all of the *Aeromonas* isolates. Plasmids were isolated from selected strains of the arsenite- and mercury-resistant organisms and transformed into *Escherichia coli* XL1-Blue MRF′, showing that the resistance genes were carried on plasmids. From the data, it was concluded that mercury and arsenite resistance could be transferred to other organisms in natural environments.

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CHAPTER I

INTRODUCTION

The Genus *Aeromonas*

Members of the genus *Aeromonas* are ubiquitous aquatic bacteria. They are Gram-negative rods that are oxidase positive and facultatively anaerobic (Holt et al., 1994). They are also resistant to vibriostatic agent 0/129 and do not require NaCl for growth (Pemberton et al., 1997). They now belong to the family *Aeromonadaceae.*

Aeromonas was considered to be in the family *Vibrionaceae* until 1986. The genera *Vibrio* and *Aeromonas* share many phenotypic characteristics (Overman et al., 1985). They are so similar that, even now in the clinical setting, *Aeromonas* is sometimes still misidentified as *Vibrio* (Abbott et al., 1998). However, despite their similarities, *Aeromonas* and *Vibrio* each have different phylogenetic histories. This conclusion was based on rRNA-DNA binding competition studies. As a consequence, *Aeromonas* was removed from the family *Vibrionaceae* and a new family, *Aeromonadaceae*, was created (Colwell et al., 1986).

The sixteen species in the genus *Aeromonas* can be divided either into phenotypic groups or DNA hybridization groups. The members of the genus are phenotypically similar and are easily confused (Valera and Esteve, 2002). There are four phenotypic groups or phenons. These groups contain the phenospecies *Aeromonas hydrophila*, *A. caviae*, *A. sobria*, and *A. salmonicida*. The *Aeromonas* genus is further broken down into as many as fourteen DNA hybridization groups (Carnahan and Altwegg, 1996).

Mesophilic aeromonads can be found in almost every aquatic environment. They are found in both polluted and unpolluted environments as well as in chlorinated drinking water distribution systems around the world (Fernández et al., 2000; Fox et al., 1990; Huys et al., 1997; Kühn et al., 1997a), raw sewage (Holmes et al., 1996), and in the oceans at the freshwater/marine interfaces (Hazen et al., 1978). Aeromonads can also be found thriving in deep groundwater (McKeon et al., 1995).

Since *Aeromonas* species are ubiquitous in aquatic environments, it is common for fish, amphibians, and reptiles to come into direct contact with these bacteria. These cold-blooded organisms can become infected with *Aeromonas* under certain circumstances. *Aeromonas* causes, among many other diseases, hemorrhagic septicemia in fish, red-leg disease in frogs, and ulcerative or necrotic stomatitis in snakes (Gosling, 1996). *Aeromonas* infections in fish are of major concern in the fish-farming industry.

A. hydrophila, *A. sobria*, *A. allosaccharophila*, *A. salmonicida*, and *A. veronii* are the major causative agents of fish infections. There is some debate as to whether *A. hydrophila* is a primary pathogen of fish or just an opportunistic pathogen of weakened fish. Whatever the case may be, *A. hydrophila* plays a major role in the development of disease in fish. *A. hydrophila* causes redsore disease, tail and fin rot, and hemorrhagic septicemias. In cultivated fish, *A. salmonicida* causes furunculosis, which are boil-like lesions in the musculature, and is particularly devastating in aquaculture. Since *Aeromonas* infections can have such devastating consequences on aquaculture, chemotherapy has been extensively used to prevent and treat the infections. However,

antibiotic-resistant strains of *Aeromonas* have emerged, making treatment more difficult (Austin and Adams, 1996).

The range of susceptible hosts for *Aeromonas* infections does not stop with fish and other cold-blooded organisms. Warm-blooded animals such as dogs, birds, and humans can also become infected with *Aeromonas*. These infections can be in the form of septicemia, pneumonia, or wound infections (Gosling, 1996). In humans, *Aeromonas* is also a probable causal agent of gastrointestinal disease. Although it has never been proven as a true causal agent, high numbers of *Aeromonas* have been isolated from the gastrointestinal tract of those presenting with the symptoms of human diarrheal disease (Joseph, 1996; Kühn et al., 1997b). Recently, the enterotoxin genes *alt*, *act*, and *ast* have been found in aeromonads associated with diarrhea (Albert et al., 2000; Shat et al., 2002). The most common *Aeromonas* infections in humans result in gastrointestinal distress (Janda and Abbott, 1996). Most of the diarrheal diseases associated with *Aeromonas* are self-limiting and do not require antibiotics (Khardori and Fainstein, 1988).

In a severe *Aeromonas* infection, *A. sobria* was associated with acute renal failure in an infant. The symptoms the child displayed were very similar to infections caused by *Escherichia coli* O157:H7. She had watery, bloody diarrhea and renal failure. However, further investigation of stool cultures showed *Aeromonas* as the only probable pathogen. The infant had been bathed in the same bathtub as had been used to clean an aquarium. The suspected organism was also found in the fish tank (Filler et al., 2000).

Extraintestinal infections commonly occur when a wound is exposed to *Aeromonas*-contaminated water. The most common *Aeromonas* wound infections in

humans are attributed to *A. hydrophila*, *A. veronii*, *A. jandaei*, *A. trota*, and *A. schubertii* (Janda and Abbott, 1996). Wound infections can progress quickly and may ultimately be fatal if the infection becomes systemic. Cellulitis, myonecrosis, and ecthyma gangrenosum are the results of wound infections and are treated with antibiotic therapy. The latter two types of infections may progress seriously enough for amputation to be required. Swimming accidents, boating accidents, alligator bites, and fishing hook accidents are ways people that have been wounded become infected with *Aeromonas* (Janda and Abbott, 1996).

Nosocomial infections from leeches are another example of opportunistic *Aeromonas* infection. Leeches are still used medicinally after some surgeries such as replants, grafts, and reconstructive surgery (DeChalain, 1996). Leeches and *A. hydrophila* are symbionts. The presence of *Aeromonas* is critical to the leeches. Aeromonads provide the hemolytic enzymes necessary to break down blood for the leech's nutrition. *A. hydrophila* from the gut of leeches has caused opportunistic infections in the treated patients (Sartor, 2002).

Not only are aeromonads carried in leeches, but they can also survive within houseflies. *A. caviae* can persist in the housefly gut for up to eight days and can be transferred to other flies and food. Houseflies can serve as mechanical vectors, spreading the organism to various environments where they may eventually act as pathogens (Nayduch et al., 2002).

Because of the known and suspected infections aeromonads cause, the National Academy of Sciences considers the organism to be an emerging microbial threat to health

(Lederberg et al., 1992). *Aeromonas* may eventually become a greater problem by causing nosocomial infections (other than from leeches) (Ko et al., 1998). *Aeromonas* may be a greater threat to human health when resistance to antimicrobial agents is considered.

Antibiotic Resistance

Antibiotics and metals normally inhibit the growth of *Aeromonas* and other microorganisms. However, these organisms can develop resistance to one or both of these groups of antimicrobial agents. Under natural conditions, antibiotic resistance may not be very important. In uncontaminated environments, there is only a low incidence of antibiotic resistance (McArthur and Tuckfield, 2000).

Antibiotics are chemicals produced by microorganisms that kill or inhibit other microorganisms. Different antibiotics have different cellular targets. For example, β-lactam antibiotics inhibit cell wall synthesis, kanamycin targets 16S rRNA, and tetracycline attacks a site of the ribosome and inhibits protein translation. Microbes can develop resistance to antibiotics through several different avenues as discussed later (Snyder and Champness, 1997).

With the widespread use of antibiotics since the 1950s, populations of multi-resistant microorganisms—microorganisms resistant to more than one antimicrobial agent—and multi-resistance genes have emerged (Davies, 1994). Antibiotics act as a selective agent. They kill or inhibit susceptible strains, allowing strains that harbor resistance genes to propagate. The resistant bacteria then comprise the majority of the

bacterial population (Levy, 1997). These resistant microorganisms are continuing to increase in frequency for clinical isolations. A relevant example is that clinical *Aeromonas* isolates in Taiwan are showing increased incidence of resistance to tetracycline, trimethoprim-sulfamethoxazole, some extended spectrum cephalosporins, and aminoglycosides compared to aeromonads isolated from the United States and Australia (Ko et al., 1996).

Bacterial resistance to antibiotics and drugs comes from a variety of different mechanisms. These mechanisms are encoded by genes on the microorganism's chromosome or by a transferable element and include the following. (1) The uptake of the antibiotic into the cell is reduced. Chloramphenicol resistance occurs in this manner (Davies, 1994). (2) The antibiotic is actively effluxed out of the cell as with β-lactams (Zhang et al., 2000). (3) The antibiotic's cellular target is modified to inhibit the binding of the antibiotic. One of the mechanisms of tetracycline resistance occurs through ribosomal protection proteins (Chopra and Roberts, 2001). (4) Enzymes inactivate the antibiotic, as is the case with β-lactamases that hydrolyze β-lactams (Walsh et al., 1998). (5) Proteins bind to the antimicrobial and sequester it (Davies, 1994). The last two mechanisms are used in resistance to sulfonamides and trimethoprim (6). There is a metabolic bypass around the affected reaction (Davies, 1994). (7) The bacterial cell overproduces the target so that some of the target may survive an antibiotic attack (Davies, 1994).

It is thought that plasmids carrying genes for multi-resistance in pathogens have occurred only in the past fifty years (Davies, 1994). Plasmids are extrachromosomal self-

replicating molecules of DNA. They can be transferred from organism to organism. It was recently demonstrated that transfer of plasmids between *Aeromonas* isolated from hospitals to *Aeromonas* isolated from aquaculture is possible (Rhodes et al., 2000). At least three different genetic groups of resistance plasmids exist in *Aeromonas* (Hedges et al., 1985). Transfer of resistance plasmids can occur between members of the same species or between organisms from different genera or even families (Davies, 1997). Antibiotic resistance genes can even be transferred across the Gram barrier; for example, plasmids from a Gram-negative organism can be transferred to a Gram-positive organism (Courvalin, 1994). On the other hand, transfer of resistance plasmids can also occur at low frequencies and with a narrow host range (Pickup et al., 1997).

Antibiotic and metal resistance can be inherent or acquired. Most members of the genus *Aeromonas*, with the exception of *A. trota*, are inherently resistant to ampicillin due to the production of ß-lactamases (Overman and Janda, 1999). The genes for ß-lactamases in aeromonads are chromosomally located (Jones and Wilcox, 1995). ß-Lactamases are enzymes that hydrolyze the amide bond of the ß-lactam ring of the penicillins and cephalosporins, rendering the antibiotics inactive (Bastarrachea, 1998). However, some *Aeromonas* have been isolated that are not ampicillin resistant (Kilpatrick et al., 1987). Antibiotic susceptibility tests have been run on the members of the genus *Aeromonas* to find patterns that differentiate between the species. The results show that the species cannot be determined from antibiotic susceptibility patterns (Kämpfer et al., 1999; Overman and Janda, 1999; Vila et al., 2002). Acquired resistance occurs when a previously susceptible organism develops resistance through a chromosomal mutation or

by acquisition of genes through plasmids or transposable elements (Murray and Hodel-Christian, 1991).

Metal Resistance

A "metal" is an element whose oxides form hydroxides with water and whose compounds form positive ions when in solution. The theory of "hard" or "soft" acids and bases more accurately describe metals. Another metal classification system is based on equilibrium constants involved in the formation of metal ion-ligand complexes. This classification system is divided into Class A, Class B, and a borderline group. The two classification schemes are similar. Hard acids (Class A) are small in size, have low polarizability, and a high positive oxidation state. These include lithium, magnesium, and lead, among others. Soft acids (Class B) are large, have low polarizability, and low electronegativity. Some of these are copper, mercury, and silver. The group of borderline metals includes iron, nickel, cobalt, copper, and zinc (Collins and Stotzky, 1989).

Metal resistance is nothing new to bacteria. In fact, bacteria had to have resistance mechanisms in order to survive the high concentrations of metals in the environment from volcanic activity 3 to 4 billion years ago (Silver et al., 1989). Some metals, unlike antibiotics, have a biological role in the metabolism of microorganisms. These metals belong to Class A (Collins and Stotzky, 1989). Calcium, cobalt, chromium, iron, potassium, magnesium, manganese, sodium, nickel, and zinc are required for life. Iron and nickel are important in redox reactions, while magnesium and zinc help to

stabilize enzymes and DNA. Complex organic molecules sometimes contain iron, magnesium, nickel, or cobalt (Nies, 1992). Metals are cofactors in many other important enzymes as well (Wackett et al., 1989). Finally, sodium and potassium help to maintain osmotic pressure (Nies, 1992).

Other metals such as silver, aluminum, cadmium, gold, lead, and mercury are nonessential for microbial survival. These metals belong to Class B (Collins and Stotzky, 1989). High concentrations of both the essential and nonessential metals are toxic to bacteria. Essential metals can be displaced from their binding sites by the nonessential metals (Hughes and Poole, 1989). High concentrations of metals can alter the conformation of proteins and the structure of nucleic acids. Oxidative phosphorylation is also disrupted and osmotic balance is interrupted (Poole and Gadd, 1989).

At the biochemical level, these metals are toxic, and this effect radiates out to the population and community level. In general, metal-polluted environments have a decreased microbial biomass, metabolic activity, and diversity (Roane and Kellogg, 1996). Microbial communities can also adapt to metal contamination. One group of researchers found very diverse groups of chromium-resistant bacteria from chromium-contaminated sludge (Francisco et al., 2002). Organisms resistant to metals are often found in most natural habitats (Arnebrant et al., 1987; Roane and Kellogg, 1996). Horizontal gene transfer of resistance plasmids has been known to occur in polluted environments. This can eventually result in restoration of species diversity in microbial communities (Rasmussen and Sørensen, 1998).

Metal resistance is found in almost every bacterial group (Silver and Misra, 1984). There are several possible mechanisms by which organisms are resistant to metals, and many of them are the same strategies employed by bacteria to eliminate the damaging effects of antibiotics. (1) The metal can be excluded from the cell by a permeability barrier (Bruins et al., 2000). (2) There is reduced uptake of a particular metal in order to protect the cellular components (Ahuja et al., 2001). (3) The metal can be actively transported away from the cell. Some organisms contain the *ars* operon that encodes an ATPase efflux pump that actively moves arsenite out of the cell (Dey and Rosen, 1995). (4) Binding proteins can sequester metals either intracellularly or extracellularly, thus blocking access to the cellular machinery. (5) Enzymes detoxify a metal to a less toxic form. One mechanism of mercury resistance involves mercuric reductase reducing Hg(II) to Hg(0). Hg(0) then diffuses out of the cell (Bruins et al., 2000; Silver and Phung, 1996). (6) The sensitivity of the cellular targets to metals can be decreased by producing metal-resistant components or creating alternative biochemical pathways that bypass the target (Bruins et al., 2000).

