



Antimicrobial resistance profile of *Aeromonas* spp. isolated from tambaqui fish (*Colossoma macropomum*)

ARTICLES doi:10.4136/ambi-agua.2936

Received: 27 May 2023; Accepted: 01 Oct. 2023

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ABSTRACT

The current study assesses the resistance profile of *Aeromonas* spp. individuals isolated from *tambaqui* fish (*Colossoma macropomum*) grown in fish farms located in São Luís Metropolitan Region - MA. In total, 114 bacterial strains belonging to species *Aeromonas* spp. were used in the experiment. Antimicrobial resistance profile was defined based on using the disk diffusion method and 10 antimicrobials. Isolates were classified as susceptible, susceptible upon increased exposure and resistant to the assessed principles; phenotypes showing resistance to multiple tested drugs (MDR) were classified. Bacterial strains presented widespread resistance to penicillin; as well as high oxacillin-, cefepime-, cefadroxil- and azithromycin-resistance rates. Ampicillin associated with sulbactam, neomycin and ofloxacin were the antimicrobials recording the highest sensitivity rate; high frequency of isolates (34.21%) were categorized as MDR phenotypes. Antimicrobial resistance was observed in the herein assessed *Aeromonas* spp. isolates, despite the lack of antimicrobials registered in Brazil to be used for *C. macropomum* fish farming purposes.

Keywords: Aeromonadaceae, bacterial resistance, fish farming.

Perfil de resistência à antimicrobianos de *Aeromonas* spp. isoladas de tambaquis (*Colossoma macropomum*)

RESUMO

Objetivou-se avaliar o perfil de resistência de *Aeromonas* spp. isoladas de tambaquis (*Colossoma macropomum*) oriundos de cultivos da Região Metropolitana de São Luís - MA.



Foram utilizadas 114 cepas bacterianas pertencentes a espécies de *Aeromonas* spp. e o perfil de resistência aos antimicrobianos foi realizado pelo método de difusão em disco com a utilização de 10 antimicrobianos. Os isolados foram classificados como sensível, sensível com exposição aumentada e resistente aos princípios avaliados e classificados os fenótipos com resistência múltipla às drogas testadas (MDR). As cepas bacterianas apresentaram resistência generalizada à penicilina; elevados percentuais de resistência à oxacilina, cefepime, cefadroxil e azitromicina. Os antimicrobianos com maior sensibilidade foram a ampicilina associada ao sulbactam, neomicina e ofloxacina e elevada frequência dos isolados (34,21%) foram categorizados como fenótipos MDR. Conclui-se que embora não existam antimicrobianos registrados no Brasil para uso no cultivo de *C. macropomum*, a resistência antimicrobiana está presente nos isolados de *Aeromonas* spp. avaliados.

Palavras-chave: Aeromonadaceae, piscicultura, resistência bacteriana.

1. INTRODUCTION

Synthetic antimicrobials play an essential role in preventing and treating bacterial infections, in protecting both human and animal health, as well as aquatic organisms produced in intensive systems. However, the use of non-authorized antimicrobials in aquaculture systems has mainly contributed to drug-resistance development in bacterial strains capable of affecting populations' health and safety, as well as the environmental ones. In addition, these substances can leave residues in animal-origin products (AOP), such as fish, and it can pose risks to human health due to their consumption (Gastalho *et al.*, 2014; De Sousa *et al.*, 2019; Pessoa *et al.*, 2019).

The emergence of antimicrobial resistance among food-borne pathogens is widely recorded in the literature; such a phenomenon is attributed to indiscriminate use of antimicrobials in animal production systems (Caniça *et al.*, 2019). Bacteria can undergo selective pressure due to the use of antimicrobials; consequently, they can function as reservoirs of resistance genes and horizontally transfer them to other bacterial species (Hussain *et al.*, 2017). Thus, bacteria presenting multidrug resistance (MDR) phenotypes are a growing concern for One Health, since they can directly transfer resistance genes to humans through AOP intake, and it can make it hard to treat infectious diseases in both humans and animals (Nhung *et al.*, 2017).

Bacterial infection outbreaks are a limiting factor for aquaculture systems, since they affect production by decreasing its yield (Watts *et al.*, 2017); consequently, they lead to significant economic losses (Figueiredo and Leal, 2008; Turker *et al.*, 2009; Zamri-Saad *et al.*, 2014). Bacteria of interest to fish farming systems are classified as opportunistic; they are found in water and in fish microbiomes, as well as can trigger bacterial infections in weakened hosts (Leira *et al.*, 2016). Despite the diversity of bacteria showing potential pathogenicity in fish, *Aeromonas* spp. stands out for its high frequency rates (Suhel *et al.*, 2011; Sebastião *et al.*, 2015).

