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Experimental co-infection by *Aeromonas hydrophila* and *Aeromonas jandaei* in pirarucu *Arapaima gigas* (Pisces: Arapaimidae)

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Abstract

In fish, bacteriosis has been widely linked with *Aeromonas* species, but co-infection by these bacteria has been little addressed. The aims of this study were to report on an outbreak of disease in pirarucu *Arapaima gigas* caused by *Aeromonas* and to investigate experimental co-infection and characterize resistance profile, virulence factors and phenotypic and molecular differentiation. Fish samples with clinical signs of bacteriosis were collected and used to study experimental co-infection. The bacterial isolates were characterized phenotypically as *Aeromonas hydrophila* and *Aeromonas jandaei*. Virulence genes *aerA*, *gcat*, *lip*, *dnase* and *hlyA* were detected using the polymerase chain reaction, while the *alt*, *act* and *ser* genes were not found. Resistance to imipenem and ceftriaxone was observed; however, all isolates were susceptible to most of the antibiotics assayed. Phenotypic tests to determine the presence of metallo-β-lactamases showed positivity only for *A. jandaei* strains. Assays for the resistance genes *kpc*, *ndm*, *imp*, *oxa-48* and *vim* showed negative results. The co-infection and pathogenicity of *A. hydrophila* in association with *A. jandaei* in *A. gigas*, established in accordance with Koch's postulate, provided experimental support for the existence of synergism between these bacteria. This has several implications relating to occurrences of this co-infection and determinants of virulence.

KEYWORDS
bacteria, culture, infection, pirarucu, resistance profile, virulence

1 | INTRODUCTION

Fish are a major source of food and income in several countries in which part of their economy is dependent on capture fisheries and aquaculture. Increasing demand for farmed fish has enabled Brazil to reach a prominent position within the development of aquaculture. Hence, it is now one of the countries with the potential to meet the increasing worldwide demand for fish, and this has led to rapid expansion of aquaculture (Nobile et al., 2020).

In northern Brazil, which is entirely within the Amazon region (together with parts of some other countries), aquaculture is based on native fish species such as *Arapaima gigas* (pirarucu), for several zootechnical reasons. This species in the family Arapaimidae, known as the giant fish of the Amazon, is one of the largest freshwater fish in the world. It has good zootechnical characteristics such as easy adaptation to artificial feed, good zootechnical performance, high growth rate (reaching up to 10 kg per year), relative resistance to handling, absence of cannibalism even when confined at

high population densities and presentation of lean intramuscular pimples (Ono et al., 2004; Pereira-Filho & Roubach, 2018; Serrano et al., 2014). However, one of the problems relating to production of *A. gigas* has been high mortality during the early stages of cultivation, caused by bacteriosis (Proietti-Junior et al., 2017). This has not been investigated with regard to its phenotypic, molecular and resistance profiles, or its virulence-determining factors.

Globally, bacterial infections represent a great threat to fish production due to the high economic losses that they cause to the fish-farming industry (Assefa & Abunna, 2018; Bentzon-Tilia et al., 2016; Figueras-Salvat & Ashbolt, 2019; Wamala et al., 2018). This threat is also seen in relation to farmed fish in Brazil, such as *A. gigas* (Proietti-Junior et al., 2017; Tavares-Dias & Martins, 2017; Zaniboni-Filho et al., 2018).

Several bacterial species have been reported to cause diseases in freshwater farmed fish around the world. The pathogens of particular importance include those of the genus *Aeromonas* (Chandrarathna et al., 2018; Fig ueras-Salvat & Ashbolt, 2019; Fowoyo & Achimugu, 2019; Wamala et al., 2018). *Aeromonas* comprises a group of ubiquitous oxidase-positive Gram-negative bacilli that are considered to be autochthonous in aquatic environments (Bentzon-Tilia et al., 2016; Figueras-Salvat & Ashbolt, 2019; Fowoyo & Achimugu, 2019). Although infections caused by more than one species of *Aeromonas* are frequent in fish farming around the world, studies on co-infections caused by such bacteria species have been little (Chandrarathna et al., 2018; Mosser et al., 2015). However, such studies have been not carried out in *A. gigas*, the giant fish from the Amazon.

Aeromonas spp. generally carry numerous virulence factor genes in their genomes. However, knowledge of *Aeromonas* species and their interspecific interactions remains poor (Chandrarathna et al., 2018; Mosser et al., 2015). Better understanding of the mechanisms relating to such interactions between bacterial infections can provide important data on the role of virulence and regarding recognition of clinical signs of this bacteriosis in fish. In addition, knowledge of the susceptibility to antibiotics presented by these bacteria is vital for proper management of diseases in fish farming, because the therapeutic agent most used for controlling *Aeromonas* infections is antibiotics.

