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ORIGINAL PAPER

High frequency of hemolytic and cytotoxic activity in *Aeromonas* spp. isolated from clinical, food and environmental in Rio de Janeiro, Brazil

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Abstract Molecular study of aerolysin and cytotonic enterotoxin genes by PCR and colony blot hybridization was performed in 117 strains of *Aeromonas* spp. isolated from different sources. Homogeneous distribution of these genes in *A. hydrophila* complex strains was observed. For *A. caviae* and *A. sobria* complex strains, aerolysin genes were more frequent than cytotonic enterotoxins genes. Of 64 *A. caviae* complex strains, only one (1.5%) amplified the 451 bp product for the *aer* gene, however, the same primers detected a 400 bp product in 50 (78%) strains. This product was sequenced and had two short regions with homology to several hemolysin genes. The genotype $aer^{+}/aerA^{+}/hly^{+}/ast^{+}/alt^{+}$ was detected in six *A. hydrophila* strains from food and environmental source. The most common genotype found in *A. hydrophila* strains was hly^{+} (85%) and $aerA^{+}$ (78.7%), while in *A. caviae* complex strains was $aerA^{+}$ (32.8%). All *A. veronii* complex *sobria* strains were $aer^{+}/aerA^{+}$. All *A. caviae* and *A. hydrophila* were positive when tested with *aer* probe using the colony blot test. Thirty-seven percent of *A. hydrophila* and 53% of *A. caviae* tested were positive for *ast* probe. Eighty-nine percent of samples were cytotoxic in Vero cells. Our data demonstrated that *Aeromonas* spp. can harbor and express virulence genes and reinforce the potential of *Aeromonas* as a human pathogen.

Keywords *Aeromonas* spp. · Aerolysin genes · PCR · Virulence factors

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Introduction

Aeromonas spp. are Gram-negative bacteria, and ubiquitous in aquatic environments, although they are also involved in a variety of human infections (Janda and Abbott 1998). *Aeromonas* spp. are widely isolated from clinical (Freitas et al. 1998; Heuzenroeder et al. 1999; Hofer et al. 2006), environmental (Sen and Rodgers 2004; Aguilera-Arreola et al. 2005), and food samples (Isonhood and Drake 2002). Some *Aeromonas* species are recognized as pathogens to poikilothermic animals and humans. *Aeromonas* spp. have been isolated from patients with diarrhea, soft tissue infections, otitis, cystitis, septicaemia and extraintestinal diseases. The pathogenesis of *Aeromonas* infections is therefore complex and multifactorial. *Aeromonas* species produce an array of virulence

factors including enterotoxins, hemolysin, exoenzymes, siderophores, flagella and secretion mechanisms.

The β-hemolysin-related aerolysin and the cytotoxic enterotoxin (Act) are pore-forming toxins able to alter cell permeability and cause secretory and inflammatory diarrhea by mechanisms that have not been completely elucidated. The *act* gene expression is regulated by GidA, which is widely distributed among human pathogens. The *gid*A mutants were avirulent in mice and presented reduced hemolytic and cytotoxic activity (Sha et al. 2004). Other types of hemolysins were also described in *Aeromonas* spp., the AHH1 homologous to *Vibrio cholerae* Hly hemolysin, AerA protein homologous to aerolysin and *A. sobria* hemolysin (ASH) (Heuzenroeder et al. 1999). *Aeromonas* also produce enterotoxins that cause morphological changes in cell lines and cause fluid accumulation in animal models. These proteins are heat-labile and heat-stable cytotonic enterotoxin codified by *alt* and *ast* gene, respectively (Sen and Rodgers 2004; Aguilera-Arreola et al. 2005).

We have investigated the distribution of putative virulence genes and demonstrated phenotypically the hemolytic as well as cytotoxic activities of clinical, food and environment *Aeromonas* spp. isolates.

