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MICROBIOLOGY

Aeromonas from farmed tambaqui from North Brazil: molecular identification and pathogenic potential

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ABSTRACT: *The aim of this study was to molecularly identify different species of Aeromonas isolated from farmed tambaqui (Colossoma macropomum) from North Brazil, and evaluate their pathogenic potential by the presence of virulence genes. From the extraction of bacterial DNA, PCR (polymerase chain reaction) of the primers 16S rDNA, aerA (cytolytic enterotoxin), ast (cytotoxic enterotoxin) and act (cytotoxic enterotoxin) were performed. Of 24 isolates evaluated, eight amplified the ast gene, one amplified the act gene, but the areA gene was not amplified in any isolate. Sequencing of the 16S rRNA primer revealed a predominance of Aeromonas jandaei specie (92%). Aeromonas taiwanensis (4%), for the first time isolated from fish in Brazil, and Aeromonas hydrophila (4%) each appeared as just one isolate. Results showed that 32% of Aeromonas isolated from farmed tambaqui have considerable pathogenic potential for systemic damage, since the selected PCR primers are encoding the most common virulence genes in Aeromonas with high pathogenic intensity.*
Key words: *Colossoma macropomum, polymerase chain reaction, virulence genes, Aeromonas taiwanensis.*

Aeromonas de tambaqui de cultivo do norte do Brasil: identificação molecular e potencial patogênico

RESUMO: *O objetivo deste estudo foi identificar molecularmente diferentes espécies de Aeromonas isoladas de tambaqui (Colossoma macropomum) de cultivo do norte do Brasil e avaliar seu potencial patogênico pela presença de genes de virulência. A partir da extração do DNA bacteriano, foi realizada a PCR (reação em cadeia da polimerase) dos primers 16S rDNA, aerA (enterotoxina citolítica), ast (enterotoxina citotóxica) e act (enterotoxina citotóxica). Dos 24 isolados avaliados, oito amplificaram o gene ast, um amplificou o gene act, mas o gene areA não foi amplificado em nenhum isolado. O sequenciamento do primer 16S rRNA revelou uma predominância da espécie Aeromonas jandaei (92%). Aeromonas taiwanensis (4%), pela primeira vez isolada em peixes no Brasil, e Aeromonas hydrophila (4%) apareceram cada uma como apenas um isolado. Os resultados mostram que 32% das Aeromonas isoladas de tambaqui de cultivo apresentam considerável potencial patogênico para danos sistêmicos, uma vez que os primers de PCR selecionados estão codificando os genes de virulência mais comuns em Aeromonas com alta intensidade patogênica.*
Palavras-chave: *Colossoma macropomum, reação da polimerase em cadeia, genes de virulência, Aeromonas taiwanensis.*

INTRODUCTION

Tambaqui (*Colossoma macropomum*) is the most native farmed fish in Brazil, with a production of 101,079 tons in 2019. From this, 73,181 tons came from the north of the country (IBGE, 2021). On the other hand, with this intensification in tambaqui production, important bacterial pathogens have been identified in these fish, with potential to cause significant economic losses in production, especially due to disease outbreaks caused by *Aeromonas* (VALLADÃO et al., 2018; HILSDORF et al., 2022).

Aeromonas bacteria can cause an infection characterized by septicemia and is one of the most common pathogens in tropical fish. This disease is responsible for high morbidity and mortality rates, causing considerable losses in aquaculture (MARINHO-NETO et al, 2019; AZZAM-SAYUTI et al., 2021), thus, the understanding of its pathophysiology is crucial to develop control strategies of this bacterial infection in farmed fish (MARINHO-NETO et al, 2019)

Aeromonas species are ubiquitous in diverse aquatic habitats. High stocking density and improper

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farm practices can increase the susceptibility of fishes to motile *Aeromonas* septicemia (MAS) outbreak. However, polymorphic phenotypes and genotypes can lead to misidentification of *Aeromonas* isolates from diseased fish (AZZAM-SAYUTI et al., 2021). So, to achieve appropriate treatment for bacterial diseases, we should first accurately identify the bacterial species, for this, conventional microbiological methods used for the identification of pathogenic bacteria are very laborious and time-consuming. Molecular methods such as polymerase chain reaction (PCR) have overcome these problems, enabling rapid and accurate identification of bacteria together with the detection of virulence genes that contribute to bacterial pathogenicity, which cannot be achieved by any other conventional microbiological method (ABOYADAK et al., 2015; KINGOMBE et al., 2010).

