Virulence determinants in genetically heterogeneous populations of Aeromonads recovered from an urban lagoon

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ABSTRACT

The diversity and distribution of *Aeromonas* spp. associated with virulence profiles from the Rodrigo de Freitas Lagoon were investigated using phylogenetic analysis of *gyrB*/*rpoB* gene sequences for speciation. The concatenated *gyrB*/*rpoB* gene sequences clustered into five species: *Aeromonas punctata*/*caviae* ($n = 37$), *A. hydrophila* ($n = 10$), *A. dhakensis* ($n = 16$), *A. jandaei* ($n = 1$) and *A. enteropelogenes*/*trota* ($n = 3$). The virulence genes (*atc*/*aerA*/*hlyA*/*asp*/*amp*) resulted in 19 virulence profiles, distributed heterogeneously among the five *Aeromonas* species. Out of the 67 isolates, 16% presented five distinct profiles carrying four virulence genes and 7% showed all genes investigated. The hemolytic genes were detected as follows: *act* 54% (37/67), *aerA* 36% (24/67), *hlyA* 26% (18/67) and proteolytic genes such as *asp* 36% (24/57) and *amp* in 85% (57/67) were widely distributed in lagoon sampling stations. Meanwhile, 88% (59/67) and 92% (62/67) of the isolates showed hemolytic and proteolytic activity, respectively. Our results demonstrated that concatenated sequences of the *gyrB* and *rpoB* genes showed to be an adequate approach for the *Aeromonas* speciation and prevalence. The high heterogeneity of virulence genes among the species resulted in several virulence profiles, as well as high percentages of hemolytic and proteolytic activity, demonstrating the necessity of further epidemiological surveys of *Aeromonas* species pathogenicity in an aquatic recreational lagoon.

Key words | *Aeromonas* species, taxonomic analysis, virulence profiles, water quality

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INTRODUCTION

Aeromonas species are Gram-negative facultative anaerobic bacilli and are ubiquitous in aquatic environments, such as freshwater, coastal waters, drinking water, wastewater, estuaries, marine waters, and even chlorinated drinking water (Piotrowska & Popowska 2014). This genus composed of 31 species and 12 subspecies are considered as opportunistic pathogens. *Aeromonas hydrophila*, *A. caviae*, and *A. veronii* biovar *sobria* strains are important human pathogens that could be involved in gastroenteritis, respiratory, and genitourinary problems, wounds, eye, skin, and soft tissue infections, sepsis, and meningitis (Piotrowska & Popowska 2015). Besides, *Aeromonas* spp. are included in the group of emerging pathogens due to their increasingly frequent presence in local and systemic infections of immunologically competent hosts (Seshadri *et al.* 2006; Igbinosa & Okoh 2013).

The taxonomy of *Aeromonas* spp. is complex. For this reason, conventional phenotypic tests do not necessarily

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correspond to results achieved by genetic methods, and this is especially evident in environmental isolates (Puthucheary *et al.* 2012). In addition, the 16S rRNA gene proved to be an unsuccessful phylogenetic marker for *Aeromonas* species identification due to their low taxonomic resolution (Nagar *et al.* 2013). Therefore, sequencing of the housekeeping genes *gyrB* and *rpoB* has been used as a suitable means for the identification and phylogenetic analysis of *Aeromonas* spp. (Soler *et al.* 2004; Küpfer *et al.* 2006; Persson *et al.* 2015).

Aeromonas spp. can express several virulence factors, such as enterotoxins, hemolysins, proteases, lipases, adhesins, hydrolases, surface proteins, flagellum, and pilus. In this way, an infection caused by *Aeromonas* spp. can be multifactorial and multifaceted (Rasmussen-Ivey *et al.* 2016; Igbinosa *et al.* 2017). It may include the expression or secretion of a number of different virulent determinants acting collectively or independently. The *asc*F determinant, which expresses type III secretion as well as the flagellin gene (*fla*), also plays a significant role in *Aeromonas* spp. pathogenicity (Körkoca *et al.* 2014). Also, the ability of *Aeromonas* species to acquire novel virulence and antimicrobial resistance genes has been increasing the spread of virulence genes and resistance determinants that are another issue of significant public health concern (Scoaris *et al.* 2008; Moura *et al.* 2012).

The *hlyA*, *aerA,* and *act* genes are the most prevalent genes expressing the production of hemolytic toxins in *Aeromonas* spp. Aerolysin encoded by the *aerA* gene is an important determinant of virulence that confers high invasiveness as epithelial cells and gastroenteritis (Körkoca *et al.* 2014; Soltan-Dallal *et al.* 2016). The presence of these determinants of hemolytic virulence may be indicative of a clinical condition related to diarrhea (Igbinosa *et al.* 2017).

Combined with the virulence hemolytic factors, extracellular proteases produced by *Aeromonas* spp. play an important role in the invasion and establishing the infection (Kobayashi *et al.* 2017). The serine protease (*Aeromonas sobria* serine protease, ASP) produced by *Aeromonas* species is a member of the kexin subfamily of serine proteases. It induces the destruction of the protein structure, compromising essential functions for host defense (Takahashi *et al.* 2014; Kobayashi *et al.* 2017).

The ASP is considered a powerful virulence factor that participates in the pathogenesis causing edema and septic shock (Imamura *et al.* 2017). In contrast, Aeromonas metalloprotease (AMP) produced by *Aeromonas* spp. is shown to be involved in elastin degradation, an insoluble protein constituent. Metalloproteases are enzymes that require a divalent metal ion for their activity. In addition to the elastolytic action, AMP presents a hydrolytic action to casein (Takahashi *et al.* 2013, 2014).