Materials and methods

Bacterial strains

A total of 117 isolates of *Aeromonas* spp. (64 *A. caviae* complex, 47 *A. hydrophila* complex and 6 *A. veronii* complex) previously isolated from feces in patients with diarrhea, also from vegetable and water samples (Freitas et al. 1998; Araújo et al. 2002; Trajano et al. 2003) were used in this study. The strains were maintained at −70 and −20°C in Skim Milk (Difco, Lab, Detroit, MI, USA) containing 20% Glycerol (Reagen, RJ, Brazil) (Table 1). The strains were subcultured on Standard II *Nahr Agar* (Merck, Darmstadt, Germany) and incubated at 28–30°C overnight. The bacterial growth was spread onto Standard II *Nahr Agar* plates incubated at 28–30°C for 24 h.

From each plate one typical colony, oxidase positive was inoculated in tubes containing the same culture medium. The cultures were tested in phenotypic and genotypic assays.

Determination of hemolytic and cytotoxic activities

The strains were tested for β-hemolytic activity on Blood Agar Base (Oxoid, Hampshire, England) supplemented with 5% washed and ressuspensed sheep erythrocytes in Phosphate Buffer Saline (pH 7.4) with incubation at 28–30°C until 72 h. The *A. hydrophila* ATCC7966, FDA110-36 and *A. caviae* A1833 strains with hemolytic activity in 24 h of growth and *A. caviae* strains (A1885, A2057, A2091 and ATCC15468) with hemolytic activity after 24 h of growth were used as positive control. Assays using Vero (ATCC CCL81) cells were performed according Couto et al. (2007). The cytotoxic-positive effect was considered if there was >50% of cell death. *A. caviae* ATCC 15468 filtrates were used as negative control and *A. hydrophila* ATCC 7966 filtrates were used as cytotoxic-positive control.

Detection of putative virulence genes

All strains were analyzed by PCR, the bacterial DNA was prepared by boiling according Falcón et al. (2006). In a final volume of 50 µl, the PCR reaction mixture consisted of 5 µl of DNA sample, 1× PCR Buffer (20 mM Tris–HCl pH 8.4 and 50 mM KCl), 3 mM $MgCl_2$, 250 µM of each dNTP(Invitrogen); 0.2 µM of each primer set (Invitrogen) and 2 U of Taq DNA polymerase (Invitrogen). The PCR assays were performed using a Thermal Cycler (PTC—100TM MJ Research, Inc.). The primers used for PCR detection of virulence genes are listed in Table 2. The PCR cycling condition was performed according to the reference for each primer set. When specified, PCR products were digested with 1.5 U *Hae*III (Invitrogen). Further identification of the PCR products obtained with HemPF/PR primers was done by sequence analysis using the same primers. The 400 and 451 bp amplicons were sequenced in both directions using the BigDye terminator mix version 3.1 (Applied Biosystems) according to manufacturer's recommendations. Electrophoresis of fluorescent products was performed in an ABI PRISM 3730 instrument (Applied Biosystems). The partial sequence obtained was compared with all *Aeromonas*

Table 1 Distribution of 117 *Aeromonas* spp. isolated from clinical, food and environment

Origin	Source	*A. hydrophila* complex	*A. caviae* complex	*A. sobria* complex	Total
Human	No diarrheic feces	–	5	–	5
	Diarrheic feces	14	28	1	43
	Wound	3	–	–	3
	Others[a]	5	5	1	11
Food	Cheese	6	11	–	17
	Vegetables[b]	12	5	1	18
	Milk[c]	1	–	–	1
Environment	Lagoon[d]	6	10	3	19
Total		47	64	6	117

[a] Non-determined origin
[b] Lettuce
[c] Pasteurized milk
[d] Superficial water

Table 2 Primers used for PCR detection of virulence genes in clinical, food and environmental *Aeromonas* isolates

