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Virulence characteristics of Aeromonas spp. in portuguese waters

Características de virulência em Aeromonas spp. em águas portuguesas

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Ana Sofia Duarte, Investigadora em Pós-doutoramento do Departamento de Biologia da Universidade de Aveiro e da Doutora Ana Cristina Esteves, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho à Lara...Simplesmente por tudo e muito mais do que é possível aqui descrever..

o júri

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palavras-chave

Aeromonas, isolados ambientais, factores de virulência, citotoxicidade, resistência antimicrobiana

resumo

Aeromonas spp. são bactérias autóctones de ecossistemas aquáticos distribuídas universalmente e tem sido descritas como importantes agentes patogénicos para o homem e peixes. Foram isoladas 35 estirpes de *Aeromonas* obtidas em fontes municipais e aquaculturas de Aveiro (Portugal). As espécies foram identificadas através de sequenciação do gene *gyrB* e descritas como *A. media* (30%), *A. hydrophila* (18%), *A. salmonicida* (18%), A*. eucrenophila* (9%), *A. allosaccharophila* (9%), *A. bestiarum* (9%), *A. punctata* (3%) e *A. molluscorum* (3%). Os isolados foram submetidos a testes para detectar características de virulência e perfis de resistência antimicrobiana. A presença dos genes de virulência foi de 94% para elastase (*ahpB*), 77% para lipases (*lip/lip/lipH3/apl-1*) e 31% para aerolisina/haemolisina (*act/aerA/hlyA*). A análise da produção de hemólise identificou 83% isolados hemolíticos a 37ºC, enquanto que a 25ºC apenas 49%. As actividades proteolíticas, lipolíticas, DNase e motilidade detectaram 89%, 83%, 94% e 43% de isolados positivos para essas actividade, respectivamente. Neste estudo a citotoxicidade em células Vero revelou a presença de 37 % de isolados citotóxicos, dos quais 91% possuíam o gene (*act/aerA/hlyA*), facto que sugere a importância deste gene para a citotoxicidade bacteriana. Quase todos os isolados foram resistentes a cefalotina e amoxicilina com ácido clavulânico, enquanto a maioria manifestou susceptibilidade (>80%) a tetraciclina, ceftazidima, cefepime, gentamicina, ciprofloxacina, ácido nalidixico, trimetoprima-sulfametoxazol, cloranfenicol e aztreonam. Foram encontradas resistências a mais do que cinco antibióticos simultaneamente em 11% dos isolados. Os nossos resultados demonstram a presença de *Aeromonas* potencialmente patogénicas e com multiresistência a antibióticos em águas Portuguesas, representando um sério risco para a saúde pública e um factor importante nas quebras de produtividade em aquaculturas.

keywords

Aeromonas, environmental isolates, virulence factors, cytotoxicity, antimicrobial resistance

abstract

Aeromonads are inhabitants of aquatic ecosystems with worldwide distribution and have being described as an important pathogen for humans and fish. A total of 35 *Aeromonas* strains collected from drinking fountains and aquacultures located in Aveiro (Portugal) were identified by *gyrB* sequencing as *A. media* (30%), *A. hydrophila* (18%), *A. salmonicida* (18%), *A. eucrenophila* (9%), *A. allosaccharophila* (9%), *A. bestiarum* (9%), *A. punctata* (3%) and *A. molluscorum* (3%). These isolates were analyzed for virulence characteristics and antimicrobial resistance patterns. The prevalence of putative virulence genes were 94% for elastase (*ahpB*), 77% for lipases (*lip/lip/lipH3/apl-1*) and 31% for aerolysin-like proteins (*act/aerA/hlyA*). The haemolyse production assay showed that 83% were haemolytic at 37ºC, while 49% were haemolytic at 25ºC. The proteolytic, lipolytic, DNase and motility activities were present in 89%, 83%, 94% and 43% of isolates, respectively. In this study the cytotoxicity assay in Vero cells showed 37% cytotoxic isolates and 91% had the *act/aerA/hlyA* gene, suggesting the major role of this gene in bacterial cytotoxicity. Nearly all isolates were resistant to cephalothin and amoxicillin/clavulanic acid, while they were typically sensitive (>80%) to tetracycline, ceftazidime, cefepime, gentamicin, ciprofloxacin, nalidixic acid, trimethoprim–sulfamethoxazol, chloramphenicol and aztreonam. Multiple resistances to more than five antibiotics were evidenced in 11% of the isolates. Our results demonstrated that potential pathogenic *Aeromonas* strains with multidrug resistance phenotypes are present in Portuguese waters, representing a real health concern and a major economic loss in aquaculture industry.

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Chapter 1. Introduction

1.1 General information

Aeromonas genus belongs to a single family of bacteria, *Aeromonodaceae*, in the order *Aeromonodales* (Martin-Carnahan & Joseph 2005). Aeromonads are Gram-negative organisms frequently motile by polar flagella. These organisms are straight, coccobacillary to bacillary bacteria with rounded ends that are oxidase- and catalase-positive, glucose-fermenting, facultative anaerobic, with resistance to the vibriostatic agent O/129 (Martin-Carnahan & Joseph 2005; Tacão *et al*., 2005; Yáñez *et al*., 2003).

Aeromonads may be divided in two major groups, according to different phenotypical characteristics, such as growth temperature, motility, production of indole, and elaboration of melanim-like pigment in tyrosine agar. The strains that grow at 35ºC to 37ºC and motile by polar flagella belong to mesophilic group and are commonly responsible for numerous human infections, which is subdivided into *A. hydrophila*, *A. caviae* and *A. sobria*. The psychrophilic group comprises non-motile strains that grow better at 22ºC to 28ºC and are responsible for causing fish infection, designated *A. salmonicida* (Janda & Abbott 1998).

1.2 Environmental distribution

Aeromonas species are waterborne bacteria that grow optimally within a temperature range of 22-35ºC. Some species can also growth in a temperature range of 0-45ºC (Mateos *et al*., 1993). These bacteria tolerate a pH range from 4.5 to 9 and optimum sodium chloride concentration is 0-4% (Isonhood & Drake 2002). With this high capacity of adaptation, several studies have demonstrated that aeromonads are naturally occurring inhabitants of aquatic ecosystems, widely distributed in nature. They can be found in many different habitats, namely freshwater, marine and estuarine waters, sewages, ground waters, untreated and treated drinking water and aquacultures, (Rahman *et al*., 2007; Nielsen *et al*., 2001; Janda & Abbott 1998; Holmes *et al*., 1995; Havelaar *et al*., 1992) but also can occur virtually in all foods, soil and feces (Razzolini *el al*., 2008; Chopra & Houston 1999). *Aeromonas* are also an important constituent of bacterial biofilms in water distribution systems due his efficiency in surface colonization (Razzolini *el al*., 2008; Sen & Rodgers 2004; Rabaan *et al*., 2001).

1.3 Pathogenicity

Members of the genus *Aeromonas* have been recognized as an important emerging human pathogen and responsible for several diseases both in human and poikilothermic animals (Scoaris *et al*., 2008; Figueras *et al*., 2005; Sen & Rodgers 2004; Yáñez *et al*., 2003; Razzolini *el al*., 2008; Rabaan *et al*., 2001; Janda & Abbott 1998).
In humans, aeromonads are mainly associated to gastrointestinal infections in healthy individuals of any age after the ingestion of contaminated water or food (Martins et al., 2006; Figueras *et al*., 2005). Extraintestinal severe illness, such as septicaemia, wound infections, cystitis, urinary tract infections, meningitis, peritonitis, respiratory tract disease and ocular infections, normally resulting from exposure to contaminated water have also been described (Huddleston *et al*., 2006; Figueras *et al*., 2005; Balaji *et al*., 2004; Janda & Abbott 1998). It has been reported that *Aeromonas hydrophila*, *A. veronii* biovar *sobria* and *A. caviae*, are the three main pathogenic aeromonads for humans (Rabaan *et al*., 2001; Sen & Rodgers 2004) to which 85% of all clinical specimens belong.

In fishes, *Aeromonas* spp. have been recognized as a significant pathogen causing hemorrhagic disease, ulcerative disease, soft tissue rot, fin rot and furunculosis, a systemic disease mainly caused by *A. salmonicida* and *A. hydrophila* . Therefore, this infections outbreak can lead to dramatic economical losses in aquaculture industry (Nam & Joh 2007; Balaji *et al*., 2004 Nielsen *et al*., 2001). Moreover, Lehane & Rawlin (2000) reported that aeromonads caused cellulites, myositis and septicaemia in humans following injuries from handling contaminated fish, working in aquacultures or keeping fish as pets.

1.4 Antibiotic susceptibility

Aeromonas spp. has been related to human gastrointestinal infection along with other syndromes (Abbott *et al*., 2003). Is known that wound infection can progress quickly into systemic infection and the fatality rate among patients with *Aeromonas* septicaemia ranges from 27.5-46%, which makes important an effective antibiotic treatment (Huddleston *et al*., 2006; Ko *et al*., 2003). Moreover, specific antibiotic therapy is used to treat animals and reduce losses in the aquaculture industry (Martinsen *et al*., 1991).

Aeromonas have a natural resistance to β-lactamics antibiotics (Walsh *et al*., 1997), since some strains can produce three different types of β-lactamases: a

cephalosporinase, a penicillinase and a metallo-β-lactamase (Pemberton *et al*., 1997). However, aeromonads have been reported to be typically susceptible to tetracyclines, aminoglycosides, trimethoprim-sulfamethoxazol, third-generation cephalosporins, chloramphenicol and quinolones (Ko *et al*., 2003; Janda & Abbott 1998; Koehler & Ashdown 1993). Although, studies have found increasing resistance to tetracyclines, trimethoprim-sulfamethoxazol and some extended cephalosporins (Scoaris *et al*., 2008; Palu *et al*., 2006 Ko *et al*., 1996), leading to the loss of antibiotic effectiveness to treat *Aeromonas* infection. Furthermore, some authors reported that environmental contamination with antimicrobials contributes to perpetuation and dissemination of antibiotic resistance genes, particularly by resistance plasmids transmission between other enteric species (Huddleston *et al*., 2006; Marchandin *et al*., 2003).

