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Detection of putative virulence traits in *Aeromonas* spp. from brazilian food chain through phenotypic tests and polymerase chain reaction

Detecção de características de virulência em *Aeromonas* spp. isoladas da cadeia alimentar brasileira através de testes fenotípicos e reação em cadeia de polimerase

Detección de factores de virulencia en *Aeromonas* spp. aisladas de la cadena alimentaria brasileña mediante pruebas fenotípicas y reacción en cadena de la polimerasa

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Emily Moraes Roges
ORCID: https://orcid.org/0000-0001-7829-3382
National Reference Laboratory for Enteric Diseases, Brazil
Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Brazil
Federal Fluminense University, Brazil
E-mail: emyrogers@gmail.com
Salvatore Siciliano
ORCID: https://orcid.org/0000-0002-0124-8070
National School of Public Health, Brazil
Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Brazil
E-mail: gemmlagos@gmail.com
Camila Domit
ORCID: https://orcid.org/0000-0001-6158-6963
Laboratory of Ecology and Conservation of Marine Mammals and Reptiles, Brazil
Federal University of Paraná, Brazil
E-mail: cadomit@gmail.com
Paulo Henrique Ott
ORCID: https://orcid.org/0000-0003-3303-1420
State University of Rio Grande do Sul, Brazil
E-mail: paulo-ott@uergs.edu.br
Lucia Helena Berto
ORCID: https://orcid.org/0000-0001-6653-0002
General Coordination of Public Health Laboratories, Brazil
E-mail: lucia.berto@saude.gov.br
Maria Helena Cosendey de Aquino
ORCID: https://orcid.org/0000-0002-5906-8101
Federal Fluminense University, Brazil
E-mail: maryhel@uol.com.br
Dalia dos Prazeres Rodrigues
ORCID: https://orcid.org/0000-0003-1101-5518
Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Brazil
E-mail: daliarodrigues@yahoo.com.br

Abstract
Aeromonads are natural inhabitants of aquatic environments and may be associated with numerous infections in humans and animals. The human disease may range from self-limiting diarrhea to a more severe form. The pathogenesis of infections are multifactorial, because of their wide variety of virulence factors. This study aimed to evaluate virulence markers in *Aeromonas* isolates and determinate their virulence profiles. There were analyzed 120 strains of *A. caviae* (n = 57) and *A. hydrophila* (n = 63) from human, animal and environmental sources between 2008 and 2012. All isolates were examined to detect extracellular virulence enzymes by phenotypic activity and the presence of virulence genes *hly*A, *aer*A, *lip*, *gcat*, *ser*, *act*, *alt* and *exu* by PCR. We observed more than 90% of positivity for at least one phenotypic virulence factors and all of them had at least two of the virulence genes measured. Among the virulence enzymes detected, the DNase was present in 93.33% of the isolates and hemolytic activity was detected in 62.5%. Collagenase and elastase were found in 13.33% and 10.83% of the strains, respectively. We found *exu* and *gcat* in 100% of the isolates, *lip* in 40.83%, *aer*A in 40.83%, *hly*A in 40%, *alt* in 19.16%, *act* in 17.5% and *ser* in 11.66%. It was possible to observe different combinations of virulence factors between the isolates showing the multifactorial virulence among the isolates. The diversity of virulence profiles found in this study hint heterogeneity in clones circulating in our environment.
Keywords: *Aeromonads*; *Aeromonas hydrophila*; *Aeromonas caviae*; Virulence genes; Virulence factors.

Resumo

Aeromonas spp. são habitantes naturais de ambientes aquáticos e podem estar associadas a numerosas infecções em humanos e animais. A doença humana pode variar desde diarreia autolimitada até uma forma mais severa. A patogênese das infecções é multifatorial, devido à sua grande variedade de fatores de virulência. Este estudo teve como objetivo avaliar os marcadores de virulência em *Aeromonas* e determinar seus perfis de virulência. Foram analisadas 120 cepas de *A. caviae* (n = 57) e *A. hydrophila* (n = 63) de fonte humana, animal e ambiental entre 2008 e 2012. Todos os isolados foram examinados para detectar enzimas extracelulares de virulência por atividade fenotípica e a presença de genes de virulência *hly*A, *aer*A, *lip*, *gcat*, *ser*, *act*, *alt* e *exu* por PCR. Observamos mais de 90% de positividade para pelo menos um fator de virulência fenotípica e todos eles tinham pelo menos dois dos genes de virulência. Entre as enzimas de virulência detectadas, DNase estava presente em 93,33% dos isolados e a atividade hemolítica foi detectada em 62,5%. A colagenase e elastase foram encontradas em 13,33% e 10,83% das cepas, respectivamente. Encontramos *exu* e *gcat* em 100% dos isolados, *lip* em 40,83%, *aer*A em 40,83%, *hly*A em 40%, *alt* em 19,16%, *act* em 17,5% e ser em 11,66%. Foi possível observar diferentes combinações de fatores de virulência entre os isolados mostrando a virulência multifatorial entre os isolados. A diversidade de perfis de virulência encontrada neste estudo sugere heterogeneidade nos clones que circulam em nosso ambiente.
Palavras-chave: *Aeromonads*; *Aeromonas hydrophila*; *Aeromonas caviae;* Genes de virulência; Fatores de virulência.

