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Antimicrobial Susceptibilities of *Aeromonas* spp. Isolated from Environmental Sources[▽]

Jennifer R. Huddleston,* John C. Zak, and Randall M. Jeter

Department of Biological Sciences, Box 43131, Texas Tech University, Lubbock, Texas 79409-3131

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Aeromonas **spp. are ubiquitous aquatic bacteria that cause serious infections in both poikilothermic and endothermic animals, including humans. Clinical isolates have shown an increasing incidence of antibiotic and antimicrobial drug resistance since the widespread use of antibiotics began. A total of 282** ***Aeromonas*** **pure cultures were isolated from both urban and rural playa lakes in the vicinity of Lubbock, Texas, and several rivers in West Texas and New Mexico. Of these, at least 104 were subsequently confirmed to be independent isolates. The 104 isolates were identified by Biolog and belonged to 11 different species. The MICs of six metals, one metalloid, five antibiotics, and two antimicrobial drugs were determined. All aeromonads were sensitive to chromate, cobalt, copper, nickel, zinc, cefuroxime, kanamycin, nalidixic acid, ofloxacin, tetracycline, and sulfamethoxazole. Low incidences of trimethoprim resistance, mercury resistance, and arsenite resistance were found. Dual resistances were found in 5 of the 104** ***Aeromonas*** **isolates. Greater numbers of resistant isolates were obtained from samples taken in March versus July 2002 and from sediment versus water. Plasmids were isolated from selected strains of the arsenite- and mercury-resistant organisms and were transformed into** ***Escherichia coli*** **XL1-Blue MRF′. Acquisition of the resistance phenotypes by the new host showed that these resistance genes were carried on the plasmids. Mercury resistance was found to be encoded on a conjugative plasmid. Despite the low incidence of resistant isolates, the six playa lakes and three rivers that were sampled in this study can be considered a reservoir for antimicrobial resistance genes.**

Members of the genus *Aeromonas* (family *Aeromonadaceae*) are gram-negative rods that are ubiquitous in aquatic ecosystems. There are currently 15 named and 2 unnamed species that can be categorized into at least 17 DNA hybridization groups (25, 51). Mesophilic aeromonads have been found in almost every aquatic environment, including chlorinated drinking water, raw sewage, groundwater, and both polluted and unpolluted streams and rivers (12, 13, 21, 26, 33, 37).

Aeromonads are major causative agents of infections in fish (5) and have been associated with human diarrheal disease and wound infections (28, 29, 34). Many opportunistic infections of wounds are the result of exposure to contaminated water. Swimming and boating accidents, alligator bites, and fishing-hook accidents have been reported as ways that people became infected with *Aeromonas* (28). Wound infections can progress quickly and may ultimately be fatal if the infection becomes systemic. Cellulitis, myonecrosis, and ecthyma gangrenosum can result from wound infections and have to be treated with antibiotics. Antimicrobial resistance in the pathogen can make these infections difficult to treat.

Environmental contamination with antibiotics and other pollutants contributes to the maintenance and spread of antibiotic resistance genes (16). One mechanism that allows the perpetuation of such genes is the spread of resistance plasmids between unrelated bacteria in natural environments (32). The transfer of genes is inferred by the fact that antibiotic- and metal-resistant strains of bacteria have been isolated from environments in which they are not known to have ever been directly exposed to metals or antibiotics (41). *Aeromonas* spp. that are resistant to both antibiotics and metals have been previously isolated from polluted and unpolluted waters (39).

Although aeromonads are widespread in aquatic ecosystems and have been designated an emerging threat to human health (35), little is known about the antibiotic and metal resistance profiles of aeromonads from freshwater systems, including shallow rural and urban playa lakes. Playa lakes are small, circular-to-oval basins that drain surface runoff waters from surrounding areas. There are more than 20,000 playa basins in the High Plains of Texas, New Mexico, Colorado, and Oklahoma (18). 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 (19). Urban playas receive runoff from the urban landscape, and the rural playas receive runoff from farmland, rangeland, or stockyards. Several studies have been conducted to analyze both the chemical and biological compositions of urban playa lakes (4, 23, 40). Westerfield (60) studied pathogens from playas and found *Aeromonas* to be present, whereas Warren et al. (59) tested *Aeromonas* isolates for antibiotic resistance but not metal resistance.

The objectives of this investigation were to (i) identify species diversity of aeromonads from lakes and rivers in the Southern High Plains of West Texas and in New Mexico, (ii) determine if the levels of metal and antimicrobial resistance in aeromonads differ between sampling locations, and (iii) determine if the resistances in selected strains are encoded by plasmids.