Molecular techniques such as polymerase chain reaction (PCR) have advantages over conventional microbiological identification methods, such as rapid diagnosis and specificity for the investigated agent (ABU-ELALA et al., 2015), as well as the use of this technique allows the study of virulence factors of the strains, allowing to determine the potential degree of pathogenicity of a strain of interest (KIM et al., 2018). Several virulence factors have been described as important in the pathogenesis of infections caused by *Aeromonas* spp., among the most studied are aerolysin (*AerA*), cytotoxic enterotoxin (*Act*), and cytotonic enterotoxin (*Ast*). Studies that evaluated the presence of genes encoding these virulence factors observed that positive genotypes were more frequent in isolates obtained from diseased fish than from healthy fish or from environmental samples (LI et al., 2011; HU et al., 2012). Therefore, the detection of genes responsible for *Aeromonas* virulence is a vital tool in establishing the potential pathogenicity of the bacteria, as these virulence genes may act alone or in synergy in the establishment of infections (IGBINOSA & OKOH, 2013).

Given the above, the present study aimed to amplify the 16S rDNA gene in order to identify, at species level, strains of the genus *Aeromonas* isolated from farmed tambaqui from the Amazon State, North Brazil, and relate to this identification, the occurrence of different virulence genes aerolysin (*aeraA*) cytotoxic enterotoxin (*act*) and cytolytic enterotoxin (*ast*); and consequently, its pathogenic potential.

MATERIALS AND METHODS

Aeromonas spp. (24 samples), were isolated from 153 juvenile tambaquis collected from 10 farms of the municipalities of Iranduba and Manacapuru, important producing municipalities in Amazonas State, Brazil. The isolation was carried out at the Embrapa Western Amazon Pisciculture Laboratory from the cranial kidney or from external lesions of the animals, as described by LEÃO et al. (2020). Animals collected for isolation were not sampled at the time of a disease outbreak.

For DNA extraction, fresh colonies were inoculated into BHI (Brain Hearth Infusion) broth for propagation. The bacteria pellets harvested were subject to genomic DNA extraction following the procedures provided by the Genomic DNA Purification Kit (Thermo Fisher Scientific) having as final product 100 µl of genomic DNA, which was stored at -20 °C. Quantification of extracted DNA was performed via Nanodrop and DNA integrity was visualized on a 0.8 % agarose gel with 1kb DNA molecular marker.

From the extracted genomic DNA, the following primers were prepared for PCR (Table 1).When performing the PCR of 16S rDNA for better results from the amplicons, the annealing temperature was changed to 59 °C, which resulted in amplification with molecular weights compatible with those expected, at approximately 800 bp. The PCR preparation, with a final volume of 25 µl, consisted of ultrapure water (16.3 µl), enzyme buffer (2.5 µl), dNTPS (1.0 µl), Taq DNA Polymerase (0.2 µl), template DNA (3.0 µl), Primer Forward (1.0 µl) and Primer Reverse (1.0 µl). The polymerase chain reaction was carried out in a Thermocycler (Applied Bioystems), in 35 cycles, with changes in the annealing temperature as indicated for each primer. After the period in the thermocycler, the PCR product was electrophoresed on a 1.5% agarose gel, and the amplicons were visualized in a transilluminator and photodocumented under UV light. At the end of the PCR of the 16s rDNA gene, the purification of this product was continued with the addition of Exo-Sap (0.3 µl) and ultrapure water (1.7 µl), with subsequent incubation in a Thermocycler (Applied Bioystems) at 37 °C for 15 min for the enzyme activation process, followed by heating at 80 °C for 15 min for the enzyme deactivation. Next, for the sequencing reaction, with a final volume of 10 µl, Big Dye buffer (1.5 µl), ultrapure water (0.3 µl), Big Dye Terminator (0.5 µl), primer F and R (0.7 µl) and the PCR product of the 16S gene (7.0 µl).

The PCR purification was carried out using exoSAP-IT (ThermoFisher; catalog number: 78200.200.UL), according to the manufacturer's recommendations. The sequencing reactions were performed in a volume of 10 µL, containing 2 µL of

Table 1 - Primers used for identification and detection of virulence genes.

