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# Pathogenic *Aeromonas* spp in Amazonian fish: Virulence genes and susceptibility in *Piaractus brachypomus*, the main native aquaculture species in Peru

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ABSTRACT

The aim of this study was to isolate and identify *Aeromonas* species, characterize their virulence genes factors, and to evaluate their lethality in *Piaractus brachypomus*. Presumptive *Aeromonas* (n = 34) were selected from 44 bacteria retrieved from diseased Amazonian fish. Nineteen isolates were chosen based on fish species, group, the location where were isolated, and colony morphology. Isolates were biochemically and molecularly identified as *Aeromonas hydrophila*, *A. dhakensis*, *A. veronii*, *A. jandaei*, and *A. caviae* using API 20E, API 20NE and the analysis of 16 S rRNA and *gyrB* genes, respectively. Eight virulences genes (*aer*, *altA*, *act*, *hly*, *ser*, *lip*, *fla ahyB*) were found in one *A. dhakensis* and two *A. hydrophila* isolates. *A. veronii*, *A. jandaei*, and another two *A. hydrophila* isolates harbored four, two and three virulence genes, respectively. All *A. caviae* were *fla* +, *ahy*B + and *lip* +, except two isolates that also presented *ser* and altA. *P. brachypomus* (9 ± 1 cm), infected intraperitoneally ($4 \times 10^6$, $4 \times 10^7$, and $4 \times 10^8$ CFU/fish), showed susceptibility to all *Aeromonas* species, each harboring a different virulence genes profile. Both *A. hydrophila* and *A. dhakensis* caused > 80% mortality at the lowest evaluated concentration ($\sim 10^6$ CFU/fish). *A. veronii* and *A. jandaei* isolates produced 20% and 70% mortality, respectively, using $\sim 10^7$ CFU/fish, but with lower intensity compared to *A. hydrophila* and *A. dhakensis.* At the highest concentration ($\sim 10^8$ CFU/fish), *A. veronii* caused 70% mortality, while *A. caviae* isolates caused mortality ranging from 50% to 80%. Remarkably, *A. jandei* resulted in 100% mortality at this level, similar to *A. hydrophila* and *A. dhakensis*. This report confirms the variety of *Aeromonas* species infecting farmed native Amazonian fish. The study also suggests that virulence genes evaluated in *Aeromonas* species may not always be an indicator of virulence. Moreover, *P. brachypomus*, an economically important aquaculture fish species could be susceptible not only to the high mentioned *A. hydrophila* but also to other less observed species such as *A. dhakensis*, *A. veronii*, *A. jandaei*, and *A. caviae*, bacteria that should be taken into account in health surveillance for the implementation of aquaculture disease prevention strategies.

## 1. Introduction

*Aeromonas* is a Gram-negative, rod-shaped bacterial genus responsible for causing diseases in animals and humans (Gonçalves Pessoa et al., 2019) *A. hydrophila* is often reported causing hemorrhagic septicemia in ornamental and aquaculture fish (Janda and Abbott, 2010; Austin and Austin, 2012). However, in recent years, fish mortality associated with aeromoniasis have been linked to other *Aeromonas* species, including *A. veronii*, *A. dhakensis*, *A. jandaei*, and *A. caviae* (Marques et al., 2016; Zhang et al., 2016; Dong et al., 2017; Baldissera

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et al., 2018; Soto-Rodriguez et al., 2018; Hoai et al., 2019; Sharma et al., 2022; Carriero et al., 2020; Alvarez-Rubio et al., 2020; Assane et al., 2021; Marcusso et al., 2014; Proietti-Junior et al., 2021; Wang et al., 2021; Xue et al., 2022). Fish infected by these species usually express clinical signs such as hemorrhages, epithelial ulcers, and eroded fins, among others (Soto-Rodriguez et al., 2018; Marinho-Neto et al., 2019).

The ability of these bacteria to produce hemolysins, proteases, lipases, and other virulence factors has been linked to those clinical signs (Beaz-Hidalgo and Figueras, 2013; Das et al., 2020; Claudiano et al., 2020a). Some of them including aerolysin (*aer*A), flagellin (*fla*), lipase (*lip*), elastase (*ela*), cytotoxic enterotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*), and hemolysin (*hly*A) facilitate the pathogenic process. For instance, aerolysin leads the formation of pores or channels, causing the breakdown of membrane permeability and consequently osmotic lysis, and finally cell death (Iacovache et al., 2016; Ran et al., 2018). According to El-Bahar et al (El-Bahar et al., 2019). and Tartor et al (Tartor et al., 2021)., *Aeromonas* isolates from fish frequently include these virulence genes. Additionally, *Aeromonas* species carrying the *fla* gene may be more harmful because this facilitates cell adhesion to the host, invasion, and biofilm formation (Gavín et al., 2003).

The characteristics hemorrhages observed in fish affected by *Aeromonas* spp are related to hemolytic genes like *hly*A which cause anemia and breakdown of the membranes of red blood cells (Tartor et al., 2021), while the expression of *act* gene causes degeneration of villi and mucus-producing cells (Tomás, 2012; Králová et al., 2016). Both are crucial for *Aeromonas* spp. pathogenicity (Das et al., 2020; Janda, 2002; Sreedharan et al., 2013).

In Brazil, the largest producer of Amazonian fish (Valladão et al., 2018), several reports that the presence of *Aeromonas* spp could threaten the aquaculture industry (Dias et al., 2016; Carriero et al., 2016; Jawahar Abraham et al., 2017; Claudiano et al., 2019; Gallani et al., 2020). In Peru, increased production of native Amazonian species for aquaculture and ornamental fish trade (Produce, 2023) may lead the same way. In this context, it is essential to know the *Aeromonas* species, their virulence factors, and the potential impact in aquaculture.

*Piaractus* sp. are one of the most produced species in South America (Valladão et al., 2018). The most frequently cultivated native species in Peru is paco, *P. brachypomus* (Produce, 2023). However, there is no information on the susceptibility of this species to *Aeromonas* sp. Therefore, the aim of this study was to isolate and identify *Aeromonas* species, characterize their virulence genes factors, and to evaluate their lethality in *P. brachypomus*.