MATERIALS AND METHODS

Sampling. Water and sediment samples were collected from four urban playa lakes (Clapp, Higinbotham, Maxey, and Stevens) in Lubbock, Texas; two rural playa lakes near Shallowater, Texas; the Pecos River in Carlsbad, New Mexico;

* Corresponding author. Mailing address: Department of Biological Sciences, Box 43131, Texas Tech University, Lubbock, TX 79409-3131. Phone: (806) 742-2226. Fax: (806) 742-2963. E-mail: jennifer.huddleston@ttu.edu.

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the North Fork of the Brazos River in Lubbock, Texas; and the South Fork of the Rio Hondo in Taos Ski Valley, New Mexico. Surface water samples were taken from the banks of the playa or river using a grab sampler. A 500-ml plastic cup attached to a 2-m 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, Pittsburgh, PA). The temperature of the sample was taken with a laboratory thermometer and 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 taken at least 50 m apart on each side.

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 a sediment surface that was submerged 10 to 15 cm below the surface of the water. Approximately 200 g of sediment was 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 due to sample date. Samples were kept cool during transport to the laboratory and processed within 12 h of collection. In the laboratory, the pH of the water was measured with a digital pH meter (Radiometer America Inc., Cleveland, OH). Combined water samples were made by mixing 1-ml aliquots of each of the four water samples for each location and sampling date. Combined sediment samples were made in the same way by mixing 1-g portions.

Media and growth conditions. The combined water and sediment samples were diluted in 0.85% NaCl to 10^{-4} and 10^{-5}, respectively, and 0.1-ml volumes were plated onto *Aeromonas* isolation medium (four replicates per sample). Preliminary data indicated that isolation of aeromonads from the sampled environments was more efficient on the medium presented here than using previously described media (24, 38, 49). The medium used in this investigation consisted of 4 g of soluble starch (Becton Dickinson, Sparks, MD), 0.25 g of NH_4Cl (Fisher Scientific), 1 g of tryptone (Becton Dickinson), 0.5 g of yeast extract (Becton Dickinson), 40 mg of the pH indicator bromothymol blue (Fisher Scientific), 15 g of granulated agar (Becton Dickinson), and 1 liter of distilled water and was adjusted to pH 8.0 with 1 N KOH. After autoclaving and cooling, 100 mg of sodium deoxycholate (Becton Dickinson), 5 ml of 0.41% L-tryptophan (Sigma-Aldrich, St. Louis, MO), and 5 ml of 0.99% L-phenylalanine (Sigma-Aldrich) were added per liter. Ampicillin, which has been used as a selective agent in previous surveys of environmental aeromonads (33, 34, 39, 50, 58, 59), was not added in order to recover all aeromonads, including the ampicillin-sensitive ones. After inoculation and incubation at 30°C for 36 h, *Aeromonas* cells appeared as light yellow, circular colonies that were 1 to 3 mm in diameter.

The diluted water and sediment samples were also plated onto 20% tryptic soy agar (TSA), which consisted of 8 g of TSA (Becton Dickinson), 12 g of granulated agar, and 1 liter of distilled water, to grow all culturable bacteria for total plate counts. Plates were incubated at 25°C for 48 h.

Presumptive identification as aeromonads. Gram-negative rod-shaped isolates were assessed as presumptive aeromonads by screening for the absence of growth in Difco nutrient broth (Becton Dickinson) supplemented with 6% NaCl (EM Science, Gibbstown, NJ), oxidase activity using oxidase reagent (Becton Dickinson), DNase using DNase test agar (Becton Dickinson) supplemented with methyl green (0.05 g per liter) (Manufacturing Chemists, Rochester, NY), resistance to 10 μg and 150 μg of vibriostatic agent 0/129 (Oxoid, Ogdensburg, NY), and anaerobic growth on 20% TSA in an anaerobic chamber (model 1025 anaerobic system; Forma Scientific, Marietta, OH) at 30°C for 72 h. *Aeromonas hydrophila* ATCC 7965^T, *A. veronii* biogroup *sobria* ATCC 9071^T, and *A. caviae* ATCC 15468^T were used as positive controls, and *Escherichia coli* DH5α and *Vibrio fischeri* 345^T (Presque Isle Cultures, Presque Isle, PA) were used as negative controls.