Resumen

Aeromonas son habitantes naturales de los ambientes acuáticos y pueden estar asociadas a numerosas infecciones en humanos y animales. La enfermedad humana puede variar desde una diarrea autolimitada hasta una forma más grave. La patogénesis de las infecciones es multifactorial, debido a su gran variedad de factores de virulencia. El objetivo de este estudio es evaluar los marcadores de virulencia en aislados de Aeromonas y determinar sus perfiles de virulencia. Se analizaron 120 cepas de *A. caviae* (n = 57) y A. *hydrophila* (n = 63) de fuente humana, animal y ambientales entre 2008 y 2012. Se examinaron todos los aislados para detectar enzimas de virulencia extracelular por actividad fenotípica y los genes de virulencia *hly*A, *aer*A, *lip*, *gcat*, *ser*, *act*, *alt* y *exu* por PCR. Observamos más del 90% de positividad para al menos un factor de virulencia fenotípico y todos ellos tenían al menos dos genes de virulencia medidos. Entre las enzimas de virulencia detectadas, DNasa estaba presente en 93,33% de los aislados y se detectó actividad hemolítica en 62,5%. Colagenasa y elastasa se encontraron en 13,33% y 10,83% de las cepas, respectivamente. Encontramos *exu* y *gcat* en todos los aislados, *lip* en 40,83%, *aer*A en 40,83%, *hly*A en 40%, *alt* en 19,16%, *act* en 17,5% y *ser* en 66%. Se observó diferentes combinaciones de factores de virulencia entre los aislados, que demuestra la virulencia multifactorial em *Aeromonas* spp. y la diversidad de perfiles de virulencia encontrados sugiere la heterogeneidad de los clones que circulan en nuestro medio.
Palabras clave: *Aeromonads*; *Aeromonas hydrophila*; *Aeromonas caviae;* Genes de virulencia; Fatores de virulencia.

1. Introduction

Aeromonas species are Gram-negative rod-shaped ubiquitous free-living organisms found in aquatic ecosystems, which can quickly colonize an exceptionally wide variety of habitats and hosts. These microorganisms have already been isolated from water, mammals, fish, invertebrates, birds, insects and soil (Chaix et al., 2017; Fernandes-Bravo & Figueras, 2020; Grilo et al., 2021).

Recognized as emerging pathogens associated with gastroenteritis worldwide, Aeromonads are also implicated as an etiologic agent in a variety of extra intestinal human diseases. The spectrum of disease severity is broad, ranging from mild diarrhea to life-threatening necrotizing fasciitis, septicemia, meningitis, cholera-like illness, and hemolytic-uremic syndrome (Fernandes-Bravo & Figueras, 2020; Gonçalves Pessoa et al., 2019). *A. caviae* and *A. hydrophila* lie between most common Aeromonads isolates from human source and are especially implicated in cases of diarrhea (Mbuthia et al., 2018). While traditionally regarded as a pathogen of immunocompromised humans; there have been several reported *Aeromonas* infections of immune competent individuals (Batra, et al., 2016).

Aeromonas can be isolated from several natural sources, particularly aquatic environments. Contact with fish and other aquatic animals develop in a continuous and almost inevitable way (Gonçalves Pessoa et al., 2019). These microorganisms are indigenous to aquatic environments and have been isolated from surface, underground, potable, bottled, residual, seawater, and irrigation waters (Fernandes-Bravo & Figueras, 2020) and have been isolated from chlorinated drinking water in many countries. They occur in distribution systems where biofilm formation protect the strains of disinfection (Skwor

et al., 2020; van Bel et al., 2020; Miyagi et al., 2021). Metagenomic studies of wastewater revealed *Aeromonas* to be one of the prevailing bacteria probably due to its capacity to regrow in the sewerage system (Gonçalves Pessoa et al., 2019).

