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MICROBIOLOGY

The presence of plasmids in *Aeromonas hydrophila* and its relationship with antimicrobial and heavy metal-resistance profiles

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ABSTRACT: *Aeromonas hydrophila is a common fish pathogen that causes extensive damage to aquaculture. To develop and implement a more adequate strategy to farm fish, it is crucial to understand the bacterial-resistance levels and their transference dynamics. The objective of this study was to analyze the resistance profile of isolated Aeromonas hydrophila to antimicrobial agents and heavy metals and draw a correlation of the observed profiles with the presence of plasmids. Resistance of the isolated bacteria to antimicrobial agents (oxacilin, gentamicin, tetracycline, and nalidixic acid) and heavy metals (cadmium, lead, copper, and manganese) was verified using the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) standards. The Multiple Antibiotic Resistance Index (MAR Index) was calculated. Plasmids were extracted by using a common methodology described elsewhere. Mann-Whitney Test, implemented in the R environment, was used to determine the correlation between resistance and plasmids presence. A high resistance to almost all antimicrobial agents and heavy metals was observed, except to gentamicin and cadmium. The MAR index results showed resistance to all antimicrobial profiles. Of the isolated bacteria, 14 showed the presence of plasmids. However, no correlation was noted between the resistance profile and the plasmid presence for these isolates, indicating that the genes responsible for resistance to microbial agents and heavy metals are present in the cromossomic DNA, which in turn suggested the possibility of gene transfer between the isolated bacteria. The resistance to heavy metals can be linked to heavy utilization of fertilizers along the Sao Francisco River.*
Key words: *aquiculture, multiresistance, megaplasmids, cromossomic DNA, integrons.*

Presença de plasmídeos em *Aeromonas hydrophila* e sua relação com perfis de resistência aos antimicrobianos e metais pesados

RESUMO: *Bactérias da espécie Aeromonas hydrophila são patógenos que atacam peixes, causando grandes prejuízos à piscicultura. Entender os perfis de resistência dessa bactéria e a capacidade da mesma em transferir tal resistência é importante para implantação de um manejo adequado na produção de peixes. Os objetivos desse estudo foram analisar a resistência de isolados de Aeromonas hydrophila à antimicrobianos e metais pesados e, correlacionar os perfis encontrados com a presença de plasmídeos. A resistência dos isolados aos antimicrobianos (oxacilina, gentamicina, tetraciclina e ácido nalidíxico) e metais pesados (cádmio, chumbo, cobre e manganês) foi verificada pelas técnicas da Concentração Bactericida Mínima (CBM) e Concentração Inibitória Mínima (CIM). Foi calculado o Índice de Resistência Múltipla aos Antimicrobianos (IRMA). Os plasmídeos foram extraídos por metodologias descritas pela literatura. A relação entre a resistência aos antimicrobianos e metais pesados com a presença de plasmídeos foi determinada pelo teste de Mann-Whitney utilizando o ambiente R. Foi observada alta resistência aos antimicrobianos e metais pesados testados, com exceção à gentamicina e cádmio. No IRMA os isolados apresentaram resistência a todos os perfis de antimicrobianos possíveis. Quatorze isolados apresentaram plasmídeos, mas não foi encontrada relação dos perfis de resistência com a presença destes, o que indica que os genes de resistência a esses compostos estejam presentes no DNA cromossômico. Porém apontam a possibilidade de transferência dos genes de resistência entre os isolados. Estes resultados apontam alta resistência dos isolados e capacidade de transmissão dessa resistência a outras bactérias. A resistência aos metais pesados, pode estar ligada ao uso de fertilizantes nas plantações localizadas próximas as margens do Rio São Francisco.*
Palavras-chave: *aquicultura, multirresistência, megaplasmídeos, DNA cromossomal, integrons.*

INTRODUCTION

Aquaculture in Brazil is an emerging activity, and future studies on sanitary and environmental impact of this activity are important (RESENDE et al., 2012). The Brazilian northeastern semi-arid region presents a vast territory and environmental conditions that favors the implementation of farming systems for tropical species. The presence of large water reservoirs like Sao Francisco River, man-made irrigation systems, and lakes as well as high temperatures all year long make this region a viable place to farm exotic fish

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species such as Nile tilapia (*Oreochromisniloticus*), becoming an important economic alternative for the region (MEURER et al., 2009).

However, the intensive fish farming activity can increase the likelihood of spreading bacterial infections (CRUZ et al., 2012). Another issue that significantly affects fish farming is effluent emissions that can contaminate fresh water with biological and chemical agents and increase the concentration of solids and nutrients derived from the unconsumed fish food (MEIRELLES, 2010).

The bacteria of genus *Aeromonas* are the main pathogen to fish, as they are under severe stress since they are opportunistic pathogens (JANDA; ABBOTT, 2010). The *Aeromonas* can be found in fresh water, rivers, lakes, estuaries, underground water, and even sewer (JANDA; ABBOTT, 2010).

