Molecular characterization of virulence factors in *Aeromonas hydrophila* obtained from fish[1]

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ABSTRACT.- Oliveira S.T.L., Veneroni-Gouveia G. & Costa M.M. 2012. **Molecular characterization of virulence factors in *Aeromonas hydrophila* obtained from fish.** *Pesquisa Veterinária Brasileira 32(8):701-706.* Laboratório de Microbiologia e Imunologia Animal, Universidade Federal do Vale do São Francisco, Campus Ciências Agrárias, Colegiado Acadêmico de Zootecnia, Rodovia BR 407 Km 12, Lote 543, Projeto de Irrigação Nilo Coelho s/n, C1, Petrolina, PE 56300-000, Brazil. E-mail: mateus.costa@univasf.edu.br

Multiple factors can be involved in the virulence processes of *Aeromonas hydrophila*. The objective of the present paper was to verify the presence of aerolysin, hidrolipase, elastase and lipase virulence genes through the polymerase chain reaction (PCR) in *A. hydrophila* isolates obtained from fish of the São Francisco River Valley, and to evaluate virulence according to the presence of these genes in Nile tilapia fingerlings. One hundred and fourteen isolates from the bacteria were used. DNA was heat extracted and PCR undertaken using specific primers described in the literature. For *in vivo* tests Nile tilapia fingerlings were used. From the PCR tests, negative isolates for all genes tested were selected, positive isolates for two genes (aerolysin and elastase) and positive for the four genes tested. These were inoculated at a concentration of 10^8 UFC/ml into the tilapias, considered as treatments; another group of animals was used as control (with inoculation of saline solution). In all, 12 distinct standards regarding the presence of virulence factors in isolates from *A. hydrophila,* were observed. Of the 114 isolates analyzed, 100 (87.72%) presented at least one of the virulence factors under study. The virulence factors were widely distributed among the *A. hydrophila* isolates. Aerolysin was the most frequent virulence factor present in the isolates analyzed. *A. hydrophila* led to the mortality of the Nile tilapia fingerlings, regardless of the absence or quantity of virulence genes tested.

INDEX TERMS: *Aeromonas hydrophila*, virulence factors, aerolysin, elastase, hidrolipase, lipase, *Oreochromis niloticus.*

RESUMO.- [**Caracterização molecular de fatores de virulência em *Aeromonas hydrophila* obtidas de peixes.**] Múltiplos fatores podem estar envolvidos nos processos de virulência de *Aeromonas hydrophila*. O objetivo do presente trabalho foi verificar a presença dos genes de virulência aerolisina, hidrolipase, elastase e lipase, utilizando a reação em cadeia da polimerase (PCR), em isolados de *Aeromonas hydrophila* obtidos de peixes do Vale do São Francisco e, avaliar sua virulência de acordo com a presença desses genes de virulência em alevinos de tilápia do Nilo. Cento e quatorze isolados foram utilizados. O DNA foi termoextraído e a PCR realizada com a utilização de inciadores específicos descritos pela literatura. Para os testes *in vivo* alevinos de tilápia do Nilo foram utilizados. Segundo os resultados da PCR, foram selecionados isolados negativos para todos os genes de virulência testados, isolados positivos para dois genes de virulência, (aerolisina e elastase) e positivos para os quatro genes avaliados. Esses isolados foram inoculados na concentração de 10^8 UFC/ml nos alevinos e foram considerados como tratamentos e, um outro grupo de animais foi utilizado como grupo controle (com inoculação de solução salina). Ao final, 12 padrões distintos, em relação a presença dos fatores de virulência nos isolados de *A. Hydrophila*, foram observados. Dentre os 114 isolados analisados, 100 (87,72%) apresentaram pelo menos um dos genes de virulência sob estudo. Os fatores de virulência foram amplamente distribuídos entre os isolados de *A. hydro-*

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phila. A aerolisina foi o fator de virulência mais frequente nos isolados analisados. *A. hydrophila* levou à mortalidade dos alevinos de tilápia do Nilo, independente da ausência ou quantidade de genes de virulência testados.