Antimicrobial sensitivity testing. Isolates were first subcultured in 20% TSA to assess the susceptibilities of presumptive aeromonads to selected antibiotics and were incubated for 24 ± 2 h at 30°C. Isolated colonies were picked from the cultures grown overnight, inoculated into 4.5 ml of 40% nutrient broth, and adjusted to an A_{560} value of 0.12 ± 0.02 (0.5 McFarland standard). Next, 5.5 μl of the culture was inoculated into 5.5 ml of 40% nutrient broth (a 1:1,000 dilution). After mixing, a 100-μl sample of the suspension was inoculated into each well of 96-well Costar microtiter plates with 200 μl of 40% nutrient broth at pH 7.0. Each well was supplemented with one of the five antibiotics, two drugs, six metals, or one metalloid. Concentrations were set up as twofold serial dilutions. The antimicrobials used were cefuroxime (Sigma-Aldrich), kanamycin sulfate (Fisher Scientific), nalidixic acid sodium salt (Sigma-Aldrich), ofloxacin (Sigma-Aldrich), tetracycline hydrochloride (Fisher Scientific), sulfamethoxazole (Sigma-Aldrich), trimethoprim (Sigma-Aldrich), K_2CrO_4 (Sigma-Aldrich), $CuSO_4$ (Fisher Scientific), $HgCl_2$ (Fisher Scientific), $NiSO_4 \cdot 6H_2O$ (Fisher Scientific), $ZnSO_4 \cdot 7H_2O$ (J. T. Baker, Phillipsburg, NJ), and $NaAsO_2$ (Sigma-Aldrich). Each antimicrobial was stored and prepared according to the instructions of the manufacturers. The plates were incubated at 30°C for 24 ± 2 h and then read using an automated plate reader (EL311sc Auto reader; Bio-Tek, Winooski, VT). An A_{550} value of >0.1 indicated growth. *A. hydrophila* ATCC 7965 was tested as a control each time the assessments were performed. Ten percent of the isolates were retested for antimicrobial resistance under the same conditions in order to verify reproducibility and accuracy. *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were also tested in accordance with the minimal quality control recommendations of the CLSI to determine the accuracy of the antibiotic and drug susceptibility tests (9).

Identification of isolates. Isolates were grouped according to isolation sources and antimicrobial resistance patterns. At least one representative isolate from each group was identified using the Biolog (Hayward, CA) identification system. Cultures were grown overnight at 30°C on Columbia agar base (EMD Chemicals Inc., Gibbstown, NJ) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO). The colonies were resuspended in prewarmed Biolog GN/GP-IF (inoculating fluid) and adjusted turbidometrically within the recommended range, and 150 μl of the suspensions was added to each well of a 96-well Biolog GN2 MicroPlate test panel. Plates were read with an automated Biolog MicroStation reader after incubation at 30°C for 16 to 24 h. Identifications were made using the Biolog gram-negative database (release 4.20).

The *gyrB* genes from three randomly chosen isolates were sequenced in order to confirm their identity as *Aeromonas* species. The *gyrB* gene from each isolate was amplified by PCR using primers GyrB3F (5′-TCC GGC GGT CTG CAC GGC GT-3′) and GyrB14R (5′-TTG TCC GGG TTG TAC TCG TC-3′) (61) and TaKaRa Ex *Taq* polymerase (Fisher Scientific) in a GeneAmp PCR System 9600 (Perkin-Elmer, Wellesley, MA). The PCR conditions were as follows: an initial denaturation step at 94°C for 2 min; 30 subsequent cycles of denaturation at 93°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min; and no final extension step. Amplicons were extracted and purified using a QIAEX II gel extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Amplicons were sequenced at the Texas Tech University Center for Biotechnology and Genomics. The sequences were compared to known aeromonad *gyrB* sequences deposited in GenBank using Vector NTI Suite 9 (March 2006).

Plasmid purification and transfer. QIAGEN Plasmid MidiKits were used according to the manufacturer's instructions to purify plasmids from representative isolates of the resistant organisms. Competent *E. coli* XL1-Blue MRF′ (Stratagene, La Jolla, CA) cells were prepared by inoculating 2 ml of a culture grown overnight in Luria broth (LB) into 50 ml of LB and incubating the cells at 37°C with aeration until the culture reached an absorbance at 600 nm of 0.4 to 0.6. LB is composed of 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 liter of distilled water and is adjusted to pH 7.0 with NaOH. The culture was then centrifuged at 12,100 × *g* for 10 min at 4°C, resuspended in 20 ml of sterile, ice-cold 50 mM $CaCl_2$, and incubated on ice for 30 min. The competent cells were divided into 200-μl aliquots, mixed gently with 20% glycerol, and stored at −60°C until use.