1.5 Virulence factors

Aeromonads possess several structural requirements necessary to promote an effective attachment (pili, flagella) and colonization (adhesions, outer membrane proteins) and to protect bacteria from host defence (S-layer, lipopolysaccharide, capsule), leading to a successful invasion of the host (Gavin *et al*., 2003; Kirov *et al*., 2002; Zhang *et al*., 2002; Merino *et al* 1996; Kirov *et al*., 1996; Bartkova & Ciznar 1994). In addition to the cell-associated virulence factors described above, *Aeromonas* are capable to produce numerous extracellular products with virulence properties such as cytotoxic and cytotonic enterotoxins, haemolysins, extracellular proteins (proteases, serine proteases, elastases [e.g. AhpB], lipases, DNAses, amylases, chitinases, xylanase, lecithinases and gelatinases). These are important molecules responsible for causing tissue damage, for overcoming host defences and for providing nutrients (Figueras *et al*., 2009; Scoaris *et al*., 2008; Figueras *et al*., 2005; Chopra & Houston 1999).

Bacteria must be able to colonize the host organism before it can cause infection. Motility is an important virulence factor for many Gram-negative pathogens and a significant invasion-related factor, since allows *Aeromonas* spp. to reach target cells where they colonize (Barnett *et al*., 1997). The swarming motility on surfaces of *Aeromonas* is associated to lateral flagella and the swarming motility in liquids is conferred by polar flagella (Kirov *et al*., 2004). The importance of lateral and polar flagella in cell adherence and biofilm formation has been recognised (Gavin *et al*., 2003; Gavin *et al*., 2002). Therefore, motile strains have a higher virulence potential and are key factors to the formation of bacterial biofilms on surfaces (e.g., water

distribution systems, fish, and surgical implants) (Scoaris *et al*., 2008; Rabaan *et al*., 2001).

One important and well characterised virulence factor of *Aeromonas* is Act, which is a cytotoxic enterotoxin (related to aerolysin) with haemolytic activity. This toxin is a pore-forming channel haemolysin able to alter cell permeability (Castilho *et al*., 2009). Act has proved to be lethal to mice when injected intravenously (Erova *et al*., 2007; Sen & Rodgers 2004; Chopra *et al*., 2000; Xu *et al*., 1998). Additionally, this cytotoxin has been shown in a study to have an important role in the pathogenesis of aeromonads, on which have been exposed the ability to activate proinflammatory cytokines and eicosanoid cascades in macrophages and a rat intestinal cell line causing tissue damage and fluid secretory response (Chopra *et al*., 2000). A study conducted by Sha *et al*. (2002), established an association of Act with bloody diarrhoea.

Another important virulence factor that contributes to *Aeromonas* spp. pathogenicity is the AhpB, a metalloprotease with caseinolytic and elastolytic activities (Cascón *et al*, 2000). The extreme importance of elastase was confirmed with a study carried out by Cascón *et al*. (2000), on which an isogenic mutant strain for *ahpB* gene presented a lethal dose (LD_{50}) one hundred times higher than wild type strain. Song and colleagues (2004) also have demonstrated the essential role of a metalloprotease in cytotoxic and haemolytic activities through activation of proaerolysin in aeromonads.

Lipases are also considered important factors for pathogenicity, since they contribute to bacterial nutrition and has been suggested their involvement in erythrocyte lysis (Chopra & Houston 1999; Pemberton *et al*., 1997). Also, *Aeromonas* produces a glycerophospholipid cholesterol acyl-transferase (GCAT) that results in production of cholysteryl esters and phospholipase activity that digests plasma membranes of host cells, which can provoke furunculosis in infected fish (Chacon *et al*., 2003; Buckley 1983). Moreover, mutants for the specific lecithinase gene (*plc*) reduced the LD_{50} in mice and fish (Merino *et al*., 1999).

Nucleases have been described as possible virulence factors, but their role in virulence is more ambiguous than the role of other degradative enzymes (Pemberton *et al*., 1997). Podbielski *et al*. (1996) reported that DNases have been involved in bacterial infections, given that it is an enzyme directly target for DNA, a macromolecule essential for the function of any host cell. In addition, the degradation of deoxyribonucleic acid yields carbon and nitrogen molecules; consequently DNases have also been considered as possible nutritional enzymes in *Aeromonas* (Scoaris *et al*., 2008).

Apart from the specific functions played by extracellular and cell associated virulence factors in *Aeromonas* spp. the exhibition of cytotoxicity in cell lines represents a virulence potential (Castilho *et al*., 2009; Ghatak *et al*., 2006;). In several studies have been suggested the importance of cytotoxicity assay to confirm the production of cytotoxins and haemolysins in *Aeromonas* strains, indicating the relevance of these extracellular virulence factors in pathogenicity (Ghatak *et al*., 2006; Balaji *et al*.,2004 Ormen & Ostensvik 2001; Mateos *et al*., 1993)

The mechanisms used by of *Aeromonas* to cause diseases in animals and humans are essentially the same and involve an extensive array of virulence factors (Figueras *et al*., 2009; Chopra & Houston 1999; Cahill 1990). Many putative virulence factors and genes encoding these characteristics have been described. It is commonly accepted that is necessary more than one extracellular enzyme for an organism to be pathogenic, but the combination of virulence factors that would be required for pathogenesis is not completely understood despite intense investigation (Scoaris *et al*., 2008). For example, studies demonstrated presumptive virulence characteristics present in *Aeromonas* strains isolated from healthy humans (Sen & Rodgers 2004). The increased complexity of this issue is probably due the regulation and expression processes involved in virulence properties, as well as to complex immune response of the host that is able to modulate the pathogenicity of aeromonads (Sha *et al*., 2004; Sha *et al*., 2005; Galindo *et al*., 2006).

Chapter 2. Objectives

Since both humans and aquatic animals can be infected by *Aeromonas*, we have conducted a survey for *Aeromonas* isolates in several aquatic sources. We have sampled aquacultures and untreated drinking waters from Aveiro district (centre of Portugal). The aeromonads isolated from these sources were identified and characterised concerning the presence of genetic determinants of virulence and virulence associated phenotypes.

Therefore, the objective of this study is to determine if there is a threat to public health or to food industry (aquacultures) due to the presence of water associated *Aeromonas* .

The specific aims that were pursuit were:

- characterise the major putative virulence factors in *Aeromonas* isolates from aquacultures and untreated drinking waters;

- develop a correlation between the existent virulence factors and *in vitro* cytotoxicity*;*

- evaluate the antimicrobial resistance patterns and infer the most effective treatment to apply;

- develop an efficient cytotoxicity assay in Vero cell line.

Chapter 3. Bacterial cytotoxicity evaluation on Vero cell line

Human and fish *Aeromonas* related diseases have been attributed principally to the presence of aerolysin-like enterotoxins with hemolytic and cytotoxicity activities, able to induce cellular membrane lysis (Castilho *et al*., 2009; Chopra *et al*., 2000). These cytotoxins have been considered a major virulence factor presented by *Aeromonas*, making essential to assess the cytotoxicity in order to evaluate the pathogenic potential. (Ghatak *et al*., 2006). Cytotoxicity assays *in vitro* make use of various cell lines and the quantitative assessment of cytotoxicity generally relies on the visual counting of cells (Castilho *et al*., 2009; Ghatak *et a*l., 2006, Balaji *et al*., 2004), leading to low reproducibility of these approaches and consequently conferring significant limitations. In the absence of a standardized cytotoxicity method data comparison obtained from different studies rise some uncertainties that can only be overcomed with a more reliable and easy cytotoxicity assay.

Resazurin is a water-soluble dye that, when added to cell cultures, allows determining cell viability by the conversion of the reduced form by mitochondria. This redox reaction is responsible for a colour of the culture medium from blue to fluorescent pink that can be easily measured by colorimetric or fluorometric spectrophotometer. Moreover, resazurin allows continuous monitoring of cultures over time, since it is an extremely stable non-toxic dye to cells (Al-Nasiry *et al*., 2007).

Vero cell line (African green monkey kidney epithelial cells) has been chosen for evaluation of cytotoxicity since it is appointed as the most sensitive cell line to aeromonads cytotoxins, according to a study carried out on four different cell lines (Ghatak *et al*., 2006).

The purpose of this report is to describe the development of an improved, more reliable and simple cytotoxicity assay in Vero cell line.

The cell free supernatant (CFS) of *Aeromonas* isolates were prepared according to Balaji *et al*., (2004) with slight alterations. Each strain was grown in LB (Luria-Bertani, Miller) at 30ºC at 150 rpm overnight. Subsequently, the cultures were centrifuged at 10,000 rpm for 10 min and supernatants were filtered through a sterile 0.2µm pore size syringe membrane filter (Orange Scientific). The CFSs were collected in sterile tubes and stored at 4ºC till use for no longer than 24h.

Vero cell growth in tissue culture flasks was performed as described previously (Ammerman *et al*., 2008). Afterwards, volumes of 100µl Vero cells suspended in

growth medium DMEM (Dulbecco´s Modified Eagle Medium, Gibco) supplemented with 10% FBS (Foetal Bovine Serum, Gibco) were distributed into a 96-well tissue culture plate ($2x10^4$ cells per well) and incubated for 24h (± 80% confluent monolayer) at 37ºC in 5% CO_2 atmosphere. Serial dilutions (1:2; 1:16; 1:64 v:v) of CFS in PBS (phosphate-buffered saline) were made and an aliquot of 100µl of filtered supernatants was added to each well. The microtiter plates were incubated at 37ºC in 5% CO_2 for 48h. As negative control, growth medium alone without cells was used; for positive control wells did not received CFS. Each sample was tested in quadruplicate. After cell treatment, the medium was removed by aspiration and 50µl of growth medium with 10% Resazurin solution (0.1mg/mL in PBS) was directly added to each well. The microtiter plates were incubated at 37ºC in 5% CO_2 until reduction of Resazurin, converting the blue dye to pink (Al-Nasiry *et al*., 2007). The absorbance was read at 570nm and 600nm wavelength in a microtiter plate spectrophotometer (Infinite 200, Tecan i-control). Results were based on cell viability, using the following formula: Viable cells % = $(OD_{cell\ treatment} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control}) \times 100$.

The CFS preparations that induced cytopathic effect at least up to 1:16 dilution in 50% or more cells were recorded as a cytotoxic positive isolate (Ghatak et al., 2006; Villari et al., 2003).

