Short Communication

Characterization of mercury-resistant clinical *Aeromonas* species

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Abstract

Mercury-resistant *Aeromonas* strains isolated from diarrhea were studied. Resistance occurs via mercuric ion reduction but *mer*A and *mer*R genes were only detected in some strains using PCR and dot hybridization. Results indicate a high variability in *mer* operons in *Aeromonas*. To our knowledge, this is the first report of mercury-resistant clinical *Aeromonas* strains.

Key words: *Aeromonas*, mercury resistance, clinical strains.

Mercury is one of the most toxic of the heavy metals. Mercury toxicity arises from the avid ligation of its compounds to the thiol groups in proteins, which results in inhibiting macromolecule synthesis and enzyme action. Bacteria have developed several mechanisms to neutralize mercury toxicity, the most common being the conversion of highly toxic mercuric ions to less toxic and volatile elemental mercury through chemical reduction. Mercuric ion resistance involves a diverse set of genes, which are widespread in both Gram-positive and Gram-negative bacteria. The bacterial *mer* operons encode a cluster of genes involved in the detection, mobilization and enzymatic detoxification of mercury (Barkay and Wagner-Döbler, 2005).

Bacteria belonging to the genus *Aeromonas* are Gram-negative metabolically versatile bacilli, able to adapt and colonize different terrestrial and aquatic ecosystems. In accordance with this ubiquity, *Aeromonas* species resistant to a great variety of antibiotics and heavy metals have been described. Some species of this genus are considered pathogens responsible for various infections in humans (Janda and Abbott, 2010). This work focused on the isolation and characterization of mercury resistant *Aeromonas* from human diarrheic feces where no other known enteropathogenic bacterium was isolated.

Forty-one clinical strains were isolated from diarrheic feces collected between July and October 2005 from patients attending the Public Health Institute at Hidalgo State, Mexico. Strains were classified as *Aeromonas* by standard bacteriological tests. Mercury resistant strains (n = 28) were further identified using the 16S rDNA-RFLP pattern (Figueras *et al.*, 2009). Species assignation were completed by PCR amplification and sequencing of *rpo*D gene. Strains not yielding an *rpo*D amplicon were identified by *gyr*A amplification and sequencing (Beaz-Hidalgo *et al.*, 2010). Using these approaches, the following species were found: *A hydrophila* (n = 11), *A. caviae* (n = 10), *A. veronii* (n = 3), *A. aquariorum* (n = 3) and *A. media* (n = 1).

The susceptibility profile to five different antimicrobial agents was tested by the Kirby-Bauer disk diffusion method (Table 1) (CLSI 2007). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25923, *Staphylococcus aureus* ATCC 27853 and *A. hydrophila* ATCC 7966 were used as reference strains.

In order to determine the minimum inhibitory concentration of mercury (MIC), cultures were grown overnight in trypticase soy broth (TSB) and then 10 μL of each culture was spotted onto brain heart infusion (BHI) (Oxoid Hampshire, England) agar plates containing 0 to 600 μg mL^{-1} HgCl$_2$. Plates were incubated at 37 °C. The lowest concentration that inhibited growth was considered the MIC. Results are shown in Table 1. Since all strains (N = 41) were able to grow in the presence of 10 μg mL^{-1} HgCl$_2$, the cutoff value for mercury resistance was set at 25 μg mL^{-1}.

All mercury-resistant *Aeromonas* strains were tested in a mercury volatilization assay by the X-ray film method

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Table 1 - Characterization of mercury-resistant *Aeromonas* species.

Strain	Specie	MIC HgCl$_2$ (µg/mL)	Volatilization assay	*mer*A gene amplification*	*mer*A gene hybridization	*mer*R gene amplification	Antibiotic resistance pattern
6610	*A. hydrophila*	400	+	+	ND	-	TET
6632	*A. hydrophila*	200	+	+	+	+	S
6479	*A. hydrophila*	300	+	+	ND	+	TET, NAL, TMP, STR
6698	*A. hydrophila*	200	+	+	ND	-	S
6689	*A. hydrophila*	200	+	-	+	+	S
6587	*A. hydrophila*	200	+	+	ND	+	TET, NAL, TMP, STR
6492	*A. hydrophila*	200	+	+	ND	-	S
6376	*A. hydrophila*	25	+	-	+	-	TET
5794	*A. hydrophila*	200	+	-	+	+	TET, TMP, STR
3510	*A. hydrophila*	200	+	+	ND	+	S
3611	*A. hydrophila*	600	+	+	ND	+	TET
6640	*A . aquariorum*	300	+	-	+	+	TET, NAL, TMP
6336	*A. aquariorum*	200	+	-	+	-	TET, NAL, TMP
3430-1	*A. aquariorum*	300	+	+	ND	+	TET, TMP, STR
6597	*A. caviae*	25	+	+	ND	+	TET, TMP
6626	*A. caviae*	300	+	+	ND	-	TET, TMP
6425	*A. caviae*	200	+	+	ND	+	TET, TMP
6436	*A. caviae*	25	+	+	ND	-	TET, TMP
6661	*A. caviae*	25	+	-	+	+	TMP
6582	*A. caviae*	25	+	-	+	+	TET, TMP
6548	*A. caviae*	25	+	-	+	-	TMP
5881	*A. caviae*	200	+	-	+	+	TMP, STR
5919	*A. caviae*	25	+	-	+	-	TET, TMP
3817	*A. caviae*	300	+	-	+	+	TET, NAL, TMP, STR
6654	*A. veronii*	200	+	-	+	+	S
5869	*A. veronii*	200	+	+	ND	-	TET, NAL, TMP
5933	*A. veronii*	300	+	+	ND	+	TET, TM, CAM
3925	*A. media*	25	+	-	+	-	TMP, STR