Aeromonas may express different virulence factors such as extracellular products including cytotoxic and cytotonic enterotoxins, hemolysins and hydrolytic enzymes, which may create disturbances and biochemical changes in the host's body and as with all pathogens, disease is the result of complex molecular interactions between bacterium, environment, and host. *Aeromonas* virulence factors are involved in the establishment of infection and can act meaningfully in the development of the disease (Barger et al., 2020, Jin et al., 2020). *Aeromonas* species may produce different toxins like as aerolysin (*aer*A), cytolytic enterotoxin (*act*), cytotonic enterotoxin (*ast*), heat labile cytotonic enterotoxin (*alt*), as well as many enzymes that are considered virulence factors, and may even possess a Type III Secretion System and extracellular hydrolytic enzymes: amylases, arilamidases, esterases, elastases, desoxirribonucleases, chitinases, peptidases and lipases (Citterio & Francesca, 2015). Although these virulence factors contribute to pathogenesis of animal and human diseases caused by *Aeromonas*, none of them alone may be responsible for all symptoms of disease stages. Because of the complexity in pathogenesis of *Aeromonas* spp, due to its multi-factorial nature, identification of multiple virulence factors of this genus, become important (Awan et al., 2018).

In Brazil, several studies indicate the occurrence of Aeromonads from aquatic ecosystem, seafood (fish, oysters, mussels and crabs), marine and estuarine animals (Castelo-Branco et al., 2015; Silva et al., 2017; Cardoso et al., 2018; Marinho-Neto et al., 2019; Vaz Rodrigues et al., 2019). Since *Aeromonas* are known as zoonotic agents, those studies are important to public health because of association with foodborne disease and extra intestinal infections).

Knowing this background we purpose investigate the production of extracellular hydrolytic enzymes and eight virulence genes in *A. hydrophila* and *A. caviae* isolated from different sources in Brazilian food chain and their relevance in public health.

2. Methodology

Strain Selection

The study was performed with 120 strains of *Aeromonas caviae* (n = 57) and *A. hydrophila* (n = 63) isolated from human, animal and environmental sources from 2008 to 2012 selected in the database of National Reference Laboratory for Enteric Diseases / Oswaldo Cruz Institute / FIOCRUZ / Brazil (Table 1).

Reisolation and biochemical confirmation of *Aeromonas caviae* and *A. hydrophila*

Strains of *Aeromonas caviae* and *Aeromonas hydrophila* kept in Buffered Nutrient Agar were streaked onto Glutamate Starch Phenol Red Agar – GSP Agar (Merk) and incubated at 37ºC (18-24h) and subcultured into Kligler Iron Agar (Oxoid) and Lysine Iron Agar (Oxoid). The biochemical characteristics at fenospecies level confirmed the resistance to O/129 (2,4 diamino, 6,7 diisopropilpteridine – Sigma Aldrich), fermentation of glucose, lactose, sucrose and arabinose, glucose gas production, ornithine and lysine decarboxylation, arginine dehydrolase, hydrolysis of esculin, growth in different concentrations of NaCl and glucose fermentation pathway in VP medium (Martin-Carnahan & Joseph, 2015).

Table 1 - Source and distribution of 120 of *Aeromonas hydrophila* and *A. caviae*.

	Aeromonas hydrophila	*Aeromonas caviae*
Human		
Blood	1	1
Lung Fragment	1	2
Secretion	1	0
Skin wound	1	0
Stools	15	20
Synovial liquid	1	0
Animal[+]		
Arctocephalus australis	0	9
Arctocephalus gazella	4	1
Bos taurus	1	0
Delphinus sp	0	1
Eubalaena australis	5	1
Gallus gallus	4	0
Genidens barbus	2	0
Kogia sima	1	0
Lagenodelphis hosei	0	7
Megaptera novaeangliae	10	13
Oreochromis niloticus	1	0
Sporophila maximiliani	1	0
Pontoporia blainvillei	3	1
Stenella coeruleoalba	7	0
Environmental		
River water	4	1

+These are the popular names of the animals listed: *Arctocephalus australis* - South American Fur Seal; *Arctocephalus gazella* - Antartic Fur Seal; *Bos taurus* - Cattle; *Delphinus* sp - Comon Dolphin; *Eubalaena australis* - Southern Right Wale; *Gallus gallus* - Chicken; *Genidens barbus* - White Sea Catfish; *Kogia sima* - Dwarf Sperm Whale; *Lagenodelphis hosei* - Fraser's Dolphin; *Megaptera novaeangliae* - Humpback Whale; *Oreochromis niloticus* - Nile Tilapia; *Sporophila maximiliani* - Great-billed Seed Finch; *Pontoporia blainvillei* - La Plata Dolphin; *Stenella coeruleoalba* - Striped Dolphin. Source: The authors.