The aim of this study was to investigate the experimental co-infection by *Aeromonas* spp. using Koch's postulate to characterize the resistance profile, virulence factors and phenotypic and molecular differentiation, after an outbreak caused by such bacteria in farmed *A. gigas*. The findings may contribute towards better understanding the effects of co-infection by *Aeromonas* species in fish.

2 | MATERIALS AND METHODS

2.1 | Collection of diseased *Arapaima gigas* samples and bacteriological analysis

After the appearance of the first external signs of bacteriosis that was compatible with those described by Dias et al. (2016), eight diseased fish were collected. They were transported to the laboratory individually in sterile plastic bags, in a water tank in an isothermal box. After cryoanesthesia, the fish were euthanized through medullar section. Samples of gill and muscle tissue were then collected for bacteriological analysis. These samples were carefully investigated, following routine laboratory standards for investigating fish samples for the presence of bacterial species.

One gram of tissue from each anatomical site was homogenized for one minute in an orbital shaker, in 10 ml of buffered peptone water (Sigma-Aldrich®) plus 1% NaCl. These homogenates were then incubated at 35–37°C for 18–24 h in a bacteriological incubator (REVCO Elite II®; Kendro Laboratory Products™, Asheville, NC, USA). The homogenates were then diluted tenfold in buffered peptone water and were plated with disposable inoculating loops (calibrated at 10 μl) on glutamate starch phenol red (GSP) agar (Sigma-Aldrich®, USA), using a streaking technique for isolation of *Aeromonas* spp. (Proietti-Junior et al., 2017).

Preliminary observation of the bacteriological growth of the respective samples showed that this was a mixed culture presenting characteristic *Aeromonas* morphotypes on GSP agar plates. After thorough examination of the morphology of the colonies, these were re-isolated on blood agar and were presented as pure cultures of β-haemolytic organisms. Each morphotype was inoculated in parallel on the following screening media: Kligler iron agar (KIA) (Oxoid®), and lysine iron agar (LIA) (Oxoid®) and nutrient agar 0.5% NaCl (DIFCO®). These were all incubated under the same conditions (35–37°C for 18–24 h). For growth on nutrient agar, oxidase tests and a sensitivity test for the vibriostatic agent O129 (2,4-diamino 6,7-diisopropyl pteridine) at concentrations of 10 μg/μl and 150 μg/μl were performed.

For differentiation of the phenospecies, different metabolic characteristics were evaluated, including: fermentation: glucose, lactose, sucrose and arabinose; glucose gas production; lysine and ornithine decarboxylation; arginine dehydrolation; esculin hydrolysis; growth at different NaCl concentrations; and evaluation of glucose fermentation pathways.

Further examination showed that the organisms were Gram-negative rods. They were motile, oxidase-positive, glucose-fermenting and resistant to the vibriostatic agent O129 (for further details, see Janda and Abbott (2010).

2.2 | Determination of antimicrobial susceptibility of *Aeromonas* spp.

Antimicrobial susceptibility testing (AST) was carried out using the disc diffusion method, in accordance with the standard proposed by Clinical and Laboratory Standards Institute (2018). We tested the following 15 antimicrobial agents (as described in Table 1): ampicillin (AMP, 10 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), cefoxitin (FOX, 30 μg), ceftriaxone (CRO, 30 μg), imipenem (IPM, 10 μg), amikacin (AK, 30 μg), gentamicin (CN, 10 μg), streptomycin (S, 25 μg), tetracycline (TE, 30 μg), nalidixic acid (NA, 30 μg), ciprofloxacin (CIP, 5 μg), trimethoprim/sulfamethoxazole (SXT, 25 μg), chloramphenicol (C, 30 μg) and nitrofurantoin (F,

100 μg) (Oxoid®, Hampshire, UK). Quality control was performed using the references *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. The antimicrobial agents tested are shown in Table 1.

2.3 | Phenotypic screening of carbapenemases

For phenotype detection and differentiation of metallo-beta-lactamase and plasmid-mediated serine carbapenemase production, the following methods were used: modified carbapenem inactivation (mCIM) and ethylenediaminetetraacetic acid (EDTA)-modified carbapenem inactivation (eCIM), in accordance with the CLSI recommendations (CLSI, 2018).

2.4 | Molecular detection of resistance genes

Purified bacterial DNA was extracted using the PureLink™ commercial kit (Invitrogen Life Technologies®, Burlington, Ontario, Canada), in accordance with the manufacturer's guidelines. Molecular detection of the class-related resistance genes of beta-lactams (extended-spectrum β-lactamases, ESBL) was performed using the polymerase chain reaction/PCR (Table 2).