TET, Tetracycline; TMP, Trimethoprim; STR, streptomycin; NAL, nalidixic acid; CAF, Chloramphenicol; ND, not done; S, sensitive to all antibiotics tested. * Amplified with primers MerA1F/MerA1R and MerA1/MerA2.

as described (Nakamura and Nakahara, 1988). All 28 mercury-resistant strains were able to reduce mercuric ions as shown by the appearance of dark spots on the X-ray film, resulting from the formation of a silver amalgam (Table 1).

The presence of *mer*A and *mer*R genes was assessed by PCR amplification with primers previously described (MerA1, MerA2, A1s-n.F and A5-n.R) (McIntosh *et al.*, 2008; Vetriani *et al.*, 2005) or designed in this work (MerA.1F: CGACTGCACCGACCAGCC, MerA.1R: AAGGTCTGCGCCGCGAGCT, MerR.1F: ATCCGBTTCTATCAGCGCAAG and MerR.1R: ACGTCCTTNRGCTTGTGCTCG). Isolation of total DNA from metal-resistant strains was done according to standard protocols (Sambrook and Russell, 2001). PCR reactions were done with PCR Master Mix (2X) (Fermentas, Lithuania) and amplifications involved standard cycles of PCR.

Using MerA.1F and MerA.1R primers 12 out of 28 strains yielded the expected 461 bp amplicon. Strains that did not yield an amplification product with these primers were subjected to a new amplification reaction with primers MerA1 and MerA2. Using these primers, amplicons with the expected size were obtained for three additional strains. In an further attempt to amplify the *mer*A gene from total DNA of thirteen negative strains, degenerate primers A1s-n.F y A5-n.R were used in new *mer*A amplification reactions. Five of the strains tested this way rendered an amplification product of the right size. However, sequencing of these fragments followed by alignment to the Gen Bank database revealed no similarity with any gene belonging to the *mer* operon. The *mer*R gene amplification assays

yielded the right amplicon in 17 mercury-resistant strains. Not all the strains positive for the *mer*A gene rendered a product for the *mer*R gene or viceversa (Table 1).

The presence of genes coding for MerA and MerR was also explored using a dot hybridization assay. DNA probes to detect *mer*A and *mer*R homologues were synthesized by PCR amplification of *A. salmonicida* AS03 total DNA with MerA1 and MerA2 or MerR.1F and MerR.1R primers, respectively, in the presence of digoxigenin-dUTP. Dot blot hybridization was conducted at high stringency according to the instructions of the manufacturer (Digoxigenin-High Prime DNA Labeling and Detection Starter Kit I; Roche Molecular Biochemicals, Indianapolis, IN). All strains produced positive hybridization signals for *mer*A but none for *mer*R gene (Table 1).

In order to know whether mercury resistance was carried by an extrachromosomal element, plasmids were extracted from all the mercury-resistant strains according to Birnboim and Doly (Birnboim and Doly, 1979) and Kado and Liu procedures (Kado, and Liu, 1981). Eight plasmid-bearing strains with sizes ranging from 3 to 53 kb were found. These strains were used as donors in conjugal transfer experiments. Matings were performed on sterile nitrocellulose filters on TSA plates using *E. coli* J53-1 Nal^R as a recipient. Transconjugants were selected on TSA plates containing 30 µg mL^{-1} nalidixic acid and 50 µg mL^{-1} HgCl$_2$. *A. salmonicida* AS03 bearing a conjugative plasmid conferring mercury resistance was used as a positive control. In this case matings were performed at 28 °C. No mercury-resistant transconjugants could be detected in any of the mating experiments. In view of these results we intended to introduce the plasmids by bacterial transformation. Transformation experiments were as described by Chung *et al.* (1993), using *E. coli* DH5α (Invitrogen CA, USA) as host for the incoming plasmids. Transformants were selected on TSA plates supplemented with 50 µg mL^{-1} HgCl$_2$. Transformation using plasmid preparations did not yield any mercury-resistant *E. coli* colonies. In agreement with these results, attempts to PCR amplify *mer*A or *mer*R genes from plasmid bands excised from agarose gels were unsuccessful. These results show that plasmids are not related with metal resistance.

