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Original Article

Antimicrobial resistance profile of *Aeromonas* spp. isolated from asymptomatic *Colossoma macropomum* cultured in the Amazonas State, Brazil

Perfil de resistência antimicrobiana de *Aeromonas* spp. isoladas de *Colossoma macropomum* assintomáticos no Estado do Amazonas, Brasil

F. A. Sebastião[a,b], C. Majolo[a], V. F. S. Martins[c], C. L. Boijink[a], F. R. Brandão[a], S. L. A. Pereira[d], R. Y. Fujimoto[e] and E. C. Chagas[a*]

[a]Embrapa Amazônia Ocidental, Manaus, AM, Brasil
[b]Universidade Federal do Amazonas, Programa de Pós-graduação em Ciência Animal e Recursos Pesqueiros, Manaus, AM, Brasil
[c]Universidade Federal do Amazonas, Laboratório de Ictiologia e Ordenamento Pesqueiro, Humaitá, AM, Brasil
[d]Embrapa Roraima, Boa Vista, RR, Brasil
[e]Embrapa Tabuleiros Costeiros, Aracaju, SE, Brasil

Abstract

Bacterial diseases are important factors that limit productivity in aquaculture. To reduce negative economic impacts, fish farmers use antimicrobials, often indiscriminately, and this action has led to bacterial resistance to drugs. The objectives of this study were to isolate and identify the main putative pathogenic bacterial species in tambaqui (*Colossoma macropomum*), establish the profile of resistance to antimicrobials by the methods of disc diffusion, and determine the minimum inhibitory concentration (MIC) values. Two hundred and ninety asymptomatic fish were collected between March and November 2015 from ten fish farms in the Amazonas state (Brazil). Of the total strains recovered from tambaqui, seven were identified as *Aeromonas* spp. by sequencing the 16S rRNA gene. These seven isolates showed resistance to ampicillin, 28% to erythromycin, and 28% to sulfonamide. Additionally, the seven isolates showed a MIC higher than the range evaluated for amoxicillin, penicillin, novobiocin, tylosin tartrate, and clindamycin, and 85% showed resistance to erythromycin. The results of this study indicate the need to increase the awareness of fish farmers and, most importantly, the government, about the lack of drug regulations for use in aquaculture, and good management practices, so the indiscriminate prophylactic and systemic use of antimicrobials be inhibited.

Keywords: *Aeromonas*, antibiotics, fish farming, tambaqui.

Resumo

As doenças bacterianas são fatores importantes que limitam a produtividade na aquicultura. Para reduzir os impactos econômicos negativos, os piscicultores utilizam antimicrobianos, muitas vezes de forma indiscriminada, e essa ação tem levado à resistência bacteriana aos medicamentos. Os objetivos deste estudo foram isolar e identificar as principais bactérias com potencial putativo para o tambaqui (*Colossoma macropomum*), e estabelecer o perfil de resistência a antimicrobianos pelos métodos de difusão em disco e valores de concentração inibitória mínima (CIM). Duzentos e noventa peixes assintomáticos foram coletados entre março e novembro de 2015, em dez pisciculturas do estado do Amazonas (Brasil). Do total de cepas recuperadas de tambaqui, sete foram identificadas como *Aeromonas* spp. pelo sequenciamento do gene 16S rRNA. Esses sete isolados apresentaram resistência à ampicilina, 28% à eritromicina e 28% à sulfonamida. Além disso, os sete isolados apresentaram CIM superior à faixa avaliada para amoxicilina, penicilina, novobiocina, tartarato de tilosina e clindamicina, e 85% apresentaram resistência à eritromicina. Os resultados deste estudo indicam a necessidade de aumentar a conscientização dos piscicultores e, principalmente, do poder público, a falta de regulamentação de medicamentos para uso na aquicultura e sobre as boas práticas de manejo, para que o uso profilático e sistêmico de antimicrobianos de forma indiscriminada seja inibido.

Palavras-chave: *Aeromonas*, antibióticos, piscicultura, tambaqui.