Primers	Virulence genes	DNA sequences (5′–3′)	PCR product (bp)	Gene bank	References
HemPF	Cytolytic enterotoxin gene (*aer*)	ccggaagatgaaccagaataagag	451	M84709	Granum et al. (1998), Chopra et al. (1993)
HemPR		cttgtcgccacatacctcctggccc			
AerA A1	*A. hydrophila* hemolysin gene (*aer*A)	gcctgagcgagaaggt	418	AF410466	Heuzenroeder et al. (1999)
AerA A2		cagtcccacccacttc			
HlyA H1	*A. hydrophila* hemolysin gene (*hly*A)	ggccggtggcccgaagatacggg	595	U81555	Heuzenroeder et al. (1999)
HlyA H2		ggcggcgccggacgagacggg			
Gid AF	*gid*A gene *act* regulate	gcctgatccacatcggcatg	608	AY333759	Sha et al. (2004)
Gid AR		tgccgatgcacttgctctcc			
Ast-F	Heat-stable enterotoxin gene	tctccatgcttcccttccact	331	AF419157	Sen and Rodgers (2004)
Ast-R		gtgtagggattgaagaagccg			
Alt-F	Heat-labile enterotoxin gene	aaagcgtctgacagcgaagt	320	L77573	Aguilera-Arreola et al. (2005)
Alt-R		agcgcataggcgttctctt			

gene sequences available in the GenBank database using the BLAST program of the National Center for Biotechnology Information. Alignment of homologous sequences was carried out using CLUSTAL W program, version 1.8 (Thompson et al. 1997).

Colony hybridization

Two probes obtained by PCR with HemPF/PR primers were used, the 451 bp product using the *A. hydrophila* vegetable source (AV22) as template and the 400 bp product from *A. caviae* ATCC 15468. We also used probes obtained by PCR with AstF/AstR primers (331 bp) and *A. hydrophila* vegetable source (AV31) as template. The strain *A. hydrophila* AH10 was used as positive control and *E. coli* K12 DH5 α as negative control. Spot colonies were replicated by placing a filter paper on the surface of the MacConkey Agar for 1 h. The filters were prepared according to Maas (1983). The hybridization was performed with the Random Prime Labelling System (Invitrogen, CA, USA).

Results

All strains of *Aeromonas* spp. tested were β-hemolytic in 24–72 h of incubation (Table 3). Among the 47 isolates of *A. hydrophila*, 42 (89.3%) produced β-hemolysis after 24 h and 5 (10.6%) after 48 h of incubation. None of *A. caviae* strains were β-hemolytic in 24 h of incubation. Five (31.25%), strains

Table 3 Genotypic and phenotypic virulence factors in *Aeromonas* spp. isolates

Species	Isolates (no. of isolates)	Phenotypic characteristic No. of strains (%)		No. of positive strains in PCR assay (%)				
		β Hemolysis production (%)	Vero citotoxin production (%)	*aer* (451 bp)	*aer*A (418 bp)	*hly* (595 bp)	*ast* (331 bp)	*alt* (320 bp)
Aeromonas caviae complex ($n = 64$)	Human							
	No diarrheic feces (5)	5 (100)	5 (100)	–	1 (20)	–	–	1 (20)
	Diarrheic feces (28)	28 (100)	23 (82)	1 (3.6)	9 (32.1)	6 (21.4)	–	6 (21.4)
	Others* (5)	5 (100)	5 (100)	–	3 (60)	2 (40)	–	–
	Food							
	Cheese (11)	11 (100)	10 (91)	–	6 (54,5)	1 (9)	1 (9)	–
	Vegetables (5)	5 (100)	5 (100)	–	2 (40)	1 (20)	1 (20)	1 (20)
	Environment (10)	10 (100)	–	–	4 (40)	–	–	–
Aeromonas hydrophila complex ($n = 47$)	Human							
	Diarrheic feces (14)	14 (100)	14 (100)	3 (21.4)	13 (93)	11 (78.5)	5 (35.7)	2 (14.2)
	Wound (3)	3 (100)	3 (100)	1 (33.3)	2 (66.6)	3 (100)	–	1 (33.3)
	Others* (5)	5 (100)	5 (100)	4 (80)	3 (60)	5 (100)	2 (40)	–
	Food							
	Cheese (6)	6 (100)	6 (100)	4 (66.6)	5 (83.3)	4 (66.6)	5 (83.3)	5 (83.3)
	Vegetables (12)	12 (100)	12 (100)	5 (41.6)	10 (83)	10 (8.3)	9 (75)	7 (58.3)
	Milk (1)	1 (100)	1 (100)	–	1 (100)	1 (100)	1 (100)	–
	Environment (6)	6 (100)	6 (100)	1 (16.6)	3 (50)	6 (100)	6 (100)	6 (100)
Aeromonas sobria complex ($n = 6$)	Human							
	Diarrheic feces (1)	1 (100)	ND	1 (100)	1 (100)	–	1 (100)	–
	Others* (1)	1 (100)	1 (100)	1 (100)	1 (100)	–	–	–
	Food							
	Vegetables (1)	1 (100)	1 (100)	1 (100)	1 (100)	–	–	–
	Environment (3)	3 (100)	3 (100)	3 (100)	3 (100)	1 (33.3)	–	–