Bacteria belonging to genus *Aeromonas* are quite numerous and acknowledged for their hemolytic and biofilm formation abilities, which can affect strains' virulence (Sebastião *et al.*, 2015; Pessoa *et al.*, 2019). Thirty-six *Aeromonas* spp. species are listed in the literature; at least 19 of them are considered emerging pathogens (Pessoa *et al.*, 2019; Fernández-Bravo and Figuera, 2020). *Aeromonas hydrophila* stands out among the aforementioned species as the most isolated one in fish farming systems, at global level, as well as for its ability to infect several hosts (Pessoa *et al.*, 2019). This bacterial species was recorded in tambaqui fish (*Colossoma macropomum*) grown in Brazil (Leão *et al.*, 2020; Pessoa *et al.*, 2020; Gallani *et al.*, 2020).

Maranhão State is the fourth largest native-fish producer in Brazil. According to report by the Brazilian Fisheries Association (Peixe BR, 2020), Maranhão State produced 40,800 tons of fish in 2020 and, unlike the rest of Brazil, where tilapia is the most produced species, the native fish grown in Maranhão State are a peculiar feature of Maranhão fish farming and its acceptability in the State's internal market, with emphasis on tambaqui fish (*Colossoma macropomum*) and its hybrids (Souza *et al.*, 2022). Thus, it is necessary to implement biosecurity techniques in fish farms in order to meet the growing demand for fish consumption and production at the local, regional, national and international levels. The prevention and treatment of bacterial infections, as well as multidrug-resistant bacteria control, stand out among the main biosecurity techniques. Therefore, the current study assesses the antimicrobial resistance profile of *Aeromonas* spp. isolated from tambaqui fish (*Colossoma macropomum*) grown in fish farms located in São Luís Metropolitan Region – MA.

2. MATERIALS AND METHODS

2.1. Ethical Aspects

All methodological procedures were carried out in compliance with ethical principles established by the Brazilian College of Animal Experimentation, as well as with the Animal Experimentation Ethics Committee (CEEA - Comitê de Ética em Experimentação Animal) of State University of Maranhão (UEMA - Universidade Estadual do Maranhão) (Protocol n. 09/2022).

2.2. *Aeromonas* spp. Bacterial Isolates

In total, 114 bacterial strains isolated from tambaqui fish (*Colossoma macropomum*) were used in the current study. The aforementioned fish individuals derived from fish farms located in São Luís Metropolitan Region - MA and they did not present clinical signs of aeromonosis. All strains were confirmed as species belonging to genus *Aeromonas*, based on staining (Gram staining) and biochemical tests (oxidase; catalase; indole; Voges Proskauer; Simmons citrate; H₂S production; urea hydrolysis; tryptophan deaminase; lysine, arginine and ornithine decarboxylation; malonate; glucose oxidation; lactose, sucrose, mannitol, adonitol, myoinositol, sorbitol, raffinose, rhamnose, maltose and melobiose fermentation; as well as esculin hydrolysis, DNase and growth at increasing NaCl concentrations [0%, 1% and 6%]), according to Holt *et al.* (1994) and to the American Public Health Association (APHA, 2001).

Thus, seven *Aeromonas* species identified through biochemical tests were assessed; they totaled 114 strains, namely: *A. veronii* by *veronii* (n= 69), *A. caviae* (n= 21), *A. sobria* (n= 6), *A. schubertii* (n= 6), *A. veronii* by *sobria* (n= 6), *A. media* (n= 3) and *A. hydrophila* (n= 3).

2.3. Antimicrobial Resistance Test

Resistance-to-antimicrobial-agents tests were carried out based on the disk diffusion method, in compliance with the protocol recommended in the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2019). The aforementioned tests used disks holding 10 antimicrobial agents that represented the following classes: penicillins (penicillin G - 10µg; oxacillin - 1µg; and ampicillin associated with sodium sulbactam - 20µg); cephalosporins (cefepime - 30µg; cefoxitin - 30µg; cefadroxil - 30µg; and ceftriaxone - 30µg); quinolones (ofloxacin - 5µg); aminoglycosides (neomycin - 300µg); and macrolides (azithromycin - 15µg).

In order to carry out the resistance test, *Aeromonas* spp. isolates were inoculated in brain and heart infusion (BHI) broth and incubated at 37°C, for 24 hours, until they showed turbidity compatible to McFarland standard n. 0.5. Sowing was carried out with the aid of a sterile cotton swab, on plates filled with Müller-Hinton agar; five minutes later, antimicrobial disks were added to the plates, based on using flamed tweezers. Plates were incubated in a bacteriological oven at 37°C, for 24 hours. After the incubation procedure was over, inhibition halos - formed

around the respective active principles - were measured with a millimeter ruler.

