Universidade de Aveiro Departamento de Biologia
2010

Maria João Mendes de Carvalho

Diversidade de *Aeromonas* sp. de diferentes ambientes em Portugal

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Prof. Doutor António Carlos Matias Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e da Prof. Doutora Maria José Félix Saavedra, Professora Associada com Agregação do Departamento de Ciências Veterinárias da Universidade de Trás-os-Montes e Alto Douro.

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Às pessoas mais importantes da minha vida: os meus Pais e o meu irmão.
Obrigada por existirem.

o júri

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palavras-chave

Águas de consumo, produtos agrícolas, diversidade, filogenia, taxonomia, resistência, antibióticos, virulência

resumo

Espécies de *Aeromonas* encontram-se distribuídas por diferentes habitats, estando especialmente relacionadas com ambientes aquáticos. O seu papel em complicações na saúde humana e animal é reconhecido. De facto, não só pelo seu potencial de virulência, mas também pelos determinantes genéticos de resistência a antibióticos que possam conter, estes organismos constituem uma preocupação na medicina humana e veterinária. Assim, é essencial o estudo da diversidade de espécies de *Aeromonas* bem como explorar as suas características fenotípicas e genéticas que podem conduzir a impactos negativos.

A água constitui um importante veículo de transmissão de microrganismos e espécies de *Aeromonas* estão amplamente distribuídas em águas tratadas e não tratadas. Em Portugal é ainda comum o consumo de águas não tratadas cuja qualidade, na maioria das vezes, não é sujeita a monitorização, como acontece por exemplo, em explorações agrícolas de gestão familiar.

Neste estudo, investigou-se a presença de *Aeromonas* em águas não tratadas para consumo. Estabeleceu-se também uma linha horizontal de colheitas de diferentes amostras de origem agrícola com o intuito de avaliar a possibilidade de a água ser uma das vias de contaminação de culturas agrícolas e animais por espécies de *Aeromonas*. Obtiveram-se 483 isolados que foram discriminados por RAPD-PCR. 169 estirpes distintas foram identificadas ao nível da espécie por análise filogenética baseada no gene *gyrB*. Verificou-se uma frequente ocorrência bem como uma diversidade considerável de espécies de *Aeromonas.* Em alguns casos, as relações genotípicas entre isolados de diferentes amostras eram muito próximas. Adicionalmente, a maioria das amostras continha diferentes espécies e estirpes distintas da mesma espécie. *A. media* e *A. hydrophila* foram as espécies mais ocorrentes. Um grupo de isolados apresentou variantes moleculares de *gyrB* diferente das conhecidas até agora, o que indica que poderão constituir espécies não descritas. O perfil de susceptibilidade da colecção de *Aeromonas* a diferentes antibióticos foi estabelecido, constituindo um perfil típico do género, com algumas excepções. Estirpes multirresistentes foram encontradas. A presença de genes *tet* e *bla* foi investigada por estudos de PCR, hibridação e, em alguns casos, de sequenciação. Como era esperado, *cphA*/*imiS* foi o mais detectado. A detecção de integrões fez-se por PCR e hibridação e a sua caracterização foi feita por sequenciação de DNA; a sua ocorrência foi reduzida. A maioria das estirpes sintetizou enzimas extracelulares com actividade lipolítica e proteolítica que potencialmente contribuem para virulência. A análise por PCR e hibridação permitiram a detecção de vários determinantes genéticos que codificam moléculas possivelmente envolvidas em processos patogénicos. Diversas espécies de *Aeromonas* apresentando características relacionadas com resistência a antibióticos e potencialmente de virulência estão frequentemente presentes em produtos para consumo humano e animal em Portugal.

keywords

Water for consumption, agricultural sources, diversity, phylogeny, taxonomy, antibiotic, resistance, virulence

abstract

Aeromonas spp. are present in a wide range of ecological niches, being mainly related to aquatic environments. Their role in human and animal health complications is recognised. In fact, not only for their putative virulence but also for the antibiotic resistance genetic determinants *Aeromonas* may harbour, these organisms constitute an issue of concern in human and veterinary medicine. Thus, it is essential to get knowledge on *Aeromonas* sp. diversity and on their genotypic and phenotypic characteristics that may lead to negative impacts.

Water constitutes a good contamination route for microorganisms and *Aeromonas* are widespread in untreated and treated waters from different sources. In Portugal there is still an extensive use of untreated water which is not regularly monitored for quality. This is often the case in family smallholding farms.