ND non-determined; – none positive results; * non-determined origin

were hemolytic in 48 h and 59 (92%) in 72 h. All *A. sobria* were β-hemolytic after 24 h. The HG4 *A. caviae* ATCC 15468 and HG5 *A. media* ATCC 33907 were hemolytic only in 48–72 h of incubation. The HG1 *A. hydrophila* ATCC 7966 reference strains and HG7 *A. sobria* ATCC 43979 were hemolytic in 24 h. Concerning the cytotoxic effects on Vero cell monolayers, alteration and intense damage to the monolayers were observed, after 24 h of incubation at 37°C, with total detachment of cells from the well bottom when inoculated with all *A. hydrophila* complex and *A. veronii* biotype *sobria* strains. However, cytotoxic effects were observed only in 48 (75%) *A. caviae* strains, even after 48 h.

The presence of genes involved with the hemolytic activity in *Aeromonas* spp. was analyzed. Of the 47 *A. hydrophila* strains, 38.3% were positive to amplify the 451 bp band using HemPF/PR primers. Ninety-one, 17, and 2.1% of *A. hydrophila* strains from food, clinical and environmental sources, respectively, were positive in the PCR assay with HemPF/PR primers. All *A. sobria* complex strains amplified the 451 bp band. Only one *A. caviae* strain isolated from the patient's diarrhoeal feces showed the 451 bp band. However, the same primer pair produced an unexpected amplicon of ~400 bp in 50 (78%) *A. caviae* strains. The 400 bp product was also found in three *A. hydrophila* strains from clinical sample

and one strain from food. All 400 bp products obtained in the PCR assay were digested with *Hae*III and produced two fragments (220 and 120 bp) visualized in the gel. The 451 bp product digestion produced a 360 bp fragment as expected (data not shown), minor fragments from the *Hae*III digestion could not be detected on the gel conditions used. The 400 and 451 bp PCR products were sequenced and the nucleotide sequences were aligned, there was no homology between them. The partial sequence of the 400 bp PCR product had two regions homologous to several hemolysin genes of *Aeromonas* spp., these two regions were separated from each other by 150 bp, the alignment showed that they overlapped with each other (consensus sequence: GGSCARGAGGTMTGTGG) (S = G, C; R = A, G; M = A, C). However, the rest of the sequence was unrelated to hemolysin gene. On the other hand, the 451 bp PCR product sequenced with the forward and reverse primer produced the expected sequence, which had homology with several hemolysin genes from *Aeromonas* spp., including *A. caviae*.

In addition, all 15 *A. hydrophila* strains and six *A. caviae* strains from different sources, which were PCR negative for HemPF/PR primers, were tested in colony blot hybridization using 451 and 400 bp PCR products as probe, all tested strains were positive for both probes. The same procedure was applied to *Aeromonas* spp. strains PCR negative for Ast-F/R primers, 37% of *A. hydrophila* and 53% of *A. caviae* tested were positive for *ast* probe (data not shown).