## 2. Materials and methods

### 2.1. Sample collection

Samples were collected in commercial aquariums and farms near to the city of Iquitos, in Peru (S3°52'43"; O 73°20'15") between March and April 2021. Ornamental fish such as *Otocynclus affinis* (otocinclo) (n = 21) (~4.5 cm), *Astyanax* sp. (mojarrita) (n = 10) (~8.5 cm), *Trachelyopterus galeatus* (novia) (n = 12) (~4.5 cm), *Calophysus macropterus* (mota) (n = 10) (~10.5 cm), and aquaculture fish *Colossoma macropomum* (gamitana) (n = 10) (~9.5 cm; ~27.5 cm), *Arapaima gigas* (paiche) (n = 10) (~16 cm), and *Brycon* sp. (sábalo) (n = 5) (~16.5 cm) were sampled. These fish presented lesions and clinical signs (haemorrages, eroded fins, erratic swim, and/or melanosis).

### 2.2. Bacteria isolation

Samples of spleen, kidney, and liver from collected fish were homogenized (fish less than 5 cm were sampled as pools of three fish), and spread-platted on trypticase soy agar (TSA, Merck, Germany) and glutamate starch red phenol (GSP) agar base (Merck, Germany) supplemented with penicillin G 100,000 IU. Plates were incubated at 28 °C for 24 h. Forty-four isolates were obtained. Presumptive *Aeromonas* bacteria were selected and stored in 20% tryptone soy broth (TSB) - glycerol at −80 °C (thirty-four isolates).

### 2.3. Characterization of the Aeromonas species

Nineteen isolates were selected from the thirty-four presumptive *Aeromonas* based on fish species, the group, the location where were isolated, and finally by the colony morphology.

#### 2.3.1. Biochemical test

Biochemical characterization was performed using Analytical Profile Index (API) 20E and 20NE (BioMerieux, Marcy-l'Étoile, France), following the manufacturer instructions with modification in incubation temperature at 28 °C. The resultant API profile was compare using API Web® (BioMerieux, Marcy l'Etoile, France). Gelatin, elastin, lipids, casein, nucleases, and hemolytic tests were performed using the protocols described by Gerhardt et al (Gerhardt et al., 1981)., the American Society for Microbiology (ASM, 2023), and Sierra (Sierra, 1957). Proteolytic enzyme expression was revealed by the production of a clear zone resulting from the degradation of casein and elastin, and the liquefaction of the gelatin. Lipolytic activity was verified by the production of precipitated crystals surrounded by a halo (Sierra, 1957).

#### 2.3.2. Molecular identification

Total genomic DNA was extracted from isolates, as described by Wilson (Wilson et al., 1987). Two genes, 16S rRNA and DNA gyrase subunit B (*gyr*B), were analyzed for species identification. The PCR was performed using the GoTaq G2 Hot Start Colorless Master Mix kit (PROMEGA®, USA). The 16S gene was amplified using the universal primers 27 F and 1492R (Lane, 1991), with an initial denaturation of 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing of 52 °C for 30 s and extension of 72 °C for 1.5 min, with a final extension at 72 °C for 5 min. The *gyr*B gene was amplified with the primers gyrB3F and gyrB14R described in Yañez et al (Yanez et al., 2003)., with an initial denaturation of 95 °C for 3 min and followed by 36 cycles of denaturation at 94 °C for 30 s, annealing of 56 °C for 30 s and extension of 72 °C for 1 min, with a final extension at 72 °C for 5 min

Amplified products were evaluated in 1% agarose gels and those with expected size were sequenced for both strands on an ABI 3500 Genetic Analyzer (Applied Biosystems), using the same primers as in the previous PCR. Consensus sequences were compared with the NCBI nucleotide database using the BLASTn tool, and results with > 98% identity were considered for species identification. Nucleotide sequences of 16 S (1300 bp) and *gyr*B (830 bp) genes were aligned separately and concatenated, using MUSCLE program (in Geneious), and including *Aeromonas* sequences available in GenBank with close identity with the sequences obtained in this study. Kimura-2 parameter (K2P) pairwise distance was calculated, and phylogenetic trees were constructed using Neighbor-Joining (NJ) method, considering 1000 replicates, with MEGA-X program (Kumar et al., 2018). *Pseudomonas aeruginosa* (CP072783) was included as outgroup.

#### 2.3.3. Virulence genes detection

The present of eight virulence genes: aerolysin (*aer*), cytotonic enterotoxin (*altA*), cytotoxic enterotoxin (*act*), hemolysin (*hly*), serine protease (*ser*), lipase (*lip*), flagellin (*fla*), and elastase (*ahy*B) were determinated. PCR amplification reactions were carried out using GoTaq G2 Hot Start Green Master Mix kit (PROMEGA®), with 0.5 μM of primers, 1.5 mM of MgCl2, in 10 μL of final volume reaction. The list of primers, annealing temperature and amplification product sizes are detailed in Table 1. PCR products were evaluated in 1.5% agarose gels using TBE (Tris-borate-EDTA) buffer, and the image was visualized under Fire Reader v10 photodocumenter (UVITEC). Results were reported as presence (+) or absence (-) of the virulence gene in isolates. In addition, *A. hydrophila* ATCC® 7966 reference strains were included in the analyses.