Presence of MBL-encoding genes (*cph*A) was determined by PCR using primers and amplification conditions previously described with minor modifications (Balsalobre et al., 2009). PCR reactions to a 25 μl final volume consisted of 1X GoTaq® Reaction Buffer (Promega, Madison, WI, USA), 2.0 mM MgCl2, 0.2 mM of each dNTP (Invitrogen™, São Paulo, Brasil), 0.5 pmol of each primer, 1.25 U of GoTaq® G2 DNA Polymerase (Promega, Madison, USA) and 50–100 ng of DNA template.

TABLE 1 Classes, antimicrobial agents and antibiotic disc codes

Classes	Antimicrobial agent	Antibiotic code
Aminopenicillins	Ampicillin	AMP
Cephems	Ceftazidime	CAZ
	Cefotaxime	CTX
	Cefoxitin	FOX
	Ceftriaxone	CRO
Carbapenems	Imipenem	IPM
Aminoglycosides	Amikacin	AK
	Gentamicin	CN
	Streptomycin	S
Tetracyclines	Tetracycline	TE
Quinolones	Nalidixic Acid	NA
Fluoroquinolones	Ciprofloxacin	CIP
Folate metabolic inhibitors	Trimethoprim/ Sulfamethoxazole	SXT
Amphenicol	Chloramphenicol	C
Nitrofurans	Nitrofurantoin	F

2.5 | Molecular detection of virulence genes

The isolates were screened for haemolysin (*aer*A and *hly*A), cytotoxin (*act*), thermolabile cytotoxic enterotoxin (*alt*), lipases (*lip* and *gcat*, glycerophospholipid cholesterol acyltransferase), serine protease (*ser*) and the DNase gene (*exu*). The recommendations for primers and protocols used have been previously described (Table 3).

2.6 | Koch's postulate: clinical signs, behavioural changes and mortality of fish

Fingerlings of healthy *A. gigas* (44.6 ± 8.3 g and 18.4 ± 1.9 cm, n = 25) were acquired from a commercial fish farm and were acclimated in the Special Laboratory for Applied Microbiology, Federal University of Amapá, Macapá, Brazil, in aquaria each containing 100 L of water. These fish were fed ad libitum with commercial extruded feed containing 45% crude protein (CP), twice a day. The fish were kept in these aquaria under constant aeration, with daily replacement of water (approximately 95%). The dissolved oxygen levels (6.3 ± 0.2 mg/L), temperature (28.4 ± 0.3°C) and pH (7.4 ± 0.6) were measured daily using a multiparameter probe (Hanna, model HI 96715®); ammonia levels (4.5 ± 1.6 mg/L) and nitrite levels (0.03 ± 0.01 mg/L) were measured using a digital device (Hanna, model HI 96715®).

2.6.1 | Preparation of *Aeromonas* spp. suspensions and subcutaneous inoculations of *Aeromonas* isolates in *Arapaima gigas*

Bacterial suspensions were prepared by transferring colonies of bacterial isolates from *A. gigas* that were involved in the outbreak (*Aeromonas hydrophila* and *Aeromonas jandaei*). As a positive control, a suspension of *A. hydrophila* from ATCC 7966 was used. These suspensions were placed in Petri dishes containing tryptic soy agar (TSA) (Sigma-Aldrich®) and were cultured at 35–37°C for 18–24 h. The material was then transferred to Falcon tubes containing 10 ml of tryptic soy broth (TSB) and was re-incubated in a bacteriological incubator (REVCO Elite II®; Kendro Laboratory Products™, Asheville, NC, USA) at 35–37°C for 18–24 h.

After incubation of the bacterial suspensions with the culturing medium, the logarithmic growth phase was measured from the turbidity that the bacterial growth caused, using a densitometer (Densichek™; BioMerieux®, Marcy l'Etoile, France). The values obtained were processed in McFarland nephelometric units, and the concentrations were adjusted through 1:10 serial dilutions to the final concentration of 3.0×10^7 CFU/ml from one tube on this scale.

Subcutaneous inoculations of the suspensions of *Aeromonas* species and of a negative control were performed in the caudal peduncle region

TABLE 2 Sequence of primers and product sizes for *Aeromonas* spp. resistance genes

Gene	Sequence 5' – 3'	Amplicon	References
bla_{KPC}-F bla_{KPC} –R	CGTCTAGTTCTGCTGTCTTG CGTTCGGCTGGATTGATTTG	798 pb	Poirel et al. (2011)
bla_{NDM}-F bla_{NDM}-R	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC	621 pb	Poirel et al. (2011)
bla_{OXA}-F bla_{OXA}-R	GCGTGGTTAAGGGTGAACAC CATCAAGTTCAACCCAACCG	438 pb	Poirel et al. (2011)
bla_{IMP}-F bla_{IMP}-R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	232 pb	Poirel et al. (2011)
bla_{VIM}-F bla_{VIM}-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390 pb	Poirel et al. (2011)
Bla_{SPM}-F Bla_{SPM}-R	AAAATCTGGGTACGCAAACG ACATTATCCGCTGGAACAGG	271 pb	Poirel et al. (2011)
CphA-F *CphA*-R	TCTATTTCGGGGCCAAGGG TCTCGGCCCAGTCGCTCTTCA	590 bp	Balsalobre et al. (2009)

TABLE 3 Primer sequence, amplification conditions and product sizes for virulence genes in *Aeromonas* spp.

