

Antibiotic and heavy metal resistance of *Aeromonas hydrophila* isolated from charal (*Chirostoma humboldtianum*, Valenciennes, 1835)

Resistencia a antibióticos y metales pesados en *Aeromonas hydrophila* aisladas de charal (*Chirostoma humboldtianum*, Valenciennes, 1835)

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ABSTRACT

Antibiotic and heavy metal susceptibilities of twenty *Aeromonas hydrophila* strains, isolated from the gastrointestinal tract of the charal (*Chirostoma humboldtianum*), an autochthonous Mexican fish, were analyzed. All strains produced β -lactamase and were resistant to penicillin and dicloxacillin, showing single peak for minimum inhibitory concentration (MIC) distributions at 2000-4000 $\mu\text{g}/\text{mL}$ and 500-1000 $\mu\text{g}/\text{ml}$, respectively. Ampicillin MIC distribution was bimodal with 20% resistant strains (125-250 $\mu\text{g}/\text{ml}$) and 80% highly resistant ones (500-4000 $\mu\text{g}/\text{ml}$). All strains were susceptible to ceftriaxone (MIC= 3.9 $\mu\text{g}/\text{ml}$) and all but one were susceptible to cefuroxime (3.9 $\mu\text{g}/\text{ml}$ and 62.5 $\mu\text{g}/\text{ml}$). All strains had a single MIC distribution pattern for lead (800-3200 $\mu\text{g}/\text{ml}$), and mercury (20 $\mu\text{g}/\text{ml}$) and were considered resistant and susceptible to these ions, respectively. Fifteen percent of the isolates were resistant to arsenite (MIC= 400-800 $\mu\text{g}/\text{ml}$) and all were susceptible to silver (MIC= 1.25-2.5 $\mu\text{g}/\text{ml}$), chromate (MIC= 93.5-375 $\mu\text{g}/\text{ml}$), and zinc (MIC=21.25-42.5 $\mu\text{g}/\text{ml}$).

Key words: *Aeromonas*, heavy metal resistance, *Chirostoma*.

RESUMEN

Se analizó la susceptibilidad a antibióticos β -lactámicos y a metales pesados en 20 cepas de *Aeromonas hydrophila*, aisladas del tracto gastrointestinal de charal (*Chirostoma humboldtianum*), un pez autóctono mexicano. Todas las cepas produjeron β -lactamasa, fueron resistentes a penicilina y dicloxacilina, y presentaron distribuciones unimodales para la concentración mínima inhibitoria (CMI) de 2000-4000 $\mu\text{g}/\text{ml}$ y 500-1000 $\mu\text{g}/\text{ml}$, respectivamente. La distribución de la CMI de ampicilina fue bimodal con 20% de cepas resistentes (125-250 $\mu\text{g}/\text{ml}$) y 80% altamente resistentes (500-4000 $\mu\text{g}/\text{ml}$). Todas las cepas fueron sensibles a ceftriaxona (CMI= 3.9 $\mu\text{g}/\text{ml}$) y todas, excepto una, fueron sensibles a cefuroxima (3.9 $\mu\text{g}/\text{ml}$ y 62.5 $\mu\text{g}/\text{ml}$). Las 20 cepas mostraron distribuciones unimodales de las CMI de plomo (800-3200 $\mu\text{g}/\text{ml}$) y mercurio (20 $\mu\text{g}/\text{ml}$) y fueron consideradas resistentes y sensibles, respectivamente, a

estos iones. Quince por ciento de las cepas fueron resistentes a arsenito (CMI= 400-800 µg/ml) y todas fueron sensibles a plata (CMI=1.25-2.5 µg/ml), cromato (CMI=93.5-375 µg/ml) y zinc (CMI= 21.25-42.5 µg/ml).

Palabras clave: *Aeromonas*, resistencia a metales pesados, *Chirostoma*.

INTRODUCTION

Aeromonas hydrophila is a Gram negative bacterium widely distributed in freshwater environments (Holmes et al., 1996). It is a well-known fish (Hazen et al., 1978; Joseph & Carnahan, 1994; Austin & Adams, 1996) and human pathogen (Altwegg & Geiss, 1989). Since some strains of *Aeromonas* are enteropathogens possessing a range of virulence factors (enterotoxins, cytotoxins, haemolysins, and invasive ability), infected fish may be vehicles of human infection (Cahill, 1990; Kirov, 1993). Antibiotic-resistant *A. hydrophila* from clinical and environmental sources have been reported (Gosling, 1986; Chang & Bolton, 1987; Koehler & Ashdown, 1993; Yamaguchi et al., 1999; Thayumanavan et al., 2003) and some are heavy metal resistant also (Miranda & Castillo, 1998).