The distribution of *Aeromonas* in aquatic environments makes emerging reservoirs in the environment of interest to public health due to their ability to contribute to the spreading of virulence and antimicrobial resistance determinants (Igbinosa *et al.* 2017; Piotrowska *et al.* 2017). Additionally, *Aeromonas* species can adhere to biotic or abiotic surfaces forming biofilms (Dias *et al.* 2018). These microorganisms have developed regulatory mechanisms for the formation of biofilms that are also associated with the production of virulence factors and offer advantages in microbial resistance (Rasmussen-Ivey *et al.* 2016).

The Rodrigo de Freitas Lagoon is an urban lagoon designed to protect aquatic communities, the natural landscape, artisanal fishing and recreational activities such as rowing, sailing, and water skiing. For decades, this lagoon has suffered several environmental impacts, mainly due to anthropogenic activities (Crespo & La Rovere 2002; Gonzalez *et al.* 2010). The constant expansion of the population has had negative impacts on the aquatic ecosystems mainly due to the disposal of domestic and chemical residues such as hormones and antibiotics widely used in human and veterinary clinics (Barba-Brioso *et al.* 2010; Nascimento *et al.* 2017). Consequently, this has severely compromised these environments and may thus expose animals and humans to pathogenic microorganisms (Staggemeier *et al.* 2017).

The main objective of this study was to investigate the distribution of *Aeromonas* species and the prevalence of virulence genotypes of the isolates from the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil.

MATERIAL AND METHODS

Studied area and sample collection

The Rodrigo de Freitas Lagoon is located in the southern region of Rio de Janeiro (22°57′02″S; 043°11′09″W) and

had its origin in the drowning of old fluvial basins generated by transgressive–regressive variations of sea level that occurred in the past 6,000 years along the Rio de Janeiro State coast. It possesses a water surface area of 2.2 km^2, an average depth of 2.8 m, a perimeter of 7.8 km, and an approximate water volume of 6,200,000 m^3. Two collections were carried out on March 11, 2015, and March 8, 2016, both during the morning hours, from six collection points (Figure 1). Sampling (1.0 L) was done in triplicate at a depth of approximately 15–20 cm below the surface in a sterile polyethylene bottle. All samples were stored on ice and transferred to the laboratory within 4 h. The physico-chemical parameters such as temperature, pH, conductivity, dissolved oxygen (DO), turbidity, and salinity of the samples were analyzed through Water Quality Checker U-10 (HORIBA). The enumeration of total coliforms and *Escherichia coli* was carried out by the defined substrate method (Colilert, IDEXX). About 3 L of each collection point were concentrated through filtration on cellulose membranes of 0.22 μM porosity (Millipore) for the *Aeromonas* spp. analyses. In the event of filter clogging, additional filters were added.

Aeromonas spp. identification

The cellulose membranes were inoculated in alkaline peptone water broth (APW; Oxoid) and incubated at 37 °C for 24 h. The culture growth was seeded on the *Aeromonas* isolation agar (AIA; Sigma-Aldrich) medium at 30 ± 2 °C for 24 h. Approximately four colonies per sample with typical *Aeromonas* morphology (green colonies with dark end centers) were inoculated in tryptone soy agar (TSA; Sigma-Aldrich) and incubated at 30 ± 2 °C for 24 h for further identification. Bacterial strains were morphologically identified using the Gram-staining reaction and standard bio-chemical tests including sulfide-indole motility, lysine iron agar, catalase, and cytochrome oxidase activity, according to Bergey's Manual of Systematic Bacteriology (Martin-Carnahan & Joseph 2005). Genomic DNA of the isolates was obtained through the Dneasy Tissue Kit (Qiagen GmgH, Hildeitialln, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) targeting a specific region of 16S rRNA gene was carried out to confirm the suggestive *Aeromonas* spp. isolates using the reaction conditions and primers (AERF: 5′-CTACTTTTGCCGGCGAGCGG-3′ and