The concerns regarding resistance to microbial agents has increased significantly once it was discovered that it can be transferred from one bacterial cell to another facilitated by plasmids. Several genes with different functions such as virulence factor determinants, resistance to anti-microbial agents, and resistance to heavy metals and genes of substrate metabolism can be carried by plasmids (SHERLEY et al., 2004). Different types of contamination by heavy metals indicated mechanisms associated with the co-selection process of the resistance to anti-microbial agents (BAKER-AUSTIN et al., 2006).

The objectives of the present study were to analyze the resistance to antimicrobials and heavy metals in isolates of *Aeromonas hydrophila* obtained from the Valley of São Francisco, Brazil as well as to verify the relationship of this resistance with the presence of plasmids.

MATERIALS AND METHODS

We analyzed 77 isolates of *Aeromonas hydrophila* from the bacterial inventory at the Animal Microbiology and Immunology Laboratory of UNIVASF, Agrarian Science campus located in Petrolina/PE/Brazil. The isolates were derived from the samples of fish tissues removed from the kidney, tegument, intestine, and lesions of tilapias (*O. niloticus*). The animals were collected at Sobradinho dam, BA-Brazil and from the Bebedouro Project of CODEVASF/PE/Brazil. These isolates were obtained from previous studies that were approved by the ethics committee of UNIVASF (protocol numbers: 0010/220515 and 0006/160812).

In order to determine the resistance of the isolated *A. hydrophila*, 4 antibiotics (oxacilin, gentamicin, tetracycline, and nalidixic acid) and 4 heavy metals (lead, copper, cadmium, and manganese) were used, following the directions of the protocol M7-A4 (NCCLS, 2005) and the directions of PATHAK and GOPAL (2005). Antibiotics and heavy metals were diluted 8-times on micro plates to concentrations of 512µg/mL to 0.25µg/mL. The BHI broth served as the negative control (-), and BHI broth with bacterial inoculums served as a positive control. The tests were performed in triplicate.

The resistances to antimicrobial agents and heavy metals were analyzed by Multiple Antibiotic Resistance Index (MAR Index) (KRUMPERMAN, 1983). Index value >0.3 indicated that the agent was a potential source of transmission of resistance genes by the isolates.

The presence of plasmids in the bacteria cultures was analyzed using two methods: a method based on alkaline lise as described by BIRNBOIM and DOLY (1979) and a method described by KADO and LIU (1981). All of the extracted plasmids were treated with 0.01mg of Rnase enzyme and then placed in a dry bath at 25°C for 20min. Plasmids were then submitted to electrophoresis using agarose gel at 0.8% concentration including 3.5µg/L ethidium bromide dye. All electrophoresis processes were executed with the inclusion of a chromosomal DNA taken from one of the isolates in the study. This chromosomal DNA was included in all of the gels used, with the objective to differentiate chromosomal DNA from mega-plasmids. Only those samples with a different band from the chromosomal DNA were classified as plasmids.

The relationship between the resistance to antimicrobial agents and heavy metals with the presence of plasmids were determined by the Mann–Whitney test using R environment (R Core Team, 2013), considering $p \leq 0.05$. The analyses were conducted individually for each agent and heavy metal.

RESULTS AND DISCUSSIONS

A high rate of resistance was observed for the tested isolates for all tested substances, except gentamycin, which was effective at 0.25–256µg/mL, with only 7 isolates resistant at all concentrations (Table 1). Cadmium presented a bactericidal activity of 0.25–512µg/mL, with only 10 resistant isolates (Table 2). Oxacilin presented the least inhibitory capability among the antibiotics tested (Table 1). According to the MAR Index recorded, all isolates presented multi-resistance to the tested antimicrobial agents, with MAR Index varying between 0.37 and 1. KO et al. (1996) reported the sensitivity of *Aeromonas*

Table 1 - Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of antibiotics against *A. hydrophila* isolates.

Concentration (µg/mL)	----------Oxacillin-------		--------Gentamicin-------		---------Tetracycline------		------Nalidíxic Acid------	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
512	-	2	-	-	-	5	-	9
256	1	-	-	1	4	9	8	12
128	1	4	1	2	10	4	13	4
64	4	3	2	3	3	5	4	3
32	2	1	3	17	4	5	3	9
16	2	-	17	18	5	3	8	9
8	-	-	17	16	3	6	10	1
4	-	-	19	5	5	3	-	3
2	-	-	6	-	5	2	4	-
1	-	-	1	1	2	-	-	-
0.5	-	-	3	1	2	-	-	-
0.25	-	-	1	6	-	1	-	-
No activity	----------67 (87%)-------		----------7 (9%)---------		---------34 (44,1%)-------		----------27 (35%)---------	
Total isolates	----------77----------		----------77----------		----------77----------		----------77----------	

-No activity.

spp. to gentamycin and sensitivity of 88% *Aeromonas* isolates from humans to the antibiotic.

Despite the fact that the antibiotics tested in this study are not permitted for use in fish farming activities (PÁDUA et al., 2012),the resistance observed can be explained by the transference of the resistance genes from the clinical specimens, including the ones reported in human infections and other mammals to environment specimens found in aquatic animals (ARAVENA-ROMÁN et al., 2012). Another explanation to the resistance observed is the contamination of the aquatic environment with urban and agricultural effluents (LUPO et al., 2012; CABELLO et al., 2013).

