Molecular detection of enterotoxins in environmental strains of *Aeromonas hydrophila* and *Aeromonas jandaei*

L. C. Balsalobre, M. Dropa, G. R. Matté and M. H. Matté

ABSTRACT

Aeromonas species are widely distributed in aquatic environments and recent studies include the genus in the emergent pathogens group because of its frequent association with local and systemic infections in immunocompetent humans. Aiming to search for virulence genes in environmental strains of *Aeromonas hydrophila* and *Aeromonas jandaei*, we designed specific primers to detect *act/hly A/aer* complex and *alt* genes. Primers described elsewhere were used to detect *ast*. Eighty-seven strains previously identified using phenotypic and genotypic tests as *A. hydrophila* (41) and *A. jandaei* (46) were analysed for the presence of the virulence genes using PCR. DNA fragments of expected size were purified and directly sequenced. Among the 41 strains of *A. hydrophila* 70.7% (29), 97.6% (40) and 26.8% (11) possessed *act/hly A/aer* complex, *ast* and *alt* genes, respectively. Among the 46 strains of *A. jandaei*, 4.4% (2), 0% (0) and 32.6% (15) were positive for *act/hly A/aer* complex, *ast* and *alt* genes, respectively. Sequencing allowed for the confirmation of amplified products using BLAST. The present work proposes a specific and rapid diagnostic method to detect the main virulence determinants of *Aeromonas*, a genus potentially pathogenic to humans.

Key words | *act/hly A/aer* complex, *Aeromonas*, *alt*, *ast*, PCR, virulence

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INTRODUCTION

Aeromonas species are aquatic organisms that cause a series of clinical manifestations, including septicaemia, skin ulcers and diarrhoea (Chopra & Houston 1999). Although the genus was discovered more than 100 years ago, only during the past four decades has its role in a variety of human diseases been proved. *Aeromonas* species were formerly considered opportunistic organisms of immunocompromised hosts, but are now implicated as aetiological agents in numerous clinical cases involving competent and immunocompetent hosts (Janda & Abbott 1998; Sechi *et al.* 2002; Awan *et al.* 2006), as was observed following the Thailand tsunami, where the genus was the main cause of skin ulcers among the survivors (Hiransuthikul *et al.* 2005). The World Health Organization (WHO 2006) considers the health significance of *Aeromonas* as moderate and the evidence that implies the relation of genus *Aeromonas* with the cause of disease in humans includes: the absence of other pathogens in symptomatic individuals, an average of symptomatic individuals higher than asymptomatic ones, identification of enterotoxins in *Aeromonas* strains, and improvement of patients' diarrhoea when effective *Aeromonas* spp. antibiotic therapy is used (Kelly *et al.* 1993). The detection of these organisms is thought to be underestimated, as diarrhoea symptoms may subside spontaneously. Recent studies have included the genus *Aeromonas* within the emergent pathogens group owing to its increasingly frequent association with local and systemic infections in humans (Di Bari *et al.* 2007).

The genus *Aeromonas* is widely distributed in water, food and soil, and it has been isolated from treated and untreated water samples, including a hospital water supply and chlorinated water (Bomo *et al.* 2004; Wu *et al.* 2006).

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In addition, its prevalence in the environment is considered a threat to public health, as infections caused by these organisms usually result from the ingestion of contaminated water or food (Sen & Rodgers 2004). *Aeromonas* has been isolated from clinical samples as the sole pathogen, where it was inferred to be the cause of diarrhoea in the studied patients (Hofer *et al.* 2006). Previous studies have shown that *Aeromonas* strains can carry virulence determinants responsible for infection in humans (Kingombe *et al.* 1999; Sen & Rodgers 2004; Razzolini *et al.* 2008). As a result of the increasing numbers of reports of the genus's occurrence, in 1998 the Environmental Protection Agency (EPA) included *Aeromonas hydrophila* in the Contaminant Candidate List of potential waterborne pathogens (EPA 2006).