Plasmid DNA (50 to 100 ng) from the plasmid purification procedure was added to 200 μl of competent *E. coli* XL1-Blue MRF′ cells. The mixture was incubated on ice for 30 min, heat shocked at 42°C for 1 min, incubated in an ice-water mixture for 5 min, and allowed to recover in 1 ml of LB at 37°C for 1 h. The cells that were transformed with plasmids presumed to encode arsenic (arsenite) resistance were inoculated onto 40% nutrient agar consisting of 3.2 g of nutrient broth supplemented with 15 g of granulated agar in 1 liter of distilled water and supplemented with 6 mM $NaAsO_2$, pH 7.0. The cells that were transformed with plasmids presumed to encode mercury resistance were inoculated onto 40% nutrient agar containing 0.025 mM $HgCl_2$, pH 7.0. Untransformed *E. coli* XL1-Blue MRF′ cells were inoculated onto 40% nutrient agar with and without the metalloid or metal as negative and positive controls for growth. All of the plates were incubated at 37°C overnight.

In order to determine if the plasmids were mobile, conjugation experiments between the resistant aeromonads and *E. coli* XL1-Blue MRF′ were performed as follows. The donor aeromonad and the recipient *E. coli* were each grown in 2 ml Luria broth and incubated overnight at 37°C with aeration. Six hundred fifty microliters of cultures of the aeromonad and *E. coli* grown overnight were mixed together, pelleted, resuspended in 200 μl of 10% glycerol, pelleted, resuspended in 20 μl of 10% glycerol, spotted onto Luria agar, and incubated at 37°C for 8 h. The cells were then resuspended in buffer, diluted, and plated onto lactose-MacConkey agar (Becton Dickinson) supplemented with 6 mM $NaAsO_2$ or 0.025 mM $HgCl_2$. Plates were incubated at 37°C overnight and observed for resistant, purple *E. coli* XL1-Blue MRF′ colonies. Purple, metal-resistant colonies were

TABLE 1. Identification of *Aeromonas* isolates to the species level by the Biolog identification system[a]

Species	Similarity index[b]	No. of isolates identified
Aeromonas spp.	Not applicable	28
A. bestiarum	0.782 ± 0.178	8
A. caviae	0.667 ± 0.118	4
A. encheleia	0.705 ± 0.248	19
A. enteropelogenes	0.575 ± 0.000	1
A. hydrophila	0.689 ± 0.154	15
A. jandaei	0.505 ± 0.000	1
A. media-like	0.602 ± 0.026	2
A. sobria	0.693 ± 0.098	7
A. veronii	0.606 ± 0.119	9
A. veronii biogroup *sobria*	0.701 ± 0.222	10

[a] Isolates were selected across all sampling times and locations ($n = 104$).
[b] 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.

tested for the absence of oxidase, and a cracking procedure (54) was used to confirm the presence of the plasmid in the transconjugant cells.

Statistical analysis. Analysis of variance (ANOVA) using Matlab (version 6.0.0.88, release 12) was used to determine significant differences ($P = 0.05$) in CFU (CFU/ml) from water versus sediment across sampling locations and sampling dates. Data were obtained by counting resistant and sensitive isolates (both redundant and independent) that were picked from each plate and inferring the CFU/ml. The colony count data were square-root transformed for all analyses. A three-way ANOVA was performed across urban sample locations, substrate (sediment versus water), and sample date (March versus July). Separate two-way ANOVAs were performed across urban sample locations and sediment versus water and urban sampling location and sampling date to determine statistical differences in numbers of resistant aeromonads. A two-way ANOVA was performed across river sample locations and sediment versus water to examine impacts on numbers of resistant aeromonads.

RESULTS

Two hundred eighty-two isolates were presumptively identified as belonging to the genus *Aeromonas*. Of these, 104 isolates were inferred to be independent isolates (not clones) based on differences in sample locations, sample types, sample dates, antimicrobial resistance phenotypes, and plasmid profiles. The identities of these 104 isolates as aeromonads were confirmed using the Biolog identification system; 76 were identified to the species level. The *Aeromonas* isolates represented 11 distinct species (Table 1). Three of the isolates (putative *A. encheleia*, putative *Aeromonas* sp., and putative *A. hydrophila*-like) were confirmed to be aeromonads by their *gyrB* gene sequences (61).