Linked Antibiotic and Metal Resistance

The microbial impacts of antibiotic and metal resistance are intertwined. In the 1970's it became apparent that metal and antibiotic resistance in bacteria were somehow linked (Allen et al., 1977; Groves et al., 1975; Marques et al., 1979; McHugh et al., 1975; Morozzi et al., 1986; Nakahara et al., 1978; Timoney et al., 1978). Genes carrying resistance to antibiotics are most often carried on plasmids. Genes for metal resistance

can be carried on plasmids as well (Rhodes et al., 2000). In fact, there are twelve plasmid-determined metal resistance loci currently identified in bacteria. They include genes for resistance to arsenic, antimony, boron, cadmium, chromium, cobalt, copper, mercury, nickel, silver, tellurium, and zinc (Summers et al., 1978). Higher frequencies of plasmids per isolate have been found in mercury-contaminated soils as compared to unpolluted soils (Rasmussen and Sørensen, 1998).

The genes for both antibiotic and metal resistances are sometimes carried on the same plasmid. Transposon Tn*21* carries both antibiotic resistance and mercury resistance genes (Liebert et al., 1999). Other Tn*21*-like transposons also occur and carry multiple antibiotic and resistance genes (Bass et al., 1999). If metal resistance genes and antibiotic resistance genes are contained on the same plasmid, as is the case with Tn*21*, then either metals or antibiotics could serve as selective agents, and all of the genes would be perpetuated in the environment (Timoney et al., 1978).

Linked antibiotic and metal resistance occurs in different environments. One of the most prevalent areas from which to isolate metal- and antibiotic-resistant bacteria are metal-contaminated environments, such as estuaries (Allen et al., 1977; Timoney et al., 1978). Polluted streams and soils also harbor the multi-resistant organisms (Marques et al., 1979; McArthur and Tuckfield, 2000). Surprisingly, unpolluted, treated drinking water was found to have bacteria with copper, lead, and zinc resistance along with multiple antibiotic resistance. It was speculated that the copper distribution pipes were co-selecting for metal and antibiotic resistance (Calomiris et al., 1984).

Metal and antibiotic resistances have also been found to be linked in bacteria from the gastrointestinal tracts of humans and primates. Studies have been done with humans and primates in whom mercury and antibiotic resistances were found together in the fecal bacteria. The humans and primates all had dental amalgam fillings composed of mercury. It is hypothesized that the mercury exerts a selective pressure, and those bacteria with both mercury and antibiotic resistances are enriched among the selected survivors when the two traits are genetically linked.. The mercury-resistant bacteria that were isolated were more likely to also have resistances to more than one antibiotic as opposed to the mercury-sensitive bacteria (Österblad et al., 1995; Wirman et al., 1997).

Clinical isolates have also exhibited antibiotic and metal resistances. For example, *Salmonella typhimurium* strains resistant to multiple antibiotics and silver nitrate were isolated from patients in a burn unit. Silver nitrate, which was being used as a topical treatment for the burns, exerted the selective pressure in this instance. The pattern of resistance to silver and multiple antibiotics was transferred to *E. coli* through mating experiments (McHugh et al., 1975).

Environmental contamination with antibiotics and other pollutants contributes to the maintenance and spread of antibiotic resistance genes (Goñi-Urriza et al., 2000). One of the characteristics that allows for the perpetuation of genes is that resistance plasmids can be spread between unrelated bacteria in natural environments (Kruse and Sørum, 1994). The transfer of genes is demonstrated by the fact that antibiotic- and metal-resistant strains of bacteria have been isolated from environments that have never been directly exposed to metals or antibiotics (Morozzi et al., 1986). Multi-resistant

microorganisms are found in pathogens and non-pathogens alike (Levy, 1997). *Aeromonas* that are both antibiotic and metal resistant have been previously isolated out of polluted and unpolluted waters (Miranda and Castillo, 1998).

Not much is known about the antibiotic and metal resistance profiles of aeromonads from environmental waters (Miranda and Castillo, 1998), especially shallow rural and urban playa lakes. Playa lakes are small circular to oval basins that drain surface runoff waters from the surrounding area. There are more than 20,000 playa basins on the High Plains of Texas and New Mexico (Gustavson et al., 1994). The biological composition of the playas is influenced by the quality of surface-water runoff, which is directly related to the way the land in the watershed is being used (Hall, 1997). Urban playas receive runoff from the city, and the rural playas receive runoff from the farmland or stockyards. Numerous studies have been done to analyze the chemical composition of the water (Arefeen, 1995; Huang, 1992; Mollhagen et al., 1992). Westerfield (1996) studied pathogens from playas and found *Aeromonas* to be present. Warren (1998) took the study further and tested *Aeromonas* isolates for antibiotic resistance but not metal resistance.

Research Objectives

The goals of this research were to: (1) isolate *Aeromonas* from four urban playa lakes, two rural playa lakes, and three streams; (2) evaluate the metal resistance patterns for each isolate; (3) determine the antibiotic resistance patterns for each isolate; and (4)

isolate plasmids from selected isolates that show resistance to metals or antibiotics. *Aeromonas* was chosen because of its growing importance in human health.

CHAPTER II

METHODS

Locations of Sampling

Four urban playa lakes and two rural playa lakes were chosen for the study. The urban lakes included the playas at Jack Stevens Park, K. N. Clapp Park, Maxey Park, and Frank Higinbotham Park. Each area contained a recreational park around the lake. All of the urban playas are within the city limits of Lubbock, Texas. Their locations are listed in Table 2.1. Urban playas were chosen in order to compare the bacteria that had been exposed to urban runoff to bacteria exposed to agricultural runoff in the rural areas. The urban playas were sampled twice, the first time on March 23, 2002 and the second time on July 11, 2002. Two rural playas were used in this investigation and they are both located east of Shallowater, Texas, just off of FM 1294 (Figure 2.1). The rural playas were both surrounded by cotton fields. The rural playas were only sampled once, on July 11, 2002, since both the rural playas were dry at the other sampling time and at most other times during the year.

Three streams were sampled. The North Fork of the Brazos River was sampled at Yellowhouse Canyon Park. This park is located on the outer edge of the city of Lubbock and is divided in half by University Avenue. This stream was sampled twice, on the same days as the urban playas. Each time the North Fork of the Brazos River was sampled, two water and sediment samples were taken on the east side of University Avenue and two on the west side. The South Fork of the Rio Hondo was sampled on

July 6, 2002. The location was two miles from the stream's headwaters in Taos Ski Valley, New Mexico. The Pecos River was sampled on July 8, 2002 in Carlsbad, New Mexico. The latter two streams were only sampled once because of their distance from Lubbock, Texas. The locations of the streams are also listed in Table 2.1.

Sampling

Surface water samples were taken from the banks of the playa or stream using a grab sampler. First a 500-mL plastic cup attached to a 2-meter pole was dipped into the water twice to rinse it. The sample was then transferred to a clean, new 8-ounce polyethylene container with a snap-on lid (Fisher Scientific). The temperature of the sample was taken with a laboratory thermometer and recorded on the side of the cup. The time of sampling was also recorded. Four water samples from each playa lake were taken—north, south, east, and west. Four samples were taken from each river site, two samples on each side and taken at least 50 m apart.

Sediment samples were also taken from each of the water sources at the same location as the water samples were taken. A garden spade was rinsed twice with the water before sampling. The sediment sample was taken from sediment surface that was submerged 10-15 cm below the surface of the water. Approximately 7 ounces of sediment were then taken and transferred to a clean, new 8-ounce polyethylene container with a snap-on lid.

All samples of the urban playas, the North Fork of the Brazos River, and the rural playas were taken on the same day to prevent discrepancies from occurring because of

sample date. The samples were kept in an air-conditioned vehicle during transport to the lab and processed within twelve hours of collection. At the lab, the pH of the water was taken with a digital pH meter. Parts of the samples from each site were then combined: each water sample was mixed well, and a one-mL volume was taken and combined with one-mL volumes from the other three samples from the same lake. One gram from each sediment sample was taken and combined with one-gram amounts from the other three sediment samples from the same lake. After combining the parts, they were mixed thoroughly and diluted in 0.85% NaCl to 10^{-4} for the water samples and 10^{-5} for the sediment samples.

The samples from the Rio Hondo and the Pecos River were plated on July 9, 2002, several days after collection. The pH was measured with pH indicator strips (colorpHast) at the time of collection. All other sampling procedures were followed as previously described.

Culture Media

After the samples were diluted, they were plated by spreading onto two different types of media. Four replicates of each dilution were plated. The first medium was 20% tryptic soy agar (TSA), 8 g of tryptic soy agar (Difco Laboratories) supplemented with 12 g of granulated agar (Difco) for every 1 L of distilled water. This medium was used nonselectively to enumerate the total culturable playa bacteria. The reduced nutrient concentration was desirable since these bacteria occur in a low-nutrient environment.

The inoculated plates were incubated at room temperature, approximately 25°C, for 96 hours. After 96 hours, the bacterial colonies on the plates were counted.

The second type of medium was used for the selective isolation of *Aeromonas* species. Many of the common media used to isolate *Aeromonas* contain ampicillin as a selective agent to reduce the number of non-aeromonads (Havelaar et al., 1987; Huguet and Ribas, 1991; Jenkins and Taylor, 1995; Rippey and Cabelli, 1979). Rippey and Cabelli's (1979) medium is thought to be better than most other types of selective media for *Aeromonas* (Moyer, 1996). However, since some strains of *Aeromonas* are sensitive to ampicillin, a modified medium from Rippey and Cabelli (1979) without ampicillin was used. The modified *Aeromonas* isolation medium consisted of 4 g of soluble starch (Difco), 0.25 of g NH_4Cl (Fisher Scientific), 1 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 40 mg of the pH indicator bromothymol blue (Fisher Scientific), 15 g of agar (Difco), and 1 L of distilled water. It was then adjusted to pH 8.0 with 1 *N* KOH (Fisher Scientific). After autoclaving and cooling, 100 mg of sodium desoxycholate (Difco), 5 mL of 0.41% L-tryptophan (Sigma), and 5 mL of 0.99% L-phenylalanine (Sigma) were added. After inoculation and incubation, *Aeromonas* appeared as light-yellow, circular colonies 1-3 millimeters in diameter.

The diluted water and sediment samples were plated onto the modified *Aeromonas* isolation medium. These plates were incubated for 36 hours at 30°C. After the period of incubation, the putative *Aeromonas* colonies were counted. Ten putative *Aeromonas* isolates from the sediment and water samples were subcultured onto 20%

TSA and grown at 30°C overnight. The original isolation plates were stored at 4°C in case they were needed for later use.

Confirming the Identity of the Isolates

After the isolates were subcultured onto 20% TSA, they were further tested to confirm their identity as *Aeromonas*. For all of the tests, *Aeromonas hydrophila* ATCC 7965, *A. veronii* biogroup *sobria* ATCC 9071, and *A. caviae* ATCC 15468 were used as positive controls. Cultures were obtained from American Type Culture Collection, Manassas, VA. *Escherichia coli* DH5α was used as a negative control. If an isolate did not appear to be *Aeromonas* after subsequent testing, it was eliminated from the list and new isolates from the original isolation plates were subcultured to replace the non-aeromonads to provide a total of ten isolates in each sampling group. If the original isolation plates did not appear to have any more putative *Aeromonas* colonies on them, the same lake was resampled within ten days of the original sampling, and samples were plated according to the above procedure to enumerate *Aeromonas* species.

To determine if an isolate was an aeromonad, all isolates kept for testing were Gram-stained. If the isolates were not Gram-negative rods, they were eliminated from the isolate list. Colony characteristics, such as color and texture, were also observed. *Aeromonas* colonies appear colorless to white on 20% TSA. The isolates were tested for oxidase using oxidase test reagent (Difco). The oxidase reagent is composed of N,N,N ,N -tetramethyl-*p*-phenylenediamine with 1% dihydrochloride. Aeromonads are

always oxidase positive, while coliforms and other enteric bacteria are not (Holt et al., 1994).

Next the isolates were tested for deoxyribonuclease activity (DNase). Members of the genus *Aeromonas* contain DNase (Pemberton et al., 1997). DNase/methyl green medium was used. The DNase base (Difco) contains 20 g of Bacto tryptose, 2 g of deoxyribonucleic acid, 5 g of sodium chloride, and 15 g of Bacto agar for every 1 L of distilled water. Methyl green (0.05 g; Manufacturing Chemists, Rochester, NY) was added to each liter of medium made. Methyl green binds to the DNA. When the DNA is degraded, the green color disappears. A clear zone appears around colonies of organisms that contain DNase.

The isolates were also tested against the vibriostatic agent 0/129 to eliminate any *Vibrio* species. Vibriostatic agent 0/129 is 2,4-diamino-6,7-diisopropylpteridine phosphate. Two test discs with different concentrations of the agent were used, 150 mg and 10 mg. They were obtained from Oxoid (Ogdensburg, NY). *A. hydrophila* ATCC 7965 was used as a resistant control and *Vibrio fischeri* 345 (Presque Isle Cultures, Presque Isle, PA) was used as a sensitive control.

The isolates were also tested for anaerobic growth. They were subcultured onto 20% TSA and incubated in an anaerobic chamber (Model 1025 Anaerobic System; Forma Scientific, Marietta, OH) at 30°C for 72 hours. Aeromonads are facultatively anaerobic, while pseudomonads have a strictly respiratory metabolism. After all of these tests had been done to confirm the identity of the isolates as *Aeromonas* sp., the isolates were tested against metals and antibiotics.

Antibiotics and Metals Susceptibility Testing

Susceptibilities to six antibiotics and two synthetic drugs were tested for each isolate. The antibiotics tested were ampicillin, cefuroxime, kanamycin, nalidixic acid, ofloxacin, and tetracycline. The synthetic drugs were sulfamethoxazole and trimethoprim. The six concentrations of the eight antimicrobials tested are shown in Table 2.2. Seven metals susceptibilities tested included arsenite, chromate, cobalt, copper, mercury, nickel and zinc in the forms of $NaAsO_2$, K_2CrO_4, $CoCl_2$ $6H_2O$, $CuSO_4$, $HgCl_2$, $NiSO_4 \cdot 6H_2O$, and $ZnSO_4 \cdot 7H_2O$, respectively. The concentrations of the metals tested are listed in Table 2.3. The procedures for the antibiotics, the drugs, and the metals susceptibility tests were based on the procedures of Kämpfer et al. (1999). The susceptibility tests were performed in Costar 96-well cell-culture cluster plates with a flat bottom and a lid. The antimicrobial was added to 40% nutrient broth and adjusted to pH 7.0. Five concentrations of each antimicrobial were prepared in 40% nutrient broth by two-fold serial dilutions of the highest concentration (Amsterdam, 1991). Two hundred μL of medium were dispensed into each well of the microtiter plates.

Two controls were used: 40% nutrient broth without any antimicrobial agents as a positive growth control and uninoculated media at all concentrations of the antimicrobial agents as negative controls for growth. The absorbance of the uninoculated wells was subtracted from the absorbance of the inoculated wells to correct for the absorbance due to media alone. This was necessary, especially for the metals, because some of them impart a color to the medium. For example, $CuSO_4$ turns the medium blue.