Among the recognized *Aeromonas* species, *Aeromonas veronii* bv. sobria, *Aeromonas caviae* and *Aeromonas hydrophila* are more frequently associated with diarrhoea in humans, representing 85% of clinical isolates. A smaller proportion of *Aeromonas jandaei*, *Aeromonas veronii* bv. veronii, *Aeromonas schubertii* and *Aeromonas trota* have also been isolated from clinical samples (Ormen *et al.* 2003). Despite the literature data, the most pathogenic species were determined to be *Aeromonas jandaei* ATCC 49568^T followed by *Aeromonas hydrophila* ATCC 7966^T (Janda & Kokka 1991) according to a pathogenicity study of 12 *Aeromonas* type strains.

Aeromonas can produce a variety of biologically active extracellular substances similar to virulence factors found in other enteropathogens, especially toxins responsible for gastrointestinal infections. *Aeromonas* virulence is multifactorial and not completely understood, but it is known that the cytotonic enterotoxins Ast and Alt, the toxin group aerolysin-hemolysin and the cytotoxic enterotoxin encoded by *act* gene play an important role in the genus pathogenesis and are directly associated with the cause of gastroenteritis in humans (Chopra *et al.* 1996; Sha *et al.* 2002). These virulence factors are chromosomally encoded and cause fluid secretion in rabbit ileal loops (Brown *et al.* 1997).

Given the importance of *Aeromonas* to public health, it is crucial to detect the potential pathogenic strains in the environment. The present work developed a rapid and specific molecular diagnostic method that can determine the main virulence factors of *Aeromonas hydrophila* and *Aeromonas jandaei* strains.

METHODS

Bacterial strains

Eighty-seven *Aeromonas* isolates were selected from the Culture Collection of the Public Health Laboratory located at the School of Public Health-SP-Brazil. The strains were isolated from different environmental water sources and have been previously identified as *Aeromonas hydrophila* (41) and *Aeromonas jandaei* (46) using molecular and biochemical techniques.

Preparation of DNA

Total chromosomal DNA from *Aeromonas* strains was extracted using the heat-shock method according to Chapman *et al.* (2001) with modifications. In brief, the strains were grown overnight in 2 ml of LB (Luria Broth) 1% NaCl and centrifuged for 5 min at 10,000 × *g*. The pellet was resuspended in 1 ml sterile MilliQ® water and centrifuged for 3 min at 10,000 × *g*. The pellet was resuspended in 200 μl of sterile MilliQ® water and the 1.5 ml microtubes were boiled for 10 min at 95°C and then immediately frozen for 30 min at −20°C. After that, tubes were centrifuged for 10 min and the supernatant was transferred to a new 1.5 ml microtube. Total DNA was stored at −20°C.

Design of primers

Primers were designed based on conserved regions of the genes and were 18 to 22 bp in length (Table 1). We experienced difficulties in choosing the GenBank sequences for hemolysin, aerolysin and *act* because of controversial nomenclature of these genes; hence, we used the genes for citotoxic enterotoxin (*act*), β-hemolysin (*hlyA*) and aerolysin (*aer*) as a group, denominated *act/hlyB/aer* complex. For *ast*, Sen & Rodgers' (2004) primers were used. For the *act/hlyB/aer* complex, genes named as cytotoxic enterotoxin, aerolysin and hemolysins (except α-hemolysin) were chosen. When designing the *alt* gene primers set we noticed that this gene presented a high similarity with the lipase gene and by studying the complete genome of *A. hydrophila* ATCC (CP000462) and *alt* (L77573) and lipase (AF092033) genes sequences,

Table 1 | Primers selected for the detection of virulence factors in *Aeromonas hydrophila* and *Aeromonas jandaei*

Primer (5′–3′)	Target gene (toxin)	Position*	Product
act/hlyA/aer complex†	*act:* cytotoxic enterotoxin	1661–2061	400 pb
F AGAAGGTGACYACCAAGAACA	*hlyA:* beta-hemolysin		
R CCACTTCACTTCACCCGGGA	*aer*: aerolysin		
ast‡	*ast*: cytotonic enterotoxin	2349–2677	328 pb
F TCTCCATGCTTCCCTTCCACT			
R GTGTAGGGATTGAAGAAGCCG			
alt λ	*alt*: cytotonic enterotoxin	181–944	764 pb
F ATCGGGGTGACCCTCACCTC			
R GGCAGGAACTCGTTGACGAAGC			