The MIC of each antimicrobial was determined for each of the 104 independent aeromonad isolates. The MICs were used to distinguish resistance and sensitivity. For chromate, cobalt, copper, nickel, and zinc, at least 90% of the isolates were inhibited at the lowest concentrations tested (Table 2). No isolates expressed resistance to these metals under the conditions tested. However, 12/104 (11.5%) of the isolates expressed either arsenic (metalloid) or mercury (metal) resistance. No individual isolate expressed resistance to both. The maximal MIC for the resistant isolates was 12 mM for arsenite and 0.1 mM for mercury (Table 2).

There were eight arsenite-resistant isolates (7.7%) from six different water sources. These isolates were able to grow at or above 6 mM $NaAsO_2$. Of these isolates, all eight were identified by Biolog as belonging to the genus *Aeromonas*, but only four could be further identified to the species level. Each of these four isolates belonged to a different aeromonad species (Table 3). Four of the 104 (3.8%) isolates expressed mercury resistance and grew in concentrations at or above 0.025 mM $HgCl_2$ (Table 3).

TABLE 2. MICs of metals and a metalloid against all *Aeromonas* isolates ($n = 104$)

Metal or metalloid	MIC (mM)				Resistant strains (%)
	Breakpoint (reference)	Range	50%	90%	
$NaAsO_2$	6 (11)	0.375–12	0.62	2.52	7.7
K_2CrO_4	1.6 (36)	0.4[a]	0.4	0.4	0
$CoCl_2 \cdot 6H_2O$	20.2 (52)	0.125–0.25	0.125	0.125	0
$CuSO_4$	12.6 (47)	0.125–0.25	0.125	0.125	0
$HgCl_2$	0.025 (42)	0.003–0.1	0.003	0.006	3.8
$NiSO_4 \cdot 6H_2O$	4.5 (52)	0.125–0.25	0.125	0.125	0
$ZnSO_4 \cdot 7H_2O$	11 (17)	0.125[a]	0.125	0.125	0

[a] One hundred percent of isolates were inhibited at the lowest concentration tested.

For the antimicrobials cefuroxime, kanamycin, nalidixic acid, ofloxacin, tetracycline, and sulfamethoxazole, at least 90% of the isolates were inhibited at the minimum concentration tested (Table 4). There were no isolates that were resistant to these antibiotics or drugs under the conditions tested. However, trimethoprim resistance was present in 27/104 (26.0%) of the isolates. Two of the isolates had an MIC of 16 μg/ml, 10 had an MIC of 8 μg/ml, and the other 15 had an MIC of 4 μg/ml (Table 4). Five of the trimethoprim-resistant isolates were also resistant to one metalloid or metal, three were resistant to arsenite, and two were resistant to mercury. No resistance to multiple antibiotics or drugs was found among the isolates.

To determine the reliability of the antibiotic susceptibility testing, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213 were included as quality control organisms. The MICs were within control limits as recommended by the CLSI (9). Ten percent of the aeromonad isolates were retested for metals and antibiotics, and the MICs for the retested isolates were determined. These MICs varied by no more than a single twofold dilution, and this variation occurred 14% of the time.

Resistant organisms were isolated from all but one of the sources, rural playa 2, from both sediment and water and in March and July 2002 (Table 5). While there was no significant three-way interaction among sample dates (March or July), substrates, and urban playa locations, there were significant differences ($P = 0.0073$) between the number of resistant *Aeromonas* strains isolated from the water and the number isolated from the sediment. The effect of sampling date on the number of resistant aeromonads, irrespective of substrate, approached significance ($P = 0.0771$) for the three-way ANOVA. In a separate two-way ANOVA, urban playa location by substrate, there was a significant difference ($P = 0.0007$) in numbers of resistant aeromonads recovered from the sediment versus water. Differences in numbers of resistant isolates approached significance ($P = 0.0696$) when urban playa locations were compared. There was no significant two-way interaction ($P =$

TABLE 3. Characterization of aeromonads that are resistant to antimicrobials, their sources of isolation, and impact of antimicrobials on cell growth ($n = 34$)