To inoculate the microtiter plates, the isolates were subcultured onto 20% TSA and incubated at 30°C for 24 ± 2 hours. Isolated colonies were picked from the overnight cultures, inoculated into 4.5 mL of 40% nutrient broth, and grown until a turbidity equivalent to a 0.5 McFarland standard was achieved. This is an A_{560} value of 0.12 ± 0.02. Once this absorbance was reached, 5.5 µL of the culture was inoculated into 5.5 mL of 40% nutrient broth (a 1:1,000 dilution). This cell suspension was used to inoculate the microtiter plates. To the 200 µL of antibiotic- or metal-containing media, 100 µL of the cell suspension was inoculated (an additional 1:3 dilution). This is about 4.2×10^4 colony forming-units (CFU) per well or 1.4×10^5 CFU/mL. The uninoculated control wells were supplemented with 100 µL of sterile 40% nutrient broth. The microtiter plates were then incubated at 30°C for 24 ± 2 hours.

After the 24-hour incubation period, the absorbance of the microtiter plates was read at 550 nm using an automated plate reader (Bio-Tek EL311sx Auto Reader, Winooski, VT). An absorbance of 0.1 or above indicated growth. *A. hydrophila* ATCC 7965 was tested as a control each time the procedure was performed. Ten percent of the isolates were re-tested for antibiotics, drugs, and metals under the same conditions in order to verify reproducibility and accuracy. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were also tested in accordance with the minimal quality control recommendations of NCCLS (2002) in order to determine the accuracy of the antibiotic and drug susceptibility tests.

For those organisms that were found to be resistant at even the highest concentration of an antibiotic, drug, or metal, another microtiter plate was prepared with

a two-fold series of higher concentrations of that particular antimicrobial agent to determine the upper limit of the resistance. This was done for all antibiotics and metals except ampicillin, since *Aeromonas* is inherently resistant to the antibiotic.

Minimal inhibitory concentrations of the antibiotics and the metals were determined for each of the isolates. The minimal inhibitory concentration (MIC) is defined as the lowest concentration at which an organism will not grow (Amsterdam, 1991). The MICs were compared to those published in previous studies and by the 2002 NCCLS standards (Tables 2.4 and 2.5). Cumulative percentage plots were also constructed, and the MICs at which 50% and 90% of the isolates were inhibited were determined.

Biolog Identification of Isolates

After all of the isolates were tested for antibiotics and metals susceptibilities, representative isolates were identified using the Biolog Identification System, Release 4.0 (Biolog, 1999). Isolates to be identified were chosen from different resistant and susceptible groups. The Biolog Identification System identifies organisms on the basis of their ability to use 95 different carbon sources. According to the Biolog users manual (1999), the results of the Biolog identification can be accepted as accurate if the similarity index is at least 0.500. The similarity index is determined by comparing the results from the unidentified isolate to known metabolic profiles of genera and species in the database.

Plasmid Purification

Several of the resistant organisms were selected for plasmid purification from them. QIAGEN Plasmid MidiKits were used according to the manufacturer's instructions (QIAGEN, 2000).

Preparation and Transformation of Competent Cells

A procedure for preparing and transforming competent cells was obtained from Dr. Michael San Francisco (Department of Biological Sciences, Texas Tech University, Lubbock, TX) and followed except for a few modifications. *E. coli* XL1-Blue MRF′ (Stratagene) was grown overnight in 2 mL of Luria broth (LB) with aeration at 37°C. LB is composed of 10 g of tryptone (Difco), 5 g of yeast extract (Difco), 5 g of NaCl (EM Science; Gibbstown, NJ), 1 L of distilled water, and adjusted to pH 7.0 with NaOH (Fisher Scientific). The overnight culture was inoculated into 50 mL of LB and incubated at 37°C with aeration until the culture reached an absorbance of 0.4-0.6 at 600 nm. The culture was then centrifuged at 10,000 rpm for 10 min at 4°C. The pellet was resuspended in 20 mL of sterile, ice-cold 50 m*M* $CaCl_2$ and incubated on ice for 30 min. The competent cells were then divided into 200-μL aliquots, mixed gently with 20% glycerol, and stored at -60°C until ready for use.

When the transformation was performed, 50-100 ng of plasmid obtained from the plasmid purification procedure were added to 200 μL of competent *E. coli* XL1-Blue MRF′ cells and incubated on ice for 30 min. The cells were then heat shocked at 42°C for 1 min. Afterwards they were incubated in an ice-water mixture for 5 min, and the

entire amount was inoculated into 1 mL of LB and incubated at 37°C for 1 hr to allow for recovery. The cells were then plated on the appropriate media. The cells transformed with plasmids thought to carry arsenite resistance were inoculated onto 40% nutrient agar with 6m*M* $NaAsO_2$, pH 7.0. The cells transformed with plasmids thought to carry mercury resistance were inoculated onto 40% nutrient agar with 0.025 m*M* $HgCl_2$, pH 7.0. Untransformed *E. coli* XL1-Blue MRF′ was inoculated onto 40% nutrient agar with and without metals as negative and positive controls for growth. All of the plates were incubated at 37°C overnight.

Data Analysis

Paired *t*-tests were done with the plate counts of *Aeromonas* obtained on modified *Aeromonas* isolation medium to determine if a significant difference existed between viable CFUs of *Aeromonas* in the water and sediment and between the two sampling times for each sample location. A 95% confidence interval was used. The paired *t*-tests were two-tailed tests, and Systat Version 9.0 was the software used.

Means and standard deviations were calculated for both the total culturable bacteria and the presumptive *Aeromonas* colonies. The data were broken down either by water source, sampling date, sample type, or a combination of the three categories.

The rest of the data analysis was in the form of descriptive statistics. For example, the percentage of resistant isolates out of the total isolates was determined.

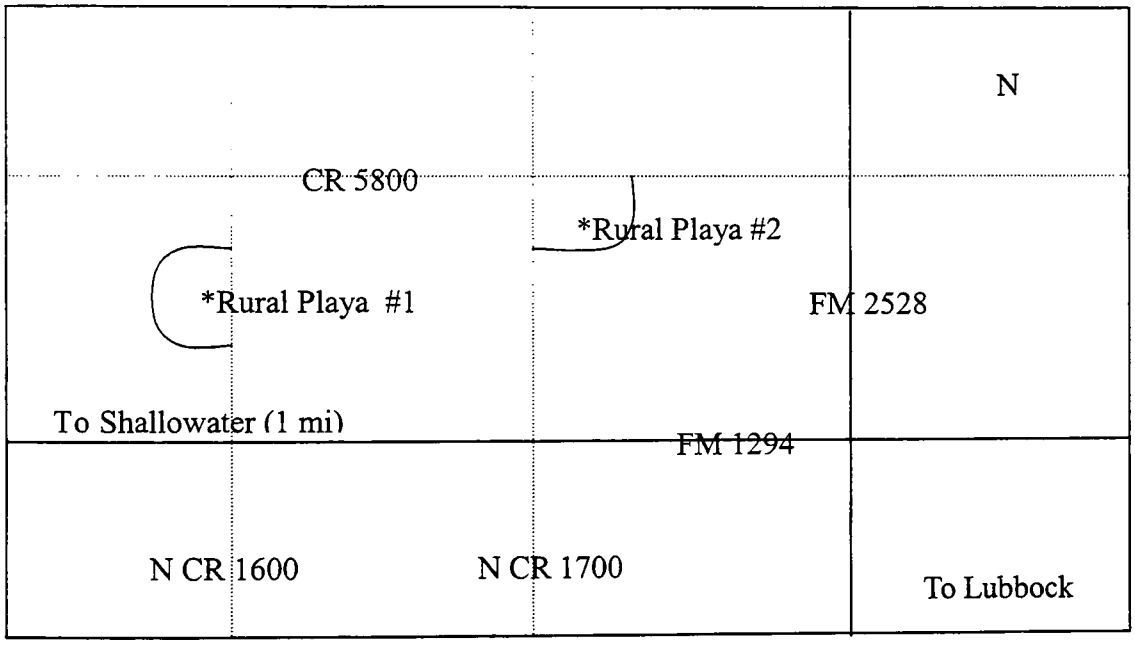

Figure 2.1. Map of rural playas. Dashed line = unpaved road. Solid line = paved road.
Scale: 1 mi = _____.

Table 2.1. Locations of sampling sites.

Sampling Site	City, State	Location
Jack Stevens Park	Lubbock, Texas	Slide Road and 73rd Street
K. N. Clapp Park	Lubbock, Texas	University Avenue and 44th Street
Maxey Park	Lubbock, Texas	Quaker Avenue and 24th Street
Frank Higinbotham Park	Lubbock, Texas	Utica Drive and 19th Street
Rural Playa 1	Shallowater, Texas	Off FM 1294; Figure 2.1
Rural Playa 2	Shallowater, Texas	Off FM 1294; Figure 2.1
North Fork of the Brazos River	Lubbock, Texas	University Avenue and Canyon Drive
South Fork of the Rio Hondo	Taos Ski Valley, New Mexico	Behind Sierra del Sol Lodge
Pecos River	Carlsbad, New Mexico	Riverside Drive, Tansill Dam

Table 2.2. Antibiotic and drug concentrations used in susceptibility testing.

Antimicrobial	Concentration (μg/mL)					
	1	2	3	4	5	6
Ampicillin	0	4.0	8.0	16.0	32.0	64.0
Cefuroxime	0	0.125	0.25	0.5	1.0	2.0
Kanamycin	0	0.25	0.5	1.0	2.0	4.0
Nalidixic Acid	0	2.0	4.0	8.0	16.0	32.0
Ofloxacin	0	0.25	0.5	1.0	2.0	4.0
Sulfamethoxazole	0	4.0	8.0	16.0	32.0	64.0
Tetracycline	0	0.031	0.063	0.125	0.25	0.5
Trimethoprim	0	0.5	1.0	2.0	4.0	8.0

Table 2.3. Metals concentrations used in susceptibility testing.

Metal	Concentration (mM)					
	1	2	3	4	5	6
$NaAsO_2$	0	0.75	1.5	3.0	6.0	12.0
K_2CrO_4	0	0.4	0.8	1.6	3.2	6.4
$CoCl_2$ $6H_2O$	0	0.125	0.25	0.5	1.0	2.0
$CuSO_4$	0	0.125	0.25	0.5	1.0	2.0
$HgCl_2$	0	0.003	0.006	0.013	0.025	0.05
$NiSO_4 \cdot 6H_2O$	0	0.125	0.25	0.50	1.0	2.0
$ZnSO_4 \cdot 7H_2O$	0	0.125	0.25	0.50	1.0	2.0

Table 2.4. Minimal inhibitory concentrations of antibiotics and drugs for Gram-negative, non-*Enterobacteriaceae*.

Antimicrobial	Concentration (μg/mL)	Reference
Ampicillin	32	NCCLS (2002)
Cefuroxime	32	NCCLS (2002)
Kanamycin	64	NCCLS (2002)
Nalidixic Acid	32	Amsterdam (1991)
Ofloxacin	8	NCCLS (2002)
Sulfamethoxazole	512	NCCLS (2002)
Tetracycline	16	NCCLS (2002)
Trimethoprim	4	NCCLS (2002)

Table 2.5. Minimal inhibitory concentrations of metals for Gram-positive and Gram-negative bacteria.

Metal	Concentration (m*M*)	Reference
$NaAsO_2$	6	San Francisco, 2002
K_2CrO_4	1.6	Marques et al., 1979
$CoCl_2$ $6H_2O$	5	Tibazarwa et al., 2000
$CuSO_4$	5	Rasmussen and Sørensen, 1998
$HgCl_2$	0.025 (variable)	Pike et al., 2002
$NiSO_4 \cdot 6H_2O$	3	Tibazarwa et al., 2000
$ZnSO_4 \cdot 7H_2O$	2.5	Große et al., 1999

CHAPTER III

RESULTS

Two hundred eighty-three organisms were isolated and identified as *Aeromonas*. The antibiotic and metal sensitivity tests were then performed. Afterwards, the organisms were divided into different groups according to their source of isolation, sample type, and resistances to metals and antibiotics. There were 58 distinct groups. One isolate from each unique group was identified to the species level using the Biolog system. The other unidentified isolates in each unique group were considered to be possible members of the same species as the identified organism. Collectively, the identified isolates represented 20.5% of the total number of bacteria isolated.

Sampling Dates, pH, and Temperature of the Water Bodies

The Brazos River and the four urban playas were sampled twice each, once in March and once in July 2002. The rural playas, the Rio Hondo, and the Pecos River were sampled once in July 2002. At the time of sampling, the pH and temperature of the water samples were measured (Table 3.1). The average pH of the water varied from 5.00-6.00 in the Rio Hondo and Pecos River, whereas the average pH of the playa lakes ranged from 7.57 in the playa at Stevens Park to 9.58 in the playa at Maxey Park. The average temperature of the water was lower in March than in July. Temperatures in March ranged from 8.75°C in the playa at Stevens Park to 12.50°C in the playa at Higinbotham

Park. July playa temperatures ranged from 26.0°C in the playa at Stevens Park to 31.0°C at Rural Playa 1.

Aeromonas Plate Counts

Putative *Aeromonas* colonies were recognized by colony morphology. On modified *Aeromonas* medium, the aeromonad colonies were 3-5 mm in diameter and were creamy yellow in color. They were circular in form, slightly raised in elevation, and had an entire margin. The colonies were smooth, opaque, and butyrous. Once the bacteria were isolated onto 20% TSA, the colonies were 3-5 mm in diameter and white in color. They were circular in form, slightly raised in elevation, and had an entire margin. These colonies were smooth, translucent, and butyrous. The modified *Aeromonas* medium was incubated aerobically at 30°C and the 20% TSA was incubated at 25°C.

The Gram stain, oxidase test, DNase test, vibriostatic 0/129 test, and test for anaerobic growth on 20% TSA were used to determine the identity of the isolates. The isolates were included in the study if they were Gram-negative rods, oxidase positive, DNase positive, resistant to the vibriostatic agent 0/129, and were able to grow anaerobically. Organisms not having all of the preceding characteristics were eliminated since they were most likely not aeromonads.

Putative *Aeromonas* colonies were counted on the modified *Aeromonas* medium. The colony-forming units per mL (CFU/mL) in the original samples were determined. Only the data for the urban playa lakes could be used for statistical analysis since they were sampled more than once. Paired *t*-tests were done on these data obtained from the

presumptive *Aeromonas* colony counts. A *t*-test compared the number of viable aeromonads between the combined sediment and the water data and showed there to be a significant difference. The mean of eight water samples was 494 CFU/mL and the mean of eight sediment samples was 94,975 CFU/mL. A 95% confidence interval was used, $n = 8$, $t = -2.412$, and $p = 0.047$. This result rejects the null hypothesis that there is no difference between the number of colony-forming units of *Aeromonas* in the water and in the sediment.

