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Detection of Fur, AmoA and pvcAB genes in *Aeromonas hydrophila* isolated from aquatic organisms and impact on bacterial growth under different iron concentrations

[*Detecção dos genes Fur, AmoA e pvcAB em* Aeromonas hydrophila *isoladas de organismos aquáticos e o impacto da presença desses genes sobre o crescimento bacteriano com diferentes concentrações de ferro*]

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ABSTRACT

Infection caused by *Aeromonas* brings great harm to fish farming. Among the factors associated with bacterial pathogenesis, iron uptake can contribute to the survival and virulence of bacteria within hosts. The aim of this study was to check the presence of genes related to iron uptake in *Aeromonas hydrophila* deriving from aquatic organisms in the São Francisco Valley and associate the presence of these genes with the ability to grow in media containing different concentrations of iron. The DNAs of 41 isolates were extracted and used in PCRs to verify the presence of the *Fur*, *AmoA* and *pvcAB* genes related to iron uptake. The growth of the isolates belonging to different genetic profiles was verified in culture media containing different iron concentrations. Two isolates were positive for the presence of the *Fur* gene, seven for the *AmoA* gene and two for the *pvcAB* gene. The growth test showed that the low availability of iron did not interfere in the growth of the isolates, nor in the isolate that did not contain any of the genes evaluated in this study, suggesting that the iron uptake's mechanisms of the tested isolates may be related to other genes and proteins.

Keywords: bacterial growth, iron uptake genes, fish farming

RESUMO

Infecções causadas por Aeromonas *trazem grandes prejuízos à piscicultura. Entre os fatores associados à patogênese bacteriana, a captação de ferro pode contribuir para a sobrevivência e a virulência das bactérias dentro dos hospedeiros. O objetivo deste estudo foi verificar a presença de genes relacionados à captação de ferro em* Aeromonas hydrophila *provenientes de organismos aquáticos do Vale do São Francisco e associar a presença desses genes com a capacidade de as bactérias crescerem em meios contendo diferentes concentrações de ferro. Os DNAs de 41 isolados foram extraídos e utilizados em PCRs para verificar a presença dos genes Fur, AmoA e pvcAB relacionados à captação de ferro. O crescimento dos isolados pertencentes a diferentes perfis genéticos foi verificado em meios de cultura contendo diferentes concentrações de ferro. Dois isolados foram positivos para a presença do gene Fur, sete para a do gene AmoA e dois para a do gene pvcAB. O teste de crescimento mostrou que a baixa disponibilidade de ferro não interferiu no crescimento dos isolados nem no isolado que não continha nenhum dos genes avaliados neste estudo, sugerindo que os mecanismos de captação de ferro dos isolados testados podem estar relacionados a outros genes e proteínas.*

Palavras-chave: crescimento bacteriano, genes de captação de ferro, piscicultura

INTRODUCTION

The aquaculture industry has been rapidly expanding to meet humanity's growing need for quality animal protein (Gobi *et al.*, 2016). According to FAO (Rakocy, 2021), Nile Tilapia (*Oreochromis niloticus* L.) is the third most cultivated freshwater species worldwide, being produced in over 100 countries (Rakocy, 2021). However, the high intensification of Tilapia

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farming can result in disease outbreaks, most of them caused by bacterial pathogens that lead to significant yield losses (Tang *et al*., 2014; Brum *et al*., 2017).

One of the main bacterial genera that affect fish is *Aeromonas*. This bacterium has been isolated from a wide variety of freshwater fish species around the world and was described as one of the infectious agents involved in bacterial septicemia in fish (Zheng *et al*., 2011). The infection occurs orally and through direct contact with lesions caused by the pathogen, after which the bacteria multiply inside the intestine producing several toxins (hemolysin, enterotoxin, cytotoxins, and proteases) that result in signs of hemorrhagic septicemia. Therefore, prophylactic and immunostimulant treatments can aid the disease's control (Ngamkala *et al*., 2010).