Phenotypic identification of enzymes

Aeromonas spp. were analyzed for the presence of virulence factors represented by the proteolytic enzymes' elastase and collagenase, the hemolytic activity and nuclease activity. The phenotypic activities investigated were described previously (Chacón et al., 20003; Lafisca et al., 2008). Briefly they were collagenase and elastase activities in BHI (Brain Heart Infusion) broth (37 ºC/18-24h) and then streaked onto Agar BHI added with 1% of collagen, Agar BHI added with 1% of elastin considering positive colonies result as a clearing zone (translucent and luminous halo) under and around the spots inoculums. Hemolysis, assayed at 37 ºC (18-24h) on TSA agar (Difco, Barcelona, Spain) containing 5% sheep blood. The presence of a clear colorless zone surrounding the colonies indicated hemolytic activity. DNase activity was tested at 37 ºC (18-24h) on DNase agar (Oxoid, Barcelona, Spain) and a halo around the colonies indicated nuclease activity.

Detection of putative virulence genes

All isolates were investigated to detect the target virulence genes hemolysin (*hly*A), aerolysin (*aerA*), cytotoxin (*act*), cytotonic heat-labile enterotoxin (*alt*), lipase (*lip*) and glycerophospholipid cholesterol acyl transferase (*gcat*), serine protease (*ser*) and DNase gene (*exu*) (Heuzenroeder et al., 1999; Chacón et al., 2003; Sen & Rodgers, 2004) using polymerase chain reaction (PCR). Aeromonads DNA was purified with DNA Dnaeasy Tissue – Qiagen. The primers and PCR conditions are listed in Table 2. The PCR was performed using a thermal cycler (Biorad MyCycler™ Thermal Cycler System with Gradient, USA). Samples were electrophoresed in 2% agarose gels (90V, 60 minutes), then stained with ethidium bromide and photographed using UV transillumination (Image Quant).

Table 2 - Polymerase chain reaction primers, annealing temperatures, and PCR products for *Aeromonas* virulence gene.

Gene	Sequence	Annealing (ºC)	Amplicon	Reference
aerA	F 5'-CCTATGGCCTGAGCGAGAAG-3' R 5'-CCAGTTCCAGTCCCACCACT-3'	56ºC	431bp	Chacón et al., 2003
lip	F 5'-CA(C/T)CTGGT(T/G)CCGCTCAAG -3' R 5'-GT(A/G)CCGAACCAGTCGGAGAA -3'	56ºC	247bp	Chacón et al., 2003
gcat	F 5'-CTCCTGGAATCCCAAGTATCAG -3' R 5'-GGCAGGTTGAACAGCAGTATCT -3'	56ºC	237bp	Chacón et al., 2003
ser	F 5'-CACCGAAGTATTGGGTCAGG -3' R 5'-GGCTCATGCGTAACTCTGGT -3'	60ºC	350bp	Chacón et al., 2003
exu	F 5'-(A/G)GACATGCACAACCTCTTCC -3' R5'-GATTGGTATTGCC(C/T)TGCAA(C/G)-3'	54ºC	323bp	Chacón et al., 2003
hlyA	F 5'-GGCCGGTGGCCCGAAGATACGGG -3' R 5'-GGCGGCGCCGGACGAGACGGG -3'	62ºC	597bp	Heuzenroeder et al., 1999
act	F 5'-AGAAGGTGACCACCAAGAACA -3' R 5'-AACTGACATCGGCCTTGAACTC -3'	55ºC	232bp	Sen & Rodgers, 2004
alt	F 5'-TGACCCAGTCCTGGCACGGC -3' R 5'-GGTGATCGATCACCACCAGC -3'	55ºC	442bp	Sen & Rodgers, 2004

Source: Authors.

3. Results

The 120 Aeromonads isolates were found to be more than 90% positive for at least one phenotypic virulence factor and all of them had at least two of the measured virulence genes (Table 3).

One hundred thirteen (94.2%) of the isolates exhibited the virulence factors examined. Of the 120 isolates, 75 (62.5%) showed hemolytic activity, and DNA nuclease activity was detected in 112 (93.3%) isolates. The proteolytic enzymes collagenase and elastase were positive in 16 (13.3%) and 15 (12.5%) isolates, respectively. Among the isolates of human origin (n=43), 97.7% of the isolates had at least one of the virulence factors. Among the isolates of animal origin (n=72), this was the case for 91.7% of the isolates. All isolates from the environment (n=4) had virulence factors. Nine different combinations of virulence factors were detected among the isolates (Table 4). Both *Aeromonas caviae* and *A. hydrophila* exhibited six profiles of virulence factors. Proteolytic activity of the enzymes collagenase and elastase was observed primarily in human sourced *Aeromonas hydrophila*, with the isolate from a skin wound sample positive for all virulence factors tested.

Table 3 - Prevalence of virulence factors and genes among *Aeromonas* isolates.

