Research Article

Virulence-Associated Genes and Antimicrobial Resistance of Aeromonas hydrophila Isolates from Animal, Food, and Human Sources in Brazil

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Aeromonads are natural inhabitants of aquatic environments and may be associated with various human or animal diseases. Its pathogenicity is complex and multifactorial and is associated with many virulence factors. In this study, 110 selected Aeromonas hydrophila isolates isolated from food, animals, and human clinical material from 2010 to 2015 were analyzed. Antimicrobial susceptibility testing was performed by the disk diffusion method, and polymerase chain reaction was conducted to investigate the virulence genes hemolysin (hlyA), cytotoxic enterotoxin (act), heat-labile cytotoxic enterotoxin (alt), aerolysin (aerA), and DNase-nuclease (exu). At least 92.7% of the isolates had one of the investigated virulence genes. Twenty different virulence profiles among the isolates were recognized, and the five investigated virulence genes were observed in four isolates. Human source isolates showed greater diversity than food and animal sources. Antimicrobial resistance was observed in 46.4% of the isolates, and multidrug resistance was detected in 3.6% of the isolates. Among the 120 isolates, 45% were resistant to cefoxitin; 23.5% to nalidixic acid; 16.6% to tetracycline; 13.7% to cefotaxime and imipenem; 11.8% to ceftazidime; 5.9% to amikacin, gentamicin, and sulfamethoxazole-trimethoprim; and 3.9% to ciprofloxacin and nitrofurantoin. Overall, the findings of our study indicated the presence of virulence genes and that antimicrobial resistance in A. hydrophila isolates in this study is compatible with potentially pathogenic bacteria. This information will allow us to recognize the potential risk through circulating isolates in animal health and public health and the spread through the food chain offering subsidies for appropriate sanitary actions.

1. Introduction

Since its first isolation in 1890, several events have discussed numerous aspects of the genus Aeromonas, and some of these, which have taken place over the past century, have been instrumental in understanding current issues about this group of bacteria [1]. Aeromonas microorganisms are highly adaptable to aquatic environments and have been described as pathogenic to humans and animals. The genus Aeromonas comprises...
more than 30 valid species, of which *A. hydrophila*, *A. caviae*, *A. media*, *A. veronii* bv. *sobria*, and *A. veronii* bv. *veronii* are of particular clinical significance [2].

They are widely isolated from clinical, environmental, and food samples where they can develop even at low temperatures and produce toxins, which significantly increase the risk of foodborne infection [3, 4].

Aeromonads have a wide geographical distribution, being able to determine infections in animals and humans [5]. Commonly found in aquatic environments, they are recognized as eventual pathogens of reptiles, fish, and some mammalian species. Recognized as emerging pathogens, their situation is privileged when natural disasters occur, having been largely isolated from skin and soft tissue infections in tsunami survivors that struck Thailand in 2004 [6]. Besides, *Aeromonas* have been recognized as a relevant etiological agent in human gastrointestinal infections, having been isolated from food and drinking water samples [7, 8].

Its virulence is multifactorial, and numerous factors have been identified in intestinal and systemic infections caused by this microorganism including endotoxins, enterotoxins, adhesins, cytoxins, hemolysins, lipases, and proteases [7, 9].

*Aeromonas* spp. have the ability to receive and transmit a set of genes located within genetic elements such as plasmids, IS elements, transposons, genomic and/or pathogen islands, and integron-associated gene cassettes. These, referred to as flexibl, can encode virulence factors, toxic compounds, and antibiotic resistance [10]. These elements are important in the rapid transfer of genetic materials into the microbial community. Environmental contamination is considered the most efficient for the selection of resistant populations as well as for the exchange of resistant genes through mobile genetic elements [11].

In recent years, the relevance of continuous isolation and identification of *A. hydrophila* observed in National Reference Laboratory for Bacterial Enteroinfections from Oswaldo Cruz Institute, especially in migratory marine mammal species that land on the Brazilian coast, has been questioned. The possibility of introducing different genetic traits through transfer to ubiquitous species in our environment is the fundamental concern. In contrast, the lack of literature in our country regarding the relevance of such microorganisms impels the need for subtyping and identification of virulence and antimicrobial drug resistance characteristics.