*e-mail: edsandra.chagas@embrapa.br
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1. Introduction

According to data from the Brazilian Association of Fish Farming (Peixe BR, 2021), fish production in Brazil reached 802,930 tonnes in 2020, which represents an increase of 5.9% compared to the previous year. Among the native fish, tambaqui is the main species produced in Brazil (IBGE, 2019) and it has great economic and social importance, either through artisanal fishing or through commercial aquaculture (Wood et al., 2018). The tambaqui, *Colossoma macropomum*, production contributed 278,671 tonnes or 34.7% of the total production (Peixe BR, 2021).

The intensification in the production of tambaqui is reflected in the increased stocking densities of fish, higher amount of food offered and excrement produced, and more frequent handling. These factors enhance the risk of outbreaks of diseases (Leira et al., 2017). In fish farms, bacterial diseases are important factors that limit productivity and can cause high mortality rates (Carraschi et al., 2011). Among the potentially pathogenic bacteria for fish, *Flavobacterium columnare*, *Streptococcus agalactiae*, and motile *Aeromonas* stand out (Sebastião et al., 2015).

The genus *Aeromonas* comprises 36 species of Gram-negative bacteria that act mainly as opportunistic pathogens (Pessoa et al., 2019). Infectious processes usually develop in fish and other marine animals under stressful conditions such as handling, transportation, poor water quality, and other improper aquaculture practices (Pessoa et al., 2020). Diseases caused by motile *Aeromonas* are a worldwide problem in fish farming and are known as motile *Aeromonas* septicemia (Gallani et al., 2020). In addition, ulcers, putrefaction, and tail rot have been observed (Leão et al., 2020). Consequently, mortality outbreaks caused by *Aeromonas* spp. are considered a major concern for world aquaculture (Pang et al., 2015) and have been responsible for high economic losses worldwide (Wang et al., 2019). In Brazil, *Aeromonas* spp. have been isolated in tambaqui in the northern (Leão et al., 2020), northeastern (Silva et al., 2018; Pessoa et al., 2020), and southeastern (Ariede et al., 2018; Ferrante et al., 2020) regions. However, despite the importance of bacterial pathogens, as far as we know, there is still no monitoring work being performed on the main bacterial pathogens that affect tambaqui and how these pathogens affect the supply chains of fish farming (Pessoa et al., 2020).

The control of bacterial diseases has been performed through the use of antimicrobials incorporated into the feed (Figueiredo and Leal, 2008). However, there are only two antibiotics approved by the Ministry of Agriculture, Livestock and Food Supply for use in Brazilian aquaculture (oxytetracycline and florfenicol) and none have been approved for tambaqui. These few options to control and prevent the diseases lead the fish farmers to use unapproved drugs or chemicals of general purpose that are not labeled for aquaculture (Portela et al., 2020). The indiscriminate use of antimicrobials led to the accumulation of residues in the aquatic environment, persisting for months, as well as favoring the selection of resistant microorganisms (Miranda et al., 2018). Residues of oxytetracycline, tetracycline, and chlortetracycline have been found in sediments; and oxytetracycline, tetracycline, and florfenicol have been identified in water and fish samples in São Paulo, Brazil (Monteiro, 2014). In addition, 36 strains of bacteria resistant to quinolones, tetracyclines, and sulfonamides were isolated and the multiple antibiotic resistance index (MAR) ranged between 0 and 0.86.

Considering the rapid growth and importance of the aquaculture industry worldwide, raising intensive system production, and the use of antibacterial agents nonregulated for fish farming, the present study aimed to identify the main putative pathogenic bacterial species in tambaqui fish farms cultured in Rio Preto da Eva (Amazonas state, Brazil). and their resistance profile to antimicrobials.

2. Materials and Methods

2.1. Fish

Asymptomatic specimens of tambaqui (n = 290; 1.110 ± 0.63 g; 32.25 ± 9.96 cm) were obtained from ten fish farms in Rio Preto da Eva (Amazonas state, Brazil) during the rainy season (March to May) and the dry season (September to November) of 2015. Fish were fed with a commercial extruded diet containing 28% crude protein (CP). Fifteen fish were sampled per farm, five fish were captured randomly from three ponds using trawl nets. Unfortunately, ten fish were lost in the last rainy season collection. The fish were transported to the Fish Culture Laboratory at Embrapa Amazônia Ocidental (Manaus, Amazonas state, Brazil) and euthanized via anesthesia using the sprinkling method in the gills of tambaqui (benzocaine solution, 100 mg L^{-1}), which was followed by medullar sectioning. Brain and kidney samples of each fish were collected aseptically.