	A. hydrophila (n=63)			*A. caviae* (n=57)		
	Human (n=20)	Animal (n=39)	Environmental (n=4)	Human (n= 23)	Animal (n=33)	Environmental (n=1)
Virulence factors						
Elastase	30%	0%	0%	21,7%	9%	0%
Collagenase	35%	10,2%	75%	0%	6%	0%
Hemolytic activity	80%	64,1%	100%	52,2%	51,5%	100%
Nuclease activity	100%	92,3%	100%	95,6%	87,9%	100%
Virulence genes*						
*aer*A	50%	56,4%	0%	56,5%	12%	0%
*hly*A	55%	28,2%	25%	13%	54,5%	100%
lip	30%	76,9%	25%	26%	12%	100%
ser	0%	20,5%	25%	8,7%	0%	100%
act	15%	66,6%	0%	8,7%	3%	0%
alt	45%	7,7%	75%	26%	0%	0%
exu	100%	100%	100%	100%	100%	100%
gcat	100%	100%	100%	100%	100%	100%

* The virulence genes are: *aerA* – aerolysin; *hly*A – hemolysin; *lip* – lipase; *ser* – serine protease; *act* – cytotoxic enterotoxin; *alt* – termolabile cytotonic enterotoxin; *exu* –DNases and *gcat* – glycerophospholipid:cholesterol acyltransferase. Source: Authors.

Table 4 - Virulence factors observed in *Aeromonas hydrophila* and *A. caviae* isolates.

Virulence factors*	*A. hydrophila*			*A. caviae*		
	HU**	AN**	EV**	HU**	AN**	EV**
col-dnase	1	3	-	-	-	-
col-dnase-ela	-	-	-	-	1	-
col-dnase-ela-hem	2	-	-	-	-	-
col-dnase-hem	4	1	3	-	-	-
col-hem	-	-	-	-	1	-
dnase	3	8	-	8	11	-
dnase-ela	-	-	-	2	1	-
dnase-ela-hem	4	-	-	4	1	-
dnase-hem	6	24	1	8	15	1

*Hem = hemolysis, Col = collagenase, DNase = nucleasse, Ela = elastase. **: HU: human; AN: animal; EV: environmental. Source: Authors.

Among the nine different combinations of virulence factors (collagenase, elastase DNase and hemolysin) in Table 4, it was possible to notice that *A. hydrophila* and *A. caviae* showed differences regarding the enzyme profile. There were 3 unique profiles in each of the *Aeromonas* species in the study. The most frequent virulence enzyme combination was dnase-hem observed in 31 strains of *A. hydrophila* and 24 of *A. caviae*. Only two *A. hydrophila* of human origin presented the four virulence enzymes investigated.

The presence of eight virulence-associated genes was analyzed by PCR. At least two of these genes were present in all *Aeromonas* isolates (Table 3). All isolates had *gcat* and *exu* genes. Hemolytic and pore-forming toxins encoding hemolysin

(*hly*A) and aerolysin (*aer*A) genes were detected in 45 and 49 of the isolates, respectively. Genes encoding the *Aeromonas* enterotoxins act and alt were detected in 23 and 21 isolates, respectively. Positive *Aeromonas* for the *lip* (49) and *ser* (14) genes were also found. The hemolytic ability of Aeromonads can usually be associated with the presence of specific genes, such as aerolysin (*aer*A), hemolysin (*hly*A), and cytotoxic entrotoxin (*act*). Of the 75 isolates that exhibited hemolytic activity (hem), twelve were not associated with these genes. The others were distributed as follows: *aer*A-hem (n=8), *act*-hem (n=2), *hly*A-hem (n=21), *aer*A-*act*-hem (n=11), *aer*A-*hly*A-hem (n=7), and *aer*A-*act*-*hly*A-hem (n=14).

The presence of nuclease activity (DNAse) was detected in 112 isolates. However, the eight phenotypically negative aeromonads were positive for the presence of the *exu* gene.

Isolates from human sources were distinguished between fecal and nonfecal origin. For genes associated with *Aeromonas* enterotoxins and hemolysins, isolates of fecal origin had a higher incidence of the *act* gene. Isolates of nonfecal origin had a higher incidence of the *aer*A, *alt*, and *hly*A genes.

Twenty-seven combinations (Table 5) of the putative *Aeromonas* virulence genes were observed. The most frequent gene combination was exu-*gcat*, which occurred in 27 isolates, followed by *exu*-*gcat*-*hly*A in 19 isolates, *act*-*aer*A-*exu*-*gcat*-*hly*A-*lip*, and *act*-*aer*A-*exu*-*gcat*-*lip* in nine isolates each. *Aeromonas hydrophila* isolates had 18 different virulence profiles, with greater diversity of profiles among isolates from animal sources. *A. caviae* had 12 virulence profiles, with greater diversity of profiles among isolates from human sources.