Results were compared to the measurement table set by the laboratory accounting for manufacturing the antimicrobial disks. Isolates were classified as susceptible, susceptible upon increased exposure and resistant to the assessed antimicrobial principles (Table 1).

Table 1. Interpretation pattern of disk diffusion test based on halo measurements, and results observed for sensitivity, sensitivity upon increased exposure and resistance of *Aeromonas* spp. isolates tested against 10 antimicrobials.

Antibiotic	Code	Classes	Concentration (μg)	Diameter zone (mm)		
				S	I	R
Penicillin G	PEN	Penicillin	10	≥ 15	NR	≤ 14
Oxacillin	OXA		1.0	≥ 18	15 to 17	≤ 14
Ampicillin associated with Sulbactam	ASB		20	≥ 15	12 to 14	≤ 11
Cefepime	FEP	Cephalosporin	30	≥ 25	19 to 24	≤ 18
Cefoxitin	CFO		30	≥ 18	15 to 17	≤ 14
Cefadroxil	CFD		30	≥ 18	15 to 17	≤ 14
Ceftriaxone	CRO		30	≥ 23	20 to 22	≤ 19
Neomycin	NEO	Aminoglycoside	30	≥ 17	13 to 16	≤ 12
Ofloxacin	OFX	Quinolone	5.0	≥ 16	13 to 15	≤ 12
Azithromycin	AZI	Macrolide	15	≥ 13	NR	≤ 12

Where in: S= susceptible; I= susceptible upon increased exposure; R= resistant; PEN= penicillin; OXA=oxacillin; ASB=ampicillin associated with sulbactam; FEP= cefepime; CFO= cefoxitin; CFD=cefadroxil; CRO= ceftriaxone; NEO=neomycin; OFX=ofloxacin; AZI = azithromycin.

The study took into consideration multiple-drug resistance (MDR) phenotypes, as well as isolates showing resistance to three, or more, antimicrobial classes many different at the same time, according to Dos Santos *et al.* (2014).

3. RESULTS AND DISCUSSION

Aeromonas spp. isolates, deriving from tambaqui fish (*Colossoma macropomum*), have shown generalized resistance to penicillin, as well as high resistance to oxacillin, cefepime, cefadroxil and azithromycin (Table 2); this finding suggested environmental contamination and/or likely use of these antimicrobials in fish farms. According to Saavedra *et al.* (2004), Costa *et al.* (2008) and Franco *et al.* (2010), *Aeromonas* spp. resistance to beta-lactam antibiotics, such as penicillin, oxacillin, cefepime and cefadroxil has been increasing in aquaculture systems (Miranda *et al.*, 2013). According to Harakaeh *et al.* (2006), the increased number of antimicrobials-resistant microorganisms is explained by the indiscriminate and excessive use of these drugs in intensive animal production systems.

Bacterial infections are one of the main causes of economic losses in intensive production systems like fish farming (Tavares-Dias and Martins, 2017; Bandeira Junior *et al.*, 2019; Pessoa *et al.*, 2019). According to Pessoa *et al.* (2020), *Aeromonas hydrophila* was the most prevalent bacterial species isolated (41.2%) from tambaqui fish, although other species were also isolated at lower rates, namely: *A. dhakensis*, *A. caviae*, *A. veronii* and *A. jandaei*. These findings have evidenced that tambaqui fish are not free from epizootic outbreaks caused by bacteria belonging to genus *Aeromonas*. Therefore, greater attention should be given to this bacterial genus in native fish species.

Table 2. Action of antimicrobials against *Aeromonas* spp. isolated from tambaqui fish (*Colossoma macropomum*), deriving from São Luís Metropolitan Region (Maranhão State).