A *t*-test was performed comparing the number of viable *Aeromonas* colony counts between March and July across all playa lakes. There was no significant difference in *Aeromonas* densities with season. The mean of March was 39,013 CFU/mL and the mean of July was 56,456 CFU/mL. A 95% confidence interval was used, $n = 8$, $t = -0.357$, and $p = 0.732$. This accepts the null hypothesis that there is no difference between the number of colony-forming units of *Aeromonas* in March and July.

Standard deviations and means of the CFU/mL were calculated for the total culturable bacteria and the presumptive *Aeromonas* colonies. The totals of all of the bacteria isolated are in Table 3.2. The mean of the total culturable bacteria across all samples, locations, and seasons was 7.9×10^6 CFU/mL with a standard deviation of 1.1×10^6 CFU/mL. The mean of the presumptive *Aeromonas* colonies across all samples, locations, and seasons was 4.9×10^4 CFU/mL with a standard deviation of 1.1×10^5 CFU/mL. Individual means of total culturable bacteria greater than 1.0×10^5 CFU/mL occurred in the sediment of the playas at Stevens Park, Clapp Park, Maxey Park, and Higinbotham Park in March and Maxey Park in July. Individual means of presumptive

Aeromonas greater than 1.0×10^5 CFU/mL occurred in the sediment of the Brazos River and the playa at Stevens Park in March and in the sediment from the playas at Clapp Park, Higinbotham Park, and the Rio Hondo in July. Individual means of presumptive *Aeromonas* less than 1.0×10^3 CFU/mL occurred in the water of all of the sampling sources.

Figure 3.1 shows the means of the putative *Aeromonas* colony counts in sediment compared across sampling location and season. The means were up to fourteen times higher in the streams in both March and July than in the rural playas or the urban playas. The lowest densities occurred in the playas in July. Figure 3.2 shows the means of the putative *Aeromonas* colony counts in water compared across sampling location and season. The highest densities of water aeromonads at 8.5×10^2 CFU/mL were found in the rural playas in July and the lowest in streams in March and July.

The individual means and standard deviations of total culturable bacteria and presumptive *Aeromonas* for each playa and stream are in Tables 3.3-3.11. The Brazos River, the Rio Hondo, the playas at Stevens Park, Maxey Park, and Rural Playa 2 had a mean of 10^6 CFU/mL of total culturable bacteria. The Pecos River and Rural Playa 1 had 10^7 CFU/mL of total culturable bacteria. The Brazos River, the Pecos River, the playas at Stevens Park, and Clapp Park contained 10^5 CFU/mL of presumptive aeromonads. Rural Playa 1 and Rural Playa 2 had less than 10^3 CFU/mL of presumptive aeromonads while the Rio Hondo contained greater than 10^5 CFU/mL.

Biolog Identification

Eleven distinct species of *Aeromonas* were positively identified using the Biolog system (Table 3.12). Of the 58 isolates tested, all isolates were identified to at least the genus *Aeromona*; 45 could be identified to the species level. A wide representation of DNA hybridization groups was found. The species were correlated with DNA hybridization groups 1, 2, 4, 5A, 7, 8, 9, 10, and 16. The playas at Maxey Park and Higinbotham Park had the greatest species diversity: seven different *Aeromonas* species were identified from each lake. One of the rural playas, the Pecos River, and the Rio Hondo had the least diversity with only two species being isolated from each source. However, these three sources were only sampled one time, while the urban locations were sampled twice.

Each location had at least two different species of *Aeromonas* present. In all, at least four different species were isolated from the North Fork of the Brazos River. Three of the isolates were *A. encheleia*. The only isolate of *A. icthiosmia* in this study was isolated from this stream in July (Table 3.13). Six different species were isolated from the playa at Clapp Park. Three of these isolates were *A. hydrophila* DNA Group 1 (Table 3.14). Five different species were isolated from the playa at Higinbotham Park. Two of these were *A. hydrophila* DNA Group 1 and were isolated in March. Two other isolates were identified as *A.veronii/sobria* DNA Group 8 and were isolated in July (Table 3.15). Six species were isolated and identified from the playa at Maxey Park. Four isolates were identified as *A.veronii/sobria* DNA Group 8 and were isolated both in March and July (Table 3.16). Four different species were isolated from the playa at Stevens Park.

A. encheleia was the most frequent species identified. *A. jandaei* was isolated and identified from this playa only (Table 3.17).

Table 3.18 describes the similarity indices of the identified isolates compared to the Biolog reference strains and the number of isolates of each species identified. An identification to species was accepted if the similarity index was greater than 0.500. A genus identification was accepted if the first ten choices were the same genus. Since Biolog identification is based on the ability of organisms to use certain carbon sources, these patterns were evaluated. Table 3.19 summarizes the utilization of 95 carbon sources by the *Aeromonas* isolates identified by the Biolog system. All of the isolates were able to utilize dextrin, glycogen, *N*-acetyl-D-glucosamine, D-fructose, α-D-glucose, maltose, ß-methyl-D-glucoside, D-gluconic acid, L-aspartic acid, and inosine. None were able to use adonitol, xylitol, D-galactonic acid lactone, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, itaconic acid, quinic acid, D-saccharic acid, sebacic acid, D,L-carnitine, γ-aminobutyric acid, phenylethylamine, or 2-aminoethanol.

Some of the identified isolates showed carbon-source utilization patterns not expected for the species. Some species of *Aeromonas* should not be able to utilize succinamic acid; however, 62% of these isolates were able to use this carbon source. There were also significant anomalies (greater than 10%) among the identified isolates with citric acid, putrescine, D-cellobiose, turanose, and propionic acid (Table 3.20).

Antibiotic, Drug, and Metal Resistance

The minimal inhibitory concentration (MIC) of each antimicrobial was determined for each of the 283 isolates. The minimal inhibitory concentrations were used to determine resistance and sensitivity. The MICs of the metals are given in Table 3.21. The minimum MIC for each of the metals was below the concentrations tested. The maximum MIC was 12 m*M* for arsenite and 0.1 m*M* for mercury.

A total of 5.30% of the isolates contained either arsenite or mercury resistance. No isolate contained more than one metal resistance. There was a wide range of MICs of arsenite. A cumulative percentage plot of the MICs of arsenite show that over 85% of the isolates were inhibited at a concentration of 3 m*M* or lower, and over 90% were inhibited at 6 m*M* or lower (Figure 3.1).

There were eight arsenite-resistant isolates from six different sources. These isolates were able to grow in 6 m*M* $NaAsO_2$ or greater (Table 3.22). Of these isolates, half of them could only be identified to genus. The other four were each from a different species. Seven of the isolates contained mercury resistance and grew in concentrations at or above 0.025 m*M* $HgCl_2$. Two of these isolates were identified as *A. encheleia*, and four of the isolates were presumed to also belong to this species. The other isolate was *A. hydrophila*-like DNA Group 2 (Table 3.23). All but one of these mercury-resistant isolates were isolated from sediment. Three of the arsenite-resistant isolates were isolated from sediment, and five were isolated from water.

For all of the rest of the metals, chromate, cobalt, copper, nickel, and zinc, 90% or more of the isolates were inhibited at the lowest concentrations tested (Table 3.21). No isolates contained resistance to these metals under the conditions tested.

The minimal inhibitory concentrations of the antibiotics and drugs are given in Table 3.24. For the antibiotics cefuroxime, kanamycin, nalidixic acid, and ofloxacin, 90% or more of the isolates were inhibited at a level less than the minimum concentration tested (Table 3.24). There were no resistant isolates under the conditions tested. The MICs of sulfamethoxazole and tetracycline were more varied than the previously mentioned antibiotics, but none of the isolates displayed high-level resistance. The MICs of trimethoprim and cefuroxime cover a wide range. Over 90% of the isolates were inhibited at 4 μg/mL or lower of trimethoprim (Figure 3.2), and almost 80% were inhibited at concentrations equal to or less than 0.5 μg/mL of cefuroxime (Figure 3.3).

Ampicillin resistance occurred in 91.52% of the isolates (Table 3.24). However, 8.48% of the isolates were sensitive to ampicillin (Table 3.25). These aeromonads consisted of nine of the eleven species isolated. All of the isolates of *A. icthiosmia* and *A. enteropelogenes* were resistant to ampicillin. At least one ampicillin-sensitive aeromonad was isolated from all four of the urban playas, Rural Playa 2, the Brazos River, and the Pecos River. The aeromonads isolated from Rural Playa 1 and the Rio Hondo were all resistant to ampicillin.

Trimethoprim resistance was present in 8.83% of the isolates. Two of the isolates were resistant to as high as 8 μg/mL while the other 23 were resistant to 4 μg/mL (Tables 3.26 and 3.27). Three of the trimethoprim-resistant isolates were also resistant to a metal,

two to arsenite and one to mercury (Table 3.28). Two of the metal-resistant isolates and one of the trimethoprim-resistant isolates were also ampicillin-sensitive as shown in Table 3.29.

Excluding ampicillin resistance, no multiple antibiotic or drug resistance was found among the isolates under the conditions tested.

Resistant organisms were isolated from all but one of the sources—Rural Playa 2—and from both sediment and water. However, the playa at K.N. Clapp Park was the source of 41% of the resistant isolates. Sediment from all sources yielded 62% of the resistant isolates. Most (70%) of the total resistant isolates came from the March sampling date. All of these data are presented in Table 3.30. About 25% of all of the isolates sampled in March contained at least one resistance, excluding ampicillin. This is in contrast to the 6% found in July.

Paired *t*-tests were done on the numbers of resistant isolates isolated from the sediment versus those isolated from the water. The *t*-test showed there to be no significant difference. The mean of the sediment was 4.25 resistant isolates per urban lake and the mean of the water was 2.75 resistant isolates per urban lake. A 95% confidence interval was used, $n = 4$, $t = 3.00$, and $p = 0.058$. This accepts the null hypothesis that there is no difference between the number of resistant *Aeromonas* isolated from the water and the sediment.

The *t*-test performed comparing the number of resistant *Aeromonas* isolated between the March and July sampling months showed there to be no significant difference. The mean of March was 6.00 resistant isolates per urban lake and the mean of

July was 1.00 resistant isolate per urban lake. A 95% confidence interval was used, n = 4, t = 1.768, and p = 0.175. This accepts the null hypothesis that there is no difference between the number of resistant isolates from March and July.

Reliability of the antibiotic and metal sensitivity testing was determined. The quality control organisms *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were tested and found to comply with the 2002 NCCLS guidelines for sensitivity and resistance. Ten percent of the aeromonad isolates were re-tested, and the MICs of the re-tested isolates were determined. These MICs varied by no more than one two-fold dilution. This variation occurred 14% of the time. This should not affect the numbers of isolates considered to be resistant or sensitive because all of the organisms that appeared to be resistant were re-tested using higher dilutions at least once.

Plasmid Isolations and Transformations

Plasmid isolations were done on eight resistant isolates (Table 3.31). These plasmid isolations were done on two arsenic-resistant isolates, two mercury-resistant isolates, two trimethoprim-resistant isolates, one arsenite and trimethoprim-resistant isolate, and one mercury and trimethoprim-resistant isolate. Three plasmids were isolated, two from arsenite-resistant isolates and one from a mercury-resistant isolate. All of these plasmids were successfully transformed into *E. coli* XL1-Blue MRF′. The *E. coli* XL1-Blue MRF′ transformants were resistant to the metals, meaning that the resistance genes were carried on the isolated plasmids.

Table 3.1. Average temperatures and pHs of sampling sites on particular dates.

Source	Sampling Date	Average pH	Average Temperature (°C)
Stevens	March 23, 2002	7.57	8.75
Stevens	July 11, 2002	7.62	26.00
Clapp	March 23, 2002	7.79	11.00
Clapp	July 11, 2002	7.98	26.75
Maxey	March 23, 2002	8.29	12.00
Maxey	July 11, 2002	9.58	28.25
Higinbotham	March 23, 2002	8.06	12.50
Higinbotham	July 11, 2002	9.33	28.75
Rural 1	July 8, 2002	7.79	31.00
Rural 2	July 8, 2002	8.32	30.50
Brazos River	March 23, 2002	8.45	9.50
Brazos River	July 11, 2002	8.67	27.00
Rio Hondo	July 6, 2002	5.00	8.00
Pecos River	July 8, 2002	6.00	30.00

Table 3.2. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from all water sources. (0.1 mL of serial dilutions plated onto 4 replicas each; TFTC = too few to count; TMTC = too many to count)

Source	Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
Brazos	March 23	Water	7.78	0.0425
Brazos	March 23	Sediment	3240	112
Brazos	July 11	Water	3.20	0.0700
Brazos	July 11	Sediment	1620	1.00
Brazos	July 17	Sediment	7880	22.5
Stevens	March 23	Water	151	0.125
Stevens	March 23	Sediment	13000	225
Stevens	July 11	Water	42.0	0.200
Stevens	July 11	Sediment	3240	2.00
Stevens	July 17	Water	14.7	0.0150
Stevens	July 17	Sediment	10200	TMTC
Clapp	March 23	Water	161	0.550
Clapp	March 23	Sediment	13000	25.0
Clapp	July 11	Water	199	1.30
Clapp	July 11	Sediment	8150	167
Maxey	March 23	Water	252	0.275
Maxey	March 23	Sediment	48800	37.5
Maxey	July 11	Water	TFTC	0.200
Maxey	July 11	Sediment	5980	TMTC
Maxey	July 17	Water	22.8	0.325
Maxey	July 17	Sediment	22400	2.00
Higinbotham	March 23	Water	918	0.350
Higinbotham	March 23	Sediment	34500	23.3
Higinbotham	July 11	Water	45.3	0.825
Higinbotham	July 11	Sediment	9880	278
Rural 1	July 8	Water	1090	1.15
Rural 1	July 8	Sediment	14900	10.00
Rural 2	July 8	Water	36.0	0.525
Rural 2	July 8	Sediment	6730	TMTC
Rio Hondo	July 6	Water	17.2	0.325
Rio Hondo	July 6	Sediment	7380	508
Pecos River	July 8	Water	257	0.225
Pecos River	July 8	Sediment	25600	62.5
		Mean	7930	49.4
		Standard deviation	1120	112