The experimental procedures used in this work were approved by the Animal Experimentation Ethics Committee (CEUA/UFGD 003/2014), and access to the genetic heritage of the animals involved in this research was approved through the AA94832 register number from the Genetic Heritage Management Council (CGEN), Ministry of the Environment (MMA).

2.2. Bacterial isolation

Head kidney and brain fragments were inoculated onto MacConkey Agar (Kasvi, Italy), modified Hsu Shotts agar [MHS; Bullock (1986)] supplemented with 2 mg L^{-1} of tryptone and trypticase soy agar (TSA; Kasvi, Italy) supplemented with 5% sheep blood and incubated at 28 °C for 24-72 h. Next, gram staining, catalase, and oxidase tests were performed. Biochemical characterization of the isolates was carried out using the API 20E kit (Bio-Merieux SA, France) and API Strep kit (Bio-Merieux SA, France).

2.3. Molecular identification

The DNeasy Blood and Tissue extraction kit (Qiagen, Germany) was used according to the manufacturer's instructions to extract the DNA of each isolate. DNA quantification was performed via fluorimetry, using the QuBit 4.0 device (ThermoFisher, USA).

The polymerase chain reaction (PCR) was performed using 2.5 µL of 10X buffer (10 mM Tris-HCl, 50 mM KCl); 0.2 µL of 25 mM DNTP; 1.0 µL 50 mM $MgSO_4$; 0.2 µL of Taq High Fidelity (Platinum® Taq DNA Polymerase, Life Technologies, NY, USA); 2.0 µL of each primer (10 pmol); DNA template 25 ng; ultra-pure water to complete 25 µL of reaction. The amplification program used was 94 °C for 2 min, 35 cycles at 94 °C for 30 seconds, temperature of 55 °C, for 30 s, 68 °C for 1.5 min, and 68 °C for 10 min for the final extension. The 16S rRNA primers used were fD1 (GAG TTT GAT CCT GGC TCA G) and rD1 (TAA GGA GGT GAT CCA GCC), as described by Weisburg et al. (1991). The generated amplicons (~1500 bp) were visualized via electrophoresis on 1% agarose gel, stained with ethidium bromide. Afterward, a 1 h run at 75 V was performed. PCR products were purified using the MinElute kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. The gene sequencing was carried out at the Multi-Generation Sequencing in Gene Expression Multiplier Laboratory (Jaboticabal, São Paulo State, Brazil), using the methodology recommended by Sanger et al. (1977).

The sequences obtained were visualized in the program Geneious (v. 2020.2.4). Using the BLASTn tool (NCBI, 2022a), each fragment (1299 bp) was compared to the sequences deposited in GenBank (NCBI, 2022b), assuming 100% coverage and identity equal to or greater than 98% with the type-strain deposited to confirm the genus of the sequence.

2.4. Antibiotic susceptibility testing and determination of multiple antibiotic resistance (MAR) indexes

The susceptibility to 9 antimicrobials was determined using the agar disk diffusion method and it is in accordance with the Clinical and Laboratory Standards Institute guidelines VET04 (Clinical and Laboratory Standards Institute [CLSI], 2020), at 28 °C. The antimicrobials tested were ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (10 µg), gentamicin (10 µg), nalidixic acid, (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), sulfonamide (300 µg) and tetracycline (30 µg). Strain susceptibility was calculated based on the bacterial halo diameter for *A. hydrophila* as follows: gentamicin susceptible (diameter between 18 and 19 mm), gentamicin resistant (diameter < 18 mm), tetracycline susceptible (diameter between 22 and 23 mm), tetracycline resistant (diameter < 22 mm) (CLSI, 2020). For the antimicrobials not listed in the aforementioned document (chloramphenicol, erythromycin, nalidixic acid, nitrofurantoin, norfloxacin and sulfonamide), the epidemiological cutoff values were: S (susceptible, diameter ≥ 20 mm); I (intermediate, diameter between 15 and 19 mm); R (resistant, diameter ≤ 14 mm), according to CLSI (2012), at 35±2 °C. Intermediate resistance was excluded from the resistance percentage calculations. For quality control, it was used *Escherichia coli* ATCC 25922 strain (CLSI, 2020) at 28 and 35 °C.