Among isolates from marine mammals with migratory habits, the most prevalent virulence profile was *exu*-*gcat*-*hly*A, which was detected in 13 isolates from *Megaptera novaeglangiae*, three from *Arctocephalus australis*, and one from *Eubalena australis*. The *exu*-*gcat* profile was found in 11 isolates of *Lagenodelphis hosei* (n=6), *Arctocephalus gazella* (n=4), and *Kogia sima* (n=1). The virulence profiles *act*-*aer*A-*exu*-*gcat*-*hly*A-*lip* and *act*-*aer*A-*exu*-*gcat*-*lip* were found in nine isolates each. The first was present in two isolates of *Pontoporia blainvillei* and seven of *Stenella coeruleoalba*, and the second was present in five isolates of *Eubalena australis* and six of *Megaptera novaeangliae*. These migratory animals tend to reproduce in tropical waters. "Grounding" probably caused by navigational error, stressful conditions, weak immunity, and disease incidence. The health status of the "grounded" animal may provide an indication of the status of the population that is at sea.

Table 5 - Virulence profiles observed among *Aeromonas hydrophila* and *A. caviae* isolates.

Virulence Profile*	*A. hydrophila*			*A. caviae*		
	HU**	AN**	EV**	HU**	AN**	EV**
act-aerA-alt-exu-gcat-hlyA-lip	2	1	-	1	-	-
act-aerA-alt-exu-gcat-hlyA-lip-ser	-	1	-	-	-	-
act-aerA-alt-exu-gcat-lip	-	1	-	-	-	-
act-aerA-exu-gcat	1	-	-	-	-	-
act-aerA-exu-gcat-hlyA-lip	-	9	-	-	-	-
act-aerA-exu-gcat-lip	-	9	-	-	-	-
act-aerA-exu-gcat-ser	-	1	-	-	-	-
act-aerA-exu-gcat-lip-ser	-	-	-	1	-	-
act-exu-gcat	-	-	-	-	1	-
act-exu-gcat-lip-ser	-	2	-	-	-	-
act-exu-gcat-lip	-	2	-	-	-	-
aerA-alt-exu-gcat	-	-	-	5	-	-
aerA-alt-exu-gcat-hlyA	1	-	-	-	-	-
aerA-exu-gcat	-	-	-	4	3	-
aerA-exu-gcat-hlyA	2	-	-	-	-	-
aerA-exu-gcat-hlyA-lip	4	-	-	-	-	-
aerA-exu-gcat-lip	-	-	-	1	1	-
aerA-exu-gcat-lip-ser	-	-	-	1	-	-
alt-exu-gcat	4	-	2	-	-	-
alt-exu-gcat-hlyA	2	-	-	-	-	-
alt-exu-gcat-hlyA-lip-ser	-	-	1	-	-	-
exu-gcat	4	8	1	7	7	-
exu-gcat-hlyA	-	-	-	1	18	-
exu-gcat-hlyA-lip	-	-	-	1	-	-
exu-gcat-hlyA-lip-ser	-	-	-	-	-	1
exu-gcat-lip	-	2	-	1	3	-
exu-gcat-lip-ser	-	3	-	-	-	-

**aerA* – aerolysin; *hly*A – hemolysin; *lip* – lipase; *ser* – serine protease; *act* – cytotoxic enterotoxin; *alt* – termolabile cytotonic enterotoxin; *exu* –DNases and *gcat* – glycerophospholipid:cholesterol acyltransferase. ** HU: human; AN: animal; EV: environmental. Source: The authors.

4. Discussion

Aeromonas have multifactorial virulence profile. Some authors associate the presence of high number of virulence factors to bacteria pathogenic potential. Knowing that, virulence markers in clinical strains point out a relation with the symptoms and it is possible to investigate which mechanisms are behind the disease. Studies reported *Aeromonas* as a cause of diarrhea in many countries around the World (Qamar et al., 2016; Wenqing et al., 2016; Mbuthia et al., 2018; del Valle de Toro et al., 2020). Some studies propose a correlation between *Aeromonas* toxin genes and potential diarrheal diseases (Teunis & Figueras, 2016). Ottavianni et al. (2011) found at least two toxin genes with distinct combinations in *Aeromonas* strains showing their pathogenic potential. Virulence factors could determine how disease develops (Tavares, et al., 2015).

Aeromonas are reported as a cause of diarrhea in many countries around the World but their incidence in human

infections worldwide is unknown. Studies therefore show that the incidence of diseases caused by Aeromonas vary by geographical location and can be related to bad hygiene habits in undeveloped regions The human gastroenteritis disease caused by *Aeromonas* can appear in the form of a self-limiting liquid diarrhea to a more severe and invasive diarrhea, which specially is a problem for young children and infants (Fernandes-Bravo & Figueras, 2020).