Chirostoma humboldtianum is a Mexican endemic fish belonging to the Atherinopsidae family (Dyer & Chernoff, 1996). It is a wild non-cultivated fish widely consumed in Mexico since long before the Spanish Conquest (Sierra & Sierra, 1977), for which, to our knowledge, there is no microbiological studies published. In this paper we report the isolation of thirty bacterial strains: *Aeromonas hydrophila* (20), *Hafnia alvei* (5), *Enterobacter cloacae* (2), *Enterobacter amnigenus* (1), *Citrobacter freundii* (1), and *Klebsiella spp.* (1) from the charal (*Chirostoma humboldtianum*) and the *A. hydrophila* susceptibility to five antibiotics and to six heavy metals.

MATERIALS AND METHODS

Bacteria isolation and identification. Fifty two fishes were collected during September of year 2000 and March 2001, from the San Felipe Tiacaque freshwater pond at Ixtlahuaca, Edo. de Mexico, Mexico. Fishes were collected in sterile polyethylene bags and brought to the laboratory in an ice chest. Samples were processed within 2 h of collection. Extraction of the intestinal tract content of the fishes was done as suggested in Thoesen (1994). First the excess of mucus was removed by cleaning the ventral surface area using a sterile paper towel, then, a 70% ethanol solution was spread over, and dried with a paper towel. After this, by using sterile surgical scissors and a scalpel, an incision was performed starting from the ventral wall towards the anus. The digestive tract was gently held using sterile forceps. Intestinal content was aseptically swabbed using sterile cotton buds, inoculated into brain hearth infusion broth (BHI, Bioxon, Mexico) and incubated at 25°C for 24 h.

Afterwards, aliquots of culture were streaked on eosin methylene blue agar and sheep blood agar (Bioxon, Mexico) and incubated at 25°C for 24 h. Thirty strains were isolated and they were identified as *A. hydrophila* (20), *Hafnia alvei* (5), *Enterobacter cloacae* (2), *Enterobacter amnigenus* (1), *Citrobacter freundii* (1), and *Klebsiella spp.* (1) by the API-20E system (bioMérieux, France). *Aeromonas hydrophila* isolates were selected for further characterization.

MIC of heavy-metals determination. The minimal inhibitory concentration (MIC) of toxic metals in nutrient agar (Bioxon, Mexico) was determined by an agar dilution procedure, as previously described (Vaca et al., 1995). In brief: Each bacterial strain was grown in nutrient broth at 25°C for 24 h with agitation. Cultures were diluted 1:3 in the wells of a Steers replicator and inoculated on nutrient agar plus serial double dilutions of each metal. Ions tested, all from Merck, were as follows: lead [Pb (CH₃COO)₂], chromate (K₂CrO₄), zinc (ZnCl₂), silver [(CH₃COO)Ag], mercury (HgCl₂), and arsenite (NaAsO₂). MIC breakpoints (µg/ml) for considering a bacterial isolate as susceptible (S) or resistant (R) were those reported in Vaca et al. (1995): lead S < 800, R >800; chromate S <750, R >750; mercury S <54, R >54; Nakahara et al. (1977): arsenite S <400, R >400; zinc S <170, R >170; and Gupta et al. (1998): silver S ≤ 34, R > 34.

MIC of β-lactams determination. MIC of β-lactam antibiotics were determined in Mueller-Hinton according with the guidelines of the NCCLS (1997). β-lactams tested, all from Sigma-Aldrich, were: ampicillin, penicillin G procainic, dicloxacillin, cefuroxime, and ceftriaxone. Ampicillin plus sulbactam (2:1) was from Glaxo.

MIC breakpoints (µg/ml) for considering a bacterial isolate as susceptible (S) or resistant (R) were those recommended by the NCCLS (1997): penicillin S ≤ 0.12, R ≥ 0.25; dicloxacillin, ampicillin, cefuroxime S ≤ 8, R ≥ 32; ceftriaxone S ≤ 8, R ≥ 64.

β-lactamase activity was detected by hydrolysis of the chromogenic cephalosporin nitrocefim (BBL) (O'Callaghan et al., 1972).

RESULTS

All strains produced β-lactamases and were resistant to penicillin and dicloxacillin, showing single peak MIC distributions at 2000-4000 µg/ml (Fig. 1A) and 500-1000 µg/ml (Fig.