The resistance of some microbial groups may vary with the environmental conditions at the location they were collected (CHEN et al., 2013; HARNISZ et al., 2015). The level of resistance manly to tetracycline, is an important bio-indicator of anthropogenic alterations HARNISZ, 2013; HARNISZ et al., 2015). In *Aeromonas* isolates, the resistance to tetracycline is observed along with other antimicrobial drugs, such as chloramphenicol (VOOLAID et al., 2012). In this study, 34 isolates (44.1%) presented with resistance to this antibiotic, showing that the environment where the fish was collected presents a major alteration in the water quality, since the resistance to antimicrobial agents can be correlated to its persistence in the environment. Drugs such as sulfas, trimethoprim, macrolides, and fluoride quinolones are commonly found in water bodies. Hydrophobic antibiotics such as tetracycline and ciprofloxacin can easily attach to other particles present in the water (VOOLAID et al., 2012). *Aeromonas* spp. exposure to hospital waste can elevate its resistance profile to antibiotics such as sulfas and, once the resistance is acquired, it can stay stable in the environment (POPOVIC et al., 2015).

A high resistance of the isolates was noted to the tested heavy metals, with cadmium presenting the best inhibitory effects, being effective up to the concentration of 0.25µg/mL (Table1). AKINBOWALE et al. (2007) analyzed the resistance to heavy metals in 90 samples of *Aeromonas* spp. and in 129 samples of *Pseudomonas* spp., where sensibility to cadmium and increased resistance to manganese, copper, and lead were observed. These results are similar to the ones observed in this study. The high resistance of the isolates to heavy metals evaluated could be linked to the use of fertilizers in the plantations along the Sao Francisco River. The agro-industrial activity along the river has created environmental issues since a long time (ZELLHUBER; SIQUEIRA, 2007).

Resistance to lead tends to be higher than to other elements such as zinc, copper, and manganese that serve as enzymatic cofactors (NEETHU et al., 2015). The resistances to zinc, copper, and lead are

Table 2 - Minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of heavy metals against *A. hydrophila* isolates.

Concentration (µg/mL)	---------Cadmium---------		----------Lead----------		----------Copper----------		--------Manganese--------	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
512	-	23	-	-	-	16	-	2
256	22	18	-	-	15	-	2	3
128	20	14	-	1	1	-	1	-
64	14	6	1	-	-	-	2	-
32	6	4	-	-	-	-	-	-
16	3	1	-	-	-	-	-	-
8	1	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-
0.5	-	-	-	-	-	-	-	-
0.25	1	1	-	-	-	-	-	-
No activity	---------10(7.7%)--------		--------76(98.7%)--------		---------61(79.2%)-------		---------72(935%)--------	
Total isolates	----------77----------		----------77----------		----------77----------		----------77----------	

-No activity.

frequently co-selected (CEYLAN; UGUR, 2012). It is expected that, in environments with high concentration of heavy metals, bacteria activate their resistance mechanisms, which are selected within the population and serve as an important indicator of environmental contamination (ROOSA et al., 2014). The heavy metals are also important indicators of anthropogenic actions over the environment (GIBBONS et al., 2014; STALEY et al., 2015).

Among the 77 isolates studied, 14 presented plasmids using alkaline lyse, with 8 isolates classified as mega-plasmids (bigger than the chromosomal DNA). Among the samples evaluated by KADO e LIU (1981), none showed plasmids, indicating that the alkaline lyse is more efficient in identifying plasmids in the *Aeromonas* isolates. The literature presents that several aquatic bacteria host a wide variety of mobile genetic elements (e.g., plasmids, integrons, transposons) that can be recombined and create bacteria that are more adapted to the environment, including the presence of antibiotics (SORUM et al., 2008; CABELLO et al., 2013). It has long been known that fish pathogenic bacteria may possess genes of antibiotic resistance that are carried by plasmids and which are responsible of transferring them to other bacteria (CABELLO et al., 2016).

No relationship was noted between the resistance profiles and the presence of plasmids. Results do not excluded the possibility of transmission of genes responsible to resistance to antibiotic and heavy metals, rather they only indicate that these genes are not present in the plasmids, but that recombination events can happen and the resistance genes can be transferred to the plasmids present in the bacteria. These results indicated that the genes form part of the chromosomal DNA or in regions of the integrons, which may be responsible for transferring the genes among isolates.

CONCLUSION

The low sensibility noted at different concentrations of evaluated antibiotics and the high resistance to heavy metals can be justified by their frequent utilization in the study region, highlighting the importance of the careful use of antibiotics in aquaculture activities and the need for controlling fertilizer use in plantations along rivers. Some isolates presented plasmids; however, the presence of these plasmids was not associated to the resistance to antibiotics and heavy metals, which indicated that the responsible genes can be present at the chromosomal DNA in the integrons regions.

DECLARATION OF CONFLICTING OF INTERESTS

We have no conflicts of interest to declare. The founding sponsors had no role in the design of the study; in the

collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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