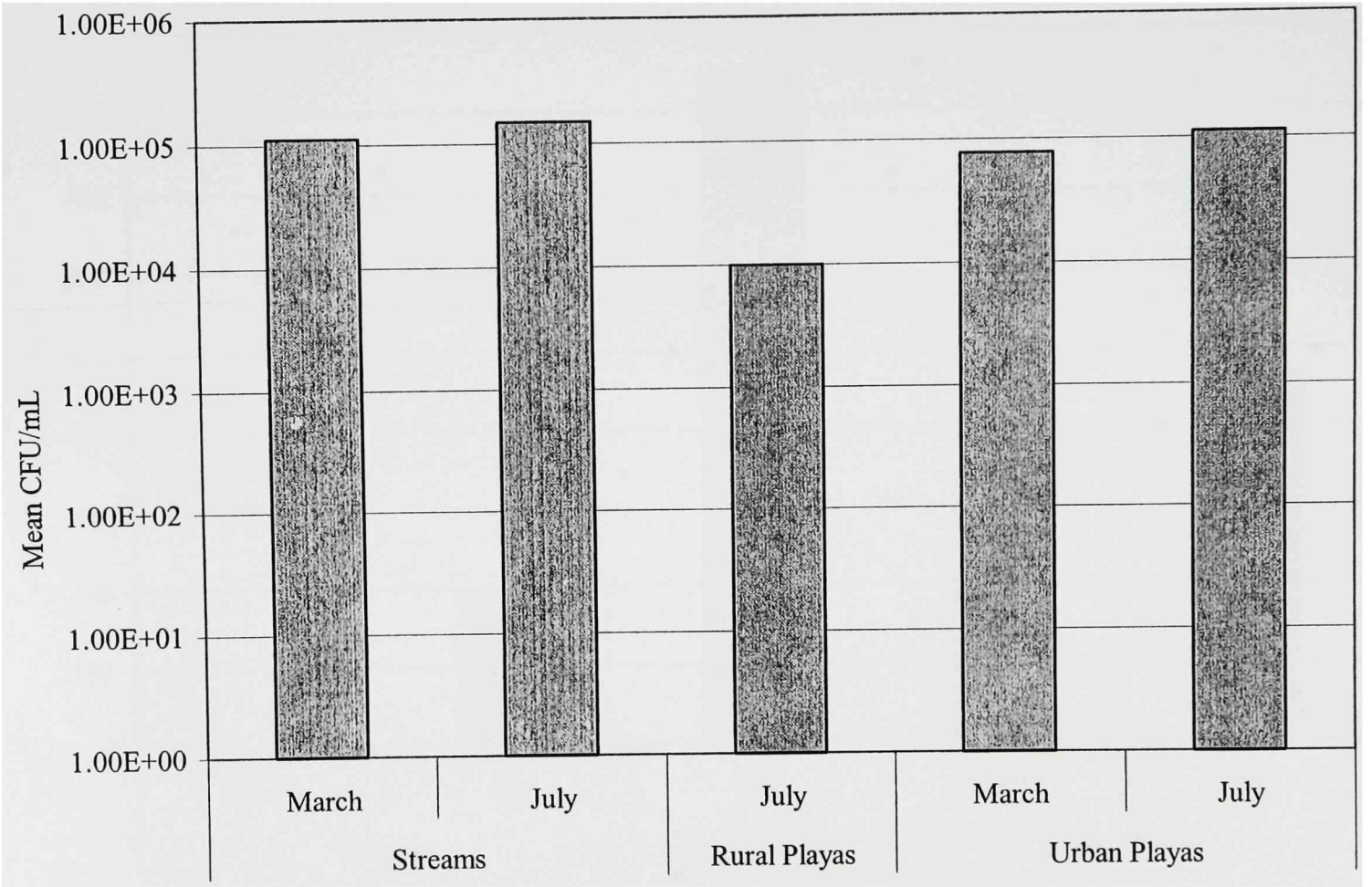

Figure 3.1. Presumptive *Aeromonas* (CFU/mL) from sediment compared across sampling location and season.

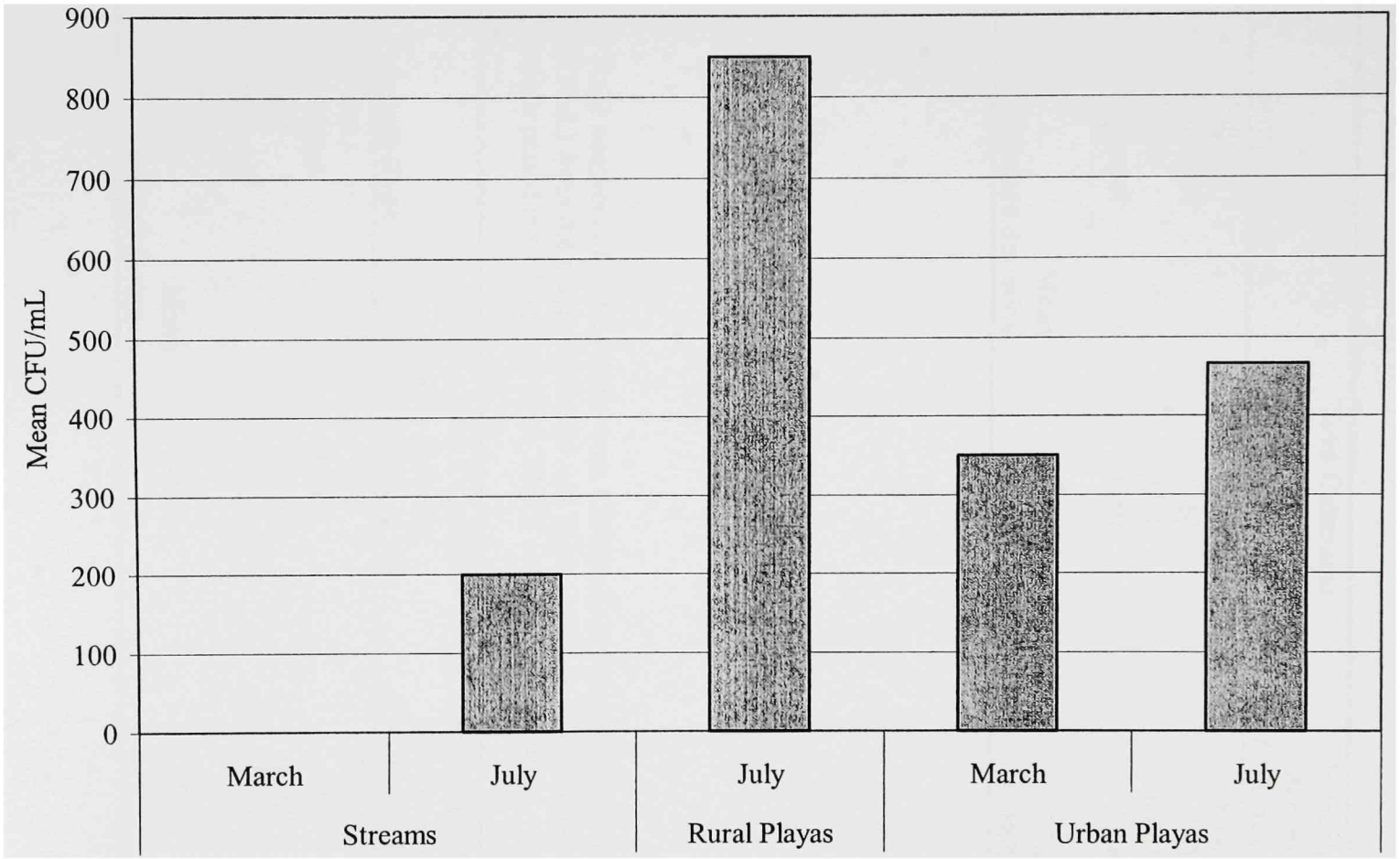

Figure 3.2. Presumptive *Aeromonas* (CFU/mL) from water compared across sampling location and season.

Table 3.3. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the Brazos River, Lubbock, TX. (0.1 mL of serial dilutions plated onto 4 replicas each)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
March	Water	7.78	0.0430
March	Sediment	3240	113
July	Water	32.0	0.0650
July	Sediment	4750	11.8
	Mean	2550	27.2
	Standard deviation	3270	48.6

Table 3.4. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the playa at Jack Stevens Park, Lubbock, TX. (0.1 mL of serial dilutions plated onto 4 replicas each; TMTC = too many to count)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
March	Water	151	0.125
March	Sediment	13000	225
July	Water	28.4	0.108
July	Sediment	6720	2.00
	Mean	4440	45.5
	Standard deviation	5750	100

Table 3.5. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the playa at K.N. Clapp Park, Lubbock, TX. (0.1 mL of serial dilutions plated onto 4 replicas each)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
March	Water	161	0.550
March	Sediment	13000	25.0
July	Water	199	1.30
July	Sediment	8150	167
	Mean	5380	48.5
	Standard deviation	6320	79.8

Table 3.6. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the playa at Maxey Park, Lubbock, TX. (0.1 mL of serial dilutions plated onto 4 replicas each; TFTC = too few to count; TMTC = too many to count)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
March	Water	252	0.275
March	Sediment	48800	37.5
July	Water	22.8	0.263
July	Sediment	12700	2.00
	Mean	15500	8.06
	Standard deviation	20700	16.5

Table 3.7. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the playa at Higinbotham Park, Lubbock, TX. (0.1 mL of serial dilutions plated onto 4 replicas each)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
March	Water	918	0.350
March	Sediment	34500	23.3
July	Water	45.3	0.825
July	Sediment	9880	27.80
	Mean	11300	75.6
	Standard deviation	16100	135

Table 3.8. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the Rural Playa 1, Shallowater, TX. (0.1 mL of serial dilutions plated onto 4 replicas each; TMTC = too many to count)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
July	Water	1090	1.15
July	Sediment	14900	10.0
	Mean	11400	5.58
	Standard deviation	7860	6.26

Table 3.9. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the Rural Playa 2, Shallowater, TX. (0.1 mL of serial dilutions plated onto 4 replicas each; TMTC = too many to count)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
July	Water	36.0	0.525
July	Sediment	6730	TMTC
	Mean	3380	
	Standard deviation	4730	

Table 3.10. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the Rio Hondo, Taos Ski Valley, NM. (0.1 mL of serial dilutions plated onto 4 replicas each)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
July	Water	17.2	0.0325
July	Sediment	7380	508
	Mean	370	254
	Standard deviation	5210	359

Table 3.11. Total numbers of culturable bacteria (CFU/mL) and presumptive *Aeromonas* (CFU/mL) from the Pecos River, Carlsbad, NM. (0.1 mL of serial dilutions plated onto 4 replicas each; TFTC = too few to count)

Date	Sample Type	Total Culturable Bacteria (CFU/mL x 10^3)	Presumptive *Aeromonas* (CFU/mL x 10^3)
July	Water	257	0.225
July	Sediment	25600	62.5
	Mean	12900	31.4
	Standard deviation	17900	44.0

Table 3.12. Biolog identification of 58 selected isolates, their sources, and number isolated.

Isolate	Source and Number of Isolates								
	Brazos	Stevens	Clapp	Maxey	Higinbotham	Hondo	Pecos	Rural 1	Rural 2
Aeromonas species	2	3	3		1			1	1
A. caviae DNA Group 4		1	1						
A. encheleia	3	1	1	1		1			
A. enteropelogenes					1				
A. hydrophila DNA Group 1			1	2	2			2	
A. hydrophila-like DNA Group 2	1					3			
A. icthiosmia	1								
A. jandaei DNA Group 9		1							
A. media-like DNA Group 5A			1	1					
A. sobria DNA Group 7	1	1		1	1				1
A. veronii DNA Group 10				1	1		1		1
A. veronii/sobria DNA Group 8				4	2		1		

Table 3.13. Comparison of numbers of *Aeromonas* isolated and identified from the Brazos River, Lubbock, TX, in March and July 2002.

Isolate	Number Isolated in March	Number Isolated in July
Aeromonas sp.	1	1
A. caviae DNA Group 4		
A. encheleia	3	
A. enteropelogenes		
A. hydrophila DNA Group 1		
A. hydrophila-like DNA Group 2	1	
A. icthiosmia		1
A. jandaei DNA Group 9		
A. media-like DNA Group 5A		
A. sobria DNA Group 7		1
A. veronii DNA Group 10		
A. veronii/sobria DNA Group 8		

Table 3.14. Comparison of numbers of *Aeromonas* isolated and identified from the K. N. Clapp Park, Lubbock, TX, in March and July 2002.

Isolate	Number Isolated in March	Number Isolated in July
Aeromonas sp.	3	1
A. caviae DNA Group 4	1	1
A. encheleia	1	
A. enteropelogenes		
A. hydrophila DNA Group 1	1	2
A. hydrophila-like DNA Group 2		
A. icthiosmia		
A. jandaei DNA Group 9		
A. media-like DNA Group 5A	1	
A. sobria DNA Group 7		
A. veronii DNA Group 10		
A. veronii/sobria DNA Group 8		

Table 3.15. Comparison of numbers of *Aeromonas* isolated and identified from Frank Higinbotham Park, Lubbock, TX, in March and July 2002.

Isolate	Number Isolated in March	Number Isolated in July
Aeromonas sp.	1	
A. caviae DNA Group 4		
A. encheleia		
A. enteropelogenes		1
A. hydrophila DNA Group 1	2	
A. hydrophila-like DNA Group 2		
A. icthiosmia		
A. jandaei DNA Group 9		
A. media-like DNA Group 5A		
A. sobria DNA Group 7	1	
A. veronii DNA Group 10		1
A. veronii/sobria DNA Group 8		2

Table 3.16. Comparison of numbers of *Aeromonas* isolated and identified from Maxey Park, Lubbock, TX, in March and July 2002.

Isolate	Number Isolated in March	Number Isolated in July
Aeromonas sp.		
A. caviae DNA Group 4		
A. encheleia	1	
A. enteropelogenes		
A. hydrophila DNA Group 1	2	
A. hydrophila-like DNA Group 2		
A. icthiosmia		
A. jandaei DNA Group 9		
A. media-like DNA Group 5A	1	
A. sobria DNA Group 7	1	
A. veronii DNA Group 10		1
A. veronii/sobria DNA Group 8	1	3

Table 3.17. Comparison of numbers of *Aeromonas* isolated and identified from Jack Stevens Park, Lubbock, TX, in March and July 2002.

Isolate	Number Isolated in March	Number Isolated in July
Aeromonas sp.	3	1
A. caviae DNA Group 4		1
A. encheleia	1	1
A. enteropelogenes		
A. hydrophila DNA Group 1		
A. hydrophila-like DNA Group 2		
A. icthiosmia		
A. jandaei DNA Group 9		1
A. media-like DNA Group 5A		
A. sobria DNA Group 7		1
A. veronii DNA Group 10		
A. veronii/sobria DNA Group 8		

Table 3.18. Accuracy of *Aeromonas* species identified by the Biolog identification system across all sampling times and locations.

Species	Similarity Index[a]	Number Identified
Aeromonas sp.	Not applicable	13
A. caviae DNA Group 4	0.728 ± 0.058	3
A. encheleia	0.701 ± 0.145	8
A. enteropelogenes	0.575 ± 0	1
A. hydrophila DNA Group 1	0.715 ± 0.129	8
A. hydrophila-like DNA Group 2	0.756 ± 0.204	5
A. icthiosmia	0.700 ± 0	1
A. jandaei DNA Group 9	0.505 ± 0	1
A. media-like DNA Group 5A	0.602 ± 0.026	2
A. sobria DNA Group 7	0.663 ± 0.087	5
A. veronii DNA Group 10	0.612 ± 0.101	4
A. veronii/sobria DNA Group 8	0.696 ± 0.152	7

[a] The similarity index was determined by averaging the Similarity Indices given by the Biolog system for each isolate belonging to the species and then determining the range of values.