The MAR index was calculated for each isolate based on the results of the disk diffusion method analysis. The MAR index for a single isolate was calculated as the number of antibiotics to which an isolate is resistant divided by the total number of antibiotics against which the isolate was tested (Zhang et al., 2015).

2.5. Determination of the minimum inhibitory concentration (MIC)

The MIC of the bacterial isolates was determined in a 96-well plate (Sensititre Avian, Thermo Fisher, USA), containing 18 antimicrobials: enrofloxacin (2-0.12 µg mL^{-1}), gentamicin (8-0.5 µg mL^{-1}), ceftiofur (4-0.25 µg mL^{-1}), neomycin (32-2 µg mL^{-1}), erythromycin (4-0.12 µg mL^{-1}), oxytetracycline (8-0.25 µg mL^{-1}), tetracycline (8-0.25 µg mL^{-1}), amoxilin (16-0.25 µg mL^{-1}), spectinomycin (64-8 µg mL^{-1}), sulphadimethoxine (256-32 µg mL^{-1}), trimethoprim/sulfamethoxazole (2/38-0.5/9.5 µg mL^{-1}), florfenicol (8-1 µg mL^{-1}), sulphathiazole (256-32 µg mL^{-1}), penicillin (8-0.06 µg mL^{-1}), streptomycin (1024-8 µg mL^{-1}), novobiocin (4-0.5 µg mL^{-1}), tylosin tartrate (20-2.5 µg mL^{-1}), clindamycin (4-0.5 µg mL^{-1}), according to the manufacturer's instructions. Briefly, the *Aeromonas* spp. were inoculated on cation-adjusted Mueller Hinton agar (BD BBL, USA), and incubated at 28 °C for 24 h. A bacterial solution at 10^8 CFU/mL was prepared for each strain and diluted 1:1000 in cation-adjusted Mueller Hinton broth (BD BBL, USA). Following, 50 µl of the bacterial dilution was inoculated into the 96-well plates and incubated for 24 h at 28 °C. The MIC values were interpreted following VET04 - CLSI (2020). It was used *E.coli* 25922 for quality control (CLSI, 2020).

3. Results

3.1. Bacterial isolation and identification

No isolates of *F. columnare* or *Streptococcus* sp. were obtained in this study. Of the total 28 isolates obtained, the most prevalent bacterial genus encountered in the Rio Preto da Eva region was *Aeromonas* spp. (57.1%), which was isolated in 80% of the fish farms. The isolates were obtained between March and November 2015 and were previously characterized and identified using biochemical kits. Of the 16 *Aeromonas* spp. isolates, nine were obtained in the rainy season in seven fish farms, while another seven isolates were obtained in the dry season, in only three fish farms in the region (Figure 1). Due to technical problems in the freezer, from the total *Aeromonas* isolates, only seven survived and were submitted to gene sequencing to confirm the identification as belonging to the *Aeromonas* genus. These are registered under GenBank accession numbers MW940897-MW940904.

A few other bacterial species were isolated; though, they are less reported in northern Brazilian fish farms: *Pasteurella pneumotropica* (n = 3), *Plesiomonas shigelloides* (n = 3), *Vibrio fluvialis* (n = 2), *Elizabethkingia meningoseptica* (n = 1), *Chromobacterium violaceum* (n = 2) and *Pseudomonas fluorescens* (n= 1).

3.2. Antibiotic susceptibility test, MAR index, and MIC

In the present study, all of the *Aeromonas* strains were susceptible to chloramphenicol, gentamicin, nalidixic acid,

nitrofuranate, norfloxacin, and tetracycline and resistant to ampicillin. Twenty-eight percent of the strains were resistant to erythromycin and sulfonamide, and 28% were classified as having intermediate resistance to erythromycin and 14% to sulfonamide. Multiple antibiotic resistance index values were between 0.11 and 0.33 (Table 1). Also, for quality control, *E. coli* 25922 were susceptible to all antimicrobials tested and the diameters were within the range reported in CLSI (2012, 2020) (Supplement Material).