Resistance of *Aeromonas hydrophila* from *Chiostoma humboldtianum*

1C), respectively. Ampicillin MIC distribution was bimodal (Fig. 1E) with 20% resistant strains (MIC= 125-250 $\mu\text{g/ml}$) and 80% highly resistant ones (MIC= 500-4000 $\mu\text{g/ml}$). Strains showed three distinguishable susceptibilities to the combination ampicillin plus sulbactam (Fig. 1B). Thirty percent of the strains had a 3.9 $\mu\text{g/ml}$ ampicillin plus sulbactam MIC (96.7-99.9 % lower than their corresponding ampicillin MIC), whereas 45% of the strains showed a 31.3 $\mu\text{g/ml}$ ampicillin plus sulbactam MIC (87.5-98.4 % lower than their ampicillin MIC) and 25% had a 125-250 $\mu\text{g/ml}$ ampicillin plus sulbactam MIC (75-87.5% lower than their ampicillin MIC). All strains were susceptible to ceftriaxone (Fig. 1D) and all but one were susceptible to cefuroxime (Fig. 1F).

All strains were resistant to lead (MIC=800-3200 $\mu\text{g/ml}$, Fig. 2A) and susceptible to chromate (MIC \leq 375 $\mu\text{g/ml}$), silver (MIC \leq 2.5 $\mu\text{g/ml}$), mercury (MIC=20 $\mu\text{g/ml}$) and zinc (MIC \leq 42.5 $\mu\text{g/ml}$) (Fig. 2 C-F). Susceptibility to arsenite showed a bimodal MIC distribution with a peak of 15% resistant strains (MIC=400-800 $\mu\text{g/ml}$), and 85% of them susceptible (MIC=200 $\mu\text{g/ml}$ Fig. 2B).

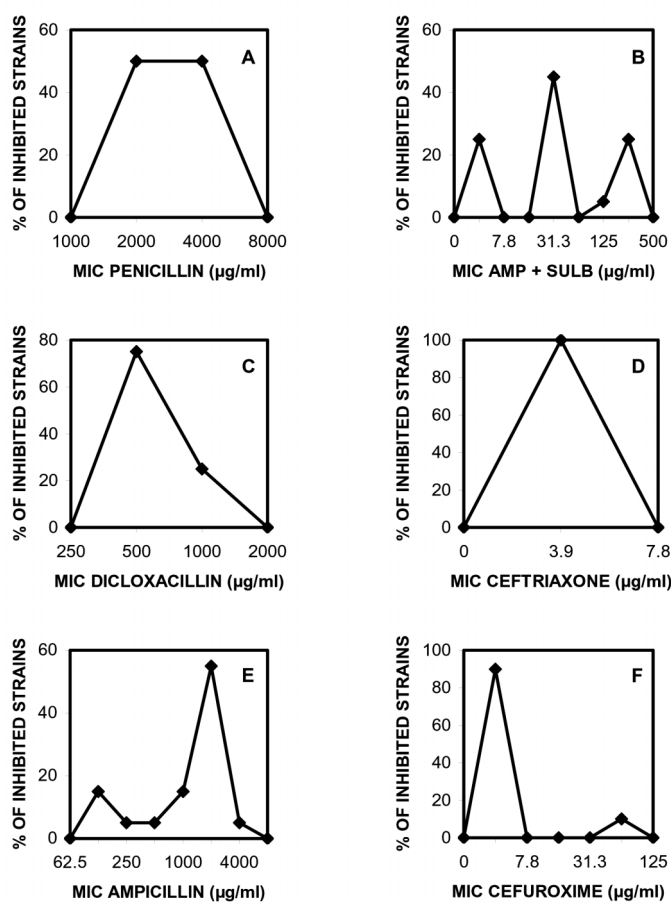


Figure 1. MIC distributions patterns of various β -lactam antibiotics for the *A. hydrophila* strains. A, Penicillin; B, Ampicillin plus sulbactam; C, Dicloxacillin; D, Ceftriaxone; E, Ampicillin; F, Cefuroxime.

DISCUSSION

Chiostoma humboldtianum is an endemic Mexican fish, widely consumed by low-statuses Mexican people, for which no microbiological studies have been published. We have found that these fishes harbored β -lactamase producer *A. hydrophila* strains resistant to penicillin, dicloxacillin, and ampicillin (Fig. 1 A, C, E). Since ampicillin MIC was 75-99.9% lowered by sulbactam (Fig. 1B) these results suggest us that β -lactamases produced by the strains probably are conventional spectrum enzymes, which do not confer resistance to cephalosporins (Payne *et al.*, 1994). This conclusion was further supported by the ceftriaxone and cefuroxime susceptibility of the strains (Fig. 1 D, F). These results are similar to those found by Castro-Escarpulli *et al.* (2003) for 82 *Aeromonas* spp. strains isolated from frozen tilapia (*Oreochromis niloticus niloticus*) purchased in local markets of Mexico City; all strains were resistant to penicillin and ampicillin, but sensitive to cefuroxime.