**Table 1**
Primers and sequences using for identification of bacteria isolates and virulence genes characterization.

| Gene | Primer | Sequence (5- 3) | Ta | Size (bp) | Reference |
|---|---|---|---|---|---|
| 16S | 27F | AGAGTTTGATCMTGGCTCAG | 52 | 1400 | Lane (1991) |
| | 1492R | TACGGTTACCTTGTTACGACTT | | | |
| *gyrB* | gyrB3F | TCCGGCGGTCTGCACGGCGT | 56 | 900 | Yañez et al. (2003) |
| | gyrB14R | TTGTCCGGGTTGTACTCGTC | | | |
| *aerA* | aerF | CAAGAACAAGTTCAAGTGGCCA | 57 | 309 | Wang (2003) |
| | aerR | ACGAAGGTGTGGTTCCAGT | | | |
| *altA* | altA-F | TGACCCAGTCCTGGCACGGC | 60 | 442 | Sen (2004) |
| | altA-R | GGTGATCGATCCACCAGC | | | |
| *act* | act-F | AGAAGGTGACCACCAAGAACA | 59 | 232 | Sen (2004) |
| | act-R | AACTGACATCGGCCTTGAACTC | | | |
| *hly* | hly-F | GCCGAGCGCCCAGAAGGTGAGTT | 60 | 130 | Wang (2003) |
| | hly-R | GAGCGGCTGGATGCGGTTGT | | | |
| *ser* | ser-F | CACCGAAGTATTGGGTCAGG | 59 | 350 | Nawaz (2010) |
| | ser-R | GGCTCATGCGTAACTCTGGT | | | |
| *lip* | lip-F | ATCTTCTCCGACTGGTTCGG | 59 | 382 | Sen (2005) |
| | lip-R | CCGTGCCAGGACTGGGTCTT | | | |
| *fla* | fla-F | TCCAACCGTYTGACCTC | 57 | 608 | Sen (2004) |
| | fla-R | GMYTGGTTGCGRATGGT | | | |
| *ahy*B | ahyB-F | ACACGGTCAAGGAGATCAAC | 57 | 540 | Sen (2005) |
| | ahyB-R | CGCTGGTGTTGGCCAGCAGG | | | |

### 2.4. Experimental infection

Juveniles of *P. brachypomus* (~ 9 cm) were obtained from a commercial fish farm and acclimatized for 3 weeks (dissolved oxygen = 6.1 mg/L; T° = 28 ± 0.5 °C; NH3 < 0.01; NO2 < 0.05). Fish were fed ad libitum with commercial diet, and daily water exchange was performed (100%). These conditions were maintained during the experiment. A sample was taken to perform microbiology procedure to assess their health status prior to challenges.

Infections were performed with ten selected *Aeromonas*, the selection was performed according bacteria biochemical characteristics, molecular identification, and virulence genes detection (Tables 2 and 3). Bacteria were grown in TSB and incubated at 28 °C overnight. Afterward, the bacterial suspension was centrifuged (5000 g for 5 min), washed three times, and resuspended in sterile saline solution in each step.

Prior to infection, fish were anesthetized in 90 mg/L of tricaine methanesulfonate salt (MS-222, Sigma Aldrich). Six hundred healthy *P. brachypomus* were intraperitoneally injected with 0.1 mL of $4 \times 10^6$; $4 \times 10^7$or $4 \times 10^8$ CFU/fish and maintained in 25 L tanks with dechlorinated water. Fish injected with 0.1 mL of saline solution were considered as a control group (40 fish). Experimental infection was performed in duplicate (twenty fish in each group) for 10 days. Moribund fish were euthanized with an overdose of MS-222 (Fluka) (AVMA, 2020). Samples of the liver, spleen, and kidney were taken to reisolate causative agents, following the methodology explained before. Ethical approval was granted prior to the commencement of this study under the "Universidad Nacional Agraria La Molina" Permit number 001–2022.

## 3. Results

### 3.1. Isolation and biochemical test

Bacteria were isolated from natural infected fish, these presented different clinical signs such as depigmentation (Fig. 1A and B), erosion of fins (Fig. 1C), hemorrhages with bilateral exophthalmia (Fig. 1D), and hemorrhages (Fig. 1E). Internally, fish showed mainly hepatic and intestinal congestion, ascites, and hemorrhages (Fig. 2A, B and C).

Presumptive *Aeromonas* genus isolates were Gram-negative, rod shaped, motile, oxidase-positive, and colonies surrounded by yellow zones on GSP agar. Colonies commonly grow abundant and pure in both TSA and GSP. All selected bacteria were positive for proteolytic activity (gelatin hydrolysis, caseinase production), except for elastase where 8 out of 19 were positive. Lipolytic activity and haemolysis showed

**Table 2**
Biochemical and molecular identification of bacteria isolated from Amazon fish.

| Fish source | Biochemical ID | | Molecular ID | | |
|---|---|---|---|---|---|
| | API 20 E | API 20 NE | Species Identification | 16S rRNA | *gyr*B |
| *Arapaima gigas* | 3046127 | 7575755 | *Aeromonas caviae* FO129–1 | OQ000931 | OQ030245 |
| *Arapaima gigas* | 3246127 | 7475755 | *Aeromonas caviae* FO129–2 | OQ000932 | OQ030246 |
| *Otocinclus affinis* | 3046127 | 7575755 | *Aeromonas caviae* FO129–3 | OQ000933 | OQ030247 |
| *Otocinclus affinis* | 3046127 | 7575755 | *Aeromonas caviae* FO129–4 | OQ000934 | OQ030248 |
| *Calophysus macropterus* | 3246127 | 7575755 | *Aeromonas caviae* FO129–15 | OQ000938 | OQ030252 |
| *Calophysus macropterus* | 3246127 | 7577745 | *Aeromonas caviae* FO129–16 | OQ000939 | OQ030253 |
| *Colossoma macropomum* | 3246127 | 7575755 | *Aeromonas caviae* FO129–19 | OQ000940 | OQ030254 |
| *Colossoma macropomum* | 3246127 | 7575755 | *Aeromonas caviae* FO129–20 | OQ000941 | OQ030255 |
| *Arapaima gigas* | 3246127 | 7577754 | *Aeromonas caviae* FO129–29 | OQ000945 | - |
| *Arapaima gigas* | 3246127 | 7575754 | *Aeromonas caviae* FO129–30 | OQ000946 | - |
| *Otocinclus affinis* | 3046125 | 7476755 | *Aeromonas dhakensis* FO129–5 | OQ000935 | OQ030249 |
| *Trachelyopterus galeatus* | 2046125 | 7556755 | *Aeromonas dhakensis* FO129–13 | OQ000937 | OQ030251 |
| *Brycon sp* | 7046127 | 7577754 | *Aeromonas hydrophila* FO129–7 | OP801419 | OP795660 |
| *Astyanax sp.* | 7046127 | 7577754 | *Aeromonas hydrophila* FO129–17 | OP801422 | OP795659 |
| *Colossoma macropomum* | 7047127 | 7577754 | *Aeromonas hydrophila* FO129–27 | OP801420 | OP795661 |
| *Colossoma macropomum* | 7047127 | 7577755 | *Aeromonas hydrophila* FO129–28 | OQ000944 | - |
| *Colossoma macropomum* | 7047127 | 7575754 | *Aeromonas hydrophila* FO129–37 | OQ000942 | - |
| *Arapaima gigas* | 7246104 | 7176755 | *Aeromonas jandaei* FO129–12 | OQ000936 | OQ030250 |
| *Trachelyopterus galeatus* | 7246124 | 7176755 | *Aeromonas veronii* FO129–14 | OP801421 | OP795658 |