The seven *Aeromonas* isolates presented a MIC value higher than the concentrations evaluated for the antimicrobials: amoxicillin (>16 μg mL^{-1}), penicillin (>8 μg mL^{-1}), novobiocin (>4 μg mL^{-1}), tylosin tartrate (> 20 μg mL^{-1}) and clindamycin (>4 μg mL^{-1}). For erythromycin, 85% of the isolates had a MIC value >4 μg mL^{-1}. Eighty-five percent of the isolates had a MIC value lower than the range evaluated for the antimicrobials oxytetracycline (<0.25 μg mL^{-1}) and tetracycline (<0.25 μg mL^{-1}). In addition, all of the isolates presented MIC values <0.5/9.5 μg mL^{-1}for trimethoprim/sulfamethoxazole. For the other antimicrobials, the responses in the isolates varied (Table 2). Results of the quality control using *E. coli* 25922 are in the Supplement Material.

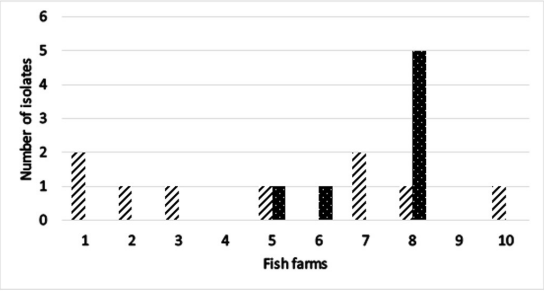

Figure 1. Records of *Aeromonas* spp. isolated from tambaqui (*Colossoma macropomum*) by fish farms in the rainy season (bars with stripes) and the dry season (bars with dots).

4. Discussion

Corroborating the global reports, the main bacterial isolates obtained from tambaqui in the present study belong to the *Aeromonas* genus (57.1% of 28 isolates). The prevalence of motile *Aeromonas* can vary by location and season, with *Aeromonas* infections being significantly higher in the summer when the temperature is elevated (Loch and Faisal, 2010; Sebastião et al. 2015). In the present study, there was no difference between the number of *Aeromonas* spp. isolates obtained in the rainy and dry seasons, since the temperature is high throughout the year in the Amazon (Northern Brazil). However, in the dry season, the prevalence of *Aeromonas* was found in greater numbers in three fish farms, and this may be related, in addition to high temperatures, to the characteristics of the rearing environment i.e., the high stocking densities and the excess of organic matter in the ponds.

To avoid economic losses, producers frequently use antimicrobials to treat these diseases, often without technical support. Currently, 73% of the major aquaculture producing countries have been reported as using oxytetracycline, florfenicol, and sulphadiazine and 55% applied erythromycin, amoxicillin, sulfadimethoxine, and enrofloxacin (Lulijwa et al., 2020; Preena et al., 2020a). In Brazil, the most common antibiotics used are florfenicol, oxytetracycline, tetracycline, and enrofloxacin (Rezende, 2012; Monteiro, 2014). However, none of them is approved by the Ministry of Agriculture, Livestock, and Food Supply for tambaqui farming, then its usage is off-label. Consequently, the emergence of resistant strains to multiple drugs in aquatic environments is a reality (Gallani et al., 2020). In the present study, 100% of the *Aeromonas* spp. isolates were resistant to ampicillin, 28% to erythromycin, and 28% to sulfonamide. Corroborating our results, Gallani et al. (2020) also reported resistance of *A. hydrophila* isolated from tambaqui against ampicillin, amoxicillin, penicillin, streptomycin, sulphazotrin, and

Table 1. Antimicrobial susceptibility profile and multiple antibiotic resistance (MAR) indexes of *Aeromonas* spp. (isolates A533, A561, A565, A568, A284, A562, A248) obtained from *Colossoma macropomum* in Rio Preto da Eva region (Amazonas state, Brazil).