Gene	Sequence	PCR conditions	Amplicon
*aer*A[1]	F 5′-CCTATGGCCTGAGCGAGAAG-3′ R 5′-CCAGTTCCAGTCCCACCACT-3′	95°C 3′-1X 94°C 1′/56°C 1′/72°C 1′-35x 70°C 5′-1X	431 bp
lip[1]	F 5′-CA(C/T)CTGGT(T/G)CCGCTCAAG-3′ R 5′-GT(A/G)CCGAACCAGTCGGAGAA-3′	95°C 3′ – 1X 94°C1′/56°C 1′/72°C 1′- 35x 72°C 5′ – 1X	247 bp
gcat[1]	F 5′-CTCCTGGAATCCCAAGTATCAG-3′ R 5′-GGCAGGTTGAACAGCAGTATCT-3′	95°C 3′ – 1X 94°C 1′/56°C 1′/72°C 1′- 35x 72°C 5′ – 1X	237 bp
ser[1]	F 5′-CACCGAAGTATTGGGTCAGG-3′ R 5′-GGCTCATGCGTAACTCTGGT-3′	95°C 3′ – 1X 94°C 1′/60°C 1′/72°C 1′- 35x 72°C 5′ – 1X	350 bp
exu[1]	F 5′-(A/G)GACATGCACAACCTCTTCC -3′ R5′-GATTGGTATTGCC(C/T)TGCAA(C/G)-3	95°C 3′ – 1X 94°C 1′/54°C 1′/72°C 1′- 25x 72°C 5′ – 1X	323 bp
hlyA[2]	F 5′-GGCCGGTGGCCCGAAGATACGGG -3′ R 5′-GGCGGCGCCGGACGAGACGGG -3′	94°C 30″/62°C 30″/72°C 2′-35x 72°C 1′ – 1X	597 bp
act[3]	F 5′-AGAAGGTGACCACCAAGAACA -3′ R 5′-AACTGACATCGGCCTTGAACTC -3′	95°C 3′ – 1X 95°C 25″/55°C 30″/72°C 1′- 25x 70°C 5′ – 1X	232 bp
alt[3]	5′-TGACCCAGTCCTGGCACGGC -3′ R 5′-GGTGATCGATCACCACCAGC -3′	95°C 3′ – 1X 95°C 25″/55°C 30″/72°C 1′- 25x 70°C 5′ – 1X	442 bp

Note: 1. Chacón et al. (2002), 2. Heuzenroeder et al. (1999), 3. Sen and Rodgers (2004).

of *A. gigas*, exactly on its lateral line. The bacterial suspension was adjusted to 3.0×10^7 CFU/ml, and, once standardized as previously described, the range was observed for up to 15 min after adjustment. This bacteria suspension was adjustment according to previous studies by Dias et al. (2016), in which 3.0×10^7 CFU/ml caused 20% of mortality in 96 h, showing that this concentration was sufficient for cause signals and symptoms of aeromonosis in *A. gigas*. In this context, for this phase of Koch's postulate, 25 fingerlings of *A. gigas* were divided into five groups of five fish each and were inoculated with 0.1 ml of the bacterial suspension per 10 g of fish weight (Table 4). The bacterial suspension for group 3 was prepared by adding equal volumes of standardized *A. hydrophila* and *A. jandaei*. The fish were analysed for clinical signs, behavioural changes and mortalities for four days after inoculation. After 96 h of experimentation, the surviving fish (*A. hydrophila* or *A. jandaei*) were euthanized by means of medullar section after cryoanesthesia and were necropsied.

2.6.2 | Re-isolation and characterization of outbreak of *Aeromonas* spp. in *Arapaima gigas*

For this phase of Koch's postulate, freshly dead fish were used for bacteriological analyses. The gills and modified swim bladders of *A.*

gigas were used to re-isolate the microorganisms. To confirm the identification of the bacteria, the same phenotypic and molecular assays previously described in items 2.4–2.5 were used for characterization of the aetiologic agents in the experimental outbreak.

3 | RESULTS

3.1 | Phenotypic evaluations of *Aeromonas* spp.

From the fingerling samples (n = 100) presenting clinical signs of aeromonosis, *A. hydrophila* and *A. jandaei* phenospecies were recovered from the gills and modified swim bladders.