*Position based on the alignment of the genes for this study.
†Primers designed in this work.
‡Primers designed by Sen & Rodgers (2004).

we observed that the *alt* gene sequence is located inside the lipase gene, when compared with the *A. hydrophila* ATCC complete genome, so the primers herein designed to amplify the *alt* gene, are also able to amplify the lipase gene. Based on the information above, different nomenclatures have been given to the same gene. Unique primers were designed for the amplification of virulence encoding genes *alt* and *act/hlyA/aer* complex. All available gene sequences for a given factor were manually aligned using BioEdit (BioEdit version 5. 0. 6; Hall 1999) and searches were performed for all primers to test the overall theoretic specificity by using the basic local alignment search tool (BLAST). All primers were synthesized by Bioneer Oligo Synthesis Report (Korea).

PCR (polymerase chain reaction) analysis

Cycling conditions were determined for the three genes separately as: 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. The expected product sizes for *ast*, *alt* and *act/hlyA/aer* complex were 328 bp, 764 bp and 400 bp, respectively. Negative controls of *Vibrio fluvialis*, *Vibrio cholerae* and *Escherichia coli* were included.

The amplified products were visualized on 1.8% agarose gels stained with ethidium bromide. Bands were purified with illustra™ GFX™ PCR DNA (GE Healthcare, UK) and Gel Band Purification Kit and sequenced directly.

RESULTS

All the strains of *A. hydrophila* presented at least one gene: 70.7% (29), 97.6% (40) and 26.8% (11) possessed *act/hlyA/aer* complex, *ast* and *alt* genes, respectively. Almost 40% (17) of *Aeromonas jandaei* strains presented at least one gene: 4.4% (2), 0% (0) and 32.6% (15) were positive for *act/hlyA/aer* complex, *ast* and *alt* genes, respectively. The occurrence of more than one gene in the same strain was observed in 78.0% (32) of *Aeromonas hydrophila* and 6.2% (1) of *Aeromonas jandaei* strains. The distribution of the virulence factors alone and in combination was calculated based on the positive strains for the occurrence of at least one gene and is described in Table 2. Sequencing allowed the confirmation of the amplified products using BLAST, showing specificity of the *act/hem/aer* complex and *alt* primers, emphasizing the capability of detecting aerolysin, β-hemolysin and cytotoxic enterotoxin genes using the same pair of primers (Figures 1 and 2). The sequences are available with GenBank accession nos. EU849093, EU849094, EU849095, EU849096 and EU849097.

DISCUSSION

The application of molecular methods in the identification of *Aeromonas* species has brought advances in the area. Molecular evolutionary studies and methods have

Table 2 | The distribution of the virulence factors alone and in association, based on the positive strains for the occurrence of at least one gene

	Species (no. of strains)	Location	*Act*[*]	*ast*	*alt*	%
Aeromonas hydrophila						
	6, 7[†]; 12–16, 19[‡]; 17, 20, 39–41, 43, 44[§]; 21[‖]; 23, 24, 26, 29, 31[¶] (21)	STE, Santana Cave, Guarapiranga Dam, Dirty Water Cave, Tietê River	+	+	−	51.2
	3 (1)	STE	+	−	+	2.4
	5[†]; 18[‡]; 22, 27, 28, 33[¶]; 45–47[§] (9)	STE, Santana Cave, Guarapiranga Dam, Tietê River	−	+	−	22.0
	38, 42, 50 (3)	Guarapiranga Dam	−	+	+	7.3
	8[†]; 25, 30, 32[¶]; 37, 48, 49[§] (7)	STE, Guarapiranga Dam, Tietê River	+	+	+	17.1
Aeromonas jandaei	96(1)	Guarapiranga Dam	+	−	−	6.2
	94 (1)	Guarapiranga Dam	+	−	+	6.2
	52[**]; 58, 71, 73, 75, 76, 79, 84, 87, 90, 93, 95, 101, 103, 104[§] (15)	Recreational Lake, Guarapiranga Dam	−	−	+	88.2

[*] *act/hlyA/aer* complex.
[†] STE, sewage treatment effluent.
[‡] Santana Cave.
[§] Guarapiranga Dam.
[‖] Dirty Water Cave.
[¶] Tietê River.
[**] Recreational Lake.

elucidated the emergence of epidemiological pathogenic bacteria associated with the transmission of disease to humans (Kingombe *et al.* 1999; Conway & Roper 2000), helping in the detection, identification, differentiation and distribution of virulence determinants responsible for the pathogenicity of microorganisms (Schrag & Wiener 1995; Wassenaar & Gaastra 2001).