Antimicrobials	Isolates						
	A533	A561	A565	A568	A284	A562	A248
AMP	R	R	R	R	R	R	R
CLO	S	S	S	S	S	S	S
ERY	S	S	R	I	S	R	I
GEN	S	S	S	S	S	S	S
NAL	S	S	S	S	S	S	S
NIT	S	S	S	S	S	S	S
NOR	S	S	S	S	S	S	S
SUL	S	R	I	S	S	R	S
TET	S	S	S	S	S	S	S
MAR index	0.11	0.22	0.22	0.11	0.11	0.33	0.11

Note: AMP: ampicillin 10 μg; CLO: Chloramphenicol 30 μg; ERY: Erytromycin 15 μg; GEN: Gentamicin 10 μg; NAL: Nalidixic acid 30 μg; NIT: Nitrofurantoin 300 μg; NOR: Norfloxacin 10 μg; SUL: Sulfonamide 300 μg; TET: Tetracycline 30 μg; I: intermediate; R: resistant; S: susceptible.

Table 2. Minimum inhibitory concentration (μg mL^{-1}) of *Aeromonas* spp. (isolates A562, A284, A248, A533, A561, A565, A568) obtained from *Colossoma macropomum*, in the region of Rio Preto da Eva (AM, Brazil).

ID	ENRO	GEN	TIO	NEO	ERY	OXY	TET	AMOX	SPE	SDM	SXT	FFN	STZ	PEN	STR	NOV	TYLT	CLI
A562	<0.12	<0.5	2	<2	>4	<0.25	<0.25	>16	32	<32	<0.5/9.5	<1	<32	>8	<8	>4	>20	>4
A284	<0.12	2	4	4	>4	<0.25	<0.25	>16	64	>256	<0.5/9.5	<1	256	>8	16	>4	>20	>4
A533	<0.12	1	1	<2	>4	<0.25	<0.25	>16	64	<32	<0.5/9.5	<1	<32	>8	<8	>4	>20	>4
A248	<0.12	1	4	4	>4	<0.25	<0.25	>16	64	>256	<0.5/9.5	>8	256	>8	16	>4	>20	>4
A561	<0.12	<0.5	1	<2	>4	<0.25	<0.25	>16	32	<32	<0.5/9.5	<1	<32	>8	16	>4	>20	>4
A565	<0.12	1	0.5	<2	4	>8	8	>16	64	<32	<0.5/9.5	<1	64	>8	16	>4	>20	>4
A568	<0.12	1	0.5	<2	>4	0.5	<0.25	>16	32	64	<0.5/9.5	<1	64	>8	16	>4	>20	>4

Note: ENRO: Enrofloxacin; GEN: gentamicin; TIO: ceftiofur; NEO: neomycin; ERY: erythromycin; OXY: oxytetracycline; TET: tetracycline; AMOX: amoxicillin; SPE: spectinomycin; SDM: sulfadimethoxine; SXT: trimethoprim/sulfamethoxazole; FFN: florfenicol; STZ: sulphathiazole; PEN: penicillin; STR: streptomycin; NOV: novobiocin; TYLT: tylosin tartrate; CLI: clindamycin.

vancomycin; intermediate resistance against gentamicin and doxycycline and were susceptible to ceftriaxone, florfenicol, and oxytetracycline. Other studies have shown that *A. hydrophila* is resistant to commercial antimicrobials, which has been attributed to the indiscriminate use of antimicrobials in aquaculture, plasmids, or horizontal gene transfer (Stratev and Odeyemi, 2016). Vivekanandhan et al. (2002) evaluated *A. hydrophila* isolated from fish in supermarkets in India and found 53.3% of resistance to tetracycline, all isolates in our study showed susceptibility.