Antimicrobial assays showed low rates of antimicrobial resistance against ampicillin, ceftazidime, amikacin, gentamicin, streptomycin, tetracycline, nalidixic acid, ciprofloxacin, trimethoprim/sulfamethoxazole, chloramphenicol and nitrofurantoin (none of the isolates) for *A. hydrophila* and *A. jandaei*. Both *Aeromonas* species demonstrated resistance to ceftriaxone and imipenem.

Phenotype detection and differentiation of metallo-beta-lactamase and plasmid-mediated serine carbapenemase production showed that *A. jandaei* produced metallo-β-lactamases (mCIM did not show any inhibition zone while eCIM demonstrated an inhibition zone of 27 mm), while *A. hydrophila* did not present production of metallo-β-lactamases (mCIM demonstrated an inhibition zone of 27 mm and eCIM demonstrated an inhibition zone of 25 mm).

3.2 | Molecular detection of resistance and virulence genes in *Aeromonas* spp.

Multiplex PCR results for the five target genes bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{OXA-48} and bla_{VIM} in the *Aeromonas* isolates involved in the outbreak were not detected. However, results indicate carbapenemase activity in phenotypic assays is associated with CphA enzyme of *A. jandaei* isolates that were positive for the presence of *cph*A gene.

Five of the eight virulence genes assayed were present in the *Aeromonas* isolates: haemolysin (*aer*A and *hly*A), lipases (*lip* and *gcat*, glycerophospholipid cholesterol acyltransferase) and the DNase gene (*exu*). The virulence genes cytotoxin (*act*), thermolabile cytotoxic enterotoxin (*alt*) and serine protease (*ser*) were not detected in any of the strains isolated.

3.3 | Koch's postulate

The fish in the negative control did not show any noteworthy changes. The fish in group 1 and group 2 which were inoculated with 3.0×10^7 CFU/ml, and the fish in the positive control showed moderate infection by *A. hydrophila* or *A. jandaei*. From the third day after the challenge, the fish began to present the following: macroscopic lesions on the integument, such as reddish edges and depigmentation in a circular pattern across the integument, tail and fins; loss of balance with erratic swimming; reduction of respiratory movements; and small lesions with irregular abrasion in the caudal region. After 96 h, the fish (5 fish per treatment) were euthanized. Necropsies were then performed, and these showed mild localized hyperaemia of organs such as the kidneys, liver and modified swim bladder (Figure 1). No deaths were observed during the experiment, in any of the three groups.

The fish inoculated with *A. hydrophila* plus *A. jandaei* showed severe infection. Anorexia was observed in the assays on the experimental infection. During the first 24 h, the fish showed changes to the characteristic coloration of the tegument, to light grey. Skin lesions could also be seen, especially at the inoculation site and on the fins. Lethargic behaviour and loss of balance with erratic swimming were observed.

By the beginning of the second day of the experiment, the fish already presented the following: haemorrhages in the opercular region; macroscopic lesions, such as reddish edges, with detachment of the skin in areas adjacent to the inoculation site; circular depigmentation along the integument, tail and fins; reduced breathing movements; erratic swimming at the surface, with difficulty in remaining at the bottom of the aquarium; and severe abrasion of the tail with irregular lesions that left the muscles exposed.

Fish inoculated with *A. hydrophila* plus *A. jandaei* presented 100% mortality within 48 h to 72 h and were immediately necropsied. These examinations invariably demonstrated macroscopic damage to the kidney, liver, spleen and gallbladder, such as splenomegaly, ascites, areas of bleeding in the liver, gallbladder enlargement, hyperaemia, swelling and necrotic haemorrhagic areas in the modified swim bladder.

4 | DISCUSSION

Currently, fish farming is the food-production industry with the fastest growth worldwide. However, this industry is threatened by bacterial diseases, particularly in tropical regions (Fowoyo &

TABLE 4 Subcutaneous inoculations of suspensions of *Aeromonas* species and negative control solution, in *Arapaima gigas*

Experimental groups	Bacteria	Final concentration
Negative control	Sterilized NaCl solution	0.85% (w/v)
Positive control	*A. hydrophila* ATCC 7966	3.0×10^7 CFU/ml
1	*A. hydrophila* (isolated strain)	3.0×10^7 CFU/ml
2	*Aeromonas jandaei* (isolated strain)	3.0×10^7 CFU/ml
3	*A. hydrophila* plus *A. jandaei* (isolated strains)	3.0×10^7 CFU/ml