In this study untreated drinking and mineral waters were assessed for their content in *Aeromonas* spp. Furthermore, a sampling scheme was designed to investigate the occurrence and diversity of *Aeromonas* sp. in different agricultural correlated sources and to assess the possibility of water being the transmission vehicle between those sources. 483 isolates were obtained and discriminated by RAPD-PCR. Identification at the species level for 169 distinct strains was done by *gyrB* based phylogenetic analysis. Results demonstrated the frequent occurrence and considerable diversity of *Aeromonas* spp. In some cases, genotypic close relations were found between isolates from different sources. Also, most samples contained different species and distinct strains of the same species. *A. media* and *A. hydrophila* were the most occurring. A group of isolates displayed *gyrB* gene sequences distinct from the previously known, indicating that they may constitute representatives of non-described species. The antibiotic susceptibility profile of the aeromonads collection was established and constituted a typical profile of the genus, although few exceptions. Multiresistance patterns were found. The presence of *tet* and *bla* genes was investigated by PCR, hybridisation and, in some cases, sequencing analysis. As expected, *cphA*/*imiS* was the most detected. Integrons were screened by PCR and hybridisation and characterised by DNA sequencing; low occurrence was recorded. The bulk of strains was able to produce extracellular enzymes with lipolytic and proteolytic activities, which may contribute to virulence. PCR and hybridisation surveys allowed the detection of distinct genetic determinants coding for molecules putatively involved in pathogenic processes.

Diverse *Aeromonas* sp. presenting distinct antibiotic resistance features and putative virulence traits are frequently present in many sources for human and animal consumption in Portugal.

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1. Introduction

This study is focused on the diversity of a collection of bacterial isolates from different environments belonging to the genus *Aeromonas* and used methodologies of different subdisciplines within Microbiology: Microbial Genetics, Molecular Microbiology, Environmental Microbiology and Evolutionary Microbiology. The knowledge here obtained may be useful in applied areas like Medical, Veterinary or Food Microbiology. In fact, the genus *Aeromonas* prompts investigations in such different topics due to its wide distribution in different ecosystems (reflecting the capacity of these specimens to adapt to broad environmental conditions), extended phylogenetic diversity and large assortment of virulence and antibiotic resistance features, culminating in an impressive array of human and animal clinical presentations.

1.1 General overview of the genus *Aeromonas*

Aeromonads constitute an heterogeneous group of bacteria virtually present in all environmental niches (Janda and Abbott, 2010*). The recent sequencing of the complete genomes of *A. hydrophila* ATCC 7966 and *A. salmonicida* subsp. *salmonicida* A449 revealed that the former is an extremely versatile organism with genetic attributes enabling its survival in different ecosystems (distinct environments and hosts) (Seshadri *et al.*, 2006) and that the latter is an organism that has undergone genetic adaptations to a specific host (Reith *et al.*, 2008). These findings testify the variability existent within the genus, whose members are tailored to survive under a wide array of environmental conditions and able to cause innumerable human and animal health complications.

1.1.1 General characteristics of the genus

The genus *Aeromonas* comprises Gram-negative bacteria appearing as straight rod cells but also as coccobacilli or with filamentous forms. Cells are 0.3-1.0 x 1.0-3.5 µm and may occur singly, in pairs or, more rarely, in short chains. The majority of specimens is motile by means of flagella with exception of few *A. caviae* and *A. media* strains and almost all *A. salmonicida* (existing species discussed below). Aeromonads can grow over a wide range of temperatures (0ºC to 45ºC), although the optimum temperature fluctuates between 22ºC and 37ºC, with the psychrophilic specimens growing at temperatures of 2ºC – 30ºC (*A. salmonicida* subspecies *salmonicida*, *A. salmonicida* subsp. *achromogenes*,

* See table 1.1 on page 10.

A. salmonicida subsp. *masoucida* and *A. salmonicida* subsp. *smithia*) and the mesophilic species optimally growing at 28ºC – 37ºC (all *Aeromonas* species, including *A. salmonicida* subsp. *pectinolytica*, except *A. salmonicida* subspecies mentioned above) (Martin-Carnahan and Joseph, 2005*). Initially thought as an attribute of several *A. salmonicida* subspecies and *A. media* strains, the production of a brown diffusible pigment has been also detected in *A. allosaccharophila* and some rare strains of other *Aeromonas