The *aer*A and *hly* genes were detected in 58 and 43.6% of the overall isolates, respectively. For *A. hydrophila* complex, the percentage of enterotoxin-positive varied from 78.7 to 85.1% using the AerA A1/A2 and Hly H1/H2 primer sets, respectively. *A. sobria* complex strains were 100% positive to *aer* and *aer*A genes. Furthermore, the *A. caviae* isolates from human, food and environmental sources showed the occurrence of the enterotoxin gene at a frequency of 20–60% to *aer*A genes and 0–40% to *hly* genes. The detection of genes that codified cytotonic enterotoxin (*ast* and *alt*) among the *Aeromonas* spp. was better observed in the *A. hydrophila* complex, specially in food source strains, just 3.1% (*ast* gene) or 12.5% (*alt* gene) of *A. caviae* isolates were positive, while 59.6% (*ast* gene) or 44.7% (*alt* gene) of *A. hydrophila* strains were positive. All strains of *Aeromonas* spp. were positive to GidA detection.

In Table 4, it is possible to observe 25 genotypes defined on the basis of PCR detection of aerolysin, cytotoxic and cytotonic enterotoxin genes. Just 6 (5%) strains, mainly in *A. hydrophila* complex strains isolated from food source, were positive for all genes (Table 4). Eighteen (15.4%) strains were positive for *ast* and *alt* genes. The *aer*A^{+}/*hly*$^{+}$ genotype was detected in 31 (66%) of *A. hydrophila* complex. The number of isolates with each hemolysin genotype was very low among the *A. caviae* complex (Table 4). One (1.5%) of the 64 strains studied presented the genotype *aer*A^{+}/*hly*$^{+}$/*ast*$^{+}$/*alt*$^{+}$. The genotype *aer*$^{+}$/*aer*A^{+} was detected in all *A. sobria* complex strains isolated from clinical, food and environmental source.

Discussion

The aerolysin and other hemolytic toxins produced by *Aeromonas* species (Abrami et al. 2000; Joseph and Carnahan 2000) were shown to play an important role in their virulence. Studies have demonstrated that aerolysin mutant strains were less virulent in assays of toxicity in vivo and in vitro (Abrami et al. 2003; Fadl et al. 2007). In addition, clinical and biological evidences highlight the aerolysin production by *Aeromonas* spp. in pathogenic processes. The PCR and colony blot hybridization assays were important tools to study the distribution of the virulent genes among the species of *Aeromonas* spp. isolated from different sources (Shaw et al. 1997; Khan et al. 1998; Kingombe et al. 1999; Sha et al. 2002, 2005). These molecular techniques may detect genes implicated in the toxins codification that have not been phenotypically detected because of sub or no expression of genes (Wang et al. 1996). However, limitations of these techniques should be considered when defining the genotypic profile and genes distribution, since PCR targeting still is not optimized for gene detection in some *Aeromonas* species, as our result suggest by detecting a non expected 400 bp product using HemPF/PR primers in *A. caviae* strains.

Studies of virulent *A. hydrophila* have contributed to identification of the virulence genes and understanding of pathogenesis mechanisms (Huang et al. 2006; Mok et al. 2006; Figueras et al. 2007). On the