**Table 3**
Presence (+) or absence (-) of virulence genes and biochemical test from *Aeromonas* isolates retrieved from Amazon fish diseased.

| Species | Isolate code | Virulence genes | | | | | | | | Biochemical test | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *fla* | *ahyB* | *lip* | *ser* | *altA* | *act* | *hly* | *aerA* | Hem | Lip | Cas | Gel | Ela |
| *A. caviae* | FO129–1 | + | + | + | - | - | - | - | - | α | + | + | + | - |
| | FO129–2 | + | + | + | - | - | - | - | - | α | + | + | + | - |
| | FO129–3 | + | + | + | - | - | - | - | - | α | + | + | + | - |
| | FO129–4* | + | + | + | - | + | - | - | - | α | + | + | + | - |
| | FO129–15* | + | + | - | + | - | - | - | - | α | + | + | + | - |
| | FO129–16 | + | + | + | - | - | - | - | - | α | + | + | + | - |
| | FO129–19 | + | + | + | - | - | - | - | - | α | + | + | + | - |
| | FO129–20 | + | + | + | - | - | - | - | - | α | + | + | + | - |
| | FO129–29* | + | + | + | - | - | - | - | - | β | + | + | + | - |
| | FO129–30 | + | + | + | - | - | - | - | - | β | + | + | + | - |
| *A. dhakensis* | FO129–5* | + | + | + | + | - | + | + | + | β | + | + | + | + |
| | FO129–13* | + | + | + | + | + | + | + | + | β | + | + | + | + |
| *A. hydrophila* | FO129–7 | + | + | + | + | + | + | + | + | α | + | + | + | + |
| | FO129–17* | + | + | + | + | + | + | + | + | β | + | + | + | + |
| | FO129–27* | + | + | + | + | + | - | + | - | α | + | + | + | + |
| | FO129–28* | + | + | + | - | - | - | - | - | β | + | + | + | + |
| | FO129–37 | + | + | + | - | - | - | - | - | α | + | + | + | + |
| *A. jandaei* | FO129–12* | + | + | - | - | - | - | - | - | β | + | + | + | - |
| *A. veronii* | FO129–14* | + | + | - | + | - | + | - | - | β | + | + | + | - |
| *A. hydrophila* (reference strain) | ATCC® 7966 | + | + | + | + | + | + | + | + | β | + | + | + | - |

* Isolates used for experimental infections. Hem: hemolysis. Lip: Lipolytic. Cas: Caseinolytic. Gel: Gelatin hydrolysis. Ela: Elastin hydrolysis.