Multiple reports on bacterial resistance to mercury including diverse bacterial groups exist in scientific literature. However, works on mercury resistance in *Aeromonas* are scarce (De J Ramaiah *et al.*, 2003; Huddleston *et al.*, 2006; McIntosh *et al.*, 2008). In this work mercury-resistant strains were characterized as: *A. hydrophila, A. caviae, A. veronii*, *A. aquariorum* and *A. media*. The first three species have been reported as the most frequently associated to diarrhea in Mexico (Aguilera-Arreola *et al.*, 2007). Some species of *Aeromonas* present high intraspecific diversity and the presence of different ecotypes has been suggested depending on the strain provenance. Therefore our strains were probably able to acquire the genes for adaptation to mercury-rich environments from diverse sources (Aguilera-Arreola *et al.*, 2005).

Different works on the isolation of bacteria resistant to heavy metals as part of normal human biota or clinical specimens exist. These works were performed using a variety of culture media (Deredjian *et al.*, 2011; Pike *et al.*, 2002). Since it has been reported that results on resistance levels are highly affected by the culture medium used (Pike *et al.*, 2002a); a direct comparison of mercury resistance among strains is not easy. In our experimental conditions strains resistance ranged between 25 and 600 µg mL^{-1}. HgCl$_2$. Bacteria growing in the presence of 25 µg mL^{-1} are true resistants as confirmed by the mercury volatilization assay and by the amplification of the *mer*A or *mer*R genes. Some of these bacteria are particularly resistant comparing to strains described recently by McIntosh *et al.* (2008). They isolated several highly resistant *A. salmonicida* strains able to grow in 270 µg mL^{-1} HgCl$_2$. Using the same medium and one of these strains (AS03), as a positive control, we found several clinical isolates able to grow between 300 to 600 µg mL^{-1} HgCl$_2$. Similar levels of resistance as observed in our aeromonad isolates have only been reported in *Bacillus subtilis* strains, which are able to grow in medium containing up to 550 µg mL^{-1} HgCl$_2$ (Kannan and Krishnamoorthy, 2006).

Many papers evidence the frequent association between the *mer* operon and antibiotic resistance determinants (Baker-Austin *et al.*, 2006; Mathema *et al.*, 2011). Given that several mercury-resistant clinical strains isolated in this work showed also resistance to tetracycline, trimethoprim, nalidixic acid and streptomycin (Table 1), a search for plasmids was implemented. We found plasmids in some mercury-resistant strains. Mercury resistance could not be transferred by conjugation or transformation with plasmid DNA, which suggests that no relationship between these mobile elements and the metal-resistance phenotype exists. This was confirmed by the lack of amplification of *mer*A or *mer*R genes using plasmid DNA as a template. Our results contrast with reports in literature where *mer* operons reside frequently in plasmids (Nakahara *et al.*, 1977). Previous work in our laboratory searching for Class 1 integrons revealed also a preferential chromosomal *vs.* plasmid localization of these elements in *Aeromonas* spp. strains (Pérez-Valdespino *et al.*, 2009).

Mercury reduction is the most common mercury detoxification mechanism in bacteria. All strains were able to reduce the mercuric ion to elementary mercury. Our results show a widespread distribution of mercury reducing activity in the clinical *Aeromonas* spp. strains studied. Despite these results, we could not amplify the *mer*A gene in around half of the strains using three different primer sets, including primers A1sn.F and A5n.R, which are widely used to demonstrate the presence of the *mer*A gene in an large diversity of bacteria (Ní Chadhain *et al.*, 2006; Skurnik *et al.*, 2010; Vetriani *et al.*, 2005). Using these primers we found

amplicons of the right size derived from some of our clinical *Aeromonas* strains. However, these amplicons showed sequences not related to *mer*A. We suggest that amplicons generated with these primers should always be sequenced in order to avoid false positive results. This is in accordance with Rochelle *et al.* (Rochelle *et al.*, 1991), who recommend confirming the presence of the *mer* genes using several techniques or probes. Due to the failure to amplify the *mer*A gene in several strains, we searched for the presence of the *mer*R gene. As observed with *mer*A, not all strains yielded the *mer*R amplicons. Considering these results, we resorted to search for *mer*A and *mer*R homologues through dot hybridization assays. All strains hybridized with the *mer*A probe but none of them hybridized with the *mer*R probe. Taken together, these results suggest a high variability of *mer* operons in clinical strains. Mercury exerts a selective pressure on microorganisms, which facilitates the selection of resistant strains carrying genetic elements conferring resistant to mercury from different bacteria to *Aeromonas*, accounting for this variability (Baker-Austin *et al.*, 2006; Smit *et al.*, 1998). Highly resistant microorganisms play a key role in mercury detoxification and mobilization in natural environments. Isolation of highly mercury-resistant *Aeromonas* from diarrheic feces might result from human exposure to water heavily contaminated with the metal, which would pose a threat to human health.

Acknowledgments

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