One of the factors that can contribute to the virulence of pathogenic bacteria inside hosts is iron uptake, which is an essential nutrient for most microorganisms. Sometimes the availability of iron can be low, therefore microorganisms have developed several iron uptake systems. The production of siderophores is an efficient strategy widely used by bacteria to uptake iron (David *et al*., 2019).

Siderophores are iron chelators that remove iron from the host's binding proteins and lead it to enter the bacterial cell through external membrane receptors (Najimi *et al*., 2008). According to Zwyno *et al*. (1992), most species of mesophilic *Aeromonas* produce a single siderophore, usually amonabactin or enterobactin. Amonabactin is the most prevalent siderophore in the species *Aeromonas hydrophila*, while in the species *A. sobria*, the siderophore that stands out is enterobactin.

Therefore, this study aimed to verify the presence of genes related to iron uptake in *Aeromonas hydrophila* isolates from fish in the São Francisco Valley, in the Brazilian, and to relate the presence of these genes with the growth capacity of this microorganism in a medium containing different concentrations of iron.

MATERIAL AND METHODS

A total of 41 *Aeromonas hydrophila* isolates were obtained from kidney, intestine, tegument, and lesions of tilapia (*Orecohromis niloticus*) and pacamãs (*Lophiosilurus alexandri*) with and without clinical symptoms and from a pool of branchonets (*Dendrocephalus brasilliensis*) intended for feeding carnivorous fish. The animals were collected at the Sobradinho/BA dam and the Codevasf/PE Bebedouro Project in the Northeast of Brazil. This study was approved by the Ethics Committee on the use of animals at Univasf with protocol No. 0010/220515.

The collected samples were seeded in Trypticase Soy Agar (TSA). Next, they were characterized by gram stain and by morphological and biochemical tests, according to Markey *et al*. (2013). In addition, part of the 16S rRNA gene was sequenced by Sanger sequencing to confirm the Genus and species. Previously extracted DNA samples (2µL) were used in PCR constituted by a mix of 1X Buffer, 2mM $MgCl_2$, 0.2mM dNTP, 0.5µM each 516F (5'- TGCCAGCAGCCGCGGTAA-3') and 13R (5'- AGGCCCGGGAACGTATTCAC–3') primer (Fredricks and Relman, 1998) and 2.5U Taq DNA Polymerase and of DNA, in a reaction with a final volume of 20µL. The conditions for amplification were initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, ended by a final extension of 7 minutes at 72 °C. PCR products were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide and visualized on a UV transilluminator. The positive samples were sent for "Sanger" sequencing in a private company.

For the characterization of genes related to iron uptake, the DNA of *Aeromonas hydrophila* isolates were heat-extracted in a final volume of 500µl and stored at -20°C according to Sá *et al*. (2013).

The presence of the *Fur* gene in *Aeromonas* spp. was verified through the PCR technique, with the primers *Fur*F (5'- AAAAGCTTATGGCAGACAACAACCAAGCG -3') and *Fur*R

(5'- CCAAGCTTCAATCGTCGTGCTTGCAGTC-3'), described by Sha *et al*. (2001), which amplify a 429bp fragment of said gene.

The PCR mix consisted of 1X enzyme buffer (10 mM Tris-HCl, pH 8.5, 50mM KCl), 2.6mM $MgCl_2$, 0.4mM dNTP, 0.4µM each primer, 2.5U of Taq Polymerase (Taq Platinum, Invitrogen®) and 5µL of unquantified DNA template, in a final volume of 25µL. The amplification cycles consisted of the following steps: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 60s, annealing at 57.7°C for 60s, and extension at 72°C for 60s. After, a final extension step of 72°C for 5 minutes was performed. PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized on a UV transilluminator.