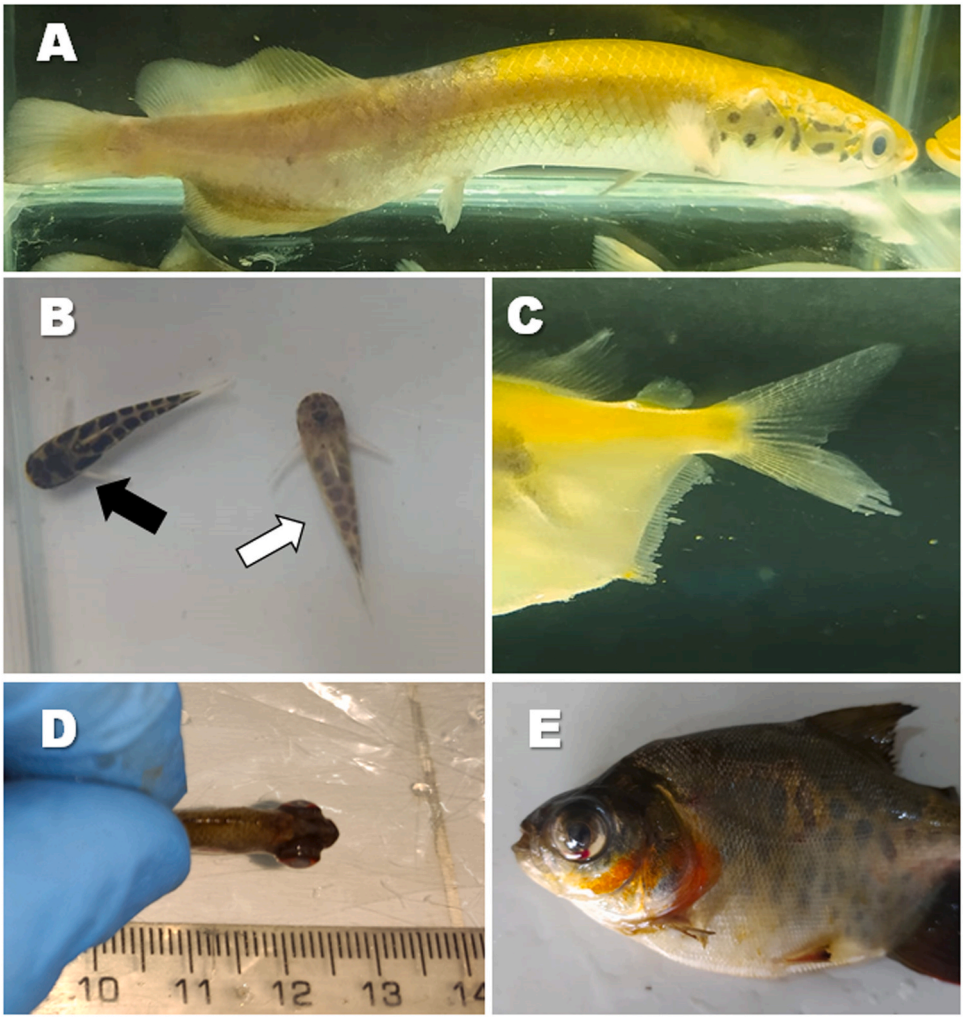



**Fig. 1.** Clinical signs in naturally infected fish. A) *Arapaima gigas*, "paiche or pirarucú", showing depigmentation on the low middle half of the body, B) *Trachelyopterus galeatus*, "novia" with apparent melanization (black arrow) comparing with a normal pigmented fish (white arrow), C) *Colosssoma macropomum*, gamitana, with eroded fins. D) *Astyanax* sp, "mojarrita" with bilateral exophtalmia. E) *C. macropomum* with ocular hemorrague and eroded fins.

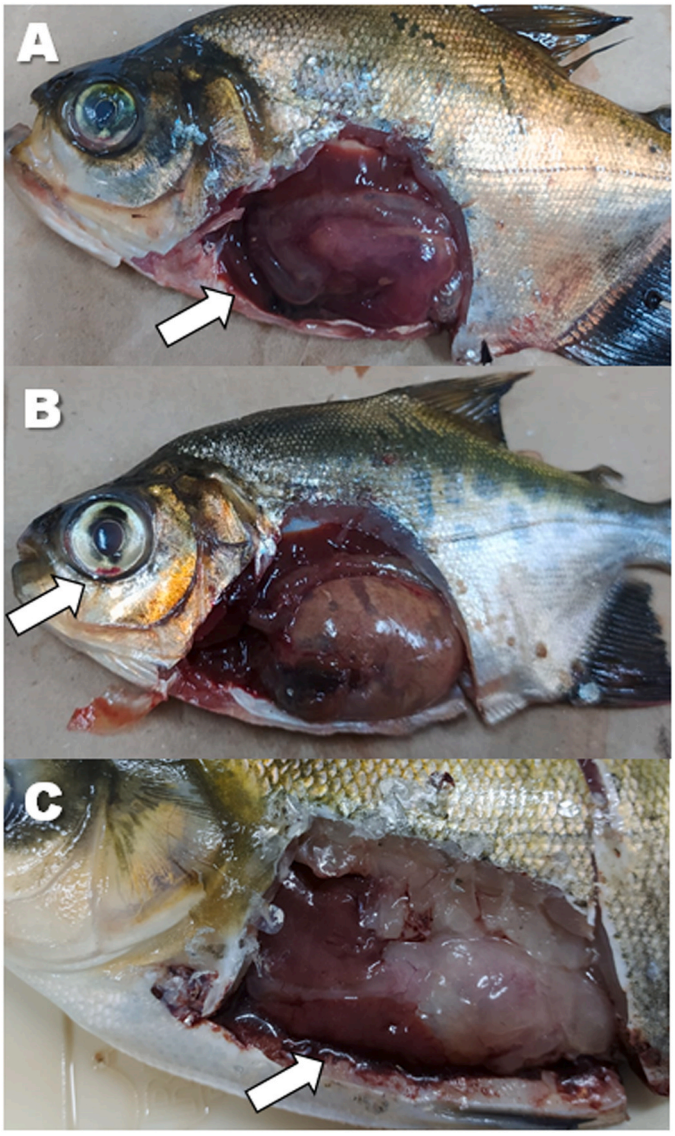



**Fig. 2.** *Colossoma macropomum* naturally infected with *Aeromonas* sp., showing internal clinical signs. A) Hemorrhages in internal organs and fluid accumulation in the intestine. B) Hiphema. C) Ascitic fluid accumulation in the peritoneal cavity.

positive results. When bacteria were evaluated, using API 20E and 20NE, different profiles were observed. However, regardless of the kit test used, all isolates were identified within the *Aeromonas hydrophila/caviae/sobria* complex. Profiles numbers are shown in Table 2.

### 3.2. Molecular identification

Based on the phylogenetic tree generated with the 16S and *gyr*B genes concatenated, the isolates were discriminated into 5 five species: *A. caviae* (n = 10), *A. dhakensis* (n = 2), *A. hydrophila* (n = 5), *A. jandaei* (n = 1) and *A. veronii* (n = 1). Isolates were clustered into clearly discriminated clades, with bootstrap values 100% (Fig. 3). The sequences obtained were registered in GenBank, with the accession numbers indicated in Table 1.

It is noteworthy that for *A. caviae* and *A. hydrophila* (with more than 2 isolates per species), no discrimination between *gyr*B sequences related to their origin (farmed and ornamental fish) was observed; whereas the two *A. dakhensis* isolates obtained in this study came from ornamental fish. Also, comparing isolates sequences, more variability was observed in *A. hydrophila* than in *A. caviae*. Thus, when aligning *gyr*B sequences of *A. hydrophila*, 60 variable sites and 8 parsimony informative (PI) sites were recorded, showing a mean distance of 0.0213 within the group. On the other hand, for *A. caviae*, 29 variable sites were recorded of which 4 were PI, and with a mean distance of 0.0125.

### 3.3. Virulence genes detection

All the isolates were positive for at least two virulence genes. The *fla* and *ahyB* genes were amplified in all isolates, the *lip* gene was present in 84% of isolates, *ser* gene present in 37% of isolates, the *act* and *alt*A were present in 31%, gene *hly* in 26%, while *aer*A was present in 21% of isolates. All eight genes were present in the reference strains *A. hydrophila* ATCC 7966 (Table 2).