Based on the current available knowledge about this microorganism, this study is aimed at investigating a group of virulence-associated genes and antimicrobial resistance profiles in *Aeromonas hydrophila* isolated from animal, food, and human sources in order to characterize circulating isolates in Brazil and contribute to the knowledge of its relevance to animal and public health.

2. Methods

2.1. Selection of *Aeromonas hydrophila* Isolated in NRLED. 110 *Aeromonas hydrophila* isolates from food (*n* = 28) (meat, fish, and chicken), animal (*n* = 52) (seabirds, marine mammals, and chelonians), and human clinical sources (*n* = 30) (Table 1) from 2010 to 2015 were analyzed at National Reference Laboratory for Enteric Diseases (NRLED), Oswaldo Cruz Institute, FIOCRUZ. The food samples were from ready-to-eat plate (meat and chicken) linked to foodborne disease. Fishes and scallops were from their natural habitat, and the marine animal isolates were obtained through monitoring programs carried out at FIOCRUZ. The human samples were obtained from patients with clinical symptoms and sent to NRLED by Public Health Laboratories.

2.2. Biochemical Characterization. Isolation and identification were performed according to Janda and Abbott [12]. The isolates were sown in Glutamate Starch Phenol-Red Agar medium (Merck) and screened in Kliger Iron Agar (Difco) and Lysine Iron Agar (Difco) and identified to the specie level by nonautomated biochemical tests. They presented a positive oxidase test, and Vibriostatic Agent O/129 test showed resistance in 10 μg and 150 μg concentrations, as shown by Martin-Carnahan and Joseph [13].

2.3. Genus *Aeromonas* Identification Using the GCAT-PCR (237 bp). The Glycerophospholipid-Cholesterol Acyltransferase (gcat) gene was amplified using a primer pair as reported previously [14]. The presence of this gene (237 bp) was visualized on 2% agarose gel (Sigma) stained with ethidium bromide.

2.4. Determination of Antimicrobial Susceptibility. Antimicrobial susceptibility testing was performed by the disk diffusion method according the Clinical and Laboratory Standard Institute (CLSI) recommendations for *Aeromonas* species (CLSI M45, 3rd ed., 2015) [15] and for *Enterobacteriaceae* (CLSI M100, 29th ed., 2019) [16] to the antimicrobials nalidixic acid (NAL) 30 μg, amikacin (AMK) 30 μg, ceftazidime (CAZ) 30 μg, cefoxitin (FOX) 30 μg, ceftriaxone (CTX) 30 μg, ciprofloxacin (CIP) 5 μg, chloramphenicol (CHL) 30 μg, gentamicin (GEN) 30 μg, imipenem (IPM) 10 μg, nitrofurantoin (NIT) 300 μg, sulfamethoxazole-trimethoprim (SXT) 1.25/23.75 μg, and tetracycline (TCY) 30 μg. *Escherichia coli* ATCC 25922 was used for quality control of the antimicrobial susceptibility test.

2.5. Polymerase Chain Reaction (PCR) of Virulence Genes. DNA extraction was performed using commercial kit (DNA DNeasy Tissue, Qiagen) following the manufacturer instructions. DNA amplification step was conducted, in order to investigate the virulence genes hemolysin (*hlyA*–597 bp) [17], cytotoxic enterotoxin (*act*–232 bp), heat-labile cytotic enterotoxin (*alt*–442 bp) [18], aerolysin (*aer*–431 bp), and DNase-nuclease (*exu*–323 bp) [19]. Eight microliters of PCR product mixed with 5x gel loading dye was loaded onto an agar gel 2% (Sigma) in 0.5x Tris-Borate-EDTA buffer, and a 100 bp DNA ladder (Invitrogen by Thermo Fischer Scientific) was used as a molecular weight marker. Gels were visualized by a UV transilluminator (ImageQuant).