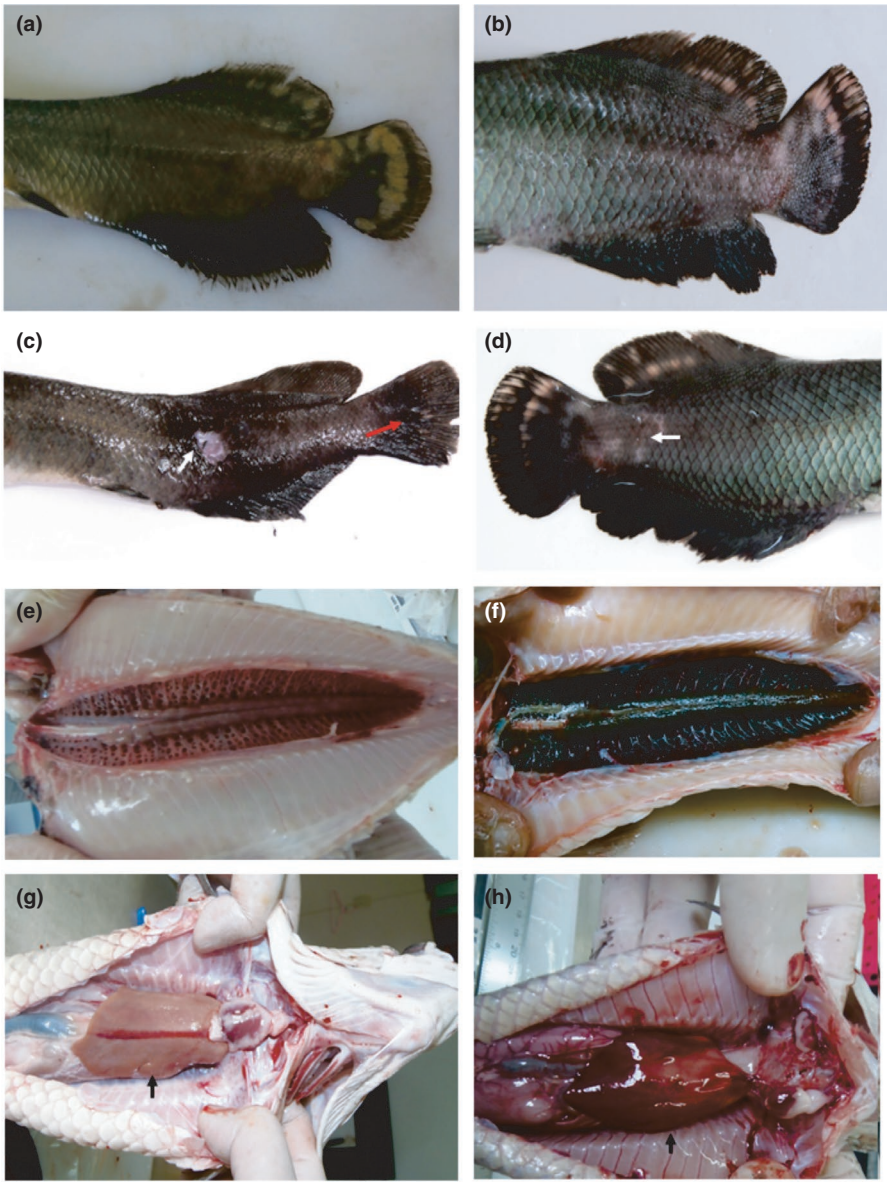

FIGURE 1 *Arapaima gigas* after subcutaneous inoculation with *Aeromonas* spp. in Koch's postulate. (a) Circular-shaped depigmentation of tail fin, caused by *Aeromonas hydrophila* (ATCC 7966). (b) Circular-shaped depigmentation of tail fin, caused by *Aeromonas jandaei*. (c) Inflammatory foci (white arrow) and haemorrhagic signs in tail fin with strong abrasion (red arrow), caused by *Aeromonas hydrophila* plus *Aeromonas jandaei*. (d) Circular-shaped depigmentation of tail fin, caused by *Aeromonas hydrophila*. (e) Modified swim bladder of control fish. (f) Localized hyperaemia and necrotic haemorrhagic areas in modified swim bladder, caused by *Aeromonas hydrophila* plus *Aeromonas jandaei*. (g) Liver (arrow) of control fish. (h) Mild localized hyperaemia in liver (arrow), caused by *Aeromonas hydrophila* plus *Aeromonas jandaei* [Colour figure can be viewed at wileyonlinelibrary.com]

Achimugu, 2019; Pridgeon & Klesius, 2012), due to co-infections by *Aeromonas*. Such problems have occurred also in *A. gigas* fingerlings rearing in high stocking density and inadequate environmental conditions (Proietti-Junior et al., 2017).