TERMOS DE INDEXAÇÃO: *Aeromonas hydrophila*, fatores de virulência, aerolisina, elastase, hidrolipase, lipase, *Oreochromis niloticus.*

INTRODUCTION

Aeromonas spp. are typically fresh water gram-negative bacteria, belonging to the *Aeromonadaceae* family, facultatively anaerobic, found in many environments, including soil and water (Nam & Joh 2007), and can also be a component of the fish microbiota (Pavanelli et al. 2008). These bacteria are important infectious agents (Janda & Abbott 2010). Similar to that which occurs in humans, the involvement of these microorganisms in fish diseases is usually associated with other conditions, and its pathogenicity appears to be related to stress of the host (Cyrino et al. 2004). However, researchers have reported *Aeromonas spp*. as emerging and primary pathogens, having highly specific mechanisms for promoting diseases (Chacon et al. 2004, Vilches et al. 2004, Sha et al. 2005, Yu et al. 2005).

Isolates with high virulence can infect healthy fish; however, the stress coming from intensive fish farming also contributes and triggers outbreaks (Suomalainen et al. 2005). The species *Aeromonas hydrophila* is the most common within the *Aeromonas* genus (Cipriano 2001, Miranda & Zemelman 2002, Santos 2010, Silva 2011). It has been isolated and identified from species with and without clinical symptoms. This species is considered to be the most virulent within the *Aeromonas* complex (Cyrino et al. 2004).

Bacteria virulence factors are related to the invasion, replication and evasion of the host's immune system, and cause injuries during pathogenesis of the disease (Vilches et al. 2004). Several researchers have described different virulence factors in *Aeromonas hydrophila*, among them antigen-O, the presence of capsules (Merino et al. 1996, Zhang et al. 2002), S-layer (Dooley & Trust 1988), exotoxins such as hemolysins and enterotoxins (Chakraborty et al. 1984), exoenzymes such as lipase, amylase and protease (Leung & Stevenson 1988, Pemberton et al. 1997) and the type III secretion system (Yu et al. 2004). The pathogenesis of *A. hydrophila* is multifactorial (Yu et al. 2004) but the disease mechanisms have not been clearly elucidated.

Molecular biology tools and techniques, such as PCR (Polymerase Chain Reaction), have been used for the identification of etiologic agents causing various diseases, enabling the identification of possible gene encoding virulence factors responsible for pathogenesis of the microorganism. In addition to molecular characterization, *in vivo* evaluation is necessary to qualify the pathogenicity (Costa et al. 2010).

The objective of this paper was to verify, by PCR, the presence virulence genes pointed by literature (aerolysin, hidrolipase, elastase, and lipase) in *Aeromonas hydrophila* isolates obtained from pacamã fish (Lophiosilurus alexandri) from the São Francisco River Valley, and to evaluate the virulence of these genes in Nile tilapia in terms of distinct parameters.

MATERIALS AND METHODS

Location. The study was conducted at the Animal Microbiology and Immunology Laboratory, linked to the Animal Science course, Agricultural Science Campus, at the Universidade Federal do Vale do São Francisco (Univasf) (Federal University of the São Francisco Valley), Petrolina/PE, Brazil.

Bacterial isolates. A total of 114 isolates of *Aeromonas hydrophila* were used in this study, from the bacteria collection of the Animal Microbiology and Immunology Laboratory at the Univasf. These isolates were obtained from the kidneys, integument, gut and injuries of pacamãs (*Lophiosilurus alexandri*).

The animals came from the Sobradinho hydroelectric dam (Sobradinho/Bahia, Brazil) and from Integrated Center for Fishery Resources (CIRPA) from Bebedouro, Pernambuco, Brazil between the 2009 and 2010 years.

Aeromonas hydrophila isolates were identified in advance by morphological, tinctorial and biochemical characteristics (Quinn et al. 1994) and by PCR for confirmation of genus and species, using the methods described by Ghatak et al. (2007). The cultures were subcultured on Tryptone Soy Agar (TSA), and then used for molecular characterization.