Table 3.19. Carbon source utilization of the isolates identified by Biolog. (+ = ≥90% of strains positive; - = ≥90% of strains negative; d = 11-89% strains positive)

Carbon source	Total	*A. jandaei* DNA Group 9	*A. icthiosmia*	*A. media*-like DNA Group 5A	*A. caviae* DNA Group 4	*A. veronii* DNA Group 10	*A. sobria* DNA Group 7	*A. hydrophila*-like DNA Group 2	*A. veronii*/ *sobria* DNA Group 8	*A. hydrophila* DNA Group 1	*A. encheleia*	*A. enteropelogenes*
Water	-	-	-	-	-	-	-	-	-	-	-	-
α-cyclodextrin	-	-	d	-	-	-	-	-	-	-	-	d
Dextrin	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+
Tween 40	+	d	+	d	+	+	+	+	+	+	+	+
Tween 80	+	d	+	d	d	+	+	+	+	+	+	+
N-acetyl-D-galactosamine	d	-	-	-	-	+	+	+	d	d	-	-
N-acetyl-D-glucosamine	+	+	+	+	+	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	d	-	-	+	+	d	d	+	d	d	+	+
D-arabitol	-	-	-	-	-	-	d	-	-	-	-	-
D-cellobiose	d	-	d	+	d	d	d	-	d	d	d	d
L-erythritol	d	-	d	-	d	d	d	d	d	d	d	d
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+
L-fucose	-	-	-	-	-	-	d	d	-	-	-	-
D-galactose	+	-	+	+	+	+	+	+	+	+	+	+
Gentiobiose	d	-	-	-	d	d	d	-	-	-	-	-
α-D-glucose	+	+	+	+	+	+	+	+	+	+	+	+
Mesoinositol	-	-	-	-	-	-	d	-	-	-	-	-
α-D-lactose	d	-	-	+	d	-	-	-	d	d	-	-

Table 3.19. Continued.

Carbon source	Total	*A. jandaei* DNA Group 9	*A. icthiosmia*	*A. media*-like DNA Group 5A	*A. caviae* DNA Group 4	*A. veronii* DNA Group 10	*A. sobria* DNA Group 7	*A. hydrophila*-like DNA Group 2	*A. veronii*/ *sobria* DNA Group 8	*A. hydrophila* DNA Group 1	*A. encheleia*	*A. enteropelogenes*
Lactulose	-	-	-	d	d	-	-	-	d	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	d	+	+	+	+	+	+	+	+	+	-
D-mannose	+	d	+	+	d	+	+	+	+	+	d	+
D-melibiose	-	-	-	d	-	-	-	-	d	-	-	-
ß-Methyl-D-glucoside	+	+	+	+	+	+	+	+	+	+	+	+
D-psicose	+	d	+	d	d	+	+	+	+	d	d	+
D-raffinose	d	-	-	d	d	d	d	-	d	-	-	-
L-rhamnose	-	-	-	-	-	-	-	d	-	-	-	-
D-sorbitol	d	-	+	-	d	d	d	d	d	d	d	-
Sucrose	d	-	+	+	+	+	d	+	d	+	+	d
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+
Turanose	d	-	+	-	d	d	d	d	d	d	d	d
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-
Pyruvic acid methyl ester	+	d	+	+	+	+	+	+	+	+	+	+
Succinic acid mono-methyl ester	d	-	+	d	d	+	+	d	d	d	d	d
Acetic acid	+	-	+	+	+	+	+	+	+	d	+	d
Cis-aconitic acid	d	-	+	d	+	+	d	d	d	d	d	-
Citric acid	d	-	+	d	+	d	d	d	d	d	d	d
Formic acid	d	-	-	-	d	-	-	d	-	d	d	-
D-glactonic acid lactone	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.19. Continued.

Carbon source	Total	*A. jandaei* DNA Group 9	*A. ichthiosmia*	*A. media*-like DNA Group 5A	*A. caviae* DNA Group 4	*A. veronii* DNA Group 10	*A. sobria* DNA Group 7	*A. hydrophila*-like DNA Group 2	*A. veronii*/ *sobria* DNA Group 8	*A. hydrophila* DNA Group 1	*A. encheleia*	*A. enteropelogenes*
D-galacturonic acid	-	-	-	-	d	-	-	-	-	-	-	-
D-gluconic acid	+	+	+	+	+	+	d	+	+	+	+	+
D-glucosaminic acid	-	-	-	-	-	-	-	-	-	-	-	-
D-glucuronic acid	-	-	-	-	-	-	-	-	-	-	-	-
α-hydroxybutyric acid	d	-	-	d	d	-	-	-	d	d	-	-
ß-hydroxybutyric acid	d	-	+	-	d	-	d	d	d	d	d	-
γ-hydroxybutyric acid	-	-	-	-	-	-	-	-	-	-	-	-
p-hydroxy-phenylacetic acid	-	-	-	-	-	-	-	-	d	-	-	-
Itaconic acid	-	-	-	-	-	-	-	-	-	-	-	-
α-ketobutyric acid	d	-	+	-	-	d	d	-	d	d	-	d
α-ketoglutaric acid	d	-	+	-	d	d	d	d	d	-	d	+
α-ketovaleric acid	-	-	+	-	-	-	-	-	+	-	-	-
D,L lactic acid	d	-	-	+	+	d	d	-	d	+	-	d
Malonic acid	-	-	-	-	-	-	-	-	d	-	-	-
Propionic acid	d	-	-	d	d	d	d	d	d	d	d	-
Quinic acid	-	-	-	-	-	-	-	-	-	-	-	-
D-saccharic acid	-	-	-	-	-	-	-	-	-	-	-	-
Sebacic acid	-	-	-	-	-	-	-	-	-	-	-	-
Succinic acid	+	-	+	+	+	+	+	+	+	+	+	+

Table 3.19. Continued.

Carbon source	Total	*A. jandaei* DNA Group 9	*A. ichthiosmia*	*A. media*-like DNA Group 5A	*A. caviae* DNA Group 4	*A. veronii* DNA Group 10	*A. sobria* DNA Group 7	*A. hydrophila*-like DNA Group 2	*A. veronii*/ *sobria* DNA Group 8	*A. hydrophila* DNA Group 1	*A. encheleia*	*A. enteropelogenes*
Bromosuccinic acid	+	d	+	d	+	+	+	+	+	+	+	+
Succinamic acid	d	-	d	-	d	d	d	d	d	-	d	d
Glucuronamide	-	-	-	-	-	-	-	-	-	-	-	-
L-alaninamide	d	-	+	d	+	d	d	d	d	d	d	+
D-alanine	d	-	+	d	+	d	d	+	d	d	d	d
L-alanine	+	-	+	d	+	+	+	+	+	d	+	+
L-alanyl-glycine	+	-	+	d	+	+	+	+	+	d	+	+
L-asparagine	+	d	+	+	+	+	+	+	+	+	+	+
L-aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+
L-glutamic acid	+	d	+	+	+	+	+	+	+	+	+	+
Glycyl-L-aspartic acid	+	d	+	+	+	+	+	+	+	+	d	+
Glycyl-L-glutamic acid	d	-	+	d	d	+	+	d	d	d	d	+
L-histidine	d	-	-	d	+	+	-	+	d	d	d	d
Hydroxy-L-proline	-	-	-	d	-	-	-	-	-	d	-	-
L-leucine	-	-	-	-	-	-	d	-	-	-	-	-
L-ornithine	d	-	d	d	+	d	d	d	d	d	d	d
L-phenylalanine	-	-	-	-	-	-	-	-	-	-	-	d
L-proline	d	-	+	d	+	d	d	d	+	d	d	+
L-pyroglutamic acid	-	-	-	-	-	d	d	-	d	-	-	-
D-serine	+	-	+	+	+	+	d	+	+	+	d	+
L-serine	+	-	+	+	+	+	+	+	+	+	+	+

Table 3.19. Continued.

Carbon source	Total	*A. jandaei* DNA Group 9	*A. icthiosmia*	*A. media*-like DNA Group 5A	*A. caviae* DNA Group 4	*A. veronii* DNA Group 10	*A. sobria* DNA Group 7	*A. hydrophila*-like DNA Group 2	*A. veronii*/ *sobria* DNA Group 8	*A. hydrophila* DNA Group 1	*A. encheleia*	*A. enteropelogenes*
L-threonine	d	-	+	d	+	d	d	d	d	d	d	d
D,L-carnitine	-	-	-	-	-	-	-	-	-	-	-	-
γ-aminobutyric acid	-	-	-	-	-	-	-	-	-	-	-	-
Urocanic acid	d	-	+	+	+	-	d	+	-	d	d	d
Inosine	+	+	+	+	+	+	+	+	d	+	+	+
Uridine	+	-	+	+	+	+	+	+	+	+	+	+
Thymidine	d	-	+	d	+	+	+	d	d	+	d	+
Phenylethylamine	-	-	-	-	-	-	-	-	-	-	-	-
Putrescine	d	-	-	d	+	-	-	+	-	d	d	d
2-aminoethanol	-	-	-	-	-	-	-	-	-	-	d	-
2,3-butanediol	-	-	d	-	d	-	-	-	-	-	-	-
Glycerol	+	d	+	+	+	+	+	+	+	+	+	+
D,L,α-glycerol phosphate	+	d	+	+	+	+	+	+	+	+	d	+
α-D-glucose-1-phosphate	+	d	+	+	-	+	+	+	+	+	d	+
D-glucose-6-phosphate	+	d	+	+	-	+	+	+	+	+	d	+

Table 3.20. Percentages of identified *Aeromonas* isolates showing significant (>10%) anomalous carbon utilization. (- indicates no carbon utilization; + indicates carbon utilization)

Carbon source	Species	Strains should be	Strains were	%
Succinamic acid	*A. encheleia* *A. hydrophila* *A. hydrophila*-like *A. caviae* *A. sobria* *A.veronii/sobria* *A. veronii*	-	+	62
Citric acid	*A. encheleia* *A. hydrophila* *A. hydrophila*-like	+	-	27
Putrescine	*A. encheleia*	+	-	13
D-cellobiose	*A. caviae* *A. sobria*	-	+	11
Turanose	*A. hydrophila* *A. veronii/sobria* *A. veronii*		+	29
Propionic acid	*A. hydrophila*-like *A. veronii/sobria* *A. sobria* *A. veronii*	+	-	13

Table 3.21. Minimal inhibitory concentrations of metals (m*M*).

Metal	Min MIC	MIC 50%	MIC 90%	Max MIC	% Resistant
$NaAsO_2$	<0.75	1.263	3.970	12	2.83
K_2CrO_4	<0.4	<0.4	<0.4[a]	<0.4	0
$CoCl_2$ $6H_2O$	<0.125	<0.125	<0.125	0.25	0
$CuSO_4$	<0.125	<0.125	<0.125	0.25	0
$HgCl_2$	<0.003125	0.00337	0.00579	0.1	2.47
$NiSO_4$ $6H_2O$	<0.125	<0.125	<0.125	0.25	0
$ZnSO_4$ $7H_2O$	<0.125	<0.125	<0.125[a]	<0.125	0

[a] 100% of the isolates were inhibited at the lowest concentration

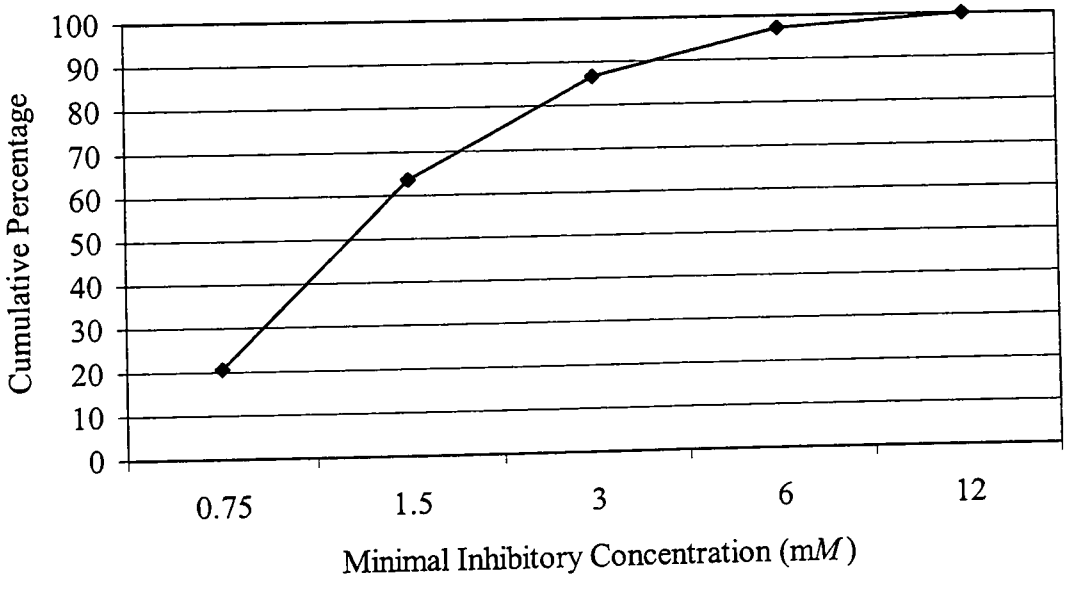

Figure 3.3. Cumulative percentage plot for the $NaAsO_2$ MICs of the 283 isolates.

Table 3.22. Isolates resistant to 6 m*M* $NaAsO_2$, their sources, identification, and ratio of cell mass grown in 40% nutrient broth with 6 m*M* $NaAsO_2$ to cell mass grown in 40% nutrient broth without $NaAsO_2$. The cell mass of each culture was measured by absorbance at 550 nm. (W = isolated from water; S = isolated from sediment)

Unique code	Source	Biolog Identification	Ratio
S2-6	Stevens (W)	*Aeromonas* sp.	0.616
C2-9	Clapp (W)	*Aeromonas* sp.	0.670
CS2-4	Clapp (S)	*Aeromonas* sp.	0.420
CS3-10	Clapp (S)	*Aeromonas* sp.	0.779
MS2-2	Maxey (S)	*A. media*-like DNA Group 5A	0.592
Y2-1	Brazos (W)	*A. encheleia*	0.647
T3-9	Hondo (W)	*A. hydrophila*-like DNA Group 2	0.563
1R3-8	Rural 1 (W)	*A. hydrophila* DNA Group 1	0.451

Table 3.23. Isolates resistant to 0.025 m*M* $HgCl_2$, their sources, and ratio of cell mass grown in 40% nutrient broth with 0.025 m*M* $HgCl_2$ to cell mass grown in 40% nutrient broth without $HgCl_2$. The cell mass of each culture was measured by absorbance at 550 nm. (W = isolated from water; S = isolated from sediment)

Unique Code	Source	Biolog Identification[a]	Ratio
CS2-1	Clapp (S)	*A. encheleia*	0.962
CS2-3	Clapp (S)	Not performed putative *A. encheleia*	0.931
CS2-9	Clapp (S)	Not performed putative *A. encheleia*	0.670
M2-8	Maxey (W)	*A. hydrophila*-like DNA Group 2	0.955
TS3-5	Hondo (S)	*A. encheleia*	0.527
TS3-8	Hondo (S)	Not performed putative *A. encheleia*	0.691
TS3-9	Hondo (S)	Not performed putative *A. encheleia*	0.917

[a] A putative species identification means that the isolate was not identified using the Biolog system. However, the organism shares the same phenotypic characteristics and source of isolation as an organism that was positively identified by Biolog.