3. Results

3.1. Biochemical and Molecular Confirmation of *Aeromonas hydrophila* Isolates. The isolates have been confirmed for
the *Aeromonas* genus by detection of the *gcat* gene present in all 110 isolates and for the *A. hydrophila* species through the use of nonautomated biochemical tests, whose results were compatible with the investigated species.

### 3.2. Distribution of *Aeromonas hydrophila* according to the Sources.

Most isolates were from marine animal source, and migratory mammals showed the highest isolation percentage (81.1%). Among the food samples, isolates from fish represented 67.8%. Human *A. hydrophila* isolates from gastrointestinal infections corresponded to 86.7%, and the sources of infections were unknown. The distribution is shown in Table 1.

### 3.3. Distribution of Virulence Genes.

Among the studied isolates, 92.7% (102) presented at least one of the virulence genes distributed among the 20 virulence profiles, highlighting 17 isolates that simultaneously presented 4 to 5 virulence genes (see Table 2). Considering the isolates of human origin among 30 *A. hydrophila*, we found 11 different virulence profiles with 1 to 5 virulence genes (see Table 3). The *act, aerA, alt, exu*, and *hlyA* genes were detected in 36, 57, 18, 70 and 47 *A. hydrophila* isolates, respectively. The frequencies of all the gene encoding virulence factors according to the source of studied Aeromonads are shown in Figure 1.
3.4. Antimicrobial Susceptibility. Almost 53.6% of the isolates were susceptible to all tested antibiotics. The remaining isolates showed resistance to at least one antimicrobial drug. Resistance to one or two antibiotics was observed in 40% of resistant isolates; however, 6.4% of resistant isolates were resistant to three or more antibiotics. Overall, twenty-one different resistance profiles were identified. Among these 51 resistant isolates, the distribution of antimicrobial resistance rates can be observed Table 4.

### Table 3: Virulence profiles of Aeromonas hydrophila isolated from human sources.

| Isolate | Source       | Virulence profile |
|---------|--------------|-------------------|
| AhH1    | Blood        | Exu               |
| AhH2    | Diarrheic stools | Act, aerA, Exu   |
| AhH3    | Diarrheic stools | aerA, Exu, hlyA  |
| AhH4    | Diarrheic stools | aerA, Exu, hlyA  |
| AhH5    | Diarrheic stools | aerA, Exu, hlyA  |
| AhH6    | Diarrheic stools | Exu               |
| AhH7    | Diarrheic stools | aerA, Exu        |
| AhH8    | Diarrheic stools | hlyA             |
| AhH9    | Diarrheic stools | hlyA             |
| AhH10   | Diarrheic stools | Act, aerA, alt, Exu, hlyA |
| AhH11   | Diarrheic stools | Act, aerA, Exu, hlyA |
| AhH12   | Diarrheic stools | aerA, alt, Exu, hlyA |
| AhH13   | Diarrheic stools | Alt, Exu, hlyA   |
| AhH14   | Diarrheic stools | Act, aerA, Exu   |
| AhH15   | Diarrheic stools | Act, alt, Exu    |
| AhH16   | Diarrheic stools | Alt, Exu, hlyA   |
| AhH17   | Diarrheic stools | Alt, Exu, hlyA   |
| AhH18   | Diarrheic stools | Exu               |
| AhH19   | Diarrheic stools | aerA, Exu, hlyA  |
| AhH20   | Diarrheic stools | aerA, Exu, hlyA  |
| AhH21   | Diarrheic stools | aerA, Exu, hlyA  |
| AhH22   | Diarrheic stools | Alt, Exu         |
| AhH23   | Diarrheic stools | Alt, Exu         |
| AhH24   | Diarrheic stools | Alt, Exu         |
| AhH25   | Diarrheic stools | Act, aerA, alt, Exu, hlyA |
| AhH26   | Diarrheic stools | Act, aerA, alt, Exu, hlyA |
| AhH27   | Diarrheic stools | Alt, Exu         |
| AhH28   | Lung          | Alt, Exu, hlyA   |
| AhH29   | Secretion     | Exu               |
| AhH30   | Synovial fluid | aerA, alt, Exu, hlyA |

*act*: cytotoxic enterotoxin; *aerA*: aerolysin; *alt*: heat-labile cytotoxic enterotoxin; *exu*: DNase-nuclease; *hlyA*: hemolysin.