*A. dhakensis* isolates were observed to harbor most of the virulence genes evaluated, followed by *A. hydrophila*. The *fla* +, *ahyB* + and *lip* + pattern was found in most of *A. caviae* isolates, as well as in two *A. hydrophila* isolates (FO129–28 and FO129–37 obtained from farmed fish carrying only the three genes). All *A. caviae* were *fla* +, *ahy*B + and *lip* +, while isolates from ornamental fish were also *ser* +(FO129–15), and *alt*A +(FO129–4). *A, jandaei* harbored only two virulence genes (*fla* and *ahy*B), while *A. veronii* (FO129–14) also presented *act ser* and *alt*A (Table 2).

### 3.4. Experimental infection

Bacteria administered intraperitoneally produced mortality in paco *P. brachypomus* with different levels of virulence. Unlike to other *Aeromonas* species evaluated, *A. hydrophila* and *A. dhakensis* caused > 80% mortality in *P. brachypomus* from the lowest concentration evaluated ($\sim 10^6$ CFU/fish). Acute mortality (100%) was observed around the first 5 days post infection at the lowest dose. However, the time of death was reduced to less than 48 h when higher bacterial loads were inoculated (Fig. 4).

*A. veronii* and *A. jandaei* isolates produced 20% and 70% mortality respectively using $\sim 10^7$ CFU/fish, but with lower intensity compared to *A. hydrophila* and *A. dhakensis* (Figure x). When the highest concentration ($\sim 10^8$ CFU/fish) was used, *A. veronii* produced 70% mortality in paco *P. brachypomus*. Similarly, *A. caviae* isolates caused from 50% to 80% mortality. Above this data, *A. jandei* was shown to cause 100% mortality at this level, similar to *A. hydrophila* and *A. dhakensis*.

Clinical signs in infected fish with all bacterial species were slightly evident being observed with greater intensity in those infected with lower bacterial load. In general, fish showed erratic swimming, belly and anus redening, hyphema, eroded fins and occasionally skin lesions. Internally, multifocal hemorrhages in the liver and intestinal congestion was observed. The clinical signs and lesions were also observed in naturally infected fish. In each group, the previously injected bacteria were re-isolated. No signs of disease and no mortality during the experiment was observed in fish of control group.

## 4. Discussion

*Aeromonas* spp are ubiquitous bacteria found in water and are part of the microbiota of Amazonian fish (Castañeda-Monsalve et al., 2019; McDonald et al., 2019), which are commonly in contact with these microorganisms in the water (ornamental fish effluents or farmed fish), wild fish species and, other animals (Gonçalves Pessoa et al., 2019; Topic Popovic et al., 2000). This presumption draws attention to the danger of introducing novel *Aeromonas* isolates via carriers, which could lead to the emergence of potential pathogens for farmed fish and the transmission of bacterial infections from species to species in aquaculture facilities.

According to numerous studies, the bacterial genus *Aeromonas* has been linked to disease in Amazonian native fish (Dias et al., 2016; Carriero et al., 2016; Jawahar Abraham et al., 2017; Claudiano et al.,

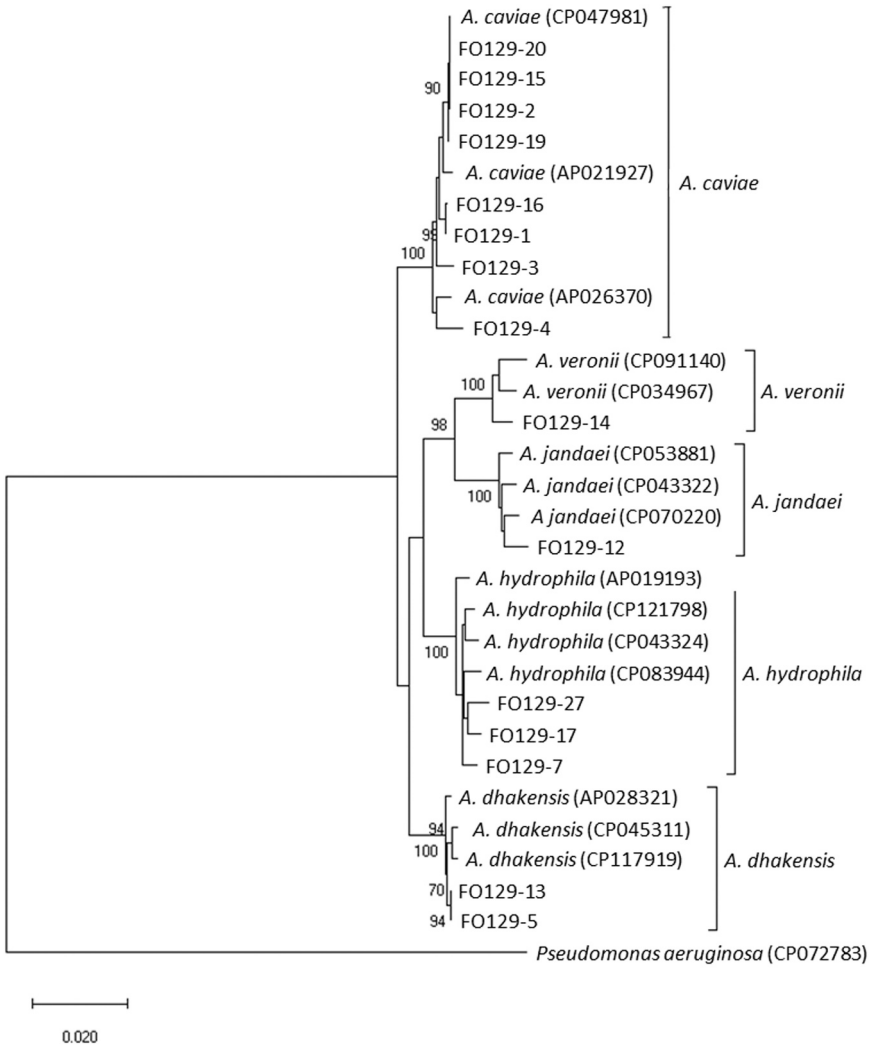



**Fig. 3.** Neighbor-Joining tree based on concatenated 16S rRNA and *gyr*B genes indicating relationship among *Aeromonas* isolates from this study.