A considerable number of studies on *Aeromonas* spp. virulence factors are characterized by utilizing classic methods, including enterotoxigenicity in suckling mice, detection of cytotoxicity in Vero cell and detection of β-haemolysis in blood agar plates (Singh & Sanyal 1992; Falcão *et al.* 1998; Alavandi & Ananthan 2003; Awan *et al.* 2006; Obi *et al.* 2007; Razzolini *et al.* 2008). Despite this, there has been an increase in research using molecular methods to reach a definitive, rapid and specific diagnosis of genetic virulence determinants. Unfortunately the confusing virulence nomenclature is becoming a problem, as

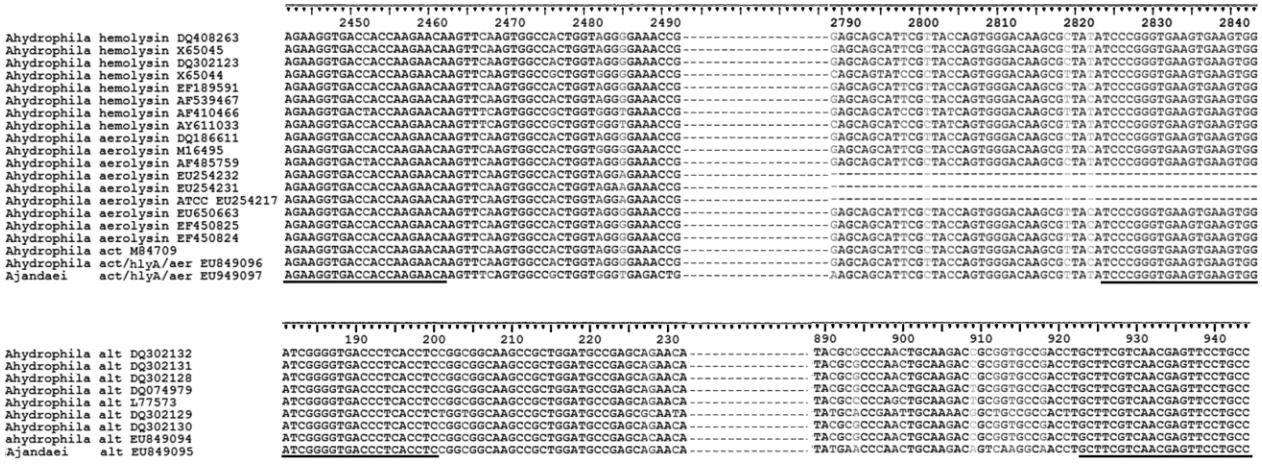

Figure 1 | Alignment of GenBank sequences and sequences found in this study of the *act/hlyA/aer* complex and *alt*; underlined sequences were regions chosen for primer design.

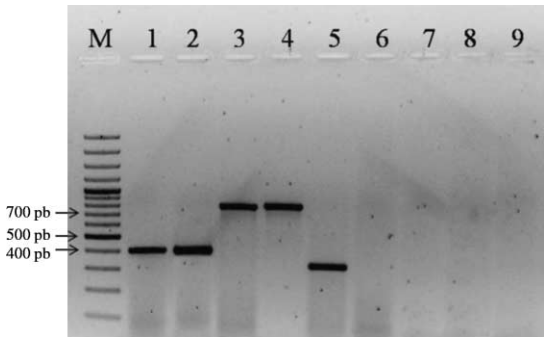

Figure 2 | Agarose gel electrophoresis of PCR products from *Aeromonas hydrophila* and *Aeromonas jandaei* virulence genes. Lanes 1, 3 and 5, *act/hlyA/aer* complex, *alt* and *ast*, respectively, from *Aeromonas hydrophila*. Lanes 2, 4 and 6, *act/hlyA/aer* complex, *alt* and *ast*, respectively, from *Aeromonas jandaei*. Lanes 7–9, *Escherichia coli* negative for the three genes. M stands for the molecular weight standard: Gene ruler® 100 bp DNA ladder Plus (Fermentas).