4. Discussion

In this present study, it was possible to observe the diversity of virulence markers presented by the isolates, through the virulence profiles. Twenty virulence profiles were observed, and each profile had one to five genes. Virulence factors of a bacterium are often associated with the damage it causes to the host. Certain studies suggest that some Aeromonas species synthesize more virulence factors and more frequently, showing clonal origin of virulence; so, only a few clones would be responsible for disease progression [20–22].

The diversity in virulence profiles of isolates and the relationship between virulence markers show that they vary according to the needs for their survival in an environment. Rasmussen-Iveyi et al. [23] mention that the expression of virulence factors is linked to gene regulation cascades associated with interactions of microorganisms with the environment in which they are found.

Aeromonas pathogenicity is complex and multifactorial and is associated with many virulence factors, and there is not a definitive link between the presence of specific toxin genes and clinical presentation [24].

In this study, the selection of isolates from different sources in the food chain allowed the observation of different virulence factor combinations, confirming the multifactorial virulence profile in Aeromonas spp. Among the tested virulence genes, hlyA, aerA, and exu were the most common genes and the exu gene was the most prevalent, present in 63.7% of the isolates. The genes aerA, exu, and hlyA showed a higher percentage of positivity among the isolates from human source, and exu was the most prevalent. Each of these genes plays an important role in the pathogenicity observed in diarrheal diseases [1, 25]. Despite Aeromonas harboring different numbers and types of virulence genes, in this study, among the five investigated genes, a correlation between clinical symptoms and gene encoding virulence factors was not observed. However, the exu gene was present at 93.3% of intestinal and extraintestinal infections. The exu gene codes for an extracellular DNase which blocks the antibacterial host defenses [26]. Its presence is associated with the microbial capacity of invasion and colonization, as well as evasion of the host immune system [9]. The high percentage of positive isolates for this gene enhances its relevance for the maintenance of Aeromonas spp. in the host.

Clinical isolates harboring a variety of toxin genes have been reported [3, 23]. Patients with different clinical manifestations brought more attention to some virulence factors, including hemolysins, enterotoxins, cytotoxic enterotoxin (act), and extracellular enzymes (proteases, amylasases, lipases, ADP-ribosyltransferases, and DNases) [21].

In intestinal infections, *alt* has been reported to be associated with loose stool, *alt* plus *ast* with watery stools, and *act* with bloody diarrhea [22]. The heat-labile cytotoxic enterotoxin *alt* is responsible for causing significant fluid secretion in the host’s cell [23]. In this study, although ten diarrheic stools samples were associated with the presence of *alt*, 15 samples of diarrheic stools were not related with *alt*. In addition, a relationship between infection and presence of gene encoding virulence factors was not observed and might be related to the limited number of isolates from extraintestinal infections. Likewise, a study completed by Wu et al. [27] found no direct association between the presence of the genes *aerA*, *hlyA*, *alt*, and *ast*, in Aeromonas isolates and development of extraintestinal infections or bacteremia.

Castelo-Branco et al. [28] observed that Aeromonads of clinical origin had fewer virulence genes than those isolated from other sources. In our study, it was possible to observe
variations in the relationship between virulence genes and source, thus indicating that the distribution of virulence genes among Aeromonas is not uniform. Except for the alt gene, observed in only one isolate (AhF1 from chicken), act, exu, and hlyA genes were found with the same frequency (44%) and aerA in 40% in the food-borne isolates. Raw seafood corresponded to 82% of the samples analyzed and had all hlyA-positive samples among isolates of food origin. Among the food and animal samples, the same percentage of act gene was observed, which is 44% and 43.6%, respectively. Rather et al. [29] found 82% positivity for the act gene among isolates from different water sources and fish. Cytotoxic enterotoxin encoded by this gene is responsible for triggering inflammatory response in host cells, plasma membrane disorders, and intestinal villus degeneration in cases of bloody diarrhea [1].