Molecular characterization of isolates. The DNA of *Aeromonas hydrophila* was heat-extracted in a final volume of 50µl. The presence of aerolysin, hidrolipase, elastase and lipase genes was verified by PCR using the specific primers shown in Table 1. PCR reactions specific for each gene are described in Table 2.

The PCRs were performed under similar conditions: the first step was denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds and an extension step at 72°C for 30 seconds. After the end of the cycles, one final extension step at 72°C for 10 minutes was added. Among the genes, the difference consisted of the annealing temperature (60.5°C for hidrolipase; 60.6°C for elastase; 58.2°C for lipase and 55.5°C for aerolysin). The products of PCR were analyzed on agarose gel 2% stained with ethidium bromide and visualized in a UV transilluminator.

Inoculation of *Aeromonas hydrophila* with different frequencies of virulence factors in Nile tilapia. Seventy two Nile tilapia fingerlings (*Oreochromis niloticus*) were used, weighing

Table 1. Primers used for detection of virulence genes

Gene	Primer	*Identification* (5' – 3')	Size in base pairs	Reference
Hidrolipase	*Lip*-F	AACCTGGTTCCGCTCAAGCCGTTG	65	(CASCON et al., 1996)
	Lip-R	TTGCTCGCCTCGGCCCAGCAGCT		
Elastase	*ahyB*-F	ACACGGTCAAGGAGATCAAC	540	(SEN, 2005)
	ahyB-R	CGCTGGTGTTGGCCAGCAGG		
Lipase	*pla/lip*-F	ATCTTCTCCGACTGGTTCGG	383 – 389	(SEN, 2005)
	pla/lip-R	CCGTGCCAGGACTGGGTCTT		
Aerolysin	*aer*-F	CCTATGGCCTGAGCGAGAAG	431	(HOWARD et al., 1987)
	aer-R	CCAGTTCCAGTCCCACCACT		

Table 2. PCR Conditions of virulence genes

Reagents	Virulence genes			
	Hidrolipase	Elastase	Lipase	Aerolysin
Buffer	1X (10 mM Tris-HCl pH 8,5, 50 mM KCl)			
$MgCl_2$ (mM)	2	1,2	1,5	2
dNTPs (mM)	0.4	0.4	0.2	0.4
Primers (pmol)	15	15	7.5	15
Taq DNA polymerase (U)	2.5	0.15	2.5	2.5
DNA template (µL)	8	4	4	8
Ultrapure water (µL)	10.0	14.6	15.75	10.0
Total (µL)	25	25	25	25

4.26±0.02g, from the Integrated Center for Fishery Resources (CIRPA) from Bebedouro, Pernambuco, Brazil. The fish were distributed in 24 tanks with a volume of 60 L in a completely randomized design with four treatments and six replications. Each experimental unit was composed of one tank with three fingerlings

Treatments consisted of inoculation in the fingerlings of *A. hydrophila* isolates negative for all genes, positive for two genes, positive for four genes and, a control treatment, in which only saline solution was inoculated. After seven days of adaptation of fishes, inoculation of *A. hydrophila* in the Nile tilapia was performed by intramuscular injection, right dorsal-lateral, in each fish from a preparation of bacterial inoculum diluted in sterile saline solution at a concentration of 10^8 CFU/ml. The isolate preparations were applied at a rate of 0.2ml/fish, and the same amount for the control treatment, consisting only of saline solution. The bacterial inoculum possessing two virulence genes (combination of lipase/aerolysin) was chosen due to its higher occurrence in the PCR analysis. The colonies used were chosen at random within the group.

The tanks had constant aeration by contact through microporous stones linked by silicone hoses to mini air compressors. Daily, in the morning (7:00 a.m.) and in the afternoon (4:30 p.m.), they were siphoned, with the removal of 40% of the water, feces and any remaining feed. The physical-chemical parameters of water were measured daily for temperature, pH, dissolved oxygen and electrical conductivity.