Gene	Primer sequence (5'- 3')	AT (C°)	Product size (bp)	Reference
16S rDNA	F: AGAGTTTGATYMTGGCTCAG R: CCGTCAATTCMTTTRAGTTT	59°	900	SEBASTIÃO, et al. (2015)
aerA	F: AACCGAACTCTCCAT R: CGCCTTGTCCTTGTA	55°	301	EL-GOHARY, et al. (2020)
act	F: GAGAAGGTGACCACCAAGAACA R: AACTGACATCGGCCTTGAACTC	55°	232	SAMAYANPAULRAJ, et al. (2020)
ast	F: TCTCCATGCTTCCCTTCCACT R: GTGTAGGGATTGAAGAAGCCG	55°	331	SAMAYANPAULRAJ, et al. (2020)

ultrapure water, 1.5 µL of Big Dye buffer, 0.5 µL of BigDye terminator v3.1 (Thermo Fisher), 1 µL of each primer and 5 µL of the purified PCR products. The cycling conditions were 96 °C for 1 min., followed by 35 cycles at 96 °C for 15 sec., 50 °C for 15 sec., and 60 °C for 4 min. The sequencing was performed using a genetic analyzer (3,500, Thermo Fisher).

Consensus sequences was obtained manually based on the alignment of forward and reverse sequences with BLAST reference sequences as well as electropherogram analysis. Sequences obtained in this study were deposited in Genbank under accession numbers descripted in table 2. The dataset was constructed using 16S sequences of 24 isolates obtained in this study, one sequence of *Aeromonas hydrophila* ATCC 7966, 35 type species of *Aeromonas*, one sequence of *Salmonella*, two of *Tolumonas* and *Pseudomonas aeruginosa* were used as an outgroup. The sequences was aligned in the MAFFT online platform (https://mafft.cbrc.jp/alignment/software/) and submitted to maximum-likelihood (ML) analysis in IQ-TREE (TRIFINOPOULOS et al., 2016). The trees were visualized in the iTOL platform (https://itol.embl.de) and edited by CorelDraw 2020 software. The alignments result in 1,587 characters and the best-fit model selected by IQ-TREE was TIM+F+I+G4. The primers selected for detection of virulence genes were properly tested with annealing temperature at 55 °C.

RESULTS AND DISCUSSION

All isolates obtained in this study from *Colossoma macropomum* infections belong to the *Aeromonas* genus. The most isolates (22) grouped with the type species *Aeromonas jandaei* with high bootstrap support, the isolate A101 grouped with the type species *A. hydrophila* and the isolate F93 grouped with *A. taiwanensis* (Figure 1).

A. jandaei is an emerging fish pathogen associated with massive mortalities in cultured freshwater fish (ASSANE et al., 2021). In Brazil, it has already been reported Nile tilapia mass mortality caused by *A. jandaei* infection in farm by ASSANE et al. (2021) and in pirarucu (*Arapaima gigas*) by PROIETTI-JUNIOR et al. (2021). Our results also revealed that, among the strains studied in this research, *A. jandaei* was, in fact, the most prevalent (91,7%) (Table 2). This result contrasts with other studies already carried out with tambaqui, where most *A. hydrophila* isolates were predominant (PESSOA et al., 2020; VALLADÃO et al., 2018; SEBASTIÃO et al., 2015).

In tambaquis under stress conditions, 34 strains were isolated recently, and were characterized as *A. hydrophila* (41.2%), *A. dhakensis* (20.6%), *A. caviae* (17.6%), *A. veronii* (11.8%) and *A. jandaei* (8.8%) (PESSOA et al., 2020), but a predominance of *A. hydrophila* is still noticed in this most recent study, despite the presence of the *jandaei* species, which is still little reported for tambaqui. In our study, only one isolate was from *A. hydrophila*, and another from *A. taiwanensi*, this last one as far as we know, not yet reported in isolations for fish in Brazil. Few studies report the isolation of *A. taiwanensis*, only, a recently reported strain of the genus was reported while studying the presence of infectious marine microbes in a lacustrine wetland in India, making this the first isolation report from the country (NANDA & SHARMA, 2020). Some *A. taiwanensis* isolate have already been described in hospitalized patients, feces and wastewater in Taiwan and Israel, described by BEAZ-HIDALGO et al. (2012), where they observed broad antibiotic resistance. Already for virulence genes, as in our study none of the strains

Table 2 - Identification of study strains and relationship between species and virulence genes.

Bp	Sample	GenBank accession number	Identification	Amplification of virulence genes		
				AerA	*Ast*	*Act*
891	A3	OM802454	*Aeromonas jandaei*	-	-	+
898	A19	OM802455	*Aeromonas jandaei*	-	-	-
865	Px26	OM802456	*Aeromonas jandaei*	-	-	+
701	A38	OM802457	*Aeromonas jandaei*	-	-	-
738	F42	OM802458	*Aeromonas jandaei*	-	-	-
900	F50	OM802459	*Aeromona jandaei*	-	-	+
541	Px52	OM802460	*Aeromonas jandaei*	-	-	-
898	Px84	OM802461	*Aeromonas jandaei*	-	-	-
899	F84	OM802462	*Aeromonas jandaei*	-	-	-
897	St86	OM802463	*Aeromonas jandaei*	-	-	+
904	F93	OM802464	*Aeromonas taiwanensis*	-	-	-
867	A96	OM802465	*Aeromonas jandaei*	-	-	-
862	A97	OM802466	*Aeromonas jandaei*	-	-	-
899	A101	OM802467	*Aeromonas hydrophila*	-	+	+
899	F103	OM802468	*Aeromonas jandaei*	-	-	-
864	A120	OM802469	*Aeromonas jandaei*	-	-	-
898	A121	OM802470	*Aeromonas jandaei*	-	-	-
898	A128	OM802471	*Aeromonas jandaei*	-	-	-
898	A130	OM802472	*Aeromonas jandaei*	-	-	-
899	F131	OM802473	*Aeromonas jandaei*	-	-	+
901	A134	OM802474	*Aeromonas jandaei*	-	-	+
899	A146	OM802475	*Aeromonas jandaei*	-	-	-
898	A151	OM802476	*Aeromonas jandaei*	-	-	+
876	A153	OM802477	*Aeromonas jandaei*	-	-	-
899	*A. hydrophila ATCC 7966*	OM802478*	*Aeromonas hydrophila*	-	+	+

*reference strain.

were positive for the cytotoxic (*act*) and cytotonic enterotoxin genes *(ast),* but all *A. taiwanensis* strains carried the genes that encode aerolysin (*aerA*), in our study, the isolate of the specie did not amplify the gene *aerA* (Table 2).

Results obtained in the detection of virulence genes were significant, where eight (33.3%) isolates showed positive amplification for *act* (seven *A. jandaei* and one *A. hydrophila*) and one (4.1%) positive for *ast* (*A. hydrophila*). The *aerA* gene did not show amplification for any isolate (Table 2).

The presence of virulence factors in some strains identified in *A. jandaei* allows bacteria to invade, colonize and destroy cells, overcoming fish immune response mechanisms (ASSANE et al., 2021). According to KIM et al. (2019), *act*, the most frequently found gene, as well as a*erA*, is also a porin, and can cause degeneration of crypts and villi in the small intestine of fish, playing an important role in infections by *Aeromonas* by inducing the beginning of the signaling process of apoptosis in eukaryotic cells, that is, its presence in an isolate means a high pathogenic potential.

Comparatively, in a study by EL-BAHAR et al. (2019), *A. hydrophila* isolated from nile tilapia showed detection of the genes a*erA* and *act*, but the gene *ast* was absent. The present study, however, differs in two aspects: *aerA* was absent and *ast* presented amplification in the isolates identified as *A. hydrophila.* Thus, it is possible to observe that the expression of such genes in *A. hydrophila* can be triggered by some circumstance, above all, as discussed by KIM et al. (2019) indicated the pathogenicity of the genus as being complex and multifactorial, involving activities of different genes acting alone or in combination with others. That is, certain genes *act* as coadjuvants for other virulence

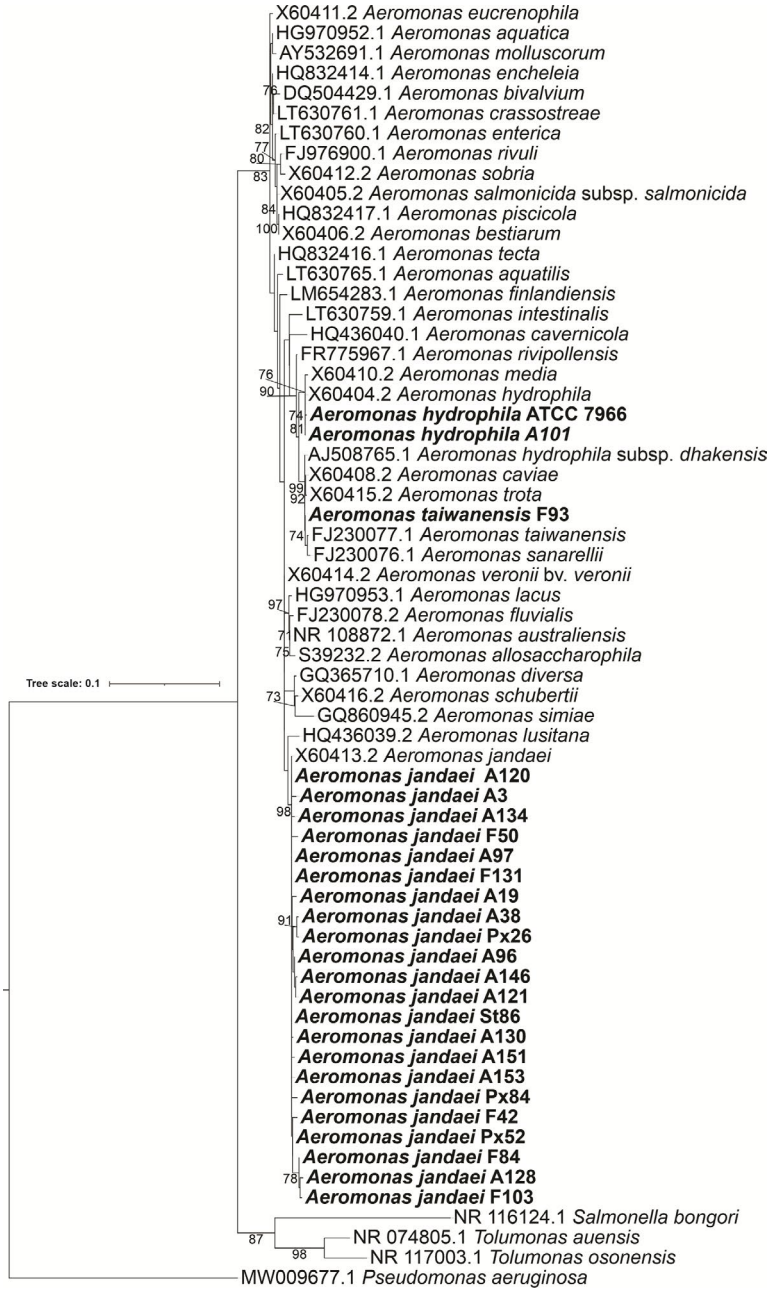

Figure 1 - Phylogenetic tree of *Aeromonas* species based on Maximum Likelihood method for 16S rRNA nucleotide sequences. Numbers indicate the bootstrap values for 1000 replicates. Isolates sequenced in this study are marked in bold. *Salmonella*, *Tolumonas* and *Pseudomonas* were used as an outgroup.

factors in *Aeromonas* an indispensable virulence factor in the mechanism of infection, which explains this differentiation of low frequencies found in the present research. In Brazil, ASSANE et al. (2021) detected neither the *act* or *ast* gene in their *A. jandaei* isolates, but *aerA* was present in all of them, being isolated from tilapia, verifying this complexity and differences for the presence of virulence genes.

The research of MAZUMDER et al. (2021) confirmed the result of the present study of positive

enterotoxin in *A. hydrophila* and *A. jandei*, they further stated that; although, the *aerA* gene is closely related to the hemolytic, cytotoxic and enterotoxic activities of the enterotoxin gene, as reported in most *Aeromonas* strains, the contradiction in results may be due to variations in the prevalence of *aerA* in relation to different geographic locations. In Brazil, *A. hydrophila* and *A. jandaei* isolated from pirarucu, *aerA* was present in all isolates, since the gene *act* was not detected in any of the strains isolated (PROIETTI-JUNIOR et al., 2021).

It can be seen; therefore, that there is a variation in relation to the fish species of the isolate, bacterial species within the genus associated with geolocation, and as the pathogenicity of *Aeromonas* strains can be directly associated with the virulence gene content, the results presented in our research revealed this potential, enabling the *Aeromonas* found as possible zoonotic agents for fish farms in the region.

CONCLUSION

Observing the results, it is possible to infer that the bacteria identified as *Aeromonas jandaei* is the most prevalent in farmed tambaquis from North Brazil, and based on the amplification of the virulence genes investigated, present a lower degree of pathogenic potential in relation to *Aeromonas hydrophila*, despite having isolated only one sample, which showed amplification of the genes *ast* and *act*, encoding cytotonic and cytotoxic enterotoxins, respectively. *Aeromonas taiwanensis*, for the first time isolated from fish in Brazil, did not amplify any of the genes evaluated. Future research should involve *in vivo* challenges to confirm the greater pathogenicity of the isolates that presented the virulence genes, in addition to broader studies, involving more virulence genes.

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTION

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The Ethics Committee for the Use of Animals (CEUA) of Embrapa Western Amazon approved the activities relevant to this work under protocol No. 04/2018, issued on 10/1/2018. Access to the genetic heritage was authorized by the Genetic Heritage Management Council (CGen) with No. AF34DA8.

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