Table 4 Genotypes detected in *Aeromonas* spp. isolates

Genotype	*Aeromonas hydrophila* complex, $n = 47$				*Aeromonas caviae* complex, $n = 64$			*Aeromonas sobria* complex, $n = 6$		
	Human source (22)	Food source (19)	E.[a] source (6)	Total (%)	Human source (38)	Food source (16)	Total (%)	Human source (2)	E. source (3)	Total (%)
aer$^+$	8	9	1	18 (38.3)	1	–	1 (1.56)	2	3	6[c] (100)
*aer*A$^+$	18	16	3	37 (78.7)	13	8	25[b] (39.1)	2	3	6[c] (100)
hly$^+$	19	15	6	40 (85.1)	8	2	10 (15.6)	–	1	1 (16.7)
ast$^+$	7	15	6	28 (59.6)	–	2	2 (3.1)	1	–	1 (16.7)
alt$^+$	3	12	6	21 (44.7)	7	1	8 (12.5)	–	–	–
aer$^+$/*aer*A$^+$	7	9	1	17 (36.2)	–	–	–	2	3	6 (100)
aer$^+$/*hly*$^+$	8	7	1	16 (34.0)	–	–	–	–	1	1 (16.7)
aer$^+$/*ast*$^+$	5	8	1	14 (29.8)	–	–	–	1	–	1 (16.7)
aer$^+$/*alt*$^+$	–	7	1	8 (17.0)	–	–	–	–	–	–
*aer*A$^+$/*hly*$^+$	15	13	3	31 (66.0)	3	1	4 (6.25)	–	1	1 (16.7)
*aer*A$^+$/*ast*$^+$	7	13	3	23 (48.9)	–	2	2 (3.1)	1	–	1 (16.7)
*aer*A$^+$/*alt*$^+$	2	10	3	15 (31.9)	1	1	2 (3.1)	–	–	–
hly$^+$/*ast*$^+$	5	13	6	24 (51.1)	–	1	1 (1.56)	–	–	–
hly$^+$/*alt*$^+$	3	10	6	19 (40.4)	1	1	2 (3.1)	–	–	–
ast$^+$/*alt*$^+$	–	11	6	17 (36.2)	–	1	1 (1.56)	–	–	–
aer$^+$/*aer*A$^+$/*hly*$^+$	7	7	1	15 (31.9)	–	–	–	–	1	1 (16.7)
aer$^+$/*aer*A$^+$/*ast*$^+$	5	8	1	14 (29.8)	–	–	–	1	–	1 (16.7)
aer$^+$/*aer*A$^+$/*alt*$^+$	–	7	1	8 (17.0)	–	–	–	–	–	–
*aer*A$^+$/*ast*$^+$/*alt*$^+$	–	9	3	12 (25.5)	–	1	1 (1.56)	–	–	–
*aer*A$^+$/*hly*$^+$/*ast*$^+$	5	11	3	19 (40.4)	–	1	1 (1.56)	–	–	–
*aer*A$^+$/*hly*$^+$/*alt*$^+$	2	8	3	13 (27.7)	–	1	1 (1.56)	–	–	–
hly$^+$/*ast*$^+$/*alt*$^+$	–	9	6	15 (31.9)	–	1	1 (1.56)	–	–	–
aer$^+$/*aer*A$^+$/*hly*$^+$/*ast*$^+$	5	6	1	12 (25.5)	–	–	–	–	–	–
*aer*A$^+$/*hly*$^+$/*ast*$^+$/*alt*$^+$	–	7	3	10 (21.3)	–	1	1 (1.56)	–	–	–
aer$^+$/*aer*A$^+$/*hly*$^+$/*ast*$^+$/*alt*$^+$	–	5	1	6 (12.8)	–	–	–	–	–	–

[a] Environment

[b] Including four isolates from environmental source

[c] Including one isolate from food source, – none positive results

other hand, clinical relevance of *A. caviae* as a putative pathogen is still not established, although this species has been described in gastroenteritis, cystitis and others clinical cases (Freitas et al. 1998; Sinha et al. 2004; Hofer et al. 2006; Al-Benwan et al. 2007). Despite of the increasing importance of *A. caviae* as a human pathogen, few studies have reported the molecular characterization of virulence genes in this species. For this reason, primers targeting *A. hydrophila* genes are also used to detect *A. caviae* genes. Consequently, the detection of *A. caviae* virulence genes can be underestimated, if we consider just PCR assays, as suggested comparing data obtained by PCR, colony blot and cytotoxic assays in *A. caviae* isolates. The PCR-based results for detection of *A. caviae* hemolysin gene indicates that HemPF/PR primer set lack specificity toward *A. caviae* hemolysin gene. Therefore, the PCR results need to be interpreted alongside phenotypic and alternative molecular methods such as colony blot hybridization and DNA sequencing.