A diet was formulated in accordance with the nutritional requirements of the species in these stage (Boscolo et al. 2002, Meurer et al. 2003), composed of 70.79% soybean meal, 16.70% corn, 5.00% soybean oil, 2.80% dicalcium phosphate, 0.20% calcitic limestone, 4.00% vitamin and mineral supplement, 0.50% salt and 0.01% butyl hydroxy toluene (BHT). It was provided free choice three times to day (8:00 a.m., 12:00 a.m. and 5.00 p.m.).

Before of the *A. hydrophila* inoculation procedure in the fish, water samples from the tanks were cultivated in blood agar to check for the presence of bacteria in the aquatic environment itself. After inoculation, the fish were observed for the formation of ulcerative lesions, and then a descriptive analysis was performed. Mortality was also assessed and the results were subjected to analysis of variance (One-way ANOVA) and the Duncan test by the Statistica 7.0 software.

At the end of the experiment, one fish from each experimental unit was used for bacterial growth in the kidney in a Tryptone Soy agar (TSA) medium. The cultivation of kidney and ulcerative lesions were also carried out in all the fish, which died as a result of the inoculation.

RESULTS

PCR

The frequency of the virulence factors investigated in 114 *Aeromonas hydrophila* isolates is shown in Table 3. The gene most frequently found in the isolates was the aerolysin gene, while the least frequent was that of hidrolipase.

Of the 114 isolates analyzed, 100 (87.72%) had at least one of the genes of the virulence factors studied. The distribution of virulence factors in the *A. hydrophila* isolates is presented in Table 4. In all, 12 distinct patterns were found for the presence of genes for the virulence factors in the *A. hydrophila* isolates studied. The association of virulence factors most frequently observed was lipase/elastase/aerolysin/hidrolipase, described in 21 isolates. The lipase / elastase / aerolysin combination was reported in 14 isolates and the presence of the aerolysin gene, in isolation, in the same quantity. An important aspect to note is that 42.98% of the isolates (49/114) had at least three of the virulence factors evaluated.

Aeromonas hydrophila inoculation with different frequencies of virulence factors in Nile tilapia fingerlings

The mean values for temperature, pH, dissolved oxygen and electrical conductivity were 27.11±0.08°C, 7.18±0.05, 7.40±0.05mg/L, 76.73±0.58µSm/cm respectively. There was no variation in these parameters among the treatments (P>0.05).

The bacterial culture showed no growth of *Aeromonas* spp. from samples of the water from the tanks. The bacterial culture from kidney and ulcerative lesion samples of fish that died during the experimental period showed bacterial growth of the *Aeromonas* spp.; however, this was not seen in the culture from kidney samples of fingerlings at the end of the experiment.

The mortality values of Nile tilapia fingerlings inoculated with *Aeromonas hydrophila* containing no gene (T2), positive for two genes (T3), positive for four genes (T4) and

Table 3. List of virulence genes present in *Aeromonas hydrophila* isolates of pacamãs collected between the 2009 and 2010 years

Genes	Number of isolates	Occurrence (%)
Aerolysin	90	78.95
Lipase	66	57.89
Elastase	53	46.49
Hidrolipase	41	35.96

Table 4. Occurrence and combinations of virulence genes in *Aeromonas hydrophila* isolates of pacamãs collected between the 2009 and 2010 years

Gene combinations	Positive isolates	Occurrence (%)
Aerolysin	17	14.91
Lipase	4	3.51
Elastase	1	0.88
Hidrolipase		
Lipase/Aerolysin	17	14.91
Aerolysin/Hidrolipase	5	4.39
Elastase/Aerolysin	4	3.51
Lipase/Elastase	3	2.63
Lipase/Elastase/Aerolysin	14	12.28
Elastase/Aerolysin/Hidrolipase	8	7.02
Lipase/Aerolysin/Hidrolipase	4	3.51
Lipase/Elastase/Hidrolipase	2	1.75
Lipase/Elastase/Aerolysin/Hidrolipase	21	18.42

inoculated only with saline solution (T1), are described in Figure 1. It may be observed that the control treatment (inoculated with saline solution) is statistically different ($P<0.05$) from treatments 2, 3 and 4.