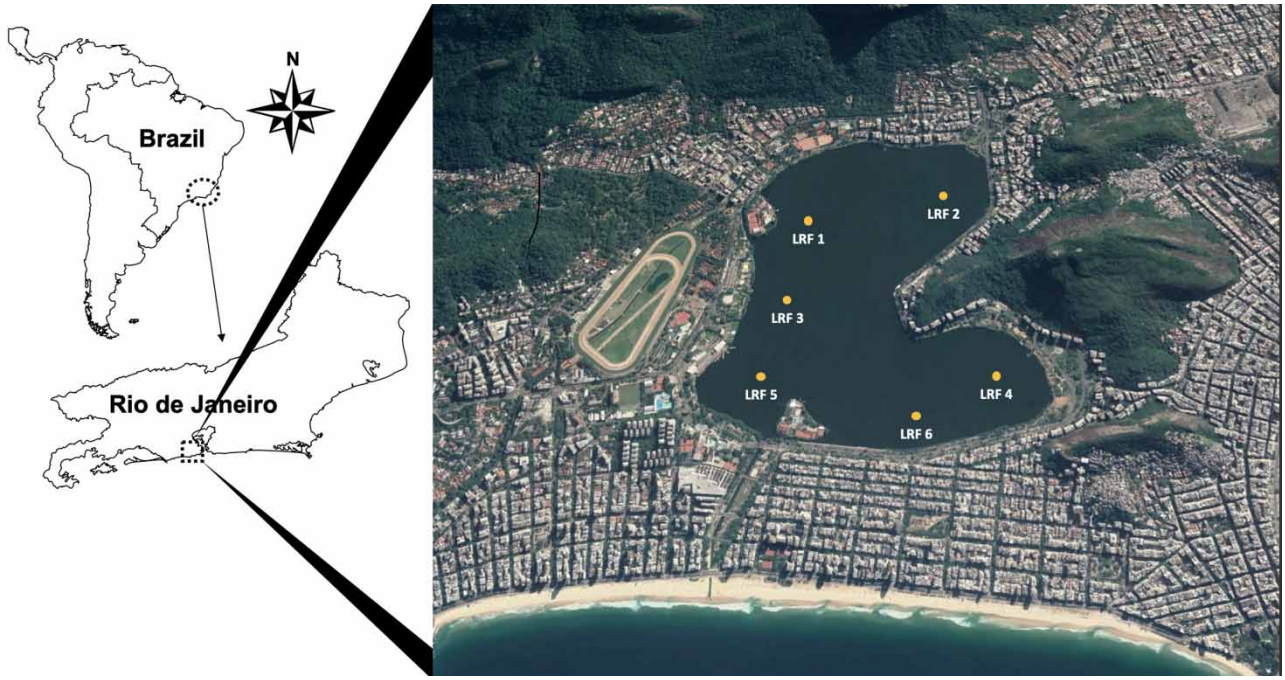

Figure 1 | Geographical location of the Rodrigo de Freitas Lagoon (22°57′22″S/43°11′09″W) in the state of Rio de Janeiro.

AERR: 5′-TGATTCCCGAAGGCACTCCC-3′) according to Lee *et al.* (2002).

Hemolytic and proteolytic activities

Hemolytic and proteolytic activities were evaluated according to Takahashi *et al.* (2014). Briefly, the cultures were spread-plated on nutrient agar plates containing 5% sheep blood and incubated at 37 °C for 48 h, and the presence of clear zones around bacterial cultures indicated hemolytic activity. For proteolytic activity, the cultures were lawn-seeded onto nutrient agar with 1% skim milk at 37 °C for 24 h and the clear zones around bacterial cultures indicate proteolytic activity. Known protease and hemolysin-positive strain *A. hydrophila* ATCC 7966, as well as negative strain *Escherichia coli* ATCC 25922, were included as controls. The reproducibility of the data for all isolates was demonstrated in triplicate.

ERIC typing and *gyrB*/*rpoB* taxonomic analysis

The genotypic analysis of the strains was investigated by amplification of the Enterobacterial Repetitive Intergenic Consensus sequence (ERIC-PCR). The ERIC2 primer was used for amplification and the conditions used were described previously (Versalovic *et al.* 1991). The amplicons were analysed by electrophoresis for 2 h at 50 V in 2% (v/v) agarose gel in 1× TAE buffer (40 mmol L^{-1} Tris base, 20 mmol L^{-1}, 1 mmol L^{-1} sodium acetate, EDTA, pH 8.0) and the standard molecular weight 100 bp DNA standard (Invitrogen Co., Carlsbad, CA, USA). The gel was photographed and analysed using ImageQuant300 (GE, Oppsala, Sweden). Fingerprint patterns were analysed using the BIONUMERICS ver. 6.6 (Applied Maths, Kortrijk, Belgium) using the Dice coefficient and the unweighted pair group method with an arithmetic mean pair group method with arithmetic average. Isolates with 100% level of similarity were considered clonally related. Subsequently, the *Aeromonas* isolates gDNA were submitted to PCR of the *gyrB* gene (gyrB-F2: 5′-GAGGACTACAGCAAGAAGGCCA-3′ and gyrB-R2: 5′-GACTTGGCCTTCTTGCTGTAGTC-3′) and *rpoB* gene (rpoB-F2: 5′-CAACTTCGTCGGTGATCACA-3′ and rpoB-R2: 5′-TGTGATCACCGACGAAGTGG-3′) (Persson *et al.* 2015), resulting in fragments of 650 bp and 560 bp, respectively. The PCR mixture was 1× MasterMix PCR (Promega Corporation); 15 pmol of each primer and approximately 20 ng of template DNA. The reactions were performed in a Mastercycler® EP (Eppendorf) thermocycler and using 94 °C for 5 min; 30 cycles at 94 °C for 40 s, 67 °C and 65 °C for 50 s, respectively, and 72 °C for 40 s and 72 °C for 5 min. The PCR product was analysed on electrophoresis gel for 1 h at 50 V on 1% (v/v) agarose gel without 1× TAE (40 mmol L^{-1} Tris base, 20 mmol L^{-1} sodium acetate, 1 mmol L^{-1} EDTA, pH 8.0) buffer and a 100 bp DNA ladder as the molecular weight standard (Invitrogen Co., Carlsbad, CA, USA). The gel was photographed and analysed using the ImageQuant 300 (GE). Sequencing reactions of *gyrB* and *rpo*B fragments were performed using the Big Dye Terminator kit by capillary electrophoresis on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Phred score sequences greater than or equal to 20 were included in the subsequent analyses. The sequence similarity analysis was performed by the BLASTn software at GenBank.