The hemolytic and cytotoxic activities have been detected in isolates of *Aeromonas* spp. (von Graevenitz 2007). In our study, all *Aeromonas* strains were β hemolytic and 90.5% of 116 strains analyzed were cytotoxic in Vero cells. These toxic activities are considered as indicative of virulence potential (Joseph and Carnahan 2000; Abbott et al. 2003; Edberg et al. 2007). In *A. caviae*, the significant percentage of positivity was related with prolonged incubation time (48 and 72 h). According to Karunakaran and Devi (1994a, b), *A. caviae* may produce toxins during the stationary phase of growing that could explain our results. In addition, our data were in agreement with Freitas et al. (1998) and Couto et al. (2007), that detected hemolytic and cytotoxic activity after incubation for 24–48 h to *A. caviae*.

High rate of positivity was detected for the genes encoding the aerolysin/hemolysin regardless of source of isolation, in comparison with the low rates of cytotonic enterotoxin gene detection. The dominant genotype was *aer*A^{+}/*hly*$^{+}$ found in 66% of *A. hydrophila* complex strains, when combining three genes the dominant genotype was *aer*A^{+}/*hly*$^{+}$/*ast*$^{+}$ found in 40.4% of *A. hydrophila* complex strains. In contrast, the genotype *aer*A^{+}/*hly*$^{+}$ was found in only 6.25% of *A. caviae* complex strains. For *A. sobria* complex, the dominant genotype was *aer*$^{+}$/*aer*A^{+} found in all strains.

When we analyzed the occurrence of these genes among the *Aeromonas* isolated from different sources, we observed homogeneous distribution in *A. hydrophila* complex strains. Although for *A. caviae* complex and *A. sobria* complex strains, these genes were detected mainly in human/food and human/environment sources, respectively. Our results were in accordance with Heuzenroeder et al. (1999); that detected the dominant *aer*A^{+}/*hly*$^{+}$ genotype mainly in *A. hydrophila* isolated from clinical and environmental sources and *A. caviae* isolated from clinical source. In the same way, they also detected low rates of *hly* gene in *A. sobria* complex. Gonzalez-Serrano et al. (2002) detected the genotypes *aer*A^{+}/*hly*$^{+}$ in all *A. hydrophila* complex analyzed, but failed to detect these genes in strains of *A. sobria* complex. In contrast, the present study observed that all *A. sobria* complex strains present the *aer*A gene. The literature has reported low percentage of detection of aerolysin genes in *A. caviae* complex strains (Kingombe et al. 1999; Wang et al. 2003). However, our findings highlight the presence of *aer*A gene in *A. caviae* complex strains isolated from clinical, food and environmental source. We found 39.1% of positivity for *aer*A gene.

In our study, the *ast* and *alt* cytotonic genes were found in 26.5 and 24.8% among the aeromonads, respectively. Sen and Rodgers (2004), detected *ast* and *alt* genes in *Aeromonas* isolated from drinking water in 30 and 43%, respectively. Aguilera-Arreola et al. (2005) observed that *ast* gene was one of the most widespread genes, it was found in 96.7% of *A. hydrophila* isolates, whereas *alt* gene was exclusively found in diarrheic isolates. The same authors found the presence of *ast* gene in 91% of *A. hydrophila* isolates from Mexico and Spain, which was absent in *A. caviae* and *A. veronii* isolates, while *alt* gene had a low incidence in *A. caviae* and *A. veronii* (Aguilera-Arreola et al. 2007).

The high rate of hemolytic activity detected in *Aeromonas* spp. is remarkable and suggests that the occurrence of hemolytic factors in *Aeromonas* spp. is sufficiently spread out, as previously reported for Janda and Abbott (1998). Although, the detected hemolytic activity can be attributed to other toxins that are hemolytic, such as lipase and lecitinase, or either, the hemolytic activity can be multifactorial in *Aeromonas* spp. Besides that, variants of hemolysin gene may exist, which has not been detected in the PCR assay. Thus, complementary studies are required

in order to improve molecular detection of hemolysin gene in *Aeromonas* strains and also to understand hemolysin gene polymorphisms.

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