In Figure 2, which shows the increase in the number of lesions after inoculation of *A. hydrophila* in the Nile tilapia, we can observe that the control treatment (inoculated with saline solution) showed no lesions throughout the experimental period, the same occurred in a 24 h period for the treatment in which the inoculum did not contain the virulence genes tested. However, the behavior of the lesions changed as of the third day, being greater in the number of fish for isolates with fewer virulence genes, but this behavior was reversed from the fifth day of the trial.

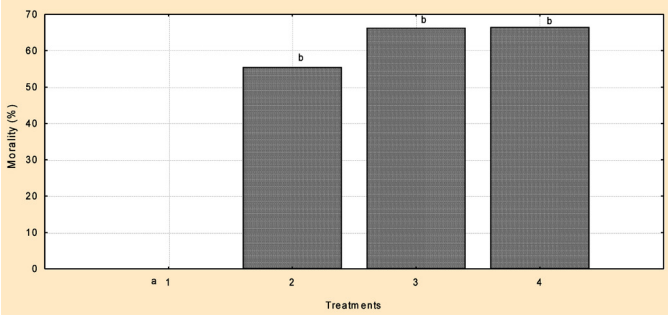

Fig.1. Mortality of Nile tilapia fingerlings after inoculation: (1) treatment inoculated with saline solution, (2) inoculated with *Aeromonas hydrophila* isolates containing no genes for virulence, (3) with two genes and (4) with four genes. Treatments with different letters (a, b) are statistically different.

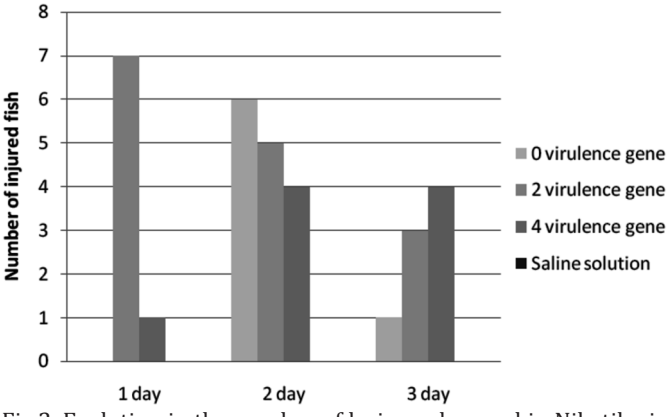

Fig.2. Evolution in the number of lesions observed in Nile tilapia fingerlings caused by *Aeromonas hydrophila* with different amounts of virulence genes.

DISCUSSION

The mean values of the physical-chemical parameters of the water tanks, such as pH, dissolved oxygen, electrical conductivity and temperature remained within the limits suitable for the Nile tilapia (Kubitza 2003).

In this study, virulence genes were identified in *Aeromonas hydrophila* isolates obtained from fish of the São Francisco River Valley by means of PCR. Due to their speed and sensitivity, tests using PCR have been used to detect the distribution of virulence genes in *Aeromonas* spp. (Yu et al. 2005). Only 12.28% (14/114) of isolates in this study showed no virulence factor, which corroborates with the data in the literature (Li et al. 2011). This fact confirms the high pathogenicity potential of *A. hydrophila* coming from fish, which suggests their high capability of causing disease in fish, especially because they were mostly isolated from diseased animals.

In this study, no statistically significant relationship was found between the presence of virulence factors and mortality among Nile tilapia caused by infection of *A. hydrophila.* This difference was only observed in relation to the control group inoculated only with saline. These results confirm the pathogenicity of the isolates regardless of the presence of the virulence factors analyzed (aerolysin, hidrolipase, lipase and elastase).

The presence of virulence factors, particularly those related to extracellular products, play an important role in the translocation of *Aeromonas* spp. in the epithelium, thus being broadly associated with bacterial virulence (Jutfelt et al. 2008). Ottaviani et al. (2011), studying *Aeromonas* spp. isolates from clinical sources (human diarrhea), did not find these relationship in the strains; although identified as the cause of acute gastroenteritis, they were considered negative for the virulence factors tested. However, the virulence of *Aeromonas* spp. may be associated with several factors, and isolates from human patients usually do not have a significant genetic relationship with the pathogens of aquatic organisms. This fact may be associated with the results reported in this study, especially considering that other virulence factors, in addition to those studied in this paper, have also been described as virulence markers in *A. hydrophila* (Li et al. 2011).