Among the animal isolates, aerA and exu were the most frequently observed genes. The exu gene was observed in 72.5% of marine animals with migratory characteristics. Among the 10 seabirds evaluated, this gene was only present in one Leucophaeus atricilla isolate. Overall, the results showed high prevalence of the gene exu among the isolates evaluated at this study. Khor et al. [30] highlight that 96% of Aeromonas sp. environmental isolates presented the exu gene, corroborating the prevalence and emphasizing the importance of this gene for the survival of the bacteria.

Pereira et al. [35] found a frequency of approximately 20% in the isolation of Aeromonas in marine mammals from the south and southeast coast of Brazil. The presence of these microorganisms in aquatic migratory animals and the fact that presenting virulence factors can also be found in isolates of human origin show zoonotic characteristics in Aeromonas spp. Several Aeromonas spp. have been reported as important

### Table 4: Antibiotic resistance patterns in Aeromonas hydrophila distributed by source.

| Antimicrobial drug       | Total (n = 110) | Human (n = 30) | Food (n = 28) | Animal (n = 52) |
|--------------------------|----------------|---------------|---------------|----------------|
|                          | N  | %     | N  | %     | N  | %     | N  | %     |
| Amikacin (AMK)           | 3  | 2.7   | 2  | 6.7   | 0  | 0     | 1  | 1.9   |
| Cefoxitin (FOX)          | 29 | 26.4  | 5  | 16.7  | 11 | 39.3  | 13 | 25.0  |
| Cefazidime (CAZ)         | 9  | 8.2   | 2  | 6.7   | 7  | 25.0  | 0  | 0     |
| Ceftriaxone (CTX)        | 7  | 6.4   | 6  | 20.0  | 1  | 3.6   | 0  | 0     |
| Chloramphenicol (CHL)    | 0  | 0     | 0  | 0     | 0  | 0     | 0  | 0     |
| Ciprofloxacin (CIP)      | 2  | 1.8   | 2  | 6.7   | 0  | 0     | 0  | 0     |
| Gentamicin (GEN)         | 3  | 2.7   | 3  | 10.0  | 0  | 0     | 0  | 0     |
| Imipenem (IPM)           | 7  | 6.4   | 2  | 6.7   | 3  | 10.7  | 2  | 3.8   |
| Nalidixic acid (NAL)     | 14 | 12.7  | 7  | 23.3  | 4  | 14.3  | 3  | 5.8   |
| Sulfamethoxazole-trimethoprim (SXT) | 4  | 3.6   | 3  | 10.0  | 1  | 3.6   | 0  | 0     |
| Tetracycline (TCY)       | 9  | 8.2   | 4  | 13.3  | 1  | 3.6   | 4  | 7.7   |

Figure 1: Percentage distribution of virulence genes in Aeromonas hydrophila according to isolation source. *FO: food; AN: animal; HU: Human. **act: cytotoxic enterotoxin; aerA: aerolysin; alt: heat-labile cytotoxic enterotoxin; exu: DNase-nuclease; hlyA: hemolysin.
zoonotic pathogens based on their virulence and antibiotic resistance profiles [12].

Data from the Brazilian Ministry of Health [36] have shown that animal-derived food, such as fish, triggers outbreaks of food- and water-borne diseases (FWBD). Water is the natural habitat of these bacteria and an important source of food contamination. Aeromonas spp. in food are a predominant feature in fish consumption, even though it has been studied in swine, chickens, and humans. It may occur in the excrement of infected animals and sick people who handle food.

Given the risk to human health, the incidence of antimicrobial resistance is alarming, particularly among A. hydrophila, A. caviae, and A. sobria, which are considered pathogens responsible for infections in both fish and humans [37]. These bacteria may be resistant by carrying intrinsic genes or by acquiring resistance markers from other microorganisms [4, 8]. Studies demonstrate that Aeromonas spp. can acquire resistance during treatment, as presented with tetracycline used in the treatment of bacterial infections in fish [38]. Increased resistance to antibiotics in Aeromonas species from different sources has been reported, showing appearance not only in isolates of clinical origin but also from other sources of isolation such as fish, food, and natural waters [4, 8].