2019; Gallani et al., 2020; Ariede et al., 2020). It has been determined that *A. hydrophila* is the primary bacterium frequently detected causing diseases. Other species, including *A. dhakensis*, *A. veronii*, *A. caviae*, and *A. jandaei*, can also infect fish and could potentially be infectious to humans (Gonçalves Pessoa et al., 2019). Presumable *Aeromonas* spp. have been linked to disease in native Amazonian fish in Peru without definitive diagnosis (Serrano-Martínez et al., 2014; Valera et al., 2018). Their identification is often a challenging work since they showed several variants and synonyms that have been reclassified in the last years (Beaz-Hidalgo et al., 2010; Fernández-Bravo and Figueras, 2020). Commercial biochemical tests have been used to aid in the identification of bacterial isolates, however, for *Aeromonas* species results are not conclusive (Abbott et al., 2003). In the current study, five species of *Aeromonas* were isolated and identified biochemically and molecularly.

Proteases produced by *Aeromonas* spp are responsible for degrading fish epithelial mucosa, leading to lesions on the skin and internal organs (Gonçalves Pessoa et al., 2019) contributing to the establishment of infection and overcoming the initial host defenses (Tomás, 2012). This has been recognized in *Aeromonas* spp as an important virulence factor (Pridgeon et al., 2013). In the current study, elastase, one of the main proteases in the genus, was both *ahy*B and biochemically positive only in *A. hydrophila* and *A. dhakensis* isolates and explain the lethality of these isolates causing mortality in *P. brachypomus* from the lowest dose (~$10^6$ CFU/fish) differing to other species (harboring *ahy*B gene but elastase negative). Similar results were found by Cascón et al (Cascón et al., 2000)., who showed that the lethality of *A. hydrophila ahy*B mutant was 100 times lesser than in elastase positive (*ahy*B gene product) wild-type strain indicating that elastase plays an important role in *Aeromonas* virulence. Other study also reported the relationship between elastase and virulence in *A. hydrophila* (Santos et al., 1987). Nonetheless, complementary studies are needed to establish this presumption.

In the other hand, *A. caviae*, *A. veronii*, and *A. jandaei* were *ahy*B positive and biochemically negative for elastase. This can be explained due to altered genetic basis that could not be amplified using the currently primers (Das et al., 2020), the temperature of incubation (Pattanayak et al., 2020), and some factors that influence genes regulation and expression, showing the multifactorial effect on virulence of these bacterial species (Galindo et al., 2006; Sha et al., 2009). Some other virulence genes detected by PCR (i.e. *ser*, *hly*) were also unable to be in accordance with phenotypic observation on substrate base test (i.e. caseinolytic positive, hemolytic strains).

The number and type of virulence genes of *Aeromonas* spp had been associated with the host mortality (El-Bahar et al., 2019; Chang et al., 2008; Youssef et al., 2023). Li et al (Li et al., 2011). found that *aer*A+ *alt*+ *ahp*+ profile in *A. hydrophila* was correlated to the most virulent isolates in zebrafish comparing six genetic profiles. In the current investigation, the eight virulence genes evaluated were present in the most lethal isolates, two *A. hydrophila* and two isolates of *A. dhakensis*, including the *aer*A and *alt* virulence genes, which are strong virulence determinants for pathogenicity (Oliveira et al., 2012). Despite this, not