Table 3.24. Minimal inhibitory concentrations of antibiotics and drugs (μg/mL).

Antibiotic	Min MIC	MIC 50%	MIC 90%	Max MIC	% Resistant
Ampicillin	<4	>64	>64	>64	91.52
Cefuroxime	<0.125	<0.125	0.871	8	0
Kanamycin	<0.25	<0.25	<0.25 [a]	<0.25	0
Nalidixic acid	<2	<2	<2 [a]	<2	0
Ofloxacin	<0.25	<0.25	<0.25 [a]	<0.25	0
Sulfamethoxazole	<4	<4	6.427	32	0
Tetracyclin	<0.03125	0.0323	0.0583	1	0
Trimethoprim	<0.5	1.311	3.890	16	8.83

[a] 100% of the isolates were inhibited at the lowest concentration.

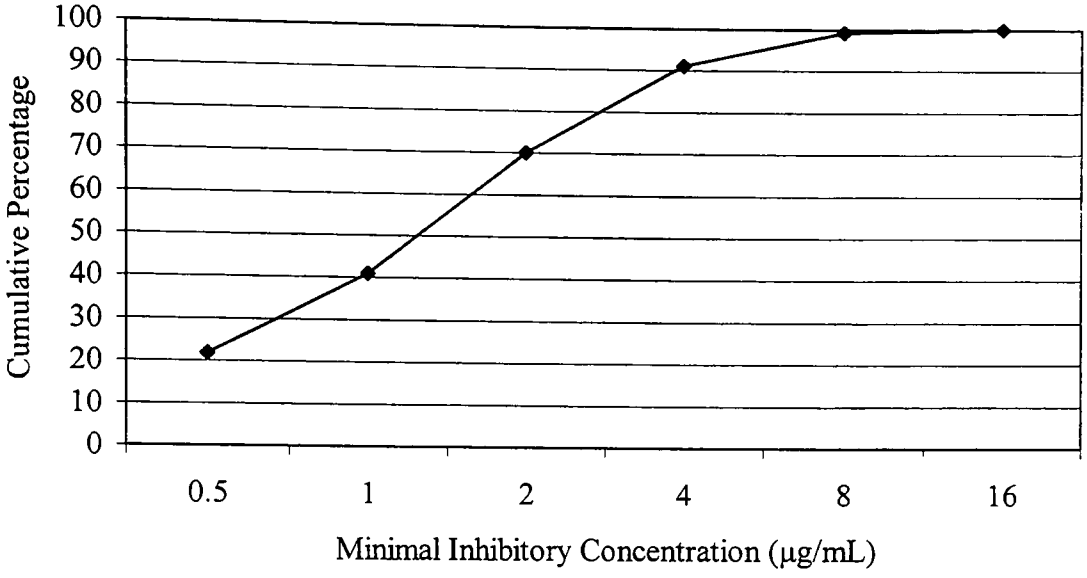

Figure 3.4. Cumulative percentage plot for the trimethoprim MICs of the 283 isolates.

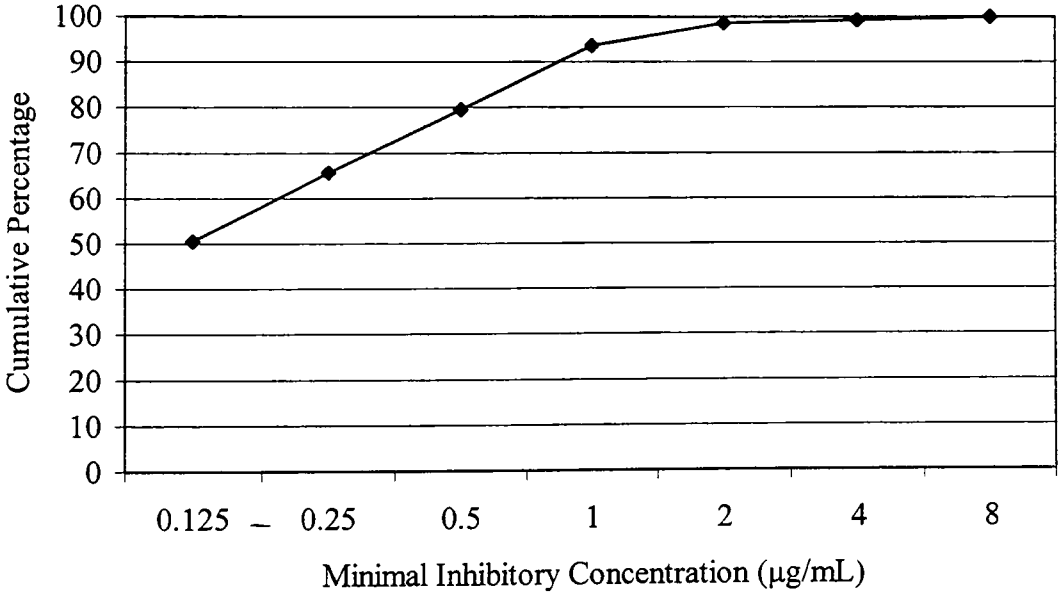

Figure 3.5. Cumulative percentage plot for the cefuroxime MICs of the 283 isolates.

Table 3.25. Ampicillin-sensitive aeromonads. (W = isolated from water; S = isolated from sediment)

Unique code	Biolog Identification [a]	Source
YS2-4	*A. hydrophila*-like DNA Group 2	Brazos (S)
C2-8	*A. caviae* DNA Group 4	Clapp (W)
C3-2	*A. caviae* DNA Group 4	Clapp (W)
H2-9	*Aeromonas* sp.	Higinbotham (W)
HS3-6	*A. veronii/sobria* DNA Group 8	Higinbotham (S)
P3-3	Not performed putative *A. veronii/sobria* DNA Group 8	Pecos (W)
P3-4	Not performed putative *A. veronii/sobria* DNA Group 8	Pecos (W)
P3-5	Not performed putative *A. veronii/sobria* DNA Group 8	Pecos (W)
P3-8	Not performed putative *A. veronii/sobria* DNA Group 8	Pecos (W)
PS3-6	Not performed putative *A. veronii* DNA Group 10	Pecos (S)
2R3-5	*A. sobria* DNA Group 7	Rural 2 (W)
MS2-5	*A. sobria* DNA Group 7	Maxey (S)
M2-5	*A. veronii/sobria* DNA Group 8	Maxey (W)
MS2-6	Not performed putative *A. sobria* DNA Group 7	Maxey (S)
M2-8	*A. hydrophila* DNA Group 1	Maxey (W)
M3-5	*A. veronii/sobria* DNA Group 8	Maxey (W)
2R3-1	*A. sobria* DNA Group 7	Rural 2 (W)
SS3B-13	*A. encheleia*	Stevens (S)

Table 3.25. Continued.

Unique code	Biolog Identification [a]	Source
SS2-3	*Aeromonas* sp.	Stevens (S)
SS2-6	Not performed putative *Aeromonas* sp.	Stevens (S)
SS2-7	Not performed putative *Aeromonas* sp.	Stevens (S)
SS2-8	Not performed putative *Aeromonas* sp.	Stevens (S)
SS2-9	Not performed putative *Aeromonas* sp.	Stevens (S)
S3-5	*A. jandaei* DNA Group 9	Stevens (W)

[a] A putative species identification means that the isolate was not identified using the Biolog system. However, the organism shares the same phenotypic characteristics and source of isolation as an organism that was positively identified by Biolog.

Table 3.26. Isolates resistant to 8 μg/mL trimethoprim, their sources, and ratio of cell mass grown in 40% nutrient broth with 8 μg/mL trimethoprim to cell mass grown in 40% nutrient broth without trimethoprim. The cell mass of each culture was measured by absorbance at 550 nm. (W = isolated from water; S = isolated from sediment)

Unique Code	Source	Biolog Identification	Ratio
C2-4	Clapp (W)	*Aeromonas* sp.	0.466
HS2-11	Higinbotham (S)	*A. hydrophila* DNA Group 1	0.391

Table 3.27. Isolates resistant to 4 μg/mL trimethoprim, their sources, identification, and ratio of cell mass grown in 40% nutrient broth with 4 μg/mL trimethoprim to cell mass grown in 40% nutrient broth without trimethoprim. The cell mass of each culture was measured by absorbance at 550 nm. (W = isolated from water; S = isolated from sediment)

Unique code	Source	Biolog Identification[a]	Ratio
YS2-3	Brazos (S)	Not performed putative *A. encheleia*	0.266
S2-3	Stevens (W)	Not performed putative *A. encheleia*	0.645
S2-5	Stevens (W)	Not performed putative *A. encheleia*	0.292
S2-6	Stevens (W)	Not performed putative *Aeromonas* sp.	0.409
S2-7	Stevens (W)	Not performed putative *A. encheleia*	0.382
SS2-3	Stevens (S)	Not performed putative *Aeromonas* sp.	0.460
SS2-4	Stevens (S)	Not performed putative *Aeromonas* sp.	0.681
SS2-6	Stevens (S)	Not performed putative *Aeromonas* sp.	0.521
C2-1	Clapp (W)	*A. media*-like DNA Group 5A	0.528
C2-3	Clapp (W)	Not performed putative *A. media*-like DNA Group 5A	0.546
C2-5	Clapp (W)	Not performed putative *A. media*-like DNA Group 5A	0.434
C2-6	Clapp (W)	Not performed putative *A. media*-like DNA Group 5A	0.482
C2-9	Clapp (W)	*Aeromonas* sp.	0.343
CS2-2	Clapp (S)	*A. hydrophila* DNA Group 1	0.600
CS2-3	Clapp (S)	Not performed putative *A. encheleia*	0.406
CS2-6	Clapp (S)	Not performed putative *A. hydrophila* DNA Group 1	0.272
CS2-7	Clapp (S)	Not performed putative *A. hydrophila* DNA Group 1	0.582

Table 3.27. Continued.

Unique code	Source	Biolog Identification[a]		Ratio
CS2-8	Clapp (S)	Not performed	putative *A. hydrophila* DNA Group 1	0.990
YS3B-2	Brazos (S)	Not performed	putative *A. icthiosmia*	0.344
YS3C-4	Brazos (S)	Not performed	putative *A. icthiosmia*	0.327
SS3-2	Stevens (S)	Not performed	putative *A. sobria* DNA Group 7	0.441
SS3B-13	Stevens (S)	*A. encheleia*		0.410
MS3-6	Maxey (S)	Not performed	putative *A. veronii* DNA Group 10	0.385

[a] A putative species identification means that the isolate was not identified using the Biolog system. However, the organism shares the same phenotypic characteristics and source of isolation as an organism that was positively identified by Biolog.

Table 3.28. Isolates with trimethoprim resistance and a metal resistance. (W = isolated from water; S = isolated from sediment)

Unique code	Biolog identification[a]	Source	Resistances
S2-6	*Aeromonas* sp.	Stevens (W)	Trimethoprim; $NaAsO_2$
C2-9	*Aeromonas* sp.	Clapp (W)	Trimethoprim; $NaAsO_2$
CS2-3	Not performed putative *A. encheleia*	Clapp (S)	Trimethoprim; $HgCl_2$

[a] A putative species identification means that the isolate was not identified using the Biolog system. However, the organism shares the same phenotypic characteristics and source of isolation as an organism that was positively identified by Biolog.

Table 3.29. Isolates with one resistance and ampicillin sensitivity. (W = isolated from water; S = isolated from sediment)

Unique code	Biolog identification[a]	Source	Resistance
M2-8	*A. hydrophila* DNA Group 1	Maxey (W)	$HgCl_2$
SS2-3	*Aeromonas* sp.	Stevens (S)	Trimethoprim
SS2-6	Not performed putative *Aeromonas* sp.	Stevens (S)	Trimethoprim

[a] A putative species identification means that the isolate was not identified using the Biolog system. However, the organism shares the same phenotypic characteristics and source of isolation as an organism that was positively identified by Biolog.

Table 3.30. Number of resistant isolates, their sample types and sources. (NS = not sampled)

Source	Sample Type		Date of Sampling		Total
	Sediment	Water	March	July	
Brazos	3	1	2	2	4
Stevens	5	4	7	2	9
Clapp	9	6	14	1	15
Maxey	2	1	2	1	3
Higinbotham	1	0	1	0	1
Rio Hondo	3	1	NS	4	4
Pecos	0	0	NS	0	0
Rural 1	0	1	NS	1	1
Rural 2	0	0	NS	0	0
Total	23	14	26	11	37

Table 3.31. Plasmid isolations from arsenite- and mercury-resistant isolates. (W = isolated from water; S = isolated from sediment).

Unique Code	Source	Biolog Identification[a]	Resistance(s)	Plasmid Isolated	Transformation of Resistant Phenotype
CS3-10	Clapp (S)	*Aeromonas* species	Arsenite	Yes	Yes
1R3-8	Rural 1 (W)	*A.hydrophila* DNA Group 1	Arsenite	Yes	Yes
CS2-1	Clapp (S)	*A. encheleia*	Mercury	Yes	Yes
TS3-9	Hondo (S)	Not performed putative *A. encheleia*	Mercury	No	NA
S2-6	Stevens (W)	*Aeromonas* species	Arsenite, Trimethoprim	No	NA
CS2-3	Clapp (S)	Not performed putative *A. encheleia*	Mercury, Trimethoprim	No	NA
SS3B-13	Stevens (S)	*A. encheleia*	Trimethoprim	No	NA
HS2-11	Higinbotham (S)	*A. hydrophila* DNA Group 1	Trimethoprim	No	NA

[a] A putative species identification means that the isolate was not identified using the Biolog system. However, the organism shares the same phenotypic characteristics and source of isolation as an organism that was positively identified by Biolog.

CHAPTER IV

DISCUSSION

Biolog Identification

All of the isolates identified by the Biolog system were in the genus *Aeromonas*. This shows that the modified *Aeromonas* medium and subsequent screening procedures were reliable in isolating only *Aeromonas* species as opposed to other genera that inhabit water or sediment. It can also be assumed that the other 225 isolates not identified by Biolog are also in the genus *Aeromonas* since they underwent the same screening procedures as those subsequently identified. For each group of resistant and sensitive isolates, a representative organism of each group was also identified by the Biolog system.

The carbon utilization patterns of the isolates were consistent with those listed in *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1993). All of these results were also consistent with those of Valera and Esteve (2002), except for the *Aeromonas encheleia* isolates. Eight of the isolates were identified as *A. encheleia* by the Biolog system. This species was originally isolated from European eels in 1995 (Esteve et al., 1995). It is expected to belong to a new DNA hybridization group 16 (Carnahan and Altwegg, 1996). Valera and Esteve (2002) tested fourteen *A. encheleia* strains, while this study only evaluated eight. Sample size could account for the discrepancies between the two studies. Since the species designation is new, it is still undergoing changes in taxonomy.