In this study, 33.3% of samples from human source, 39.3% from food, and 63.5% from animal source were susceptible to all antimicrobials. All isolates isolated from marine animals from 2010 to 2013 were susceptible to all antimicrobial drugs, a condition that changed from 2014 on marine mammals and seabirds. Among 21 samples (2014 to 2015), 19 were resistant to at least one antimicrobial drug.

Multidrug resistance was observed in 16.7% of isolates from the human source, including fluoroquinolone and carbapenem. In food origin samples, the resistance profile was present in isolates since 2010, and one fish isolate was multi-resistant (CAZ, CTX, IPM, NAL, and TCY). It has also been perceived that the fish farms analyzed either lacked proper water management or there was no management at all, with the consequent water and fish contamination. Lack of water management may cause disease and even death in fish. Because of faulty knowledge and inadequate manpower, producers use antimicrobials indiscriminately, causing the proliferation of antibiotic-resistant or even multidrug-resistant bacteria to antimicrobials [39]. According to Souza et al. [40], there is very scanty information on fish farming management and its consequences on water quality and on the health of fish in fish farms.

Aeromonas spp. are usually isolated from patients suffering from traveller’s diarrhea. Hofer et al. [41] detected the pathogen as the cause of traveller’s diarrhea in 18 (2%) out of 863 patients. A study on the outbreak of diarrhea in the town of São Bento do Una PE, Brazil, revealed that 114 (19.5%) out of the 582 coprocultures performed among the 2170 registered cases were caused by Aeromonas spp. [41].

Aeromonads were initially described as susceptible to tetracycline, chloramphenicol, cephalosporins, aminoglycosides, and quinolones [4, 12]. However, chromosomal inducible β-lactamases are recognized as a major mechanism of resistance to antimicrobials in Aeromonas spp. These enzymes are widely distributed among Aeromonas microorganisms, those of class C, as they confer resistance to cephalosporins and cefoxitin [4, 42]. Considering this, the cefoxitin resistance found in this study could be justified by the probable action of the enzyme.

Quinolone resistance has also been reported in Aeromonas. Among the studied isolates, 12 were resistant to nalidixic acid and 2 to ciprofloxacin. Sinha [43] reported high levels of intrinsic resistance to antimicrobial drugs. Resistance to these drugs may be related to gyr genes of chromosomal origin and qnr of plasmid origin. Previous studies [44–46] have identified Aeromonas spp. showing resistance to quinolones in domestic and free-living animals, hospital effluents, and wastewater. These results suggest the role of Aeromonads in the dissemination of antimicrobial resistance.

The presence of cephalosporin-, quinolone-, and carbapenem-resistant isolates are among the isolate points to the search for genes that characterize antimicrobial resistance. Acquiring and spreading antibiotic resistance genes (ARG) are of particular significance, as it is important for the health of humans and animals [10]. Aeromonads may become a reservoir of gene encoding resistance to antimicrobial drugs; studies showed the spread of tetracycline-resistant plasmids between A. hydrophila and E. coli as well as between human and aquaculture in different geographical regions [47]. The study of the resistance in different levels, such as phenotype, genotype, genomic, and epidemiological level, has turned into an important approach to understand the origin of the antibiotic resistance and its relationship with horizontal gene transfer in the genus Aeromonas spp., which is a pathogen related to public health problems [4].

5. Conclusion

In conclusion, the observation of varying virulence profiles shows the ability of Aeromonas hydrophila to adapt to the conditions of its environment. The Aeromonas isolates in this study present virulence and antimicrobial resistance aspects compatible with potentially pathogenic species capable of transferring the genes responsible for antibiotic resistance to other pathogenic microorganisms in humans and throughout the food chain which is a risk to human and animal health.

Data Availability

The Excel spreadsheet data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.
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