Particularly in Brazil, incidence ranges from 2,6% to 19,5% in cases of diarrhea (Assis et al., 2014). In our study, 29% (n=35) of isolates were from diarrhea cases. Among these 35 isolates, the most frequent combinations of virulence factors were dnase-hem and dnase-ela-hem. In relation to virulence genes, the most frequent associations were *exu-gcat* and *exu-gcat-hlyA*. Although it is not possible to predict whether a particular *Aeromonas* strain can induce diarrheal illness, it is possible that certain virulence factors of *Aeromonas* species such as proteases, hemolysins and lipases are associated with their pathogenicity in cases of diarrhea (Tomás, 2012).

The production of extracellular enzymes and proteases in *Aeromonas* spp. allows them to survive in different ecosystems. More than a matter of virulence, it is a mechanism of survival and response to environmental stress (Citterio & Biavasco, 2015; Zeynep & Comhur, 2018). All isolates from fish samples showed collagenase activity, indicating a possible association between the presence of this enzyme and the pathogenicity of *Aeromonas* to *Genidens barbus* and *Oreochromis niloticus*.

Collagenase has a role in pathogenicity of certain microorganisms, including some admittedly pathogenic *Aeromonas* species and along to the enzyme elastase, are present in infections of skin and tissue. They may compromise cartilage, bone and mucosal structures (Citterio & Biavasco, 2015; Barger et al., 2020; Jin et al., 2020). These two enzymes standing in *A. hydrophila* from skin infection, in this study, suggests the association of these enzymes to Aeromonads wound disease.

A. caviae and *A. hydrophila* regularly produce DNase (Khor et al., 2018). Nucleases contribute to the establishment of infection exceeding the initial host defenses or by providing nutrients to cellular proliferation (Tomás, 2012). Likewise, DNases may be associated with cell damage and tissue destruction besides the evasion of host defenses, playing a crucial role in microbial invasion and colonization (Li et al., 2015; Pang et al., 2015). Our findings, in which all strains exhibited the *exu* gene, corroborate the importance of this virulence gene for the maintenance of Aeromonads in various hosts and also in the aquatic environment.

The hemolytic activity is correlated with *Aeromonas* group of aerolysins and hemolysin comprising several genes, among them *aerA*, *hly*A and *act* (Hoel, et al., 2017). Aerolysins are proteins capable of altering the permeability of blood cells, as well as other eukaryotic cells (and consequently promoting osmotic lysis) due to polymerization of the structure induced by binding to a membrane specific glycoprotein site (Gonçalves Pessoa et al., 2019). The enterotoxin *act* is the most cytotoxic virulence factor of *Aeromonas* and induces multiple effects including hemolytic, cytotonic, and cytotoxic activities (Rasmussen-Ivey et al., 2016; Miyagi et al., 2021). Among our results, the research of hemolytic activity in plaque presented the second largest number of positive isolates, present in a special way among *A. hydrophila* from human source. Of the genes associated with hemolytic activity, *aer*A, *hly*A and *act*, were found in higher number of isolates of *A. hydrophila*, suggesting higher virulence in this species.

Aeromonads' extracellular enzymes role in pathogenicity is still to be determined, they represent a big potential to adapt to environmental changes. Extracellular proteases contribute to the metabolic versatility that allows Aeromonas to persist in different habitats and that facilitate ecological interactions with another organism (Tomás, 2012). It has been described that the genus Aeromonas produces a wide range of exotoxins. However, all toxins described are not produced by all strains, although strains may possess their genes. Furthermore, some strains only express toxin genes in certain growth conditions. Two main types of enterotoxins have been described in Aeromonas spp. cytotoxic and cytotonics (Gonçalves Pessoa et al., 2019; Fernandes-Bravo & Figueras, 2020; Tomás, 2012).

Cytotonic enterotoxins do not produce degeneration of the epithelium and have mechanisms of action similar to those of the choleric toxin, since they increase the cyclic adenosine monophosphate (cAMP) levels and prostaglandins in intestinal epithelial cells. Aeromonas species produces cytotonic enterotoxins that show differen molecular weights and variable reactivity to the choleric antitoxin (Tomás, 2012; Rasmussen-Ivey et al., 2016; Miyagi et al., 2021).