The cytotoxicity results obtained with this method are represented in figure 1. These data establishes a clear difference between cytotoxic strains and non-cytotoxic isolates, revealing high sensitivity and producing more accurate results than other viability assays. This is due to the fact that Resazurin may detect low cell concentration with reproducible and sensitive signal (Nociari *et al*., 1998; Ahmed *et al*., 1994). Moreover, this method provides the straightforwardly information of slightly different cytotoxicity levels for cytotoxic CFS. This method allows identifying cytotoxic strains with the added advantages of minimising time and labour that are usually associated to cell counting by microscope observation, as a large number of samples can be assessed in a short period of time. Additionally, the potential bias of human interpretation related to methods based on visual determination may be avoided. Finally, due the fact that Resazurin is a non-toxic viable dye this method offers the advantage to keep the cells in culture allowing further analysis or observe changes over time.

In conclusion, this study reveals that Resazurin assay is highly sensitive, reliable, easy to perform, economical and non-toxic method to evaluate the bacterial cytotoxicity potential based on cell viability.

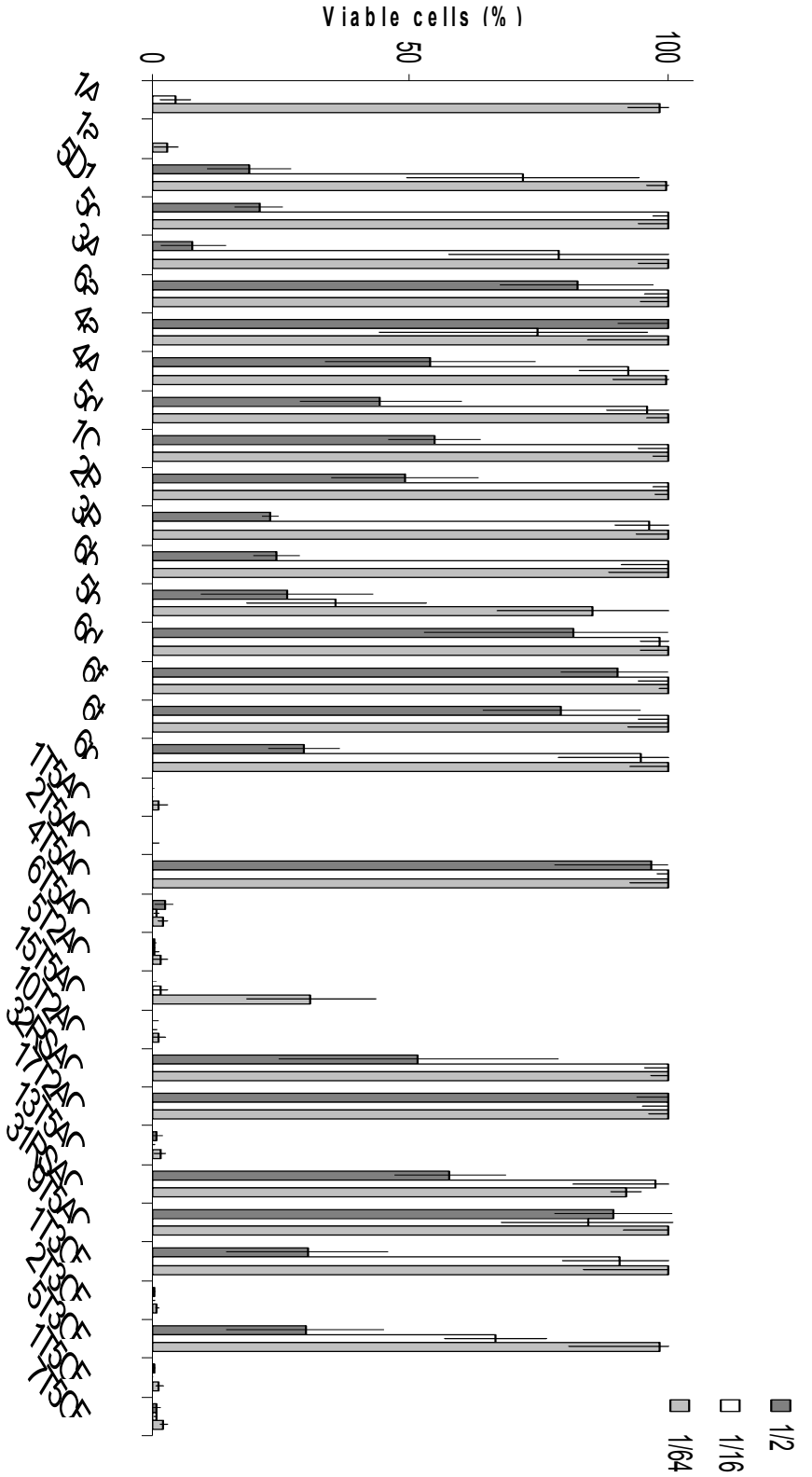

Figure 1. Cytotoxicity potential based on cell viability (results are given as percentage and the line inside bars correspond to the standard deviation) by Resazurin assessment

of 35 *Aeromonas* cell free supernatant with 1/2, 1/16 and 1/64 dilutions. As positive control the cells did not received CFS and the scale 0-100 indicates the percentage of living of living cells after the respective treatment. Cytopathic effect in 50% or more cells at least up to 1:16 CFS dilution was recorded as cytotoxic positive *Aeromonas* isolate.

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Chapter 4. Virulence factors of *Aeromonas* spp. in portuguese waters

4.1. Introduction

Aeromonas spp. are Gram-negative, oxidase-positive, facultative anaerobic, rod-shaped bacteria (Martin-Carnahan & Joseph 2005). These organisms are widely distributed in aquatic environments (Janda & Abbott 1998). Some *Aeromonas* species are responsible for a variety of human and fish infections (Scoaris *et al*., 2008; Figueras *et al*., 2005; Sen & Rodgers 2004). In the last decade aeromonads have been considered as an emergent pathogen of gastrointestinal disease in humans and normally associated to ingestion of contaminated water or food (Figueras *et al*., 2005; Yáñez *et al*., 2003).

The virulence of *Aeromonas* is complex and multifactorial, with an involvement of a number of virulence determinants, such as cytotoxic and cytotonic enterotoxins, haemolysins (AerA, HlyA, Act), proteases, lipases, elastases (AhpB), DNases and cell associated virulence factors like S-layers, pili, polar and lateral flagella, outer membrane proteins and plasmids (Figueras *et al*., 2009; Pablos *et al*., 2009; Scoaris *et al*., 2008Gavin *et al*., 2003; Kirov *et al*., 2002).

Several studies also reported the importance of aeromonads in biofilm formation in drinking water systems and highlighted his relevance in public health hazard due the ability to produce a range of virulence factors (Scoaris *et al*., 2008; Kirov *et al*., 2004; Rabaan *et al*., 2001).

Some of the toxins have been already characterised but their exact role in *Aeromonas* pathogenicity remains completely understood, probably due the regulation and expression of the various virulence factors (Scoaris *et al*., 2008). This fact requires further exploration to better understand the involvement of each putative virulent gene along with phenotypical activities in *Aeromonas* pathogenicity based on cytotoxicity *in vitro*. Moreover, this study was undertaken to determine the prevalence of potential pathogenic *Aeromonas* in portuguese waters as well to evaluate the antimicrobial resistance patterns.

4.2. Materials and methods

4.2.1 Bacterial strains

Seven water samples from aquacultures and six from drinking fountains were collected in Aveiro district. Water was collected into sterilized bottles and kept at 4ºC until analysis. 100mL Samples (100mL) were filtered through 0.45 um pore size sterile filter (Orange Scientific). Filters were placed onto agar plates selective for *Aeromonas* (GSP, glutamate starch phenol-red, Merck) supplemented with ampicilin (50µg/mL) and without supplement. Plates were incubated at 30ºC for 24h. Presumptive *Aeromonas* (yellow colonies) were selected to establish pure cultures in TSA (trypticase soy agar, Merck) plates and subjected to further analysis. Individual *Aeromonas* colonies were purified and stored in 20% glycerol at -80 ◦C.

4.2.2 Genotyping (rep-PCR)

To obtain genomic DNA templates for REP-PCR method we picked up one fresh colony from each strain, deposited into a microtube with 100µl sterile distilled water and adjusted turbidity to 1 in McFarland scale. After 1 minute centrifugation at 12000 rpm, 1µl cellular suspension was used as DNA template.

The repetitive extragenic palindromic sequence polymerase chain reaction (REP-PCR) method was used in this study for molecular typing of *Aeromonas* spp. isolates (Szczuka & Kaznowsk 2004, with slight modifications). The primers used are described in table I in annexes. The reaction mixture consisted in: 3mM $MgCl_2$ (MBI Fermentas); 160µM dNTP Mix (MBI Fermentas); 20% of 5x Green GoTaq Flexi Buffer (Promega); 5% DMSO (Dimethylesulfoxide, EuroBio, France); 0.6µM each primer (Rep1R-I and Rep2-I); 0.2 U *Taq* polymerase (Promega); 1µl of template DNA (cellular suspension) and filled up to 25µl with sterile double-distilled water. The PCR cycle conditions are described in table II in annexes. All PCR reactions were performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA).

The amplification products were electrophoresed on 1.5% agarose gels in 1xTBE buffer (Tris-Borate-EDTA, Sigma) at 80V during 180 minutes. The molecular weight marker Gene Ruler DNA Ladder Mix (MBI Fermentas, Vilnius, Lithuania) was loaded in all gels. Gels were stained with ethidium bromide. Gel images were acquired using a Molecular Imager FX^{TM} system (Bio-Rad Laboratories, Hercules, CA, USA). Banding patterns were carried out using GelCompar II (version 3.0; Applied Maths, Kortrijk, Belgium) software. Similarity between fingerprints was calculated with the

Dice coefficient. Cluster analysis was performed using the unweighted pair-group method with average linkages (UPGMA) (Tacão *et al.,* 2005).

4.2.3 DNA isolation and purification

Chromosomal DNA from all strains was isolated using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) with adapted instructions. 1/8 of the recommended reagent quantities were used, and an additional step of incubation for 1h at 37ºC with lysozyme solution (10mg/mL, EuroBio, France) was added (at the beginning of the lyses procedure) in order to improve lyses. DNA was ressuspended in 25µL of TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0) and stored at -20ºC until further utilization.

4.2.4 Molecular identification (*gyr*B)

The primers gyrB3_F and gyrB14_R (table I in annexes) were used to amplify a *gyrB* fragment according to Yáñez *et al*. (2003). PCR amplification was performed using a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Each reaction was performed in a final volume of 25µl, containing: 3mM $MgCl_2$ (MBI Fermentas); 10% of 10x *Taq* buffer with $(NH_4)_2SO_4$ (MBI Fermentas); 8µM dNTP Mix (MBI Fermentas); 0.3µM from each primer (gyrB3F and gyrB14R); 0.5U *Taq* DNA polymerase (MBI Fermentas); 1µl of DNA and end up with sterile double-distilled water.