Phylogenetic analysis

The *Aeromonas* spp. isolates were initially screened by ERIC-PCR to check the individuality of lineages and to determine the clonal variation among them. Subsequently, concatenated *gyrB* and *rpoB* genes (1,008 bp) were aligned by ClustalW of MEGA 7 software (Kumar *et al.* 2016). The phylogenetic tree was carried out by the neighbor-joining algorithm (Saitou & Nei 1987) based on the distance calculated by the Kimura-2 method (Kimura 1980). Bootstrap analysis, with 1,000 replications, provided confidence estimates for tree topologies.

Detection of virulence genes

The virulence genes were screened by PCR using primers and reaction conditions listed in Table 2. PCR analysis was carried out in 25 µl amplification reaction mixtures containing 1× PCR MasterMix (Promega Corporation), 15 pmol of each primer, and about 20 ng of DNA template. The cycling conditions consisted of an initial step of 95 °C for 5 min and 30 cycles of amplification at 95 °C for 30 s, an annealing temperature specific for each primer set (Table 1) for 30 s, 72 °C for 1 min, and a final elongation at 72 °C for 6 min. The reference strain *A. hydrophila* ATCC 7966 and *Klebsiella pneumoniae* ATCC 13883 were

Table 1 | Sequences of specific primers for virulence genes detection (*act*, *aer*A, *hly*A, *asp* and *amp*)

Primers	Sequence (5′–3′)	Target gene	Annealing (°C)	Fragment size (bp)	Reference
act-F	AGAAGGTGACCACCAAGAACA	*act*	55	232	Khor *et al.* (2015)
act-R	AACTGACATCGGCCTTGAACTC				
aer-F	CCTATGGCCTGAGCGAGAAG	*aer*A	55	431	Körkoca *et al.* (2014)
aer-R	CCAGTTCCAGTCCCACCACT				
hlyA-F	GGCCGGTGGCCCGAAGATACGGG	*hly*A	62	597	Igbinosa & Okoh (2013)
hlyA-R	GGCGGCGCCGGACGAGACGGG				
AP-165	CCCTCCAACAGCAACTTCTGGAACCTGGTG	*asp*	58	322	Takahashi *et al.* (2014)
AP-166	TCCGGGTAGGCGGACATCAGCAGCGCCATG				
ASMP-03	AGGACGCCACCGGCCCGGGGGGCAA	*amp*	60	550	Takahashi *et al.* (2014)
ASMP-04	GACCAGCCAGTCGTTGCTCCCCTT				

used as positive and negative PCR control, respectively. PCR products were loaded onto a 1% agarose gel and were separated by electrophoresis at 50 V for 1 h in 1× TAE buffer with a 100 bp DNA ladder as the molecular weight standard (Invitrogen Co., Carlsbad, CA, USA). The gels' analyses were done as described above.

RESULTS

Physicochemical and microbiological parameters

In both collections, the pH values varied between 7.36 and 8.20 among the six points. The turbidity presented a higher elevation at the LRF 1 point and a lower one at LRF 6, and levels of DO varied between 4.41 and 6.70 mg/L. The temperature remained between 21.5 °C and 27.4 °C, and the salinity showed values between 0.9% and 1.32%. The total coliform and *E. coli* counts presented values above the limits of Brazilian water quality guidelines (<2,500 MPN/100 mL) (Table 2).

Identification of *Aeromonas* spp.

A total of 154 possible *Aeromonas* colonies, based on morphological characters in the AIA medium, were isolated from six water samples from the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil. The conventional biochemical tests showed that 56% (87/154) of isolates were Gram-negative rod-shaped and facultative, oxidase, catalase, lysine decarboxylase, and indole positive, all suggestive of the *Aeromonas* genus. Out of the 87 isolates, 88% (77/87) were motile and 47% (41/87) produced H_2S. Subsequently, the confirmation of putative *Aeromonas* spp. by 16S rRNA-PCR showed a single fragment of approximately 954 bp in 87% (76/87) of the isolates, also revealed in the reference strain *A. hydrophila* ATCC 7966, used as a positive control of the reaction.

ERIC-PCR of isolates

ERIC-PCR profiles of the 76 isolates revealed five clusters with 20 strains at the 100% level of similarity. The strains within each group with identical fingerprints obtained at the same point and with the same virulence profile were considered belonging to the same clone and were excluded from the phylogenetic analysis (9/76). Out of the 11 isolates, four (P5803, P5809, P5795 and P5819) from different collection sites but with the same virulence profile were maintained. In addition, five other isolates (P5758, P5802, P5740, P4753 and P4752) from the same point and two others (P5091 and P5090) from different sites presented close ERIC types; however, different virulence profiles were also used in the analysis (Figure 2).

Detection of virulence genes

Among the 67 (100%) isolates, the occurrence of the hemolytic genes demonstrated the presence of the *act* gene 55%