Cytotonic heat-labile enterotoxin (*alt*) has a similar mechanism of action to cholera toxin by increasing the levels of cAMP and prostaglandins in intestinal epithelial cells (Tomás, 2012). The *alt* gene was positive in six *A. caviae* from human source and did not show positive result among animal source and environmental isolates. The 15 *A. hydrophila alt*$^{+}$ were distributed among the three sources, with a higher percentage of positivity among those of environmental origin (60%, n=3), but with a higher number of positives among those of human origin (n=15, 43%).

Lipases interact with human leukocytes and can affect the immune system functions due to the release of free fatty acids generated by the lipolytic action (Gonçalves Pessoa et al., 2019). The enzyme Glycerophospholipids-cholesterol acyltransferase (GCAT) digests the erythrocytes membrane and causes lysis. GCAT was characterized as one of the most lethal virulence factors in some Aeromonads. Coded by the gene *gcat*, this enzyme can complex with LPS, becoming more toxic than its free form (Gonçalves Pessoa et al., 2019). Tomás (2012) associated the presence of genes lip and *gcat* to disease establishment and nutrition of bacteria. Chacón et al. (2003), suggested that the *gcat* gene would be present in all *Aeromonas* species. The gene was later found to be highly conserved among the clinical and environmental isolates of *Aeromonas* species (Li et al., 2015; Pang et al., 2015; Hoel, et al., 2017; Khor et al., 2018; Nwaiwu, 2019). Thus, many researchers have used the gene as a specific *Aeromonas* genus marker (Mendes-Marques, et al., 2013). The results found in our study confirm this aspect of the *gcat* gene, considering that all isolates independently of species and source of isolation presented *gcat*$^{+}$.

Mendes-Marques et al. (2012) found no lip gene in clinical strains but found it in all environmental isolates concluding that its main activity is directly connected with the extracellular survival of Aeromonads. However, our results show 27% of clinical isolates and 40% of environmental isolates positive for the lip gene.

The serine protease released by certain strains of *Aeromonas*, assists the microorganism invasion breaking down the host's barriers and it's able to cleave plasma proteins such as fibrinogen generating potential complications such as septic shock and blood clotting disorder (Senderovich et al., 2012).

It was possible to observe in this study the diversity of virulence markers presented by the strains, through virulence profiles. It should be noted that the strains of human origin presented greater diversity of profiles. A comparison between the virulence profiles found in human and environmental strains shows that they present common profiles, and the environmental profiles contain virulence markers distributed among the human strains. All virulence markers of the environmental isolates were found in the human isolates. This association between the virulence factors found in environmental and human strains and at the same time the absence of common profiles points to the adaptive capacity of *Aeromonas* spp. associated with the multifactorial virulence profile.

When observing the most frequent virulence profiles among isolates of animal origin it is possible to notice the presence of markers of hemolytic characteristic, lipases and nucleases. These markers denote the pathogenicity and invasiveness of *Aeromonas* spp. in these animals. Studies (Peixoto et al., 2012; Castelo-Branco et al., 2015; Cardoso et al., 2018; Marinho-Neto et al., 2019; Vaz Rodrigues et al., 2019) found *A. hydrophila* and *A. caviae* among the main etiologies in sick fish and mammals. The isolated strains of *A. hydrophila* were mostly producers of protease, elastase, hemolysis and enterotoxin in phenotypic tests and the strains of *A. caviae* were producers of protease only.

Certain studies suggest that some species of *Aeromonas* synthesize more virulence factors and more often, suggesting the clonal origin virulence, thus, few clones were responsible for disease progression (Pang et al., 2015; Gonçalves Pessoa et al., 2019). Systematic studies on the distribution of genes coding for Aeromonads virulence factors allow us to evaluate the

virulence potential of these microorganisms circulating in our environment.

More extensive genomic and epidemiological investigations will allow us to ascertain the frequency of core and accessory genes in environmental, animal and clinical *Aeromonas* isolates. Sometimes we will be able to detect novel virulence factors through genomic surveillance and estimate the virulence potential of clinical isolates through sequence analysis alone.

5. Conclusion

Among the Aeromonads isolates was possible to observe that more than 90% were positive for at least one phenotypic virulence factors and all of them had at least two of the virulence genes measured. This study shows that pathogenic Aeromonas can be detected in various types of samples. According to our observations, when such potentially virulent Aeromonads occur in human, animal and environmental samples, they can present risks to public health. The diversity of virulence profiles found in this study hint heterogeneity in clones circulating in our environment. The distribution of virulence genes and factors among isolates denotes that different arrangements of virulence determinants may occur in different subpopulations of *Aeromonas* spp. The occurrence of identical profiles among strains of human and animal origin may indicate a zoonotic transmission. These findings serve as evidence for the presence of pathogenic *Aeromonas* by strains with high virulence in the food chain. The results also confirm the need for monitoring of Aeromonads and further studies on their virulence determinants to minimize the health risks posed by this pathogen.

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