A. enteropelogenes and *A. icthiosmia* were each identified once. *A. enteropelogenes* is most likely a synonym for *A. trota*. This is based on 16S rRNA analysis (Collins et al., 1994). However, *A. trota* is characteristically sensitive to ampicillin (Carnahan et al., 1991; Overman and Janda, 1999). The *A. enteropelogenes* isolate from this study was resistant to ampicillin at over 64 μg/mL. *A. icthiosmia* is probably a synonym for *A. veronii* (Collins et al., 1994).

A. hydrophila was also isolated frequently. This species is known to cause septicemia, myonecrosis, wound infections, *Aeromonas* arthritis, peritonitis, and has been associated with hemolytic uremic syndrome. (Janda and Abbott, 1996). The other isolates A. *veronii*, *A. jandaei*, and *A. caviae* have been associated with human wound infections and septicemia. *A. media* and *A. sobria* have been associated with human diarrheal disease (Joseph, 1996).

Antibiotic and Drug Resistance

All isolates were tested for antibiotic and drug resistance, and their MICs were determined. Ampicillin resistance was prevalent with over 91% of the isolates having an MIC of greater than or equal to 64 μg/mL. Ampicillin is a semisynthetic penicillin in the β-lactam group that impedes the synthesis of the cell wall (Madigan et al., 2000). This resistance was not surprising since most strains of *Aeromonas* are inherently resistant to the antibiotic. Ko and colleagues (1996) found 99.6% of their clinical isolates to be resistant to ampicillin while other studies have shown up to 100% resistance (Vila et al., 2002).

The only other antibiotic or drug the *Aeromonas* isolates showed resistance to was trimethoprim. Trimethoprim is a drug that blocks folate biosynthesis and indirectly disrupts several metabolic pathways including purine biosynthesis (Snyder and Champness, 1997). This study differs from most others in that trimethoprim was considered alone instead of in conjunction with sulfamethoxazole. Kämpfer el al. (1999) tested 217 clinical and non-clinical *Aeromonas* isolates against trimethoprim alone and found none of them to be resistant. This is in contrast to the 8.83% found in this study. Resistance to sulfamethoxazole also differed between the two studies. Sulfamethoxazole is a folic acid analog that inhibits a different enzymatic step in folate biosynthesis than trimethoprim (Madigan et al., 2000). This study showed no sulfamethoxazole resistance, while a published value was close to 26% (Kämpfer el al., 1999).

No other resistances were found to cefuroxime, kanamycin, nalidixic acid, ofloxacin, or tetracycline. Cefuroxime is a cephalosporin in the β-lactam group and inhibits cell wall synthesis (Madigan et al., 2000). Other studies with cefuroxime also show a low incidence of resistance. These studies range from 0 to 1.38% of isolates displaying resistance (Kämpfer et al., 1999; Overman and Janda, 1999; Vila et al., 2002). Warren (1998) did a similar study in aeromonads from the Lubbock playas. One isolate out of 151 aeromonads was cefuroxime resistant (Warren, 1998).

The observation that none of the isolates have kanamycin resistance is supported by Kämpfer el al. (1999), who also did not find the resistance among their *Aeromonas* isolates. Kanamycin blocks translation by targeting 16S rRNA (Snyder and Champness, 1997).

The widespread nalidixic acid sensitivity in this study is in contrast to the 43% resistance reported by Vila et al. (2002). However, Miranda and Castillo (1998) report finding less than 4% of their *Aeromonas* isolates to be resistant to nalidixic acid. One of the first nalidixic acid-resistant aeromonads was isolated in 1987 (Chang and Bolton, 1987). Other nalidixic acid-resistant isolates have been reported since then (Ko et al., 1996; Vila et al., 2002). Nalidixic acid is a quinolone. It targets DNA gyrase and blocks replication of DNA (Snyder and Champness, 1997).

No resistance to ofloxacin, also a quinolone, was detected either in this study or previous studies (Kämpfer el al., 1999; Overman and Janda, 1999; Vila et al., 2002). Warren (1998) also found no ofloxacin resistance. Susceptibility of the isolates to ofloxacin was not unusual. Quinolone resistance is due only to chromosomal mutations. Members of the genus *Aeromonas* do not have chromosomal resistance genes to quinolones as they do to ampicillin. These chromosomal genes, if present by mutation, are not transferable. Furthermore, since quinolones are synthetic and are not naturally present in environmental waters, there is no natural selection for resistant phenotypes (Goñi-Urriza et al., 2000). In fact, quinolone sensitivity is so common in aeromonads that these antibiotics are the first choice for the treatment of *Aeromonas* infections in humans (Jones and Wilcox, 1995).

Tetracycline resistance was also found to be low compared to previous studies. Tetracycline inhibits protein synthesis by blocking proper functioning of the 30S ribosomal subunit (Chopra and Roberts, 2001). Kämpfer et al. (1999) found

approximately 10% of their isolates to be resistant whereas 0% is reported here. Warren (1998) reported 2.6% of his isolates to be tetracycline resistant.

In general, the isolates included in this study showed an equivalent or much lower resistance than *Aeromonas* isolates in other studies. A possible explanation of these findings is that some of the earlier studies included clinical isolates that could have previously been exposed to antibiotics (Kämpfer el al., 1999; Ko et al., 1996; Vila et al., 2002). In other studies done solely with environmental aeromonads, they have been isolated them from waters highly polluted by industrial effluent or raw sewage (Goñi-Urriza et al., 2000). The six playa lakes and three streams in this study receive runoff from surrounding areas. None of the water bodies are polluted from a single source. However, the runoff can contain low levels of pollutants or even antibiotics. According to the results of this study and the low incidence of antibiotic resistance present (aside from ampicillin), the six playa lakes and three streams that were sampled could possibly be considered a reservoir for antibiotic and metal resistance genes. The resistant aeromonads from the environment could opportunistically infect humans and cause an infection that would be difficult to treat.

Metal Resistance

Not much has been done to evaluate the metal resistance profiles of aeromonads from unpolluted water sources. Many of the bacteria used in past studies to characterize metal resistance were isolated from waters polluted with metals (Allen et al., 1977; Francisco et al., 2002; Miranda and Castillo, 1998; Timoney et al., 1978). These bacteria

studied were rarely aeromonads. None of the playas or streams in this study are highly polluted with metals. However, some metals could have been introduced into these waters inadvertently. Pesticides and domestic wastes could be a source of metals for all of the water bodies (Collins and Stotzky, 1989). The Rio Hondo could be contaminated with a variety of metals from past mining attempts. This past mining near the Rio Hondo could explain why three isolates from the stream were resistant to mercury and one was resistant to arsenite.

Only resistances to arsenite and mercury were found. Resistance to arsenite was found in 2.83% of the isolates, while 2.47% of the isolates contained mercury resistance. None of the isolates contained more than one metal resistance. Miranda and Castillo (1998) found between 29-40% of their aeromonads to be mercury-resistant. They also found 3.6-8.3% resistance to chromium and 41-62% resistance to copper. This is in stark contrast to what was found in this study. A possible explanation is that Miranda and Castillo sampled from freshwater sources polluted with sewage and effluent from irrigation water in Chile. Versteegh et al. (1989) reported that copper inhibits *Aeromonas* at a concentration as low as 10 μg/L. *Aeromonas* isolates are also reportedly much more sensitive to copper than are other bacteria, such as coliforms and fecal streptococci (Versteegh et al., 1989). Inherent sensitivity of *Aeromonas* species to copper is supported by the findings of Calomiris et al. (1984).

Resistant Isolates from Sediment

It is clear from this study that there is a significant increase in recovery of viable aeromonads from the sediment as opposed to the water. In general, higher numbers of bacteria are found in sediment than in water (Cavallo et al., 1999). More resistant organisms were also isolated from the sediment than the water in this study. However, this difference was not statistically significant. An increased number of resistant isolates from the sediment could arise from the presence of a selective pressure for the resistant phenotypes. The metals settle to the bottom of the lake and into the sediment. Arefeen (1995) studied the concentration of metals of the water and sediments of eleven playa lakes in Lubbock. All of the sediments had significantly higher concentrations of metals than the water did. He compared the concentrations in water to the EPA drinking water standards. The only two playas that both Arefeen (1995) and this study had in common were Clapp and Higinbotham. Both Clapp and Higinbotham were within the EPA guidelines for arsenic. Clapp was above the EPA standard in chromium concentrations and Higinbotham was above in mercury. All of the concentrations of metals in both the sediment and the water were well below those concentrations used in this study to determine sensitivity and resistance (Arefeen, 1995).

Besides metals being present at low concentrations in the playas, another factor to consider is whether the metals are in toxic or nontoxic forms. It is not known which form or forms of the metals are in the playas. For example, *Aeromonas* species in lakes are not able to withstand concentrations of mercury as well as those in the sea because the extra chloride in the sea water binds to the mercury, making it less toxic (Babich and Stotzky,

1979). The environment in which the metal is present may play a role in the toxicity. In lab experiments, mercury-resistant oral streptococci could not be differentiated from their sensitive counterparts on some types of media supplemented with equal concentrations of mercuric chloride, such as mitis-salivarius agar or Columbia agar (Pike et al., 2002). The pH of the environment can cause changes in toxicity. As pH increases, copper toxicity also increases, but nickel toxicity decreases (Collins and Stotzky, 1989).

None of the metal-resistant isolates, including mercury, came from Higinbotham. This is somewhat unexpected, since Arefeen (1995) found elevated levels of mercury present. In fact, Clapp was the source of three of the seven mercury-resistant organisms. Seven years have passed since Arefeen's study (1995) and this one. The dynamics of the lake may have changed in this time period.

In this study, a temporal difference in the number of viable aeromonads present in the urban playa lakes could not be determined. Sampling was done during two months, March and July. According to previous studies, warmer temperatures yield more bacteria and more *Aeromonas* than cooler ones (Cavallo et al., 1999; Holmes et al., 1996; Warren, 1998).

Interestingly, the playa at Clapp Park produced the most resistant organisms. Over 40% of all resistant aeromonads came from Clapp. Out of all isolates from Clapp, 37.5% of them were resistant to at least one antimicrobial. The reasons for this are not apparent. The previous study by Arefeen (1995) did not show high metals concentrations, except for chromium, that would provide a selective pressure. The playa is urban and does not receive runoff from livestock yards that may contain antibiotics.

No direct selective pressure that increases the likelihood of resistant phenotypes has been identified for the playa at Clapp Park.

Most of the resistant aeromonads in this study belong to the species *Aeromonas encheleia*. Earlier studies report that the most commonly isolated resistant aeromonad is *A. hydrophila* (Janda et al., 1996; Warren, 1998). *A. encheleia* has been identified and characterized only recently (Esteve et al., 1995). The lack of a taxonomic category for *A. encheleia* before 1995 is a plausible explanation for this contradiction in findings. In addition, multiple isolations of the same organism from the same sample may have skewed this result.

Plasmid Isolations and Transformations

One of the first studies to suggest that plasmid-mediated antibiotic resistance occurs in environmental waters was that of Kelch and Lee (1978). Since then, the successful transfer of plasmids between bacteria in aquatic environments in both the water and sediment has been demonstrated (Genthner et al., 1988; Sandaa and Enger, 1994). Plasmids encoding resistance to arsenite or mercury were successfully isolated from *Aeromonas* isolated in this study and transformed into *E. coli* XL1-Blue MRF′. Plasmids mediating these resistances have been previously reported. The first report of arsenite resistance encoded on a plasmid was by Novick and Roth (1968). The plasmid was isolated from *Staphylococcus aureus*. Plasmids from other bacteria, such as *E. coli*, also carry determinants for arsenite resistance. An *ars* operon located on the plasmid encodes for an oxyanion pump that transports arsenite, arsenate, and antimonite out of the

cell (Kauer and Rosen, 1992). This pump functions much the same way as the H^+-translocating ATPase (Dey and Rosen, 1995). In order to determine if the plasmids isolated in this study have the homologous *ars* operon, more studies would need to be done.

In a previous study, an *A. hydrophila* isolate from river sediment was found to have five plasmids and resistance to mercury (Trevors, 1986). However, Trevors (1986) was not able to conclusively determine whether the resistance genes were carried on any one of the plasmids or located on the chromosome. Other plasmids carrying mercury resistance have been found in *Pseudomonas fluorescens* and *Acinetobacter* sp. isolated from estuary sediment (Olson et al., 1979). Bale et al. (1988) showed that large mercury-resistance plasmids could be transferred to and from *Pseudomonas* species in a natural river environment. Many of the mercury resistance genes carried on plasmids are in the *mer* (mercury resistance) operon from Tn*10* and Tn*501* (Silver and Phung, 1996). This operon codes for organomercurial lyase, which hydrolyzes the bond between mercury and carbon, and mercuric ion reductase, which reduces Hg(II) to Hg(0). The Hg(0) then diffuses out of the cell (Bruins et al., 2000; Silver and Phung, 1996). It is not known whether the plasmid encoding mercury resistance in this study contains the *mer* operon. It is plausible that the plasmid may not contain the *mer* operon. Dahlberg et al. (1997) isolated plasmids encoding mercury resistance from marine environments that did not have any homology to the *mer* operon.

The trimethoprim-resistant isolates did not have plasmids present that could be isolated using the plasmid purification kit. In these isolates, the resistance could be

chromosomally encoded. Goñi-Urriza et al. (2000) found all of the antibiotic-resistance genes in their isolates of *Aeromonas* to be chromosomally encoded. These resistances included nalidixic acid and tetracycline, among others. Hedges et al. (1985) were able to isolate plasmids encoding trimethoprim resistance in *Aeromonas*. However, these plasmids also carried genes for resistance to other antibiotics such as tetracycline and sulfonamides. Trimethoprim resistance was carried by integrons in *Aeromonas* without plasmids (Schmidt et al., 2001). This suggests that antibiotic resistance can be disseminated in the microbial community by DNA elements other than plasmids. Many Gram-negative genera do contain mobile gene cassettes or integrons that encode for antibiotic resistance. These integrons can be transferred between different species of bacteria without plasmids if the circular forms are stable (Hall, 1997). Besides plasmids and integrons, other mechanisms of transferring resistance genes could be occurring. These may include transduction (bacteriophages) or transformation (Séveno et al., 2002).

The Absence of Linked Antibiotic and Metal Resistance

Out of 283 isolates tested, only three contained more than one type of resistance, excluding ampicillin resistance (Table 3.34). There were no definite patterns of resistance to more than one antimicrobial. No plasmids were isolated from the aeromonads with more than one resistance. Previous studies have shown multiple resistances to be present on plasmids (Calomiris et al., 1984; Ghosh et al., 2000). However, this was not the case in this study. The absence of selective pressures in the

environmental water sources studied is most likely the key to the absence of the multiple-resistance plasmids among the aeromonads.

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