each publication uses its own nomenclature or does not emphasize this problem. For example, Kannan *et al.* (2001) verified the presence of the aerolysin gene in clinical strains of *A. hydrophila*, *A. caviae*, *A. veronii*, *A. schubertii* and *A. jandaei*. However, we did BLAST searches with their primers and found they showed homology to the cytotonic enterotoxin Alt, indicating that the products yielded in that study were from the *alt* gene and not the aerolysin gene. In the present work, the *act/hlyA/aer* complex of *A. hydrophila* can detect the three previously described virulence factors.

Previous studies have addressed the molecular detection of virulence factors in *Aeromonas* spp. by using oligonucleotide probes or PCR detection. Comparing the results obtained in this work with those present in the literature, we have found a higher number of *Aeromonas hydrophila* strains positive for the genes. Sen & Rodgers (2004) rarely found the *act* gene in *Aeromonas hydrophila* when compared with other species in the study, *ast* gene was found only in *A. hydrophila* and *alt* gene had a variable distribution among the species. Albert *et al.* (2000) found only one *A. hydrophila* strain among clinical isolates, which did not demonstrate the presence of *alt*, *ast* and *act* genes. Pollard *et al.* (1990) analysed 33 strains from different *Aeromonas* species using classical methods of haemolysis detection and PCR for aerolysin gene and found only one *Aeromonas sobria* strain positive for haemolysis production and only one *A. hydrophila* strain positive for the aerolysin gene. All the other strains were negative for both assays.

To our knowledge this is the first study that detects the *act/hlyA/aer* complex and a high number of positive strains for *alt* in *Aeromonas jandaei*. Few studies have included this species when searching for virulence factors and its importance to public health is still controversial, although some authors have emphasized its association with disease in humans and its capability of producing virulence factors (Hsu *et al.* 1981; Singh & Sanyal 1992; Esteve *et al.* 2003; Longa *et al.* 2005). Despite the use of *Aeromonas hydrophila* sequences to design *act/hlyA/aer* complex primers, two strains of *Aeromonas jandaei* yielded the expected product. Based on this, one can infer that this primer set can also be used to detect *act/hlyA/aer* complex in *Aeromonas jandaei*. *Ast* gene was not found in *A. jandaei* strains, corroborating reports that, except for one *A. encheleia* strain, *ast* was only found in *A. hydrophila* (Sen & Rodgers 2004).

It is important to note that the strains used in this study are environmental. Thus, both the percentage of positive strains found herein and the combination of the virulence factors are of concern vis-à-vis the potential virulence of *Aeromonas*. According to Albert *et al.* (2000), strains possessing *ast* and *alt* were associated with watery stools, as they were more frequent in clinical than in environmental samples. The opposite occurred with *ast* alone. *Act* was found to be associated with bloody diarrhoea and the gene was never found alone, as in the present work, except for one *Aeromonas jandaei* strain.

Virulence in *Aeromonas* has been associated with disease in humans. The wide distribution of the species highlights the importance of environmental sample surveys for virulence genes to determine potential pathogenic strains, which represent a risk to human health. Such surveillance requires a rapid and specific method, as the method proposed herein. This methodology may be useful to detect virulence genes in clinical *Aeromonas* strains, becoming an important instrument in the understanding of their epidemiology, distribution and association with disease.

CONCLUSIONS

This study describes a molecular method that can characterize the principal genetic virulence determinants of environmental and clinical strains of *Aeromonas*

hydrophila and *Aeromonas jandaei*. The distribution of these two species was also observed in various environmental sources and using the diagnostic method described herein we were able to show the presence and correlation of the virulence factors within the studied strains. Based on this, one can conclude that potential pathogenic strains are present in the environment, based on the detection of virulence factors in these strains, which may pose a risk to human health.

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