The reaction mixture is described in table II in annexes, with exception of 3 isolates due unspecific banding pattern, the annealing temperature was changed to 59.5 ºC for 30s. Amplified products were analysed by electrophoresis on 1% gel agarose in 1xTBE buffer (Sigma). After 1 hour the gels were stained with ethidium bromide and scanned on a Molecular Imager FX^{TM} system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were conserved at -20ºC until further analysis.

4.2.5 Detection of virulence genes

Aeromonas strains were screened for genes encoding the virulence factors lipase (*lip/ pla/ lipH3/apl-1*), aerolysin-like proteins (*act/aerA/hlyA*) and elastase (*ahpB*) by PCR (Sen & Rodgers, 2004, Kingombe *et al*., 1999 and Cascón *et al*., 2000), with slight modifications. Primers used for PCR detection are listed in table I in annexes.

All PCR reactions were performed in a final volume of 25µl using a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). In brief, each lipase and elastase reactions consisted of: 3mM $MgCl_2$ (MBI Fermentas); 10% of 10x *Taq* buffer with $(NH_4)_2SO_4$ (MBI Fermentas); 8µM dNTP Mix (MBI Fermentas); 0.3µM from each primer (LipF/LipR and AhpB_F1/AhpB_R1); 0.5U *Taq* DNA polymerase (MBI Fermentas) and filled up with sterile double-distilled water. The aerolysin-like genes reaction consisted with the same PCR reagents but in different quantities, briefly: 2.5mM $MgCl_2$; 10% *Taq* buffer; 3.2µM dNTPs; 0.4mM each primer; 0.5U of *Taq* DNA polymerase; 1µl of DNA and double-distilled water to final volume of 25µl. The PCR conditions are described in table II in annexes.

The target fragments were detected by electrophoresis on 1.5% gels in 1xTBE buffer (Sigma) for 1 hour and stained with ethidium bromide. Amplicons sequences were determined in order to confirm the amplification of target genes.

4.2.6 Sequencing and sequence analysis

For PCR products (*gyrB,* aerolysin-like, lipases, elastase) purification we used the JETQUICK PCR Product Purification Spin Kit (Genomed, LOhne, Germany) according to the manufacturer instructions. An altered step of loading the columns with double-distilled water at 65ºC for 1 min was included before final centrifugation to improve purification according to Henriques *et al*. (2006).

Purified products were used as templates for sequencing reactions and carried out by STAB-VIDA (Oeiras, Portugal). The obtained nucleotide sequences were submitted to confirmation and correction using the DNA analysis software FinchTV version 1.4.0 (Geospiza,Inc) and then compared to the GenBank nucleotide data library using BLAST software (Altschul *et al*., 1997), to determine the closest phylogenetic relatives.

The molecular identification of the strains was carried out by sequence alignment with reference taxa present in databases and carried out by the CLUSTAL W2 program. Phylogenetic trees using the Neighbour-Joining method (Saitou & Nei 1987) were produced with PAUP* 4.0 beta version software (Swofford 2003).

4.2.7 Antimicrobial susceptibility

The antibiotic resistance patterns were determined by the agar disk diffusion method on Mueller-Hinton agar (Merck, Darmstad, Germany). Isolates were tested for

resistance against a panel of 12 antibiotics, including representatives of the most important classes: β-lactamics (amoxicillin/clavulanic acid 30µg, aztreonam, 30µg; ceftazidime, 30µg; cefepime, 30µg; cephalothin, 30µg; imipenem, 10µg), aminoglycosides (gentamicin, 10µg) tetracyclines (tetracycline, 30µg), quinolones (ciprofloxacin, 5µg; nalidixic acid, 30µg), phenicols (chloramphenicol, 30µg) and trimetropim/sulfadiazina association (trimethoprim–sulfamethoxazol, 25µg).

The diameters of inhibition zones of the Mueller-Hinton Agar according to NCCLS (Merck) were measured after 24h incubation at 30ºC and the susceptibility/resistance breakpoints were those defined by the CLSI (Clinical Laboratory Standards Institute) for *Aeromonas* (CLSI 2005).

4.2.8. Determination of virulence associated phenotypes

4.2.8.1 Haemolytic activity

Haemolytic activity was assayed by culturing *Aeromonas* strains onto gelose Columbia with 5% sheep blood (Biomerieux). Incubations proceed at 25ºC and 37ºC for 24h. *A. hydrophila* (CECT 839^T) strain was used as a positive control. Haemolytic positive isolates were identified by the presence of clear (β-haemolysis) or diffuse (α-haemolysis) halos around the colonies (Guerra *et al*., 2007, with modifications).

4.2.8.2 Lipases

The presence of extracellular lipases was tested by the tween-calcium method according to Michelim *et al*. (2005), with minor modifications. Each strain was inoculated onto TSA with 1% tween 20 (v/v). The plates were incubated at 30ºC for 24h and positive lipase activity was confirmed by observing the formation of a precipitate around the colonies.

4.2.8.3 Proteases

Proteolytic activity was assayed by inoculation of each strain on TSA with 1% skim milk (w/v) plates. After incubation for 24h at 30ºC, the presence of extracellular proteases was revealed by the formation of transparent halos around the colonies (Sechi *et al*., 2002, with some modifications). For positive control we used the strain *A. hydrophila* (CECT 839^T).

4.2.8.4 DNases

For DNase detection the isolates were inoculated onto D-Nase agar (HI-Media) supplemented with 0.01% toluidine blue O (Merck) and incubated for 24h at 30ºC. The strain *Escherichia coli* ATCC 25922 was used as negative control, as positive control we used *Staphylococcus aureus* ATCC 6538-P/ *Bacillus cereus* ATCC 11778. The degradation of deoxyribonucleic acid was indicated by the presence of a pink halo surrounding the strains (Scoaris *et al*., 2008, with modifications).

4.2.8.5 Motility

Bacteria motility of each *Aeromonas* strain were tested in a semi-solid agar medium (TSBA- Trypticase soy broth containing 5g/L of Agar) according to Murinda *et al*. (2002). Inoculations were made by the stab method with a straight needle and incubation was at 30ºC for 48h. Positive motile strains were manifested as turbidity progressed laterally and downwards from the stab inoculation line and non-motile isolates grew only in the inoculated area.

4.2.9 Cytotoxicity assessment

The cytotoxicity potential of all *Aeromonas* isolates was tested in Vero cell line (African green monkey kidney epithelial cells) according to Chapter 3.

4.3. Results

4.3.1. Molecular identification of *Aeromonas* isolates

A total of 70 isolates isolated from aquacultures and drinking fountains were selected from GSP plates and subjected to genotyping using REP-PCR (figure 2). A total of 48 different REP patterns were detected, 21 from drinking waters and 27 from aquacultures. Isolates were identified by *gyrB* sequencing which allowed defining phylogenetic relationships within the *Aeromonas* spp. isolates (figure 3). The most frequent species was *A. media* (30.3%), followed by *A. hydrophila* (18.2%) and *A. salmonicida* (18.2%), *A. eucrenophila* (9.1%), *A. allosaccharophila* (9.1%), *A. bestiarum* (9.1%), *A. punctata* and *A. molluscorum*, both with 3.0% frequency. We were unable to amplify the *gyrB* fragment from several (15) presumptive *Aeromonas* isolates, selected after growth in GSP. Sequencing of the 16S rDNA confirmed that the majority of these isolates do not belong to the *Aeromonas* genus. Nevertheless, 2 of these isolates were identified as *A. salmonicida* (6T5AQ) and a second as *Aeromonas* sp. (5T3CF),

totalizing 35 *Aeromonas* isolates that were included in this study. These data show the low selective capacity of the GSP medium for aeromonads.

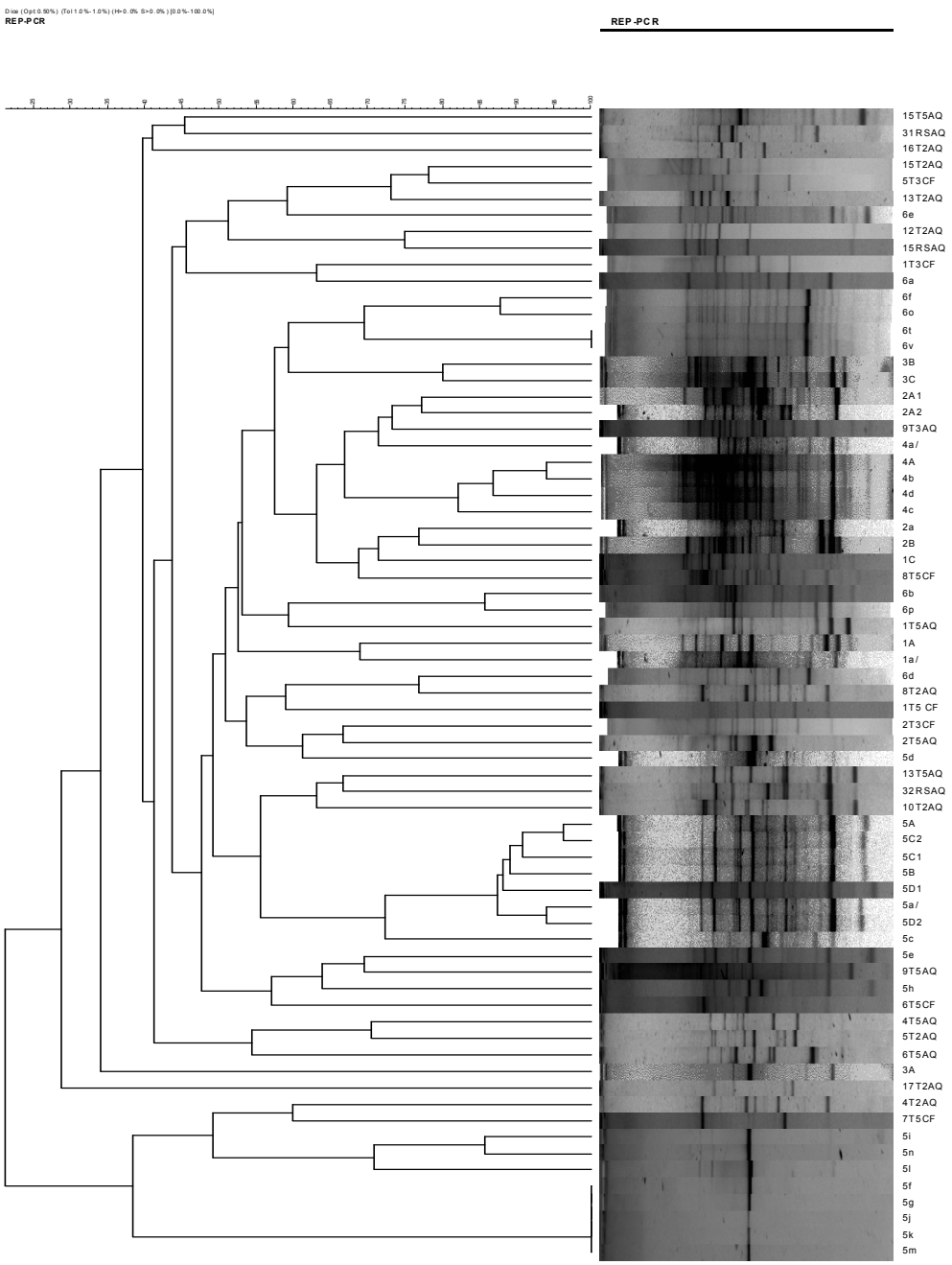

Figure 2. Dendogram showing genetic relatedness of 70 presumptive *Aeromonas* spp. isolates determined by analysis of REP-PCR fingerprint patterns using Dice similarity coefficient and UPGMA cluster methods. Isolates belonging to the same strain typically

clustered together at similarity values greater than 80% (similarity value based on identical strains already identified).