Some studies have reported a positive correlation between higher numbers of virulence genes, harbored in *Aeromonas* spp. isolates, and their potential for determining diseases (Albert et al. 2000; Sha et al. 2002; Chang et al. 2008). Heuzenroeder et al. (1999) proposed that for *A. hydrophila* the presence of two genes might indicate their virulence in animals. These results confirm that this species has a larger matrix of virulence genes when compared with other species of clinical relevance (Aguilera-Arreola et al. 2007). The strains of *A. hydrophila* investigated here in large part had two or more genes (68.42%), although in different combinations, which highlights their potential for pathogenicity, confirming the studies of Li et al. (2011) which demonstrated that the virulence properties of *A. hydrophila* are highly correlated with the presence of specific virulence genes, such as aerolysin, enterotoxin and protease.

Aerolysin was the factor most commonly described in *A. hydrophila* isolates in this study, in agreement with other papers (Nam & Joh 2007, Nawaz et al. 2010, Li et al. 2011). According to Heuzenroeder et al. (1999), the presence of aerolysin is a strong indication of virulence in pathogenic isolates of *Aeromonas* spp. Biscardi et al. (2002), studying the occurrence of *A. hydrophila* in 84 water samples, isolated six strains present in mineral water considered as cytotoxic and having the aerolysin gene; among 12 isolates of thermal waters, seven were cytotoxic and 11 contained this

gene. Ottaviani et al. (2011), studying strains of *Aeromonas* spp. isolated from foods and from the environment (surface water) in Italy, also found a higher percentage of the aerolysin gene, which was more prevalent in environment isolates. In trout, Nam & Joh (2007) also indicated a higher prevalence of aerolysin in pathogenic isolates. Aerolysin is also considered the major virulence factor in *A.veronii* isolates for catfish (Nawaz et al. 2010).

The genes encoding elastase and lipase were identified in A. hydrophila isolates of the São Francisco River Valley pacamas. These genes are commonly found in isolates of *Aeromonas* spp. (Sen 2005). The lipases and hiydrolipases are considered important virulence factors in *Aeromonas* spp. because they alter the structure of the cytoplasmic membrane of the host and thus exacerbate its pathogenicity, especially if the aerolysin gene is present (Nawaz et al. 2010). These genes are important extracellular factors for colonization of host tissues and their necrosis (Cascon et al. 2000). Song et al. (2004) demonstrated the potential of the association of elastase with the damage caused by aerolysin in cell cultures. The capacity of extracellular enzymes to cause lysis to "feed" the bacterial cells in proliferation is very important for *Aeromonas* spp. (Cascon et al. 2000). The lipase gene was also described as an important virulence factor in *Aeromonas* spp. isolates from trout (Nam & Joh 2007).

Different *A. hydrophila* isolates led to the development of lesions in tilapia, regardless of the number of virulence genes tested. It was observed that after the third day, the number of injuries grew due to a smaller number of virulence genes present in *A. hydrophila.* A reduction in the number of lesions was observed from the third day on in animals inoculated with *A. hydrophila,* without and with two virulence factors. However, after five days of testing, in the animals inoculated with isolates containing four virulence factors, the number of injuries remained constant; this fact is directly related to mortality, which was greater in animals inoculated with *A. hydrophila* containing a greater number of virulence genes.

CONCLUSIONS

Virulence factors are widely distributed among *Aeromonas hydrophila* isolates, indicating the pathogenic potential for aquatic organisms of the São Francisco River Valley.

Aerolysin was the most frequent virulence factor in the isolates analyzed.

A. hydrophila has the capability of causing mortality in Nile tilapia fingerlings, regardless of the presence and quantity of the virulence genes tested.

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