Source	Location	Sample mo	Biolog identification	Resistance(s)	MIC		Plasmid transformation of resistant phenotype
					μg/ml	mM	
Brazos River	Sediment	March	*A. encheleia*	Trimethoprim	4.0		
Brazos River	Sediment	March	*A. bestiarum*	Trimethoprim	4.0		
Brazos River	Sediment	March	*A. veronii*	Trimethoprim	4.0		
Brazos River	Sediment	July	*A. veronii* biogroup *sobria*	Trimethoprim	4.0		
Brazos River	Water	March	*A. encheleia*	Arsenite		6.0	
Clapp Playa	Sediment	March	*A. encheleia*	Mercury		0.1	Yes
				Trimethoprim	8.0		
Clapp Playa	Sediment	March	*A. hydrophila*	Trimethoprim	8.0		
Clapp Playa	Sediment	March	*Aeromonas* sp.	Arsenite		12.0	
Clapp Playa	Sediment	March	*A. encheleia*	Trimethoprim	4.0		
Clapp Playa	Sediment	March	*Aeromonas* sp.	Mercury		0.1	No
				Trimethoprim	4.0		
Clapp Playa	Sediment	July	*Aeromonas* sp.	Arsenite		12.0	Yes
Clapp Playa	Water	March	*A. media*-like	Trimethoprim	8.0		
Clapp Playa	Water	March	*A. encheleia*	Trimethoprim	4.0		
Clapp Playa	Water	March	*A. encheleia*	Trimethoprim	8.0		
Clapp Playa	Water	March	*Aeromonas* sp.	Trimethoprim	16.0		
Clapp Playa	Water	March	*A. caviae*	Trimethoprim	4.0		
Clapp Playa	Water	March	*Aeromonas* sp.	Arsenite		6.0	
				Trimethoprim	8.0		
Higinbotham Playa	Sediment	March	*A. hydrophila*	Trimethoprim	16.0		No
Higinbotham Playa	Sediment	March	*Aeromonas* sp.	Trimethoprim	4.0		
Hondo River	Sediment	July	*A. encheleia*	Mercury		0.05	No
Hondo River	Water	July	*A. bestiarum*	Arsenite		6.0	
Maxey Playa	Sediment	March	*A. hydrophila*	Trimethoprim	4.0		
Maxey Playa	Sediment	March	*A. media*-like	Arsenite		6.0	
Maxey Playa	Sediment	March	*A. hydrophila*	Trimethoprim	4.0		
Maxey Playa	Sediment	July	*Aeromonas* sp.	Trimethoprim	4.0		
Maxey Playa	Water	March	*A. bestiarum*	Mercury		0.1	
Rural playa 1	Water	July	*A. hydrophila*	Arsenite		12.0	Yes
Stevens Playa	Sediment	March	*Aeromonas* sp.	Trimethoprim	8.0		
Stevens Playa	Sediment	March	*A. encheleia*	Trimethoprim	8.0		
Stevens Playa	Sediment	March	*A. hydrophila*	Trimethoprim	8.0		
Stevens Playa	Sediment	July	*A. encheleia*	Trimethoprim	4.0		
Stevens Playa	Sediment	July	*A. encheleia*	Trimethoprim	4.0		No
Stevens Playa	Water	March	*Aeromonas* sp.	Trimethoprim	8.0		
Stevens Playa	Water	March	*Aeromonas* sp.	Arsenite		12.0	No
				Trimethoprim	8.0		

0.0898) between urban playa location and substrate for the numbers of resistant aeromonads, indicating that all urban playas sampled exhibited the same response to season and substrate. There was a significant difference ($P = 0.023$) between the number of resistant *Aeromonas* isolates in March versus July across all urban playa lakes and also a significant difference ($P = 0.0177$) between the numbers of resistant isolates recovered from each urban playa lake by date. Numbers of resistant aeromonads were higher in March than in July. However, there was no significant two-way interaction between the two sampling dates and playa locations ($P = 0.1881$). There was no significant difference in the numbers of resistant aeromonads between sediment and water for the rivers sampled ($P = 0.1846$) or between river locations ($P = 0.4505$).

TABLE 4. MICs of antibiotics and drugs for all *Aeromonas* isolates ($n = 104$)

Antibiotic or drug	MIC (μg/ml)				Resistant strains (%)
	Breakpoint[a]	Range	50%	90%	
Cefuroxime	32	0.125–8	0.15	0.93	0
Kanamycin	64	0.25[c]	0.25	0.25	0
Nalidixic acid	32	2[c]	2	2	0
Ofloxacin	8	0.25[c]	0.25	0.25	0
Sulfamethoxazole	512	4–32	4	4.35	0
Tetracycline	16	0.031–1	0.04	0.06	0
Trimethoprim	NA[b]	0.5–16	0.91	4.6	26.0

[a] MIC breakpoints as given in reference 9.
[b] NA, not available. CLSI (9) gives an MIC for trimethoprim-sulfamethoxazole (4/76 μg/ml) but not for trimethoprim alone.
[c] One hundred percent of isolates were inhibited at the lowest concentration tested.