Table 2 | Physical–chemical and microbiological parameters of collection points

Parameters	Standard CONAMA 357/05 class II brackish water	LRF 1		LRF 2		LRF 3		LRF 4		LRF 5		LRF 6	
		Collect 1	Collect 2	Collect 1	Collect 2	Collect 1	Collect 2	Collect 1	Collect 2	Collect 1	Collect 2	Collect 1	Collect 2
pH	6.5 and 8.5	7.6	7.8	7.5	8	7.65	8	7.43	7.5	7.36	7.5	7.36	8.2
Temperature (°C)	–	27.4	23.4	27.2	24.5	27.1	23.3	27.1	23.3	23.7	23.3	21.5	22.7
Dissolved oxygen (mg/L)	>4.0	4.77	4.7	4.75	6.7	5.21	5.2	4.52	4.6	4.41	4.9	4.7	5
Conductivity (mS/cm)	–	21.8	15.4	21.2	15.8	21.2	15.4	21.4	15.4	21.3	15.5	20.7	15.3
Turbidity (UNT[a])	–	17	13	11	4	12	6	8	8	8	4	6	3
Salinity (‰)	≥0.5 and 30	1.32	0.9	1.27	0.9	1.27	0.9	1.28	0.9	1.28	0.9	1.26	0.9
Total coliforms (MPN/100 mL)[b]	2,500	6,867	10,112	24,196	10,112	7,915	10,112	19,863	10,112	10,112	10,112	11,199	9,606
E. coli (MPN/100 mL)[b]	2,500	4,106	10,112	5,794	10,112	3,725	10,112	24,196	10,112	7,215	10,112	8,664	9,606

[a]Nephelometric turbidity units.
[b]Most probable number.

Figure 2 | Dendrogram showing genetic relationships among 67 *Aeromonas* species based on one ERIC primer. Cluster analysis was performed by the UPGMA method on a Jaccard's similarity matrix.

(37/67), *aerA* gene 36% (24/67), and *hlyA* 26% (18/67). According to the distribution of the virulence genes, the *aerA* gene appeared in isolates from four of the five points examined (LRF 1, 2, 3, and 5) as well as the *hlyA* gene (LRF 1, 3, 4, and 5). The *act* gene appeared in isolates from the five points examined. The proteolytic *asp* gene occurred in 36% (24/67) of isolates from LRF 1, 2, 3, 4, 5,

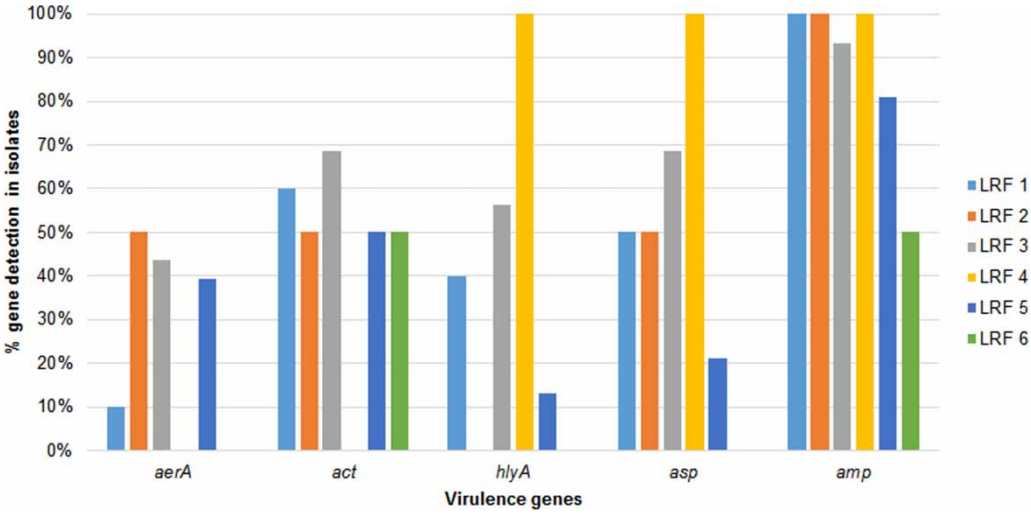

Figure 3 | Prevalence of virulence genes according to collection points.

and *amp* gene in 85% (57/67) from all six points. Seven percent (5/67) of the isolates presented all five genes and 1% (1/67) did not reveal either of them (Figure 3).

Phylogenetic analysis associated with virulence profiles

The concatenated *gyrB/rpoB* gene sequences are clustered into five different species (*A. punctata/caviae* ($n = 37$), *A. hydrophila* ($n = 10$), *A. dhakensis* ($n = 16$), *A. jandaei* ($n = 1$) and *A. enteropelogenes/trota* ($n = 3$)). The sequences were assigned to any species if the best matching reference sequence available in the database showed ≥97% homology. The virulence genes (*atc*, *aerA*, *hlyA*, *asp*, and *amp*) resulted in 19 profiles with heterogeneous distribution among the isolates. The profile IV (*amp*) was the most prevalent among the isolates 22% (15/67) followed by profile IX (act/amp) 18% (12/67) which were present only in *A. punctata/caviae* strains from LRF1 ($n = 5$), LRF 2 ($n = 1$), LRF 3 ($n = 4$), LRF 5 ($n = 15$) and LRF 6 ($n = 2$). The profiles XIX (*aerA/hlya/act/asp/amp*), XIII (*aerA/act/amp*), XIV (*hlyA/act/asp/amp*), and III (*aerA*) from points LRF 5, LRF 3 and LRF 1 showed distribution among the four species (Figure 4). *A. hydrophila* presented five profiles, 33% (3/10) of the strains exhibited the profile XIX (*aerA/hlya/act/asp/amp*), followed by profiles XIV (*hlya/act/asp/amp*), XII (*act/asp/amp*), XV(hlya/*asp/amp*), and X (*act/asp*) from points LRF1, LRF 2, LRF 3, LRF 4, and LRF 5. *A. dhakensis* also showed great diversity of virulence profiles (XIX, XVIII, XVI, XIV, XIII, XII, XI, VI, V, III, I) from different collection points. It is noteworthy that strains containing profiles XIX and I were isolated from the LRF 5. The three strains of *A. enteropelogenes/trota* presented profiles V (*act/aerA*) and III (*aerA*) and both of them from LRF 5. A unique strain of *A. jandaei* belonging to profile XV was isolated from LRF 1 (Figures 4 and 5).