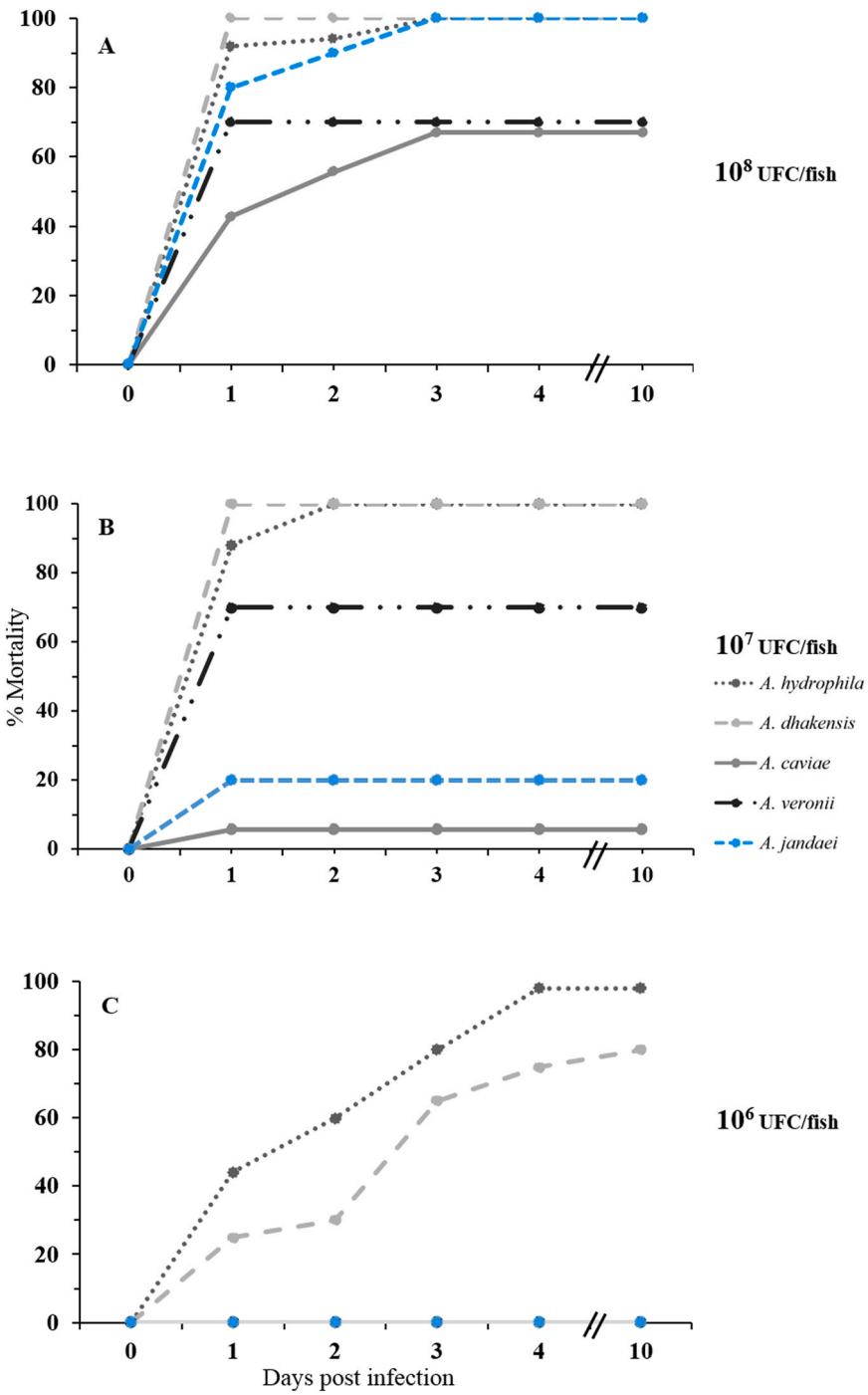



**Fig. 4.** Mortality of *Piaractus brachypomus* using different bacterial loads of *Aeromonas* species isolated from Amazon fish diseased.

all the isolates studied fulfilled with those characteristics. Some isolates of *A. hydrophila* that harbored fewer virulence genes caused similar mortality (100%) even at the lowest dose (~ $10^6$ CFU/fish).

Different outcomes were pointed by other authors, in Amazonian fish, higher *A. hydrophila* loads ($10^7$ - $10^{10}$ CFU/fish) have been used to cause > 80% mortality (Dias et al., 2016; Gallani et al., 2020; Claudiano et al., 2020b; Cueva-Quiroz et al., 2020; Bozzo et al., 2023). However, the susceptibility and age of the host, different physicochemical parameters, and different isolates, among others factors could affect the performance of *A. hydrophila* to cause 100% mortality using less bacterial load than the previous studies mentioned. In addition, other virulence factors that could act synergistically to produce clinical diseases (Oliveira et al., 2012) improving the pathogenic characteristics of these pathogens could be involved.

*A. veronii* and *A. jandaei* have been also associated with diseases in tropical fish (Dong et al., 2017; Xue et al., 2022; Bozzo et al., 2023; Li et al., 2020; Raj et al., 2019). In Amazonian fish, these bacteria have been reported causing mortalities in *Arapaima gigas* (Proietti-Junior et al., 2021) and *C. macropomum* (Gonçalves Pessoa et al., 2019; Mielke et al., 2022). However, it seems that these bacteria are less lethal than the previous ones. Dong et al (Dong et al., 2017). even suggests that *A. veronii* or *A. jandaei* alone at low density and no stress inducer may not kill the host. This is related to the high load of bacteria necessary to induce mortality in *P. brachypomus* in the current study.

*A. caviae* was the most frequent bacteria isolated from disease fish in this study (10/19), and it was expected that it might be of similar

virulence to *P. brachypomus* as other species, since *A. caviae* produce disturbance of hepatic and renal antioxidant/oxidant equilibrium contributing to the pathophysiology of the disease in fish (Baldissera et al., 2018). However, it was necessary to use a relatively high bacterial load (> 10 7 CFU/fish) also to induce high mortality in *P. brachypomus*. According to Dong et al (Dong et al., 2017)., it is possible that the contribution of stressful variables (such as environmental stress, handling stress, or the synergistic effect of co-infections) may be required to increase its lethality.

Finally, our report revealed the variety of species that are infecting farmed Amazonian native fish. For the first time, the presence of *A. hydrophila*, *A. dhakensis*, *A. jandaei*, *A. veronii* and *A. caviae* in fish farms in the Peruvian Amazon was confirmed. This study also suggests that virulence genes evaluated in *Aeromonas* species may not always be an indicator of virulence. Additionally, there is not always a relation between the genes harbored and their phenotypic expression which could be determinants to the mortality rates in fish. Moreover, *P. brachypomus*, an economically important aquaculture fish species could be susceptible not only to the high mentioned *A. hydrophila*, but also to other less observed species such as *A. dhakensis*, *A. veronii*, *A. jandaei*, and *A. caviae*, bacteria that should be taken into account in health surveillance for the implementation of aquaculture disease prevention strategies.

## CRediT authorship contribution statement

**Maurício Laterça Martins**: Conceptualization. **Caren Arteaga**: Data curation, Writing - original draft preparation. **Gabriela Cordero**: Methodology. **Marco Medina-Morillo**: Data curation, Project administration. **Marco Medina-Morillo**: Visualization, Investigation. **German Murrieta Morey**: Supervision. **Giovanna Sotil**: Software, Validation. **Jefferson Yunis-Aguinaga**: Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Jefferson Yunis Aguinaga reports financial support was provided by Universidad Científica del Sur. Jefferson Yunis Aguinaga reports a relationship with Institute of the Sea of Peru that includes: employment. Nothing to declare.

## Data availability

Data will be made available on request.

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# Update


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# Corrigendum to "Pathogenic *Aeromonas* spp in Amazonian fish: Virulence genes and susceptibility in *Piaractus brachypomus*, the main native aquaculture species in Peru" Aquacult. Rep. 33 (December) 2023

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The authors regret that the article above mentioned has an incomplete affiliation of one of the co-authors, Giovnna Sotil. The additional affiliation of Giovanna Sotil is: Facultad de Ciencias Biológicas, Universidad Nacional Mayor de San Marcos, Lima, Perú.

Second, the authors regret that the article above mentioned contained an unintentional mistake in the CRediT authorship contribution statement. In order to sincerely respect academic ethics and contribution to the research work, the authors request to correct the CRediT authorship contribution statement as follows.

**Marco Medina-Morillo**: Conceptualization, Investigation, Project administration, Writing – review & editing. **Giovanna Sotil**: Methodology, Formal Analysis, Investigation, Writing - Original Draft. **Caren Arteaga**: Investigation, Data curation. **Gabriela Cordero**: Investigation. **Maurício Laterça Martins**: Verification. **German Murrieta Morey**: Investigation. **Jefferson Yunis-Aguinaga**: Investigation, Writing – review & editing.

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