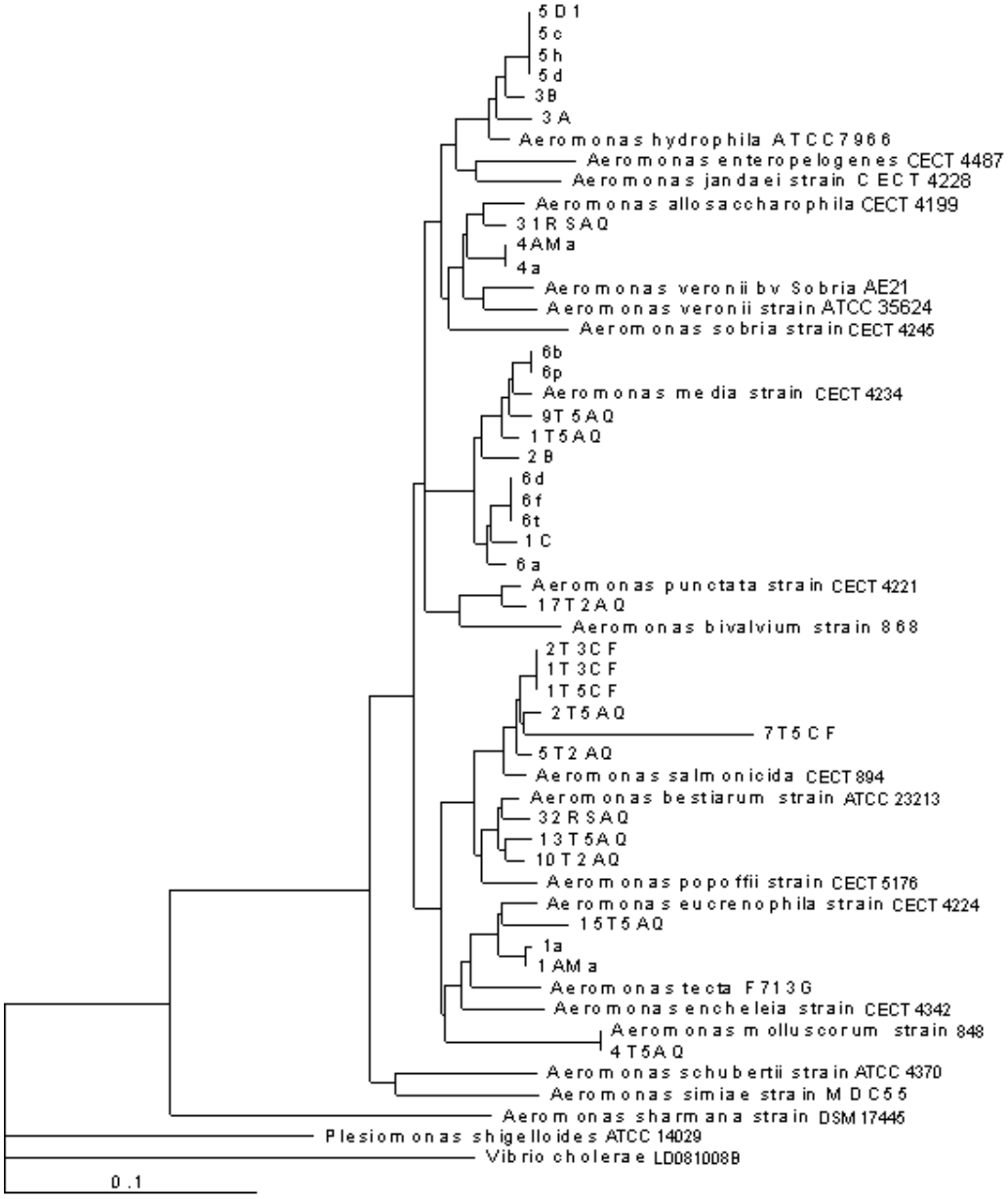

Figure 3. Phylogenetic tree based on *gyrB* DNA fragment sequences, showing relationships of the genus *Aeromonas* (constructed by the neighbour-joining method with the PAUP program; *Plesiomonas shigelloides* and *Vibrio cholerae* were used as outgroups; the scale bar represents 0.1 nucleotide changes per site).

4.3.2. Genetic detection of putative virulence factors

Thirty-five environmental *Aeromonas* strains were screened for aerolysin-like genes by PCR. We used the primers AHCF1/AHCR1 specially designed for the amplification of a 232bp fragment (Kingombe *et al*., 1999). These genes are responsible for the expression of cytotoxic enterotoxins and hemolysins, which are important virulence factors (Chopra *et al*., 2000; Cahill 1990). In total, 31% of the isolates exhibited the aerolysin-like genes (figure 4). In this study, 53% of the aquaculture water strains presented the aerolysin-like gene, while only 11% of the drinking water isolates yielded the gene (figure 5).

Studies suggest that elastase, a metalloprotease, is essential for causing disease both in humans and fish (Song *et al*., 2004; Cascón *et al*., 2000). We investigated de prevalence of *ahpB* gene in the *Aeromonas* isolates (figure 4). Results show an impressive 94% of occurrence, which may represent a potential risk promoted by these waters.

The vital role that lipases play in aeromonads pathogenicity has been reported (Chopra & Houston 1999; Pemberton *et al*., 1997). Therefore we have screened for the presence of lipase gene in our 35 isolates. A set of primers (LipF/LipR) designed by Sen & Rodgers (2004) was used. We observed 77% incidence of the *pla*/*lip*/*lipH3*/*apl-1* genes in the *Aeromonas* strains studied (figure 4).

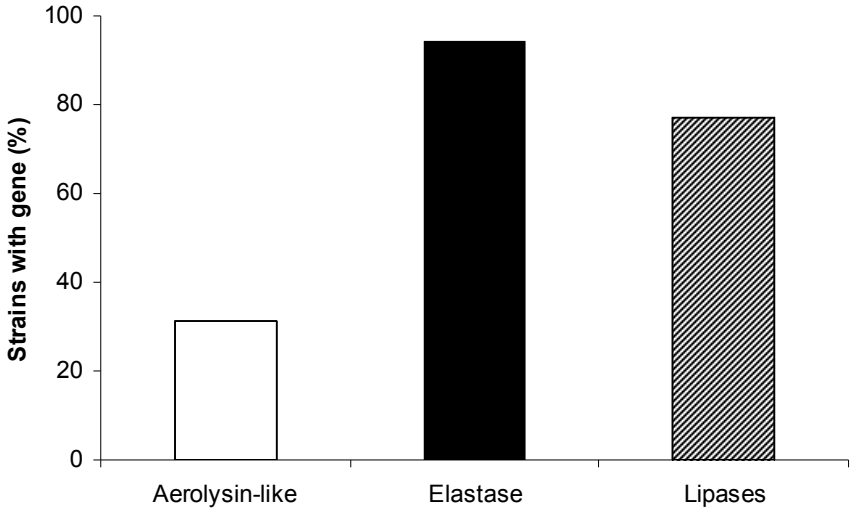

Figure 4. Frequency of putative virulence genes in *Aeromonas* spp. isolated from drinking fountains and aquacultures (results are given as percentage of total *Aeromonas* strains isolated).

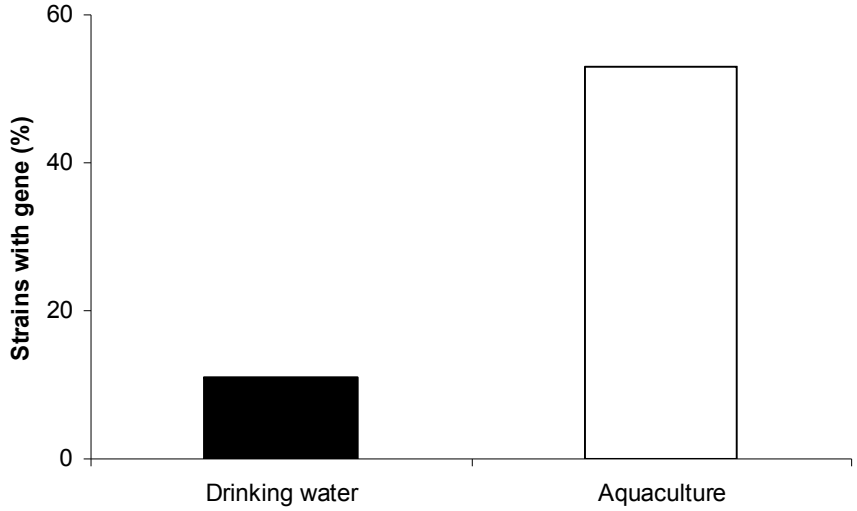

Figure 5. Frequency of aerolysin-like gene in *Aeromonas* spp. isolated from drinking fountains and aquacultures, drawing attention to the major genetic difference between water groups (results are given as percentage of total *Aeromonas* strains isolated).