<i>Aeromonas</i> spp. species	Tested Isolates (n)	Antimicrobial Resistance									
		PEN (%)	OXA (%)	ASB (%)	FEP (%)	CFO (%)	CFD (%)	CRO (%)	NEO (%)	OFX (%)	AZI (%)
<i>A. veronii</i> by <i>veronii</i>	69	100	94.20	4.34	8.70	17.40	78.26	8.70	-	-	34.79
<i>A. caviae</i>	21	100	100	-	14.29	-	71.42	14.29	28.58	-	-
<i>A. sobria</i>	6	100	100	-	50	-	50	50	-	-	50
<i>A. schubertii</i>	6	100	100	-	-	50	100	-	-	-	50
<i>A. veronii</i> by <i>sóbria</i>	6	100	100	-	-	-	50	-	-	-	50
<i>A. media</i>	3	100	100	-	100	-	-	-	-	-	-
<i>A.</i> <i>hydrophyla</i>	3	100	100	-	100	-	-	100	-	-	100

Where in: PEN= penicillin (10 µg); OXA= oxacillin (1.0 µg); ASB= ampicillin associated with sulbactam (20 µg); FEP= cefepime (30 µg); CFO= ceftioxin (30 µg); CFD= cefadroxil (30 µg); CRO= ceftriaxone (30 µg); NEO= neomycin (30 µg); OFX= ofloxacin (5.0 µg); AZI=azithromycin (15 µg); % resistant isolates' rate.

Only two antimicrobial drugs have their use approved by the Ministry of Agriculture, Livestock and Food Supply (MAPA) to treat bacterial infections in fish, namely: oxytetracycline - used to treat salmonids and catfish; and florfenicol - used to treat trout and tilapia. Both drugs are orally administered through fish feed (Sindan, 2018). Among these drugs, florfenicol is the antimicrobial drug most often used in aquaculture systems to treat several bacterial diseases in fish (Zhang *et al.*, 2020). However, according to Miranda *et al.* (2013) and Pessoa *et al.* (2019), antimicrobial drugs are misused as prophylactics and therapeutics in fish farms, a fact that contributes to the selection of resistant bacteria, as well as to the selection and dissemination of their respective antimicrobial resistance-encoding genes.

Ampicillin associated with sulbactam (penicillin), neomycin (aminoglycoside) and ofloxacin (quinolone) were the antibiotics showing the best activity against *Aeromonas* spp. isolates in the present study, i.e., the ones that presented the highest sensitivity to (Table 2). High sensitivity to antimicrobial “ampicillin” may be associated with the presence of compound “sulbactam”, which is an irreversible inhibitor of most beta-lactamases found in penicillin-resistant organisms, such as *Aeromonas* sp., and it indicates synergistic interaction between these components.

Resistance to ampicillin is expected in most *Aeromonas* spp. species, in association with the production of beta-lactamase enzymes (Miranda *et al.*, 2013); this antimicrobial drug is used as a selective agent in culture media used to grow this bacterium (Jacobs and Chenia, 2007). Costa *et al.* (2008) observed high ampicillin resistance levels in bacteria isolated from catfish (*Rhamdia quelen*) grown in Santa Maria County, Rio Grande do Sul State, Brazil. Franco *et al.* (2010) assessed bacterial isolates extracted from shrimp (*Litopenaeus vannamei*) and recorded 12.5% isolates' sensitivity to ampicillin.

Quinolones are drugs widely used to treat furunculosis, among other infections caused by *Aeromonas* spp., in fish (Smith *et al.*, 2010). However, according to Alcaide *et al.* (2010), there is concern about the indiscriminate use of quinolones, since resistance to this antibiotic emerges from the inhibition of nucleic acid synthesis. Akinbowale *et al.* (2008) observed sensitivity of bacteria isolated from a wide variety of fish species and aquaculture environments in Australia, in tests conducted with ciprofloxacin, which is a quinolone type. Costa *et al.* (2008) recorded 84.31% (n= 43) sensitivity to nalidixic acid (another quinolone) in 15 *Aeromonas* spp. isolates extracted from catfish (*Rhamdia quelen*). These findings are consistent with those observed for

the same class of antimicrobial drugs in the present study.

Franco *et al.* (2010) assessed bacterial isolates extracted from shrimp (*L. itopenaeus vannamei*) and recorded 81.2% isolates' sensitivity to neomycin. Akinboale *et al.* (2008) observed sensitivity to gentamycin in bacteria isolated from a wide variety of fish species and aquaculture environments in Australia; gentamycin belongs to the class of aminoglycosides. The aforementioned findings are similar to the one observed in the present study.

High frequency of isolates (n= 39; 34.21%) were categorized as multidrug resistance (MDR) phenotypes, since they were resistant to three antimicrobials of different classes, at the same time (Table 3). Penicillins and cephalosporins, as well as penicillin, oxacillin and cefadroxil, were the antimicrobial classes and antimicrobial drugs recording the highest resistance rates, respectively.

Table 3. Multidrug-resistance profile of *Aeromonas* spp. species isolated from tambaqui fish (*Colossoma macropomum*) grown in São Luís Metropolitan Region – Maranhão State.