Synergistic effects have been reported in occurrences of co-infection with *A. hydrophila* and *A. veronii*, in comparison with the effects of each individual strain (Chandrarathna et al., 2018; Mosser et al., 2015). These co-infections by *Aeromonas* spp. present synergism that involves production and utilization of specific virulence factors and exoproducts. This shows that some bacteria benefit from others, leading consequently to increased intensification of their virulence (Chandrarathna et al., 2018; Kotob et al., 2016; Mosser et al., 2015) and causing mortality among farmed fish. Hence, co-infections have great importance, given their characteristics of complexity, complications and secondary infections due to synergistic effects. This was observed in the current study, with *A. gigas* that was experimentally infected by *A. hydrophila* and *A. jandaei*, which led to a natural outbreak of disease and mortality among these farmed fish.

Aeromonosis is one the most destructive infectious disease in farmed fish, causing great problems in relation to production (Bentzon-Tilia et al., 2016; Dias et al., 2016; Figueras-Salvat &

Ashbolt, 2019; Wamala et al., 2018), as occurred with *A. gigas* in the present study. However, so far, only a few reports have indicated that *A. jandaei* is pathogenic to farmed fish.

The pathogenesis of the disease caused by *Aeromonas* spp. relates to virulence of polygenic or multifactorial origin, involving a complex interaction between these bacteria and host fish (Janda & Abbott, 2010; Pablos et al., 2010). Therefore, knowledge of the genetic diversity of these bacteria can help in investigation of outbreaks, in epidemiological research, in selecting strains for vaccine development and for identifying virulent *Aeromonas* strains that are responsible for outbreaks of mobile *Aeromonas* septicaemia (MAS) in fish (Pang et al., 2015; Rasmussen-Ivey et al., 2016).

Aeromonas species can present antimicrobial resistance associated with the structural components of the bacterial cell (Fowoyo & Achimugu, 2019) and other factors. Antibiotic resistance in these bacteria can be manifested in an intrinsic, natural or acquired manner. Genetic changes can lead to resistance within microbial communities, and indiscriminate use of antibiotics in fish farms has led to development of antibiotic-resistant bacteria (Pereira et al., 2017).

In contrast, we found that the susceptibility profiles of *A. hydrophila* and *A. jandaei* isolated from *A. gigas* were compatible with indigenous species that had not been subjected to selective pressure through use of antimicrobials or through anthropic interference in the environment where they grow naturally. Our eCIM and mCIM tests showed that *A. jandaei* expressed metallo-β-lactamase and *A. hydrophila* expressed serine carbapenemase, and these results were compatible with the intrinsic resistance of this bacterial genus (Balsalobre et al., 2009). In addition, showed carbapenemase activity associated with CphA enzyme in *A. jandaei*, while bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{OXA-48} and bla_{VIM} were not detected. Several β-Lactamase encoding genes (*cph*A, *cep*H and *amp*H) may be detected in chromosome of *Aeromonas* spp.; however, carbapenemase activity is usually related to CphA-producing isolates (Avison et al., 2004; Balsalobre et al., 2009; Rossolini et al., 1996). Balsalobre et al. (2009) reported similar results regarding the presence of *cph*A gene, where 97.6% and 100% of *A. hydrophila* and *A. jandaei* isolates from southeastern Brazil were positive for *cph*A gene, respectively. Carbapenemase activity was observed in 87.8% and 10.9% of the CphA-producing *A. hydrophila* and *A. jandaei* isolates, respectively, and contrarily to our results. Therefore, these results indicate that in farming of *A. gigas*, antibiotics have not used as chemotherapeutic agents because of the following factors: (i) only a limited number of antimicrobial agents have been approved for controlling and treating bacteriosis, because little information on bacteria in fish farming is available; (ii) prescription of antibiotics only by veterinarians; and (iii) medicated rations have a high cost, which make it difficult for fish farmers to have access to them.

In general, *Aeromonas* spp. carry numerous virulence factor genes in their genomes. However, the presence of a gene does not necessarily equate to its expression, which may depend on the context of the host or the niche for infection (Chandrarathna et al., 2018; Mosser et al., 2015). Virulence factors in *Aeromonas* species are also associated with the structural components of the bacterial cell and with exotoxins that are secreted during bacterial metabolism. Thus, the nature of virulence is complex and apparently varies between strains of *Aeromonas* (Fowoyo & Achimugu, 2019; Janda & Abbott, 2010; Li et al., 2011; Perretta et al., 2018; Rasmussen-Ivey et al., 2016).

It has been reported that the majority of *A. hydrophila* isolated from *Ictalurus punctatus* produces haemolysins and enterotoxins. These toxins are responsible for lethality, hemolysis and enterotoxigenicity (Fowoyo & Achimugu, 2019). Virotyping to characterize the pathogenic potential of the isolates was performed in the present study using eight virulence genes that were selected to help in describing how the bacterial infection became established. This took into consideration that there is a direct correlation between the number of virulence factors present in isolates and their pathogenic potential.