All *A. hydrophila* strains reported here were resistant to lead, showing a single-peak MIC distribution pattern (800-3200 $\mu\text{g/ml}$, Fig. 2A). This result is in good agreement with similar single-peak MIC distributions for Pb found by Nakahara *et al.* (1977) in clinical Gram-negative bacterial strains (at 1600-3200 $\mu\text{g/ml}$) and Vaca *et al.* (1995) in a group of strains (at 800-1600 $\mu\text{g/ml}$), mostly Gram-positive, isolated from soil adjacent to a busy traffic high way from Mexico City highly polluted by automobile exhausts containing Pb (9.6-29.3 ppm) derived from gasoline.

The presence of toxic inorganic ions in the environment usually inhibits the exposed microorganisms but may also select variants able to tolerate high concentrations of the ions (Doelman, 1987).

Although in this work concentrations of heavy metals present in the water or in the fishes were not measured, the fact that all *A. hydrophila* strains isolated were lead-resistant suggests that bacterial populations present at this pond had been subject to a strong selection by lead-containing pollutants. The mechanisms of bacterial resistance to lead are complex and they are not due to a single point mutation. Lead-resistant bacteria have been isolated from metal polluted soils; resistance is due to the extracellular exclusion (*Pseudomonas marginalis*), or to the intracellular accumulation (*Citrobacter freundii*) of the metal (Roane, 1999). ATPases responsible for expulsion of lead have been described in *Staphylococcus aureus* and *Escherichia coli* (Rensing *et al.*, 1998). However, the first detailed mechanism of resistance to lead described so far is the one reported for *Ralstonia metallidurans* CH34 (formerly *Alcaligenes eutrophus* CH34) (Borremans *et al.*, 2001). In *R. metallidurans*, resistance to lead is conferred by the *pbr* locus, which is an operon, inducible by lead, divergently transcribed that includes five structural and one regulatory gene. The products of these genes participate in

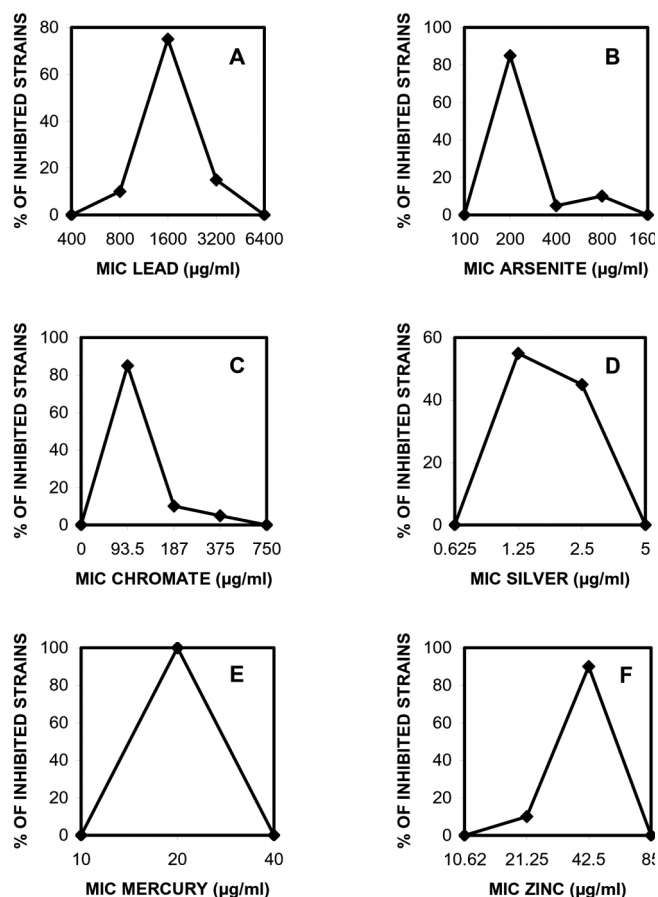


Figure 2. MIC distribution patterns of various metals for the *A. hydrophila* strains. A, Lead acetate; B, Sodium arsenite; C, Potassium chromate; D, Silver acetate; E, Mercury chloride; F, Zinc chloride.

the capture, expulsion and intracellular accumulation of lead (Borremans et al., 2001).

Bacterial antibiotic and heavy metal resistance determinants commonly reside on plasmids, and selective pressure for one resistance indirectly leads to selection for the others, as the responsible genes are linked.

Since many low-statuses farmer people consume these fish and usually swim in this freshwater pond, contact with the antibiotic and heavy metal resistant *Aeromonas* possess an important human health risk. In addition, passage of these bacteria from fish to humans would permit the horizontal gene transfer from *Aeromonas* to other bacteria harbored by the exposed persons.

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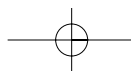
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