Hemolytic and proteolytic activities

The hemolytic and proteolytic activities presented heterogeneous results among the five *Aeromonas* species identified (Table 3). Eighty-eight percent (59/67) of isolates had hemolytic activity. Of these 59, 83% (49/59) exhibited at least one of the three hemolytic genes studied and 17% (10/59) did not reveal the investigated genes. The remaining 12% (8/67) had neither hemolytic activity nor any of the three genes studied (*A. punctata/caviae* ($n = 7$); *A. dhakensis* ($n = 1$)). Ninety-three percent (62/67) of isolates showed proteolytic activity. Ninety-two percent (62/67) revealed at least one of the two genes investigated and 7% (5/67) did not present any genes. Of the five remaining isolates that did not present proteolytic activity, 80% (4/5) did not present any of the two proteolytic genes (*A. dhakensis* ($n = 2$); *A. enteropelogenes/trota* ($n = 2$)) and 20% (1/5) exhibited the two genes studied (*A. hydrophila*).

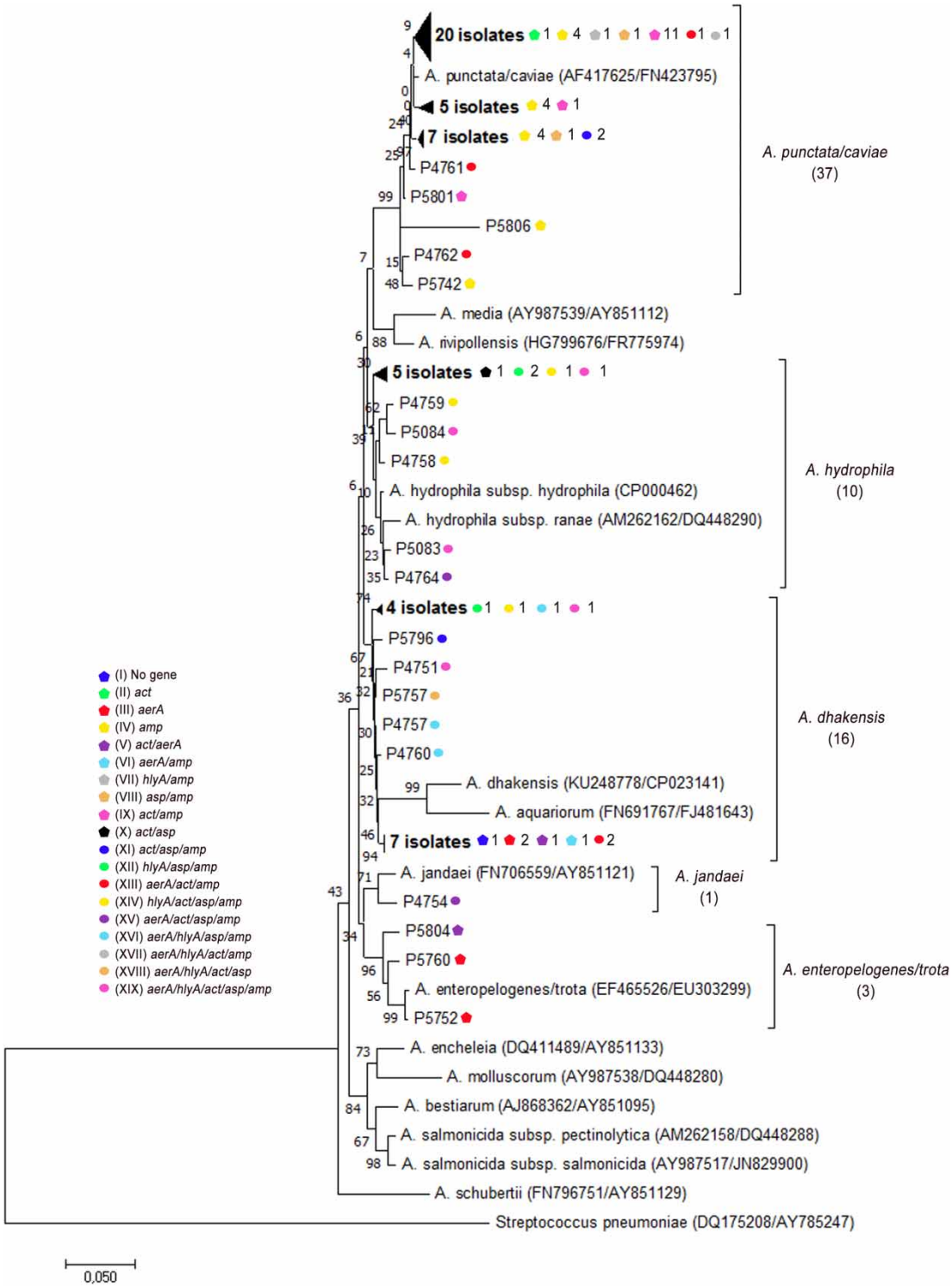

Figure 4 | Neighbor-joining tree constructed from concatenated partial *gyrB–rpoB* (1,008 bp) sequences obtained from environmental *Aeromonas* spp. from the Rodrigo de Freitas Lagoon, compared to reference sequences obtained from GenBank. The colorful points indicated the virulence profiles. Numbers at the nodes indicate bootstrap values as the percentage of 1,000 replicates. The scale bar indicates 5% sequence divergence. Please refer to the online version of this paper to see this figure in color: http://dx.doi.10.2166/wh.2019.288.