Plasmid isolations and transfer. Plasmid isolations were conducted on eight resistant isolates. Three plasmids were isolated, two from arsenite-resistant isolates and one from a mercury-resistant isolate (Table 3). All of these plasmids were successfully transformed into *E. coli* XL1-Blue MRF′ cells. The *E. coli* transformants with the plasmids from the arsenite-resistant isolates were resistant to 6 mM $NaAsO_2$, and the transformants with the plasmid from the mercury-resistant isolate were resistant to 0.025 mM $HgCl_2$. indicating that the resistance genes were carried on the isolated plasmids. The plasmid from the mercury-resistant aero-

TABLE 5. Average numbers of antimicrobial-resistant *Aeromonas* isolates in response to their habitat and sample date

Source	No. of resistant isolates (10^3) (CFU/ml) ± SD				
	Sample type		Date of sampling		Avg
	Sediment	Water	March	July	
Brazos River	6.5 ± 3.5	0.005 ± 0.005	1.5 ± 1.5	5.0 ± 5.0	3.3 ± 4.1
Stevens Playa	160.0 ± 140.0	0.1 ± 0.1	150.1 ± 149.9	10.0 ± 10.0	80.1 ± 127.2
Clapp Playa	30.0 ± 20.0	0.3 ± 0.3	25.3 ± 24.7	5.0 ± 5.0	15.2 ± 20.5
Maxey Playa	20.0 ± 10.0	0.05 ± 0.05	15.1 ± 15.0	5.0 ± 5.0	10.0 ± 12.2
Higinbotham Playa	10.0 ± 10.0	0.05 ± 0.05	10.1 ± 10.0	0.0 ± 0.0	5.0 ± 8.6
Hondo River	10.0 ± 0.0	0.1 ± 0.0	NS[a]	5.1 ± 5.0	5.1 ± 5.0
Pecos River	0.0 ± 0.0	0.0 ± 0.0	NS[a]	0.0 ± 0.0	0.0 ± 0.0
Rural playa 1	0.0 ± 0.0	0.1 ± 0.0	NS[a]	0.05 ± 0.05	0.05 ± 0.05
Rural playa 2	0.0 ± 0.0	0.0 ± 0.0	NS[a]	0.0 ± 0.0	0.0 ± 0.0
Avg no. of resistant CFU/ml	33.1 ± 75.3	0.09 ± 0.2	40.4 ± 88.0	3.3 ± 5.8	

[a] NS, not sampled.

monad, but not the two arsenite-resistant aeromonads, could be transferred to *E. coli* XL1-Blue MRF′ cells through conjugation, indicating that the mercury resistance genes were carried on a conjugative plasmid.

DISCUSSION

The carbon utilization patterns and biochemical results of the 104 presumptive *Aeromonas* isolates in this study were consistent with those published previously (1, 22, 46). The identifications were confirmed by amplifying and sequencing the *gyrB* genes from three isolates. These results verified that the *Aeromonas* isolation medium and subsequent screening procedures used in this investigation were reliable in distinguishing *Aeromonas* species from other genera that inhabit water or sediment. Although many different types of selective media have been developed for the isolation of aeromonads, there are conflicting results as to which medium is the most efficient and easiest to use (3, 48). The lack of NaCl in this medium serves to inhibit *Vibrio* spp. from growing quickly and being confused with *Aeromonas* spp. In addition, the concentrations of tryptone and yeast extract were 20 to 25% of what was used in previously published formulations (20, 49). A nutrient-limited medium aids in the isolation of environmental bacteria (7, 10). The specificity of the medium used in this study for the detection of aeromonads is high, as demonstrated by the Biolog results. All 104 representative isolates tested were positively identified as belonging to the genus *Aeromonas*.

There is no standard protocol for testing for sensitivity or resistance to metals. We chose an incubation temperature of 30°C and a decreased concentration of nutrient broth to optimize the growth of the environmental aeromonads. Antibiotic and drug testing were performed under the same conditions to maintain consistency with the previously performed metal tests. The only tested antibiotic or drug to which the *Aeromonas* isolates showed resistance was trimethoprim. This study differs from most others in that trimethoprim was tested alone rather than in combination with sulfamethoxazole. Kämpfer et al. (30) tested 217 clinical and nonclinical *Aeromonas* isolates against trimethoprim and sulfamethoxazole individually and found none of them to be resistant to trimethoprim, in contrast to the 26.0% found in this study. However, Kämpfer et al. (30) used 16 μg/ml trimethoprim as a breakpoint concentration, whereas this study used 4 μg/ml. If 16 μg/ml were used as the breakpoint in this study, 1.9% (2/104) of the isolates would be considered resistant. In addition, no sulfamethoxazole resistance was found in this study, whereas Kämpfer et al. found 36% (30). Imziln et al. (27) tested 953 *Aeromonas* isolates against trimethoprim and sulfamethoxazole separately and found 2.5% to be resistant to 5 μg/ml trimethoprim and 16% to be resistant to sulfamethoxazole. It is uncertain how many of the 953 isolates tested by Imziln et al. (27) were independent *Aeromonas* isolates.