4.3.3. Phenotypic determination of virulence factors

All strains were tested for a range of virulence factors: production of extracellular lipases, proteases and DNases, motility, hemolytic activity and cytotoxicity. The presence of these virulence properties (figures 6, 7 and 8) in our isolates is a strong indicator of potential health threat in water samples, since they can promote tissue damage, provide nutrients and overcome the host defence (Figueras *et al*., 2009; Scoaris *et al*., 2008; Figueras *et al*., 2005; Chopra & Houston 1999). The results of lipolytic activity showed 83% of strains able to produce extracellular lipases (figure 6). In total, 89% of the strains exhibit extracellular proteolytic activity and 94% extracellular DNase activity (figure 6). Moreover, the motility activity was present in 43% of the isolates (figure 6). The most notable difference seems to be the presence of motile *Aeromonas* in untreated drinking waters (87%) against a total of 12% in aquaculture strains (Figure 7).

Cytotoxicity analysis was carried out on a Vero cell line. The test revealed that 59% of aquaculture isolates were cytotoxic to Vero cells, while only 17% of the drinking water strains induced loss of viability on Vero cells (figure 6).

An important characteristic that confers virulence to *Aeromonas* strains is the production of hemolysins (α- and β-hemolysins) (Razzolini *et al*., 2008). Concerning hemolytic activity, all strains were assayed at 37ºC and 25ºC in order to assess the

pathogenic potential towards humans and fish hosts (figure 8). All *Aeromonas* strains grew at 37ºC; furthermore 83% exhibit hemolytic (α-hemolysin) at this temperature. β-hemolysin production was verified in 26% of the isolates studied. At 25ºC the hemolytic activity of isolates was 49% (α-hemolysin, 49% and β-hemolysin, 43%).

Results of the detection by PCR of the selected putative virulence genes, phenotypical characterization and identification from each *Aeromonas* strain are shown in table III in annexes.

One isolate [*A. salmonicida* (2T3CF)] exhibit all three genes screened as well as all virulence factors tested (α- and β-hemolysin active at 37ºC and 25ºC, extracellular proteases, DNases, lipases, motility and cytotoxicity). In contrast, one isolate [*A. salmonicida* (1T3CF)] did not show evidences of the presence of any of the virulence genes investigated or phenotypical activity tested. Among all isolates the $ahpB^{+}/lip^{+}$ genes combination were the most frequent with 71% of presence; furthermore, 26% of the isolates yielded the three genes. The gene combination aerolysin-like $genes^{+}/ahpB^{+}/lip^{-}$ were only observed in two isolates (both *A. eucrenophila*, isolates 1A and 1a). We have observed an apparent association between the cytotoxicity activity, the production of hemolysins at 37ºC and 25ºC and the presence of aerolysin-like $genes^{+}/ahpB^{+}$ (10/13 isolates). However, two isolates (*A. hydrophila* and *A. Media,* isolate 5h and 1T5AQ respectively) were cytotoxics, exhibiting the aerolysin-like $genes^{-}/ahpB^{+}/lip^{+}$ combination but not hemolytic activity. Moreover, we verified that majority of isolates (83%) exhibited hemolytic activity at 37ºC. The isolates that not showed hemolyses at 37ºC also did not presented hemolytic activity at 25ºC. Curiously, only 38% of all isolates that were hemolytic yielded the aerolysin-like gene. Seventy-seven per cent of all isolates were simultaneous proteolytic, lipolytic and produced DNase, independent of the genotype, cytotoxicity and/or hemolytic activity presented by the strains.

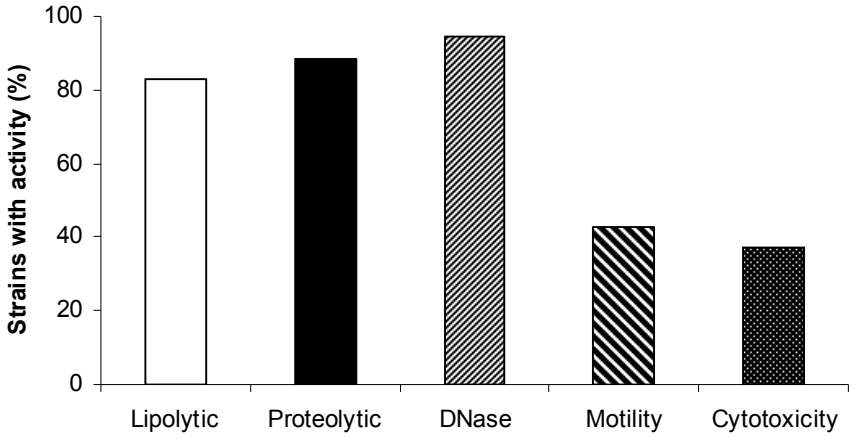

Figure 6. Frequency of virulence factors in *Aeromonas* spp. isolated from drinking fountains and aquacultures (results are given as percentage of total *Aeromonas* strains isolated).

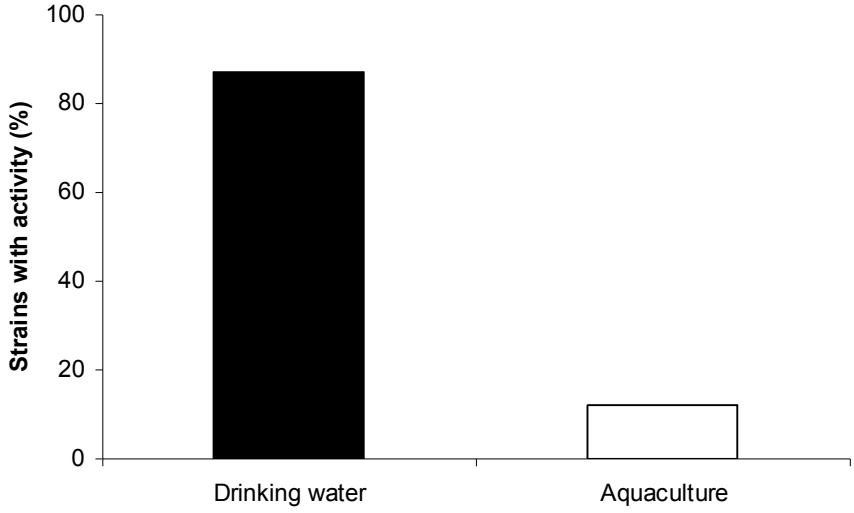

Figure 7. Frequency of motility activity in *Aeromonas* spp. isolated from drinking fountains and aquacultures, drawing attention to the major phenotypical difference between water groups (results are given as percentage of total *Aeromonas* strains isolated).

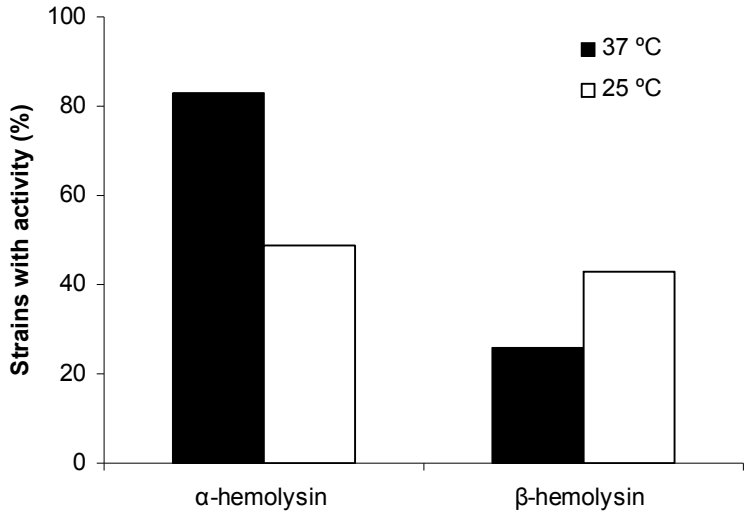

Figure 8. Occurrence of hemolytic activity at 37ºC and 25ºC in *Aeromonas* spp. isolated from drinking fountains and aquacultures (results are given as percentage of total *Aeromonas* strains isolated).

4.3.4. Antimicrobial resistance patterns

Antibiotic susceptibility patterns were determined for each of the 35 aeromonad isolates. Figure 9 shows that most strains were resistant to amoxicillin/clavulanic acid and cephalothin (94% and 97%, respectively). In addition, the highest resistance encountered was to imipenem with 31% of resistant isolates. In contrast, the isolates were less resistant for nalidixic acid (14%), aztreonam (14%), cefepime (11%), tetracycline (9%), trimethoprim–sulfamethoxazol (9%), chloramphenicol, ciprofloxacin and gentamicin each with 6% of resistant strains; moreover the most active antibiotic was ceftazidime which presented 3% of resistant isolates.

Results (table 1) show that more than 50% of the drinking water isolates displayed resistances to 2 antibiotics, while 35% of aquacultures strains presented resistance for 3 antimicrobials. We also verified the presence of multiresistant isolates: 4 isolates (5c, 5h, 1T3CF and 5T3CF) exhibited resistance to 6 or 7 antibiotics.

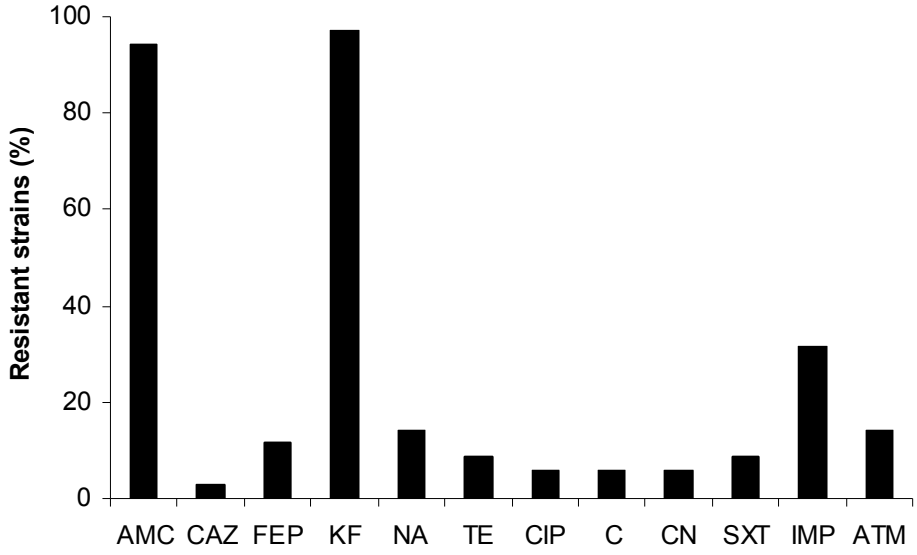

Figure 9. Resistance to antibiotics of *Aeromonas* isolated from drinking fountains and aquacultures (results are given as percentage of total *Aeromonas* strains isolated).
Antibiotic abbreviations: AMC= amoxicillin/clavulanic acid; CAZ= ceftazidime; FEP= cefepime; KF= cephalothin; NA= nalidixic acid; TE= tetracycline; CIP= ciprofloxacin; C= chloramphenicol; CN= gentamicin; SXT= trimethoprim–sulfamethoxazol; IMP= imipenem; ATM= aztreonam.