For the amplification of the AmoA and pvcAB genes, primers were designed using the Primer3Plus program (Untergasser *et al*., 2007). Then, the quality was verified using the OligoAnalyzer (Owczarzy *et al*., 2008). The primers' specificity was analyzed by observing the score and E-value on the website (Altschul *et al.* 1990).

For the design of primers for amonabactin, the gene sequence described by Barghouthi *et al*. (1991) was used. For the design of primers that amplify the pioverdin-like protein (*pvcAB*), the sequence (AHA3281) described by Seshadri *et al*. (2006) was used.

For the design of primers for amonabactin, the gene sequence was described by Barghouthi *et al*. (1991).

The presence of *AmoA* and *pvcAB* genes in *Aeromonas hydrophila* was evaluated by PCR using the primers described in Table 1. In the reaction, the following reagents were used: 1X enzyme buffer (10mM Tris-HCl Ph 8.5; 50mM KCl), 2.6mM $MgCl_2$, 0.4mM of dNTPs, 0.4µM of each primer, 2.5U of Taq DNA polymerase (Taq Platinum, Invitrogen®), and 7µL of template DNA in a final volume of 25µL. Amplification cycles consisted of an initial denaturation at 95°C for 7 minutes, followed by 35 cycles of denaturation at 95°C for 60 seconds, specific primer annealing at 58°C for 60 seconds and extension at 72 °C for 60 seconds, followed by a final extension at a temperature of 72°C for 90 seconds. The amplification products of the isolates were visualized in 1.5% agarose gel electrophoresis stained with ethidium bromide.

Table 1. Primer sequence of the amonabactin (AmoA) gene and the pioverdin-like protein *(pvcAB).*

Gene	Sequence (5'- 3')	Base pairs
AmoA	F: TCGCCCGAATTCTTGTTTAC R: CGGGTACATAGAGGCAGGAG	211pb
pcvAB	F: TCACCTATCGCATCAAGCAG R: CCACTGGTGGGCATAGAAGT	242pb

F: Forward Primer; R: Reverse Primer

A positive sample for each gene was purified on a cellulose column (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit, Invitrogen). Purified samples were sent for sequencing in a private laboratory (CPqAM-Fiocruz).

The electropherograms generated by sequencing were submitted to the *base calling program* Phred (Ewing and Green, 1998). Sequences with Phred quality above 20 were analyzed with the Basic Local Alignment Search Tool-BLAST (Altschul *et al.* 1990) for gene confirmation (Percent identity greater than 98%).

To assess the relation of *Fur, AmoA* and *pvcAB* genes with the growth of *Aeromonas hydrophila* in environments with iron restriction, one positive isolate for the presence of each gene evaluated and one isolate used as a negative control, which did not have any gene analyzed, were subjected to different concentrations of ethylene diamine hydroxyphenyl (EDDHA), a chemical iron chelator used in studies of bacterial siderophores. For this, a saline solution was used to obtain a bacterial suspension with an optical density of 0.104nm (10^8 CFU), being measured in a spectrophotometer at a wavelength of 580nm. From this suspension, 100µL were transferred to a tube containing 9.9mL of

Mueller Hinton broth (MH) and, subsequently, 10µL of this inoculum were placed in each well of the plate containing the EDDHA dilutions in the final concentrations: 100µM, 50µM, 25µM, 12.5µM, 6.25µM, 3.12µM, 1.56µM, 0.78µM, 0.39µM, 0.19µM, 0.097µM and 0.049µM.
The plates were submitted to 28°C for 24 hours. After this period, an aliquot of 10µL was removed from each well and seeded on the surface of Agar Mueller Hinton, incubating again to 28°C for 24 hours to determine the Minimum Bactericidal Concentration (CBM). As negative control (-), six microplate wells containing only MH broth were used, and as positive control (+), six microplate wells containing MH plus bacterial inoculum were used. Assays were performed in triplicate for each of the tested isolates (Freire *et al*., 2018).