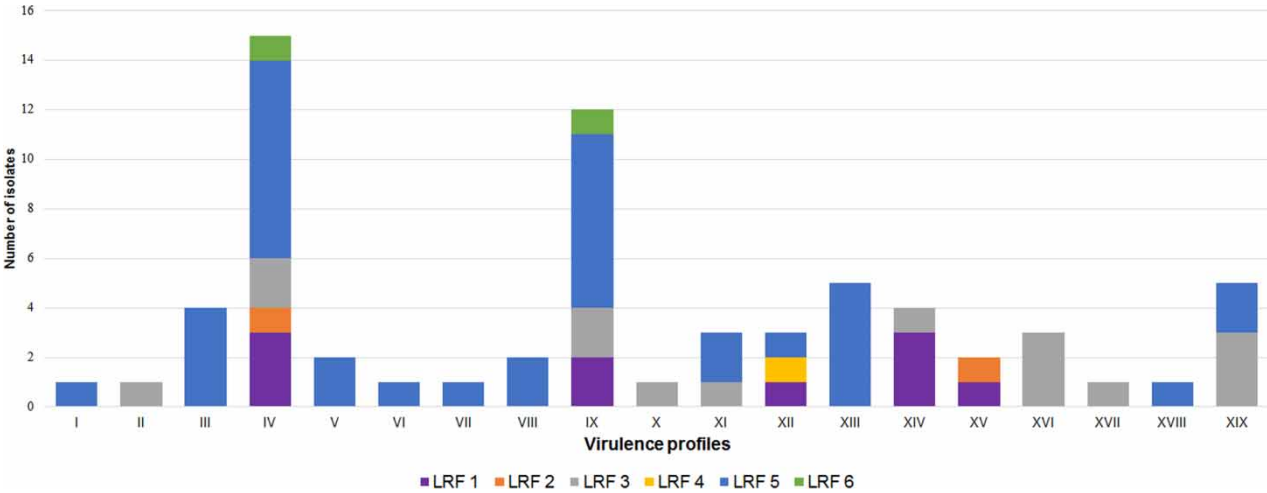

Figure 5 | Distribution of virulence profiles according to collection points. (I) no gene; (II) *act*; (III) *aerA*; (IV) *amp*; (V) *act/aerA*; (VI) *aerA/amp*; (VII) *hlyA/amp*; (VIII) *asp/amp*; (IX) *act/amp*; (X) *act/asp*; (XI) *act/asp/amp*; (XII) *hlyA/asp/amp*; (XIII) *aerA/act/amp*; (XIV) *hlyA/act/asp/amp*; (XV) *aerA/act/asp/amp*; (XVI) *aerA/hlyA/asp/amp*; (XVII) *aerA/hlyA/act/amp*; (XVIII) *aerA/hlyA/act/asp*; (XIX) *aerA/hlyA/act/asp/amp*.

Table 3 | Hemolytic and proteolytic activity of *Aeromonas* spp. strains

	No. of positive strains (%)	
Species	**Hemolytic activity**	**Proteolytic activity**
A. hydrophila ($n = 10$)	100	92
A. dhakensis ($n = 16$)	94	89
A. enteropelogenes/trota ($n = 3$)	100	33
A. jandaei ($n = 1$)	100	100
A. punctata/caviae ($n = 37$)	76	100

DISCUSSION

The wide distribution of *Aeromonas* species in aquatic environments indicates that their possible interactions with animals and humans are continuous and inevitable, allowing their opportunistic pathogenicity.

In this paper, we demonstrated that the isolation by *Aeromonas* selective culture media, as well as the phenotypical identification of *Aeromonas* spp., revealed limitations since of the 154 initial isolates, only 76 were confirmed as belonging to this genus by PCR amplification of the 16S rRNA gene. Conventional phenotypic tests do not necessarily correspond to the results obtained by the molecular methodology, as already evidenced in *Aeromonas* spp. (Ørmen *et al.* 2005; Puthucheary *et al.* 2012). Discrepancies in the identification of *Aeromonas* spp. resulting from a poor correlation between phenotypic schemes and molecular approaches are well documented in the literature (Kozinska 2007; Beaz-Hidalgo *et al.* 2010).

The physical–chemical parameters analysed showed small variations in temperature, pH, OD, and conductivity (Table 2). However, these variations do not alter the growth of *Aeromonas* spp., considering that although the optimum growth temperature is around 28 °C, they can multiply in more extreme temperatures between 5 °C and 41 °C (Popoff 1984). Additionally, studies have shown that the *Aeromonas* spp. prevalence was higher in the highest water temperature, where the maximum count was 4.0×10^5 CFU/g (gram) in sediment samples (Seidler *et al.* 1980; Pathak *et al.* 1988). Other relevant data in our study are related to salinity, where its decrease during the second collection may be associated with the intensification of freshwater inflow due to the rainfall season. This issue may also be related to the upsurge of *E. coli* levels in five of the six points investigated. In addition, the decrease in salinity levels may also justify the growth of *Aeromonas* spp. from LRF 1, 3, and 5.