Table 1. Prevalence of antibiotic multiresistant *Aeromonas* spp. isolated from drinking fountains and aquacultures

Number of resistances displayed per strain	**Strains from drinking waters (%)**	**Strains from aquaculture (%)**
1	0	12
2	56	29
3	17	35
4	17	12
5	0	0
6	11	0
7	0	12

4.4. Discussion

Aeromonas spp. are autochthonous to aquatic environments and in the last decades have been considered both fish and human pathogens (Figueras *et al*., 2005; Yáñez *et al*., 2003). The virulence of *Aeromonas* is complex and multi-factorial involving a range of specific determinants (Chacon *et al*., 2003; Janda & Abbott 1996; Pemberton *et al*., 1997). To better understand the exact role of virulence factors in pathogenicity and the potential risk associated with the presence of *Aeromonas* in water, we screened for specific putative virulence genes, for virulence phenotypes as well as for cytotoxicity *in vitro*.

In this study, we used the AHCF1/AHCR1 primers to detect aerolysin-like genes (Kingombe *et al*., 1999). Act toxin is related to aerolysin (AerA), both are channel pore-forming toxins with hemolytic, cytotoxic and enterotoxic activities, while HlyA is a non-channel forming hemolysin. These toxins along have being implicated in *Aeromonas* pathogenesis and are considered to be a good indicator of virulent strains (Pablos *et al*., 2009; Sharma *et al*., 2005). This low frequency of aerolysin-like genes in drinking fountains can be explained by the fact that 56% of drinking waters isolates belong to *A. media* and *A. allosaccharophila* species. These share the characteristic of lacking the aerolysin/hemolysins genes, as already described by Chacón and colleagues (2003). Moreover, none of the *A. hydrophila* strains, which represented 1/3 of total isolates in drinking waters, yield the aerolysin-like gene (Sen & Rodgers, 2004). Nevertheless, *A. hydrophila* is considered the major *Aeromonas* pathogen to humans and fishes (Sen & Rodgers 2004; Sha *et al*., 2002; Rabaan *et al*., 2001; Handfield *et al*., 1996; Chopra & Houston 1999), which indicate that the aerolysin-like genes perhaps are not the only or essential genes involved *in vivo* pathogenicity.

We have found a direct relationship between the presence of aerolysin-like genes and hemolytic activity: all isolates that yielded aerolysin-like genes also express extracellular hemolytic activity (α- and/or β-hemolysin) at 37ºC and 25ºC. The exception was isolate 4T5AQ (*A. molluscorum*) that only exhibited α-hemolysin at 37ºC. However, we observed that hemolysin production was not exclusive of isolates that presented the aerolysin-like genes, thus 75% of strains without these genes also expressed hemolytic activity, supporting the idea of other enzymes acting as hemolysins, which is also reported by several authors (Castilho *et al*., 2009; Scoaris *et al*., 2008; Chopra & Houston 1999; Fiore *et al*., 1997).

In addition, all isolates that expressed the aerolysin-like genes also exhibit the *ahpB* in their genotype. Furthermore, only one strain (A. eucrenophila, isolate 15T5AQ) did not express extracellular proteolytic activity. The presence of extracellular proteases (elastase encoded by *ahpB* as well as others that may be secreted), have been correlated to proaerolysin activation and subsequently leading to a higher β-hemolytic activity (Song *et al*., 2004).

In our study, the elastase gene (*ahpB*) was the most frequently gene detected (94%). This frequency is higher that that described by Guerra and colleagues (2007), in *Aeromonas* isolates from patients with acute gastroenteritis. This gene encodes a metalloprotease with caseinolytic and elastolytic activity involved in hemolytic and cytotoxic activities. Several studies reported that isogenic mutants for *ahpB* were less virulent (Song *et al*., 2004; Cascón *et al*, 2000). The high frequency of this gene found in the water strains investigated demonstrates the high pathogenic potential in these water samples. Extracellular proteolytic activity (present in 89% of the strains studied) was almost coincident with the incidence of *ahpB* gene. One isolate (*A. allosaccharophila*, isolate 4A) was protease positive but does not exhibit the *ahpB* gene, suggesting that other proteolytic enzymes are present, presumably a serine protease which is known to have caseinolytic activity (Cascón *et al*., 2000). Three isolates [*A. eucrenophila* (15T5AQ), *A. punctata* (17T2AQ) and *Aeromonas* sp., isolate (5T3CF)] although having *ahpB* did not exhibit extracellular proteolytic activity. This can be justified by the lack of a serine protease, which is the major caseinolytic enzyme of *Aeromonas* spp. and is important in speeding up the process of the pro-AhpB form, as a result we had a lower proteolytic activity (Cascón *et al*., 2000) which could explain our non-proteolytic isolates.

It has been suggested that the presence of aerolysin-like toxins may not be sufficient for virulence or are not the only important factors for pathogenesis (Sen & Rodgers 2004). Therefore, the presence of several other virulence factors such as DNases, proteases and motility were assessed.

DNase activity seems to play a secondary role in *Aeromonas* pathogenesis, possibly with a role in bacterial nutrition. In the present study only 2 isolates (A. salmonicida, isolate 1T3CF and *Aeromonas* sp., isolate 5T3CF) did not show DNases production, which corroborates with other studies confirming that this is a very frequent characteristic among aeromonads (Castro-Escarpulli *et al*., 2003). Forty three percent of isolates were motile, with significant preponderance within drinking water isolates.

Motility in *Aeromonas* spp. is associated to flagella, which have been shown to facilitate adherence in epithelial cells that potentially contributes to host cell invasion, enhancing the enteropathogenic potential (Kirov *et al*., 2004; Kirov 2003; Gavin *et al*., 2002). Flagella also have a major role in biofilm colonization, particularly in pipes of water distribution systems (Scoaris *et al*., 2008; Villari *et al*., 2003), which may be related to the higher frequency of motile strains collected in drinking fountains.

Many studies reported the fundamental role of lipases in providing nutrients to bacteria and in *Aeromonas* pathogenesis (Castro-Escarpulli *et al*. 2003; Chacon *et al*., 2003; Chopra & Houston 1999; Merino *et al*., 1999; Pemberton *et al*., 1997). Lipases genes were present in 77% of the strains investigated, while the lipolytic activity was found in 83% of all isolates (3 isolates exhibited lipolytic activity without *lip/ pla/ lipH3/apl-1*). The primers used are specific to *lip/ pla/ lipH3/apl-1* of *Aeromonas* (Sen & Rodgers 2003) and are unable to amplify the glycerophospholipid cholesterol acyl-transferase (GCAT) gene, which is targeted by another group of primers (Sen & Rodgers 2003). Consequently, the GCAT may be the lipase responsible for those positive lipolytic activities.

Interestingly our results show a correlation between extracellular lipases and hemolytic activity. It has been suggested by some authors the possible function of GCAT as a lipase or phospholipase in digesting the erythrocyte plasma membrane acting as hemolysins. Nevertheless, its role as virulence factor in humans is still unknown (Castilho *et al*., 2009; Scoaris *et al*., 2008; Guerra *et al*., 2007; Chopra & Houston 1999; Pemberton *et al*., 1997). Our results support that theory: 95% of isolates that do not have aerolysin-like genes but produced extracellular lipases also exhibit hemolytic activity, particularly at 37ºC; those strains that simultaneously don't have lipolytic activity nor have aerolysin/hemolysins genes, were not able to express hemolytic activity. Furthermore, the higher production of these hemolytic lipases at 37ºC suggests that lipases or phospholipases could have a significant role in human pathogenicity and should be taken into count in further studies.

All *Aeromonas* strains studied were able to grow at 37ºC. Furthermore, we verified a higher hemolytic activity at that temperature, contrasting to what was reported by Mateos *et al*. (1993). The fact that these environmental strains are able to grow and express virulence factors at 37ºC, seem to indicate some kind of adaptation to homeothermic hosts. Considering these results one can hypothesise that these potential

virulent strains present in aquacultures and drinking fountains (which may act as reservoirs) could have human origin.

Rahman and colleagues (2007) reported the importance of hemolysin and cytotoxin production in human gastroenteritis and soft-tissue infection in fish by *Aeromonas*. In our study we found that 91% of isolates with aerolysin-like genes had cytotoxicity activity against Vero cells line. In fact, the expression of aerolysin-like proteins associated to *ahpB* and to proteolytic activity seems to be the most important factors for cytotoxicity. Furthermore only 3/13 cytotoxic strains [*A. hydrophila* (5h), *A. media* (1T5AQ) and *A. salmonicida* (6T5AQ)] did not have the aerolysin-like genes, agreeing with Ormen & Ostensvik (2001) which demonstrated that 83% of cytotoxic isolates to Vero cells carried out the aerolysin-like gene. A study carried out by Martins and colleagues (2007) showed the existence of a cytotoxin able to induce apoptosis in Vero cells, the vacuolating cytotoxic factor (VCF), which has caseinolytic activity but not hemolytic activity. This VCF may explain why there are 3 cytotoxic isolates (2 non-hemolytics) proteolytic positive but without aerolysin-like genes, supporting the idea of a cytotoxin distinct from the aerolysin-related proteins. Additionally, it seems that hemolytic activity is not directly involved in cytotoxicity: 18 isolates without aerolysin-like genes, but with hemolytic activity at 37ºC and/or at 25ºC, did not manifested cytotoxicity in Vero cells. Nacescu *et al*. (1992) postulated a correlation between hemolytic activity and the pathogenic potential of *Aeromonas*. Therefore, it is not possible to state that these 18 isolates are not pathogenic strains.

Altogether, nearly 1/3 of our *Aeromonas* isolates are cytotoxics and exhibit hemolytic activity, which demonstrates the potential of fountains and aquacultures waters to induce gastrointestinal and soft-tissue diseases in humans and fishes.