RESULTS

All isolates evaluated were confirmed as belonging to the *Aeromonas hydrophila* species. Among the 41 isolates analyzed for the presence of the *Fur* gene by amplification of the 439bp fragment, only 2 of these samples (4.88%) were positive. For the amonabactin gene, seven samples were positive, while for the pioverdin-like protein gene, three isolates were positive.

The analysis of the electropherograms generated by the sequencing to confirm the *Fur*, *AmoA* and *pvcAB* genes showed good quality (Phred value above 20). Through the BLAST tool, it was possible to observe that all isolates showed homology with the evaluated genes.

Regarding the growth test of isolates containing different genetic profiles in iron-restricted environments, the low availability of this micronutrient did not interfere with the growth of the evaluated isolates, not even in those that did not contain the evaluated genes.

DISCUSSION

The presence of the three genes analyzed in the evaluated isolates, which are related in the literature with the iron uptake capacity, could demonstrate greater virulence capacity through the greater potential for iron uptake, as this micronutrient is essential for bacterial metabolism and permanence of the microorganism in the host.

Iron is required for several essential cellular functions such as oxygen storage and catalysis in the electron transport process. Due to its insolubility, several microorganisms have developed mechanisms for iron uptake, which are directly linked to bacterial virulence. The survival and competitive growth of bacteria in the host require varied adaptive responses by these microorganisms. Therefore, iron uptake is considered one of the most important adaptive responses to bacterial pathogenesis (Litwin and Calderwood, 1993).

The ferric uptake regulatory protein *Fur* regulates other genes that are related to iron uptake, in addition to Mg^{2+} and Mn^{2+} uptake, it is responsible for regulating a hemoglobin uptake system, but regardless of the presence of this gene, production of siderodophores may occur by microorganisms (Santander *et al*., 2012).

Ebanks *et al*. (2013) found, working with *Aeromonas salmonicida*, that the Fur gene plays an important role in the regulation of iron homeostasis, being also essential for virulence and normal growth under conditions of iron abundance.

In the work carried out by Fernandez *et al*. (1998), in order to determine whether siderophore production is widely distributed among *A. Salmonicida* isolates and to investigate the presence and variability of iron-regulated outer membrane proteins, the authors tested high concentrations of EDDHA, an EDDA analogue, and verified that the minimum inhibitory concentration was 558 -1300µM. Likewise, evaluating the growth of *A. salmonicida* at low iron concentrations, Thode *et al*. (2015) used the 2,2'-dipyridyl iron chelator to create unlimited iron conditions, and bacterial growth was clearly affected when the growth medium contained 100 or 500µM 2,2'-dipyridyl, and so decided by using the amount of 50µM, resulting in a decrease in growth. Yao *et al*. (2016), working with *A. hydrophila*, evaluated that bacterial growth happened more slowly when 150 µM 2, 2′-dipyridyl (DIP) were added when compared to the control strain.

These results suggest that the iron uptake mechanisms of the *Aeromonas hydrophila* can also be related to other specific genes and proteins. To overcome iron-restricted conditions,

most pathogens have developed several mechanisms to attract high-affinity iron, as this micronutrient is essential for its permanence in the host and necessary to allow them to establish an infection (Drechsel and Jung, 1998; Bailão *et al.*, 2014).

The wide variety of methods of acquisition of iron complexed by bacteria through siderophores, proteins, and heme groups, or in free form, where iron atoms are internalized without being associated with other molecules (Carrizo-Cháves *et al.*, 2012), can justify the large adaptability in environments with different concentrations of iron of the evaluated isolates.

CONCLUSION

The presence of the *Fur*, Amonabactin and pioverdin-like protein was verified in the studied isolates, which could indicate virulence of these isolates through greater potential for iron uptake, further studies evaluating the virulence of these isolates may confirm this hypothesis. In the growth test in iron-restricted environments, the low availability of this micronutrient did not interfere with the growth of the evaluated isolates, regardless of the genetic profile, which demonstrates that other genes are probably also contributing to the trait.

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