Piotrowska et al. (2017) reported that of the 104 isolates of *Aeromonas* spp. from urban effluents in Poland, 23% were resistant to tetracycline, 68% to chloramphenicol, and 36% to gentamicin, which is contrary to the results of the present study for these three antimicrobials (100% susceptibility). Corroborating the results of the present study, Yang et al. (2018) reported that 95.24% of their *A. hydrophila* isolates were resistant to ampicillin. However, they observed 88.89% resistance to tetracycline, contrary to our results, where all isolates were susceptible. This difference in susceptibility to antimicrobials could be related to the non-exposure of fish to these substances. Resistance to antibacterial drugs may be acquired through the mutation of a chromosomal gene that modifies the structure of the ribosomal target or via the infection of the cell with a resistant R-factor plasmid (Sekkin and Kum, 2011). Multiple antibiotic resistance occurs with the accumulation of resistant R plasmids or transposons of genes, with each coding for resistance to a specific agent, and/or by the action of multidrug efflux pumps, each of which can pump out more than one drug type (Preena et al., 2020a). Demonstration of R-factor transfer to fish pathogens was first shown with certain strains of *A. salmonicida* (Romero, Feijoó and Navarrete, 2012). In addition, transferable R-factor plasmids in drug-resistant strains were shown with *A. hydrophila, Vibrio anguillarum,* marine *Vibrio sp., Edwardsiella tarda* and *Pasteurella piscicida* (Defoirdt et al., 2011). Multiple antibiotic resistance index values can be calculated by using disc diffusion/agar dilution methods, thereby generating the antibiogram profile (Preena et al., 2019). MAR index can be calculated for each fish farm following Krumperman (1983), thereby determining the extent of antibiotic exposure, where a MAR index higher than 0.2 indicates the background of high-level antibiotic exposure (Preena et al., 2020b).

In the present study, the isolate A561 presented multiple resistance to ampicillin and sulfonamide (MAR index = 0.22), and the isolate A565 was resistant to ampicillin and erythromycin (MAR index = 0.22), and the isolate A562 showed multiple resistance to ampicillin, sulfonamide, and erythromycin (MAR index = 0.33). Notably, all of them belonged to the same tank.

Many farmed fish, such as carp, salmon, tilapia, catfish, ornamental fish, and crustaceans, such as shrimps, have been reported to possess multiple antibiotic resistant pathogens (Watts et al., 2017). Multiple antibiotic resistance indexes higher than 0.4 and 0.2 for *Pseudomonas* and *Aeromonas*, respectively, from catfish, were considered a high risk of antibiotic contamination (Nguyen et al., 2014). Karunasagar et al. (1994) reported mass mortality in *Penaeus monodon* larvae caused by *Vibrio harveyi* strains with multiple resistances to cotrimoxazole, chloramphenicol, erythromycin, and streptomycin.

Besides agar disk diffusion, measurement of minimum inhibitory concentration (MIC) is the other acceptable method for determining the antimicrobial susceptibility profile of pathogens. MIC indicates the minimum concentration required to inhibit bacterial cell division (Preena et al., 2020b). In the case of aquatic isolates, MIC values can be interpreted following epidemiologic cut-off values based on the frequency of distribution of susceptibility results or to describe drug MIC distribution of bacteria isolated from other animals (Miller and Reimschuessel, 2006). In the present study, the seven isolates showed a MIC value higher than the range evaluated for amoxicillin, penicillin, novobiocin, tylosin tartrate, and clindamycin, and 85% for erythromycin. All these antimicrobials belong to antibiotic classes that have already been reported to present multiple antibiotic resistance worldwide (Yan and Gilbert, 2004; Defoirdt et al., 2011).

Nevertheless, it should be mentioned that the amount of antibiotics and other compounds used in aquaculture differs significantly between countries. For example, Defoirdt et al. (2011) reported variations between the use of 1 g of antibiotic per metric ton of production in Norway to 700 g per metric ton in Vietnam. Additionally, the discrepancies between testing methods and measurement of zone sizes by individual scientists also represent a possible source of inter-laboratory variation (Sekkin and Kum, 2011).

5. Conclusions

The most prevalent bacteria isolated in this work were *Aeromonas* spp. It was also observed that, even in asymptomatic fish, putative pathogenic bacterial isolates with multidrug resistance to the antimicrobials ampicillin, erythromycin, and sulfonamide were identified. As such, the results of this study represent a warning of the need for government assistance on drug regulations for use in native fish, as well as the good management practices among fish farmers are encouraged, and the indiscriminate prophylactic and systemic use of antimicrobials must be inhibited.

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Supplement Material

Supplementary material accompanies this paper.

Table S1. Disk diffusion quality control ranges testing at 28±2 °C (24-28 h) and 35±2 °C (24-28 h) for Escherichia coli ATTCC 25922.
Table S2. MIC quality control ranges (µg mL-1) testing at 28±2 °C (48 h) for Escherichia coli ATTCC 25922.

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