Aeromonas hydrophila and *A. jandaei* that were isolated from *A. gigas* were positive for genes relating to expression of *hly*A and *aer*A cytotoxic exotoxins, *gcat* and *dnase* protease coders and the lipase *lip*. Expression of these factors indicates that potential for pathogenicity exists (Janda & Abbott, 2010; Perretta et al., 2018; Rasmussen-Ivey et al., 2016). Hence, experimental inoculation of *A. hydrophila* and/or *A. jandaei* that were isolated from an outbreak in *A. gigas* resulted in the similar clinical signs. Massive concentrations of specific virulence factors were then rapidly produced, which led to sudden mortality among the fish.

In *A. gigas* inoculated subcutaneously with *A. hydrophila* (ATCC 7966) and with *A. hydrophila* and *A. jandaei*, we observed the characteristic symptoms of natural infections caused by these bacteria in this host. Clinical signs of involvement of the respiratory system, hemorrhages in the opercular region, reduced breathing movements and erratic swimming at the surface, with difficulty in remaining at the bottom of the aquarium, were observed in all *A. gigas* individuals that were inoculated with *A. hydrophila* plus *A. jandaei*. These characteristics seem to be common of infection by *Aeromonas* species because they were also previously reported in other studies (Dias et al., 2016; Kumar et al., 2016; Proietti-Junior et al., 2017; Samayanpaulraj et al., 2019).

We did not observe any deaths of *A. gigas* inoculated experimentally with *A. hydrophila* or *A. jandaei*. However, during examinations of *A. gigas* inoculated with an association of *A. hydrophila* and *A. jandaei*, there was 100% of mortality of fish, and we observed the presence of severe macroscopic damages in muscle tissue and in the modified swim bladder, which is used for air breathing. Therefore, these findings suggest that co-infection by both *Aeromonas* species probably started due to skin micro lesions, which led to septicaemia with severe impairment of the respiratory system of *A. gigas*, which has air breathing (Brauner et al., 2004). After long-duration transportation, this would lead to mortality, because this severe damage to the respiratory system of this fish would impede its air breathing. In the aquaculture industry, the transportation includes the lack of feeding and also multiple stressors, which can alter immune response of the fish, decreasing their susceptible to infections (Masud et al., 2019). For this reason, long-duration transport may cause immune disturbances in *A. gigas*, which are detrimental to resistance to infectious diseases for this fish;

thus, we highlight the need for improves during the management of transport procedures to reduce the impact of diseases.

Since we selected only healthy specimens of *A. gigas* for Koch's postulate, the results obtained are representative of infection under natural conditions. Therefore, our experimental results showing co-infection by *A. hydrophila* plus *A. jandaei* in *A. gigas* confirmed that both bacteria were responsible for the outbreak of infection and the mortality of this fish that occurred after transportation.

Respiratory system impairment, which was the most common symptom that we observed in *A. gigas* infected with both strains, had not previously been correlated with *Aeromonas* co-infection, probably due to lack of studies. In addition, other factors also need to be considered: (i) pathogenicity mechanisms depending on the response of the host to stress, which result in the appearance of clinical signs; and (ii) the fact that *Aeromonas* species are opportunistic pathogens that may act in combination with other aetiological agents, through activating the bacterial quorum-sensing mechanism (Janda & Abbott, 2010).

In conclusion, our results provide experimental support for the existence of synergism between *A. hydrophila* and *A. jandaei*, and indicate several implications relating to infection and determination of its virulence in *A. gigas*. These results relating to co-infections may aid fish farmers in making early identification of bacteriosis due to *Aeromonas*, which would enable appropriate treatment for avoidance of economic losses in production of this Amazon fish. Since these pathogens present a persistent threat to the growth of *A. gigas* fingerlings, there is an urgent need to establish effective management and treatment for prevention of bacterial diseases in fish farming. Thus, we suggest improvements in stocking density used for *A. gigas* fingerlings and environmental quality as preventive measures, as well as in quarantining during this early stage of this fish, along with effective vaccination programs, should be implemented to mitigate these bacterial diseases.

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CONFLICT OF INTEREST

The authors declare that they did not have any conflict of interest.

AUTHOR CONTRIBUTIONS

A.A.P.J., D.P.R. and M.T.D. supervised the study, reviewed the manuscript and contributed to conceptualized the study and to methodology. A.A.P.J., L.S.L., E.M.R., Y.C.R. and K.V.B.L. worked on experimental and data analysis. A.A.P.J., D.P.R. and M.T.D. wrote the original draft of the manuscript.

ETHICS STATEMENT

This study was conducted in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee for Animal Use (CEUA/UFAC: No. 23107.009564/2014-29) of the Federal University of Acre and the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN No. A7EC29D), in accordance with the research regulations of the Brazilian Ministry of the Environment (MMA).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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