Also, the hemolytic activity occurred in 88% (59/67) of the isolates. Out of these 59 isolates, 10 did not present any of the hemolytic genes investigated. The other 12% (8/67) that did not show hemolytic activity were identified as *A. punctata/caviae* and *A. dhakensis*. It is important to emphasize that negative PCR results for both the hemolytic

and proteolytic genes analyzed do not exclude pathogenicity, since at least some strains of *Aeromonas* have a wide range of virulence factors not evaluated by us that allow them to overcome host defenses and establish an infection. Hoel *et al.* (2017) demonstrated the presence of β-hemolysis in 91% of their isolates, and this result was highly related to the species identified. Besides, not all *A. media* and *A. punctate/caviae* isolates had hemolytic activities including the *A. punctata/caviae* reference strain.

In our study, the proteolytic virulence genes screened *asp* and *amp* were present in 87% (66/71) and 94% (71/76) isolates with proteolytic activity, respectively. This activity was also shown in the majority of isolates of *A. hydrophila*, *A. veronii* bv. *sobria*, and *A. punctata/caviae* from patients hospitalized with acute gastroenteritis in Rio Grande do Sul, Brazil (Guerra *et al.* 2007).

Our results revealed data not yet shown regarding the diversity of *Aeromonas* spp. in the waters of the Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil. The phylogenetic position of concatenated sequences of *gyrB* and *rpoB* genes clustered the isolates with reference sequences of the respective species. *Aeromonas punctata* (formally *A. caviae*) was prevalent with 37 isolates (55%) followed by 10 isolates (15%) of *A. hydrophila*, 16 isolates (24%) of *A. dhakensis* (formally *A. aquariorum*), three isolates (4%) of *A. enteropelogenes* (formally *A. trota*), and one isolate (2%) of *A. jandaei* (Figure 3). As in our study, the concatenated sequence of *rpoD/gyrB* genes increased the resolution and allowed an unequivocal speciation of the isolates from aquarium water in Sri Lanka (Jagoda *et al.* 2014).

Of the 31 recognized species of the genus *Aeromonas*, a subgroup of four species is most frequently implicated in human infections and comprises the species *A. hydrophila*, *A. caviae*, *A. veronii* bv. *sobria*, and *A. dhakensis* (Janda & Abbott 2010; Teunis & Figueras 2016). *Aeromonas punctata/caviae* was also prevalent in studies in Europe, the United States, and India, followed by *A. hydrophila* and *A. veroni* bv. *sobria* (Albert *et al.* 2000; Sinha *et al.* 2004; Borchardt *et al.* 2009). These data are in agreement with a study in Spain, where between 1989 and 1999 these three species were responsible for 90% of *Aeromonas* associated with gastroenteritis (Velasco-Muñoz *et al.* 1999). It is important to emphasise that our study revealed the prevalence of these same potentially pathogenic species in the lagoon, which indicates a possibility of risks to human health.

Although our sampling points were distinct, the isolation of these five species from all collection points suggests that they are widely spread in this lagoon. These data are worrying since recently a study showed evidence suggestive of colonization and successful infection by lineages *Aeromonas* genus in humans, transmitted by water (Khajanchi *et al.* 2010). Voss *et al.* (1992) also found that 13 of 28 wound and soft tissue infections over a 4-year period were associated with water-related infection and 43% of the total infection could be directly related to lakes or river water.

In this study, the spreading of virulence genes into five species of *Aeromonas* genus showed that 99% (66/67) of the isolates presented at least one of the genes studied, whereas 12% (5/67) including *A. hydrophila* and *A. dhakensis* presented all virulence genes studied. A survey carried out in ornamental fish breeding sites showed results similar to those presented here, where all isolates had heterogeneous virulence profiles (three to seven virulence genes) (Guerra *et al.* 2007). Studies of virulence genes have been used as a practical approach to evaluate the genetic potential of *Aeromonas* spp. expressing virulence factors (Puthucheary *et al.* 2012). Here, we demonstrated high heterogeneity of virulence factors among the five species identified, which resulted in 19 virulence profiles. This diversity was also demonstrated in different geographic regions such as Sri Lanka, China, United States, and Korea (Nawaz *et al.* 2010; Hu *et al.* 2012; Yi *et al.* 2013; Jagoda *et al.* 2014).

Due to the complex pathogenesis of species of *Aeromonas* genus, none of the factors associated with virulence should be exclusively responsible for symptoms or early infections (Albert *et al.* 2000). In our study, the heterogeneity of virulence profiles mainly in *A. hydrophila*, *A. caviae*, and *A. dhakensis* leads us to suggest the high pathogenicity of these species in the lagoon waters. In fact, the presence of the *aerA*, *alt*, *act*, *eprCAI*, and *ahp* genes could be seen as an indicator of virulence in both clinical and environmental isolates (Sha *et al.* 2002; Wang *et al.* 2008).

CONCLUSION

In conclusion, our results revealed that *gyrB* and *rpoB* genes' concatenated sequences proved to be an adequate approach for genetic identification of *Aeromonas* species as well as in

their prevalence in the urban lagoon. The heterogeneity in the distribution of genes associated with virulence among the species resulted in several profiles and high percentages of hemolytic and proteolytic activity, indicating the pathogenic potential of these species.

Finally, our data suggest that the lagoon waters must fulfill high-quality requirements aiming at the improvements of preventive actions regarding possible negative impacts on public health. The occurrence of species frequently implicated in human infections and their high probability of transmission in aquatic environments reinforce the relevance and continuity of this investigation.

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The authors declare that there is no conflict of interest.

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