Antimicrobials are indicated for severe and chronic case of *Aeromonas* gastroenteritis as well as for extra-intestinal infections. *Aeromonas* infections can progress quickly, and may be fatal (Ko *et al*., 2003). Antimicrobial resistance in *Aeromonas* strains can make these infections difficult to treat, making pertinent to determine antibiotic resistance patterns.

In our study we found some differences in susceptibility to antibiotics between aquacultures isolates and drinking fountains, but in general they were sensitive (> 85% susceptible isolates) to tetracycline, third and fourth-generation celphasporins (ceftazidime and cefepime), aminoglycosides (gentamicin), quinolones (ciprofloxacin and nalidixic acid), trimethoprim–sulfamethoxazol, chloramphenicol and aztreonam.

Overall, our results are consistent with some previous studies (Janda & Abbott 1998; Koehler & Ashdown 1993) that documented similar antibiotic susceptible profiles. Nearly all strains were resistant to first-generation celphasporins and as well to amoxicillin/clavulanic acid which is in agreement with the antimicrobial resistance profiles reported by Koehler & Ashdown (1993) and Guerra and co-authors (2007). In opposition to the results obtained by Lupiola-Gomez *et al*. (2003) and Ko *et al*. (1996) we have found a high resistance to imipenem. Based in our results, ceftazidime seem to be the best choice to treat *Aeromonas* infections.

Eleven percent of the environmental strains tested were resistant to 6 or more antibiotics, similar to what was reported by other studies (Guerra *et al.*, 2007), indicating that multidrug-resistant phenotypes is a regular feature of *Aeromonas* spp., principally considering their ability to efficiently receive and spread plasmids carrying antibiotic resistances between unrelated bacteria in environment (Guerra *et al*., 2007; Huddleston *et al*., 2006; Marchandin *et al*., 2003).

Taking in consideration our results as well as data from other authors (Scoaris *et al*., 2008; Goni-Urriza *et al*., 2000), that claim that the increasing and probably indiscriminate use of antibiotic may contribute to develop drug resistance in bacteria, it seems that more restrictive policies regarding the use of antibiotics in untreated drinking waters and aquacultures are necessary.

4.5. Conclusion

In this chapter, we demonstrated that potentially pathogenic *Aeromonas* with antibiotic multiresistant phenotypes are present in drinking fountains and aquacultures in Portugal, indicating a public health risk and a major factor in productivity loss in aquaculture industry. We were also able to show that, aerolysin-like genes seem to represent a key role in *Aeromonas* cytotoxicity.

Chapter 5. General conclusions

In conclusion, our results based on virulence factors evaluation support the assumption that potential pathogenic *Aeromonas* strains are present in Portuguese waters, representing a real health concern and a major economic loss in aquaculture industry. Moreover, the antibiotic resistance patterns indicate the existence of multidrug-resistant phenotypes in these waters. These may act as reservoirs of resistance genes and virulence determinants in environment, a fact that can not be ignored. In addition, we concluded that ceftazidime may provide an effective antibiotic therapy against *Aeromonas* infection.

The present work also highlights the fundamental role of aerolysin-like genes in cytotoxicity pathogenesis *in vitro*. The screening of this particular gene may be a valuable tool to evaluate the virulence potential of *Aeromonas* strains. However, it seems in some away that the expression of *ahpB* gene and protease activity also contributes for cytotoxicity, elucidating the multi-factorial nature of *Aeromonas* spp. pathogenicity. Therefore, further studies are needed to better characterise specific pathogenic determinants in order to completely understand the virulence mechanism.

With this study we also develop a more sensitive, accurate and non-toxic cytotoxicity assay in Vero cell line able to assess a large number of samples with less effort and minimising time comparably to cell counting by microscope observation plus the advantage of keeping the cells in culture allowing further analysis or the detection of changes over time.

Chapter 6. References

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Chapter 7. Annexes

Table I. Sequence of the primers used for amplification of the different target genes.

Target genes	Primers	DNA sequences (5´-3´)	Expected PCR product (bp)
Partial *gyrB* gene	gyrB3_F gyrB14_R	TCCGGCGGTCTGCACGGCGT TTGTCCGGGTTGTACTCGTC	1100
Lipase, *lip/ pla/ lipH3/apl-1*	LipF LipR	ATCTTCTCCGACTGGTTCGG CCGTGCCAGGACTGGGTCTT	382
Aerolysin-like proteins	AHCF1 AHCR1	GAGAAGGTGACCACCAAGAACA AACTGACATCGGCCTTGAACTC	232
Elastase, *ahpB*	AhpB_F1 AhpB_R1	GGAACGTCAAGACTGGCAAGT CGATCAGGGAGCCTGCGGCT	746
Repetitive extragenic palindromic sequence, *REP*	Rep1R-I Rep2-I	III ICG ICG ICA TCI GGC ICG ICT TAT CIG GCC TAC	-

Table II. PCR conditions for each target gene

Target genes		Amplification condition
REP	1 Cycle	Initial denaturation: 95ºC – 7min
	35 Cycles	Denaturation: 94ºC – 1min Annealing: 40ºC – 1min Extension: 65ºC – 8min
	1 Cycle	Final extension: 65ºC – 16min
gyrB	1 Cycle	Initial denaturation: 94ºC – 3min
	35 Cycles	Denaturation: 94ºC – 30s Annealing: 55ºC – 30s Extension: 72ºC – 45s
	1 Cycle	Final extension: 72ºC – 15min
lip/ pla/ lipH3/apl-1	1 Cycle	Initial denaturation: 95ºC – 5min
	25 Cycles	Denaturation: 95ºC – 25s Annealing: 55ºC – 30s Extension: 72ºC – 1min
	1 Cycle	Final extension: 70ºC – 15min
Aerolysin-like proteins	1 Cycle	Initial denaturation: 95ºC – 10min
	25 Cycles	Denaturation: 95ºC – 15s Annealing: 57.9ºC – 30s Extension: 72ºC – 30s
	1 Cycle	Final extension: 70ºC – 15min
ahpB	1 Cycle	Initial denaturation: 94ºC – 2min
	40 Cycles	Denaturation: 94ºC – 1min Annealing: 58ºC – 30s Extension: 72ºC – 1:50min
	1 Cycle	Final extension: 72ºC – 15min

Table III. Incidence of virulence genes and phenotypic expression among *Aeromonas* isolates.

Isolates	Source	Strain Identification (*gyrB*)	Aerolysin-like genes	Lipases *lip/lip/lipH3 /apl-1* genes	Elastase *ahpB* gene	37 ºC		25 ºC
						α-Hemolysis	β-Hemolysis	α-Hemolysis
1A	Fountain	*A. eucrenophila*	+	-	+	+	-	+
1a	Fountain	*A. eucrenophila*	+	-	+	+	-	+
5D1	Fountain	*A. hydrophila*	-	+	+	+	+	+
5c	Fountain	*A. hydrophila*	-	+	+	+	+	+
3A	Fountain	*A. hydrophila*	-	+	+	+	+	+
6a	Fountain	*A. media*	-	+	+	+	-	-
4a	Fountain	*A. allosaccharophila*	-	-	+	-	-	-
4A	Fountain	*A. allosaccharophila*	-	-	-	-	-	-
5d	Fountain	*A. hydrophila*	-	+	+	+	+	+
1C	Fountain	*A. media*	-	+	+	+	-	-
2B	Fountain	*A. media*	-	+	+	+	-	-
3B	Fountain	*A. hydrophila*	-	+	+	+	+	+
6b	Fountain	*A. media*	-	+	+	+	-	-
5h	Fountain	*A. hydrophila*	-	+	+	-	-	-
6d	Fountain	*A. media*	-	+	+	+	-	-
6f	Fountain	*A. media*	-	+	+	+	-	-
6t	Fountain	*A. media*	-	+	+	+	-	-
6p	Fountain	*A. media*	-	+	+	+	-	-
1T5AQ	Aquaculture	*A. media*	-	+	+	-	-	-
2T5AQ	Aquaculture	*A. salmonicida*	+	+	+	+	-	+
4T5AQ	Aquaculture	*A. molluscorum*	+	+	+	+	-	-
6T5AQ	Aquaculture	*A.Salmonicida*	-	-	+	+	-	+
13T5AQ	Aquaculture	*A. bestiarum*	+	+	+	+	+	+
5T2AQ	Aquaculture	*A. salmonicida*	+	+	+	+	+	+
15T5AQ	Aquaculture	*A. eucrenophila*	+	+	+	+	-	+
10T2AQ	Aquaculture	*A. bestiarum*	+	+	+	+	+	+
32RSAQ	Aquaculture	*A. bestiarum*	-	+	+	+	-	+
17T2AQ	Aquaculture	*A. punctata*	-	+	+	+	-	-
31RSAQ	Aquaculture	*A. allosaccharophila*	-	-	+	+	-	-
9T5AQ	Aquaculture	*A.media*	-	+	+	+	-	-
1T3CF	Aquaculture	*A. salmonicida*	-	-	-	-	-	-
2T3CF	Aquaculture	*A. salmonicida*	+	+	+	+	-	+
5T3CF	Aquaculture	*Aeromonas sp.*	-	-	+	-	-	-
1T5CF	Aquaculture	*A. salmonicida*	+	+	+	+	-	+
7T5CF	Aquaculture	*A. salmonicida*	+	+	+	+	+	+

β-Hemolysis	Protease test	Lipase test	DNase test	Motility test	Cytotoxicity *Vero* cells
	+	-	+	+	+
+	+	+	+	+	+
+	+	+	+	+	-
+	+	+	+	+	-
+	+	+	+	+	-
+	+	+	+	-	-
-	+	-	+	+	-
-	+	-	+	+	-
-	+	+	+	-	-
+	+	+	+	+	-
-	+	+	+	+	-
-	+	+	+	+	-
+	+	+	+	+	-
-	+	-	+	-	+
-	+	+	+	+	-
-	+	+	+	-	-
-	+	+	+	+	-
-	+	+	+	-	-
-	+	+	+	-	+
-	+	+	+	-	+
+	+	+	+	-	-
-	+	+	+	-	+
+	+	+	+	-	+
+	+	+	+	-	+
-	-	+	+	-	+
+	+	+	+	-	+
+	+	+	+	-	-
-	-	+	+	-	-
-	+	+	+	-	-
-	+	+	+	+	-
-	-	-	-	-	-
-	+	+	+	+	+
+	-	-	-	-	-
-	+	+	+	-	+
+	+	+	+	-	+
+					