No isolates were found to be resistant to cefuroxime, kanamycin, nalidixic acid, ofloxacin, or tetracycline. Other studies with cefuroxime, kanamycin, and ofloxacin showed either no or a low incidence of resistance (30, 44, 57, 59). The incidence of tetracycline resistance was found to be low compared to those reported in previous studies. Kämpfer et al. (30) found approximately 10% of their isolates to be resistant to tetracycline. Warren et al. (59) reported 2.6% of their isolates from two of the same Lubbock playas that were sampled in this study to be tetracycline resistant. The susceptibility to nalidixic acid found here is in contrast to the 43% resistance reported by Vila et al. (57). However, Miranda and Castillo (39) reported finding less than 4% of their *Aeromonas* isolates to be resistant to nalidixic acid.

The environmental isolates included in this study showed equivalent or much lower resistance compared to *Aeromonas* isolates reported in other studies depending upon the antibiotic tested. A possible explanation for these findings is that some of the previous studies included clinical isolates that could have been previously exposed to antibiotics (30, 31, 57). In other studies done solely with environmental isolates, aeromonads were isolated from waters that are highly polluted by industrial effluent or raw sewage (16). The six playa lakes and three rivers sampled in this study receive storm water runoff from surrounding areas. None of the water bodies sampled in this study were polluted with sewage effluent.

The higher densities of resistant aeromonads in sediment than in the water columns from the urban playas could reflect the influences of environmental contaminants in the sediments selecting for increased antimicrobial resistance. Arefeen (4) found higher concentrations of arsenic and mercury in the

sediment than in the water of Lubbock urban playas, although the concentrations were determined to be below regulatory levels. These low levels of metals accumulating in the playa sediments could be exerting a weak selection for the development and persistence of resistant *Aeromonas* populations. None of the playas or rivers in this study are known to be highly polluted with metals (4).

The greater prevalence of resistance phenotypes among the independent isolates obtained in March (26/46; 56.5%) versus July (8/59; 13.6%) remains unexplained. This same seasonal trend was seen among both water and sediment isolates. Higher densities of *Aeromonas* isolates have been isolated from water during warmer versus cooler temperatures by Gavriel et al. (15) and Parveen et al. (45). Warren et al. (59) also isolated higher densities of aeromonads in the summer in the Lubbock urban playas. In this study, the average total aeromonad populations across all water bodies sampled showed only a modest increase (less than 2.4-fold) from March to July, suggesting that differential reproduction of sensitive and resistant subpopulations cannot completely account for these results. Water runoff into the playas was also excluded as a factor due to the lack of variability in rainfall preceding the two sampling dates.

Little research has been done previously 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 (2, 14, 39, 53) and were rarely aeromonads. Miranda and Castillo (39) found 39.3% of aeromonads from raw drinking water supplies with less than 10^2 coliforms/100 ml to be mercury resistant, 3.6% to be chromium resistant, and 57% to be copper resistant, which is in stark contrast to the findings of this and other studies (8, 56).

Plasmids encoding resistance to arsenite or mercury, but not trimethoprim, were successfully isolated from *Aeromonas* isolates and transformed into *E. coli* XL1-Blue MRF′ cells (Table 3). Trevors (55) isolated an *A. hydrophila* isolate from river sediment that contained five plasmids and that was resistant to mercury. However, it was not conclusively determined 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 isolates of *Pseudomonas* spp. and *Acinetobacter* spp. from aquatic environments (6, 43).

Out of 104 *Aeromonas* isolates tested, only 5 (4.8%) expressed more than one type of resistance, i.e., to trimethoprim and either arsenite or mercury. There were no definite patterns of resistance to more than one antimicrobial. While previous studies have shown multiple resistances to occur in *Aeromonas* isolates (39), that was not the case in this study. The apparent absence of strong selection in the environmental water sources that were investigated here is most likely the key to the absence of the multiple resistance plasmids among the aeromonads. The higher levels of antibiotic resistance detected in *Aeromonas* spp. from polluted freshwater environments suggest that there may be a threshold level of heavy metals necessary to select for populations of *Aeromonas* isolates that contain multiple resistance plasmids.

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