<i>Aeromonas</i> spp. species	Number of isolates	Antibiotics
<i>A. veronii by veronii</i>	3	PEN-OXA-CFD-AZI
<i>A. veronii by veronii</i>	3	PEN-OXA-CFD-AZI
<i>A. veronii by veronii</i>	3	PEN-OXA-CFD-AZI
<i>A. veronii by veronii</i>	3	PEN-OXA-CFD-AZI
<i>A. veronii by veronii</i>	3	PEN-OXA-FEP-CFD-AZI
<i>A. veronii by veronii</i>	3	PEN-OXA-CFO-CFD-AZI
<i>A. sóbria</i>	3	PEN-OXA-FEP-CRO-AZI
<i>A. veronii by sóbria</i>	3	PEN-OXA-CFD-AZI
<i>A. caviae</i>	3	PEN-OXA-FEP-NEO-CFO-CFD-CRO
<i>A. caviae</i>	3	PEN-OXA-NEO-CFD
<i>A. hydrophyla</i>	3	PEN-OXA-FEP- CRO-AZI
<i>A. schubertii</i>	3	PEN-OXA-CFO-CFD-AZI
<i>A. schubertii</i>	3	PEN-OXA-CFD-AZI

Where in: PEN= Penicillin (10 µG); OXA= Oxacillin (1.0 µG); FEP= Cefepime (30 µG); CFO= Cefoxitin (30 µG); CFD= Cefadroxil (30 µG); CRO= Ceftriaxone (30 µG); NEO= Neomycin (30 µG); and AZI= Azithromycin (15 µG).

Aeromonas spp. species presented different resistance profiles in the antimicrobial susceptibility test (Table 3), with emphasis on the multidrug resistance observed for the following isolates: *A. veronii by veronii* (PEN-OXA-FEP-CFD-AZI), *A. sobria* (PEN-OXA-FEP-CRO-AZI), *A. caviae* (PEN-OXA-FEP-NEO-CFO-CFD-CRO), *A. hydrophyla* (PEN-OXA-FEP-CRO-AZI) and *A. schubertii* (PEN-OXA-CFO-CFD-AZI). Resistance profiles observed for fourth-generation cephalosporin (cefepime) in *A. veronii by veronii*, *Aeromonas sobria*, *A. caviae* and *A. hydrophyla* is a finding of particular clinical-epidemiological interest.

The high frequency of isolates showing MDR profile in the current study represents the risk of spreading antimicrobial resistance to humans through contaminated fish intake. Food contamination with MDR bacteria can take place at different production chain stages, and it makes it hard to define the antimicrobial resistance origin (Oniciuc *et al.*, 2019). However, according to Khan *et al.* (2018), indiscriminate use of antimicrobial drugs is the main cause of resistant microorganisms' selection in bacterial populations and of resistance induction in certain bacterial species.

According to Hirsch *et al.* (2006), the circulation of multi-resistant *Aeromonas* species in fish can increase the risks of disseminating resistance genes through plasmids or transposons, and it implies the need of continuously monitoring antimicrobial resistance, mainly in intensive production systems.

Antibiotic-resistant bacteria transfer to humans through contaminated food is an emerging public health issue. The literature has fewer studies focused on investigating antibiotic resistance in bacteria isolated from aquatic animals than studies focused on investigating this topic in land-based food-producing animals. The present study provides further support to the hypothesis that there is potential risk of transferring resistant bacteria to humans through fish intake.

4. CONCLUSIONS

Based on findings in the current study, it may be concluded that:

- Despite the lack of antimicrobial drugs registered in Brazil to be used for *Colossoma macropomum* culture purposes, antimicrobial resistance was observed in *Aeromonas* spp. isolated from tambaqui fish.
- Multiresistance to three or more antimicrobials observed in high frequency in evaluated *Aeromonas* spp. raises concern about fish farming in São Luís Metropolitan Region - MA, as well as about human health, given the likelihood of human infections caused by resistant bacteria due to fish intake.
- Given the clinical and epidemiological implications of *Aeromonas* spp. species investigated in the present study, as well as the identification of antimicrobial drug resistance and multidrug resistance profiles, it is worth emphasizing the urgent need of both warning about and clarifying these pathogens' significance for Public Health. This knowledge can help to establish preventive infection control guidelines in intensive fish farming carried out in Maranhão State and countrywide.

5. ACKNOWLEDGEMENTS

To the Pro-Rectorate for Research (PPG) of the State University of Maranhão for awarding the scientific initiation bursary for carrying out this investigation (Process BIC-11248/22). This study was funded by the Support Foundation for Research and Scientific and Technological Development of the State of Maranhão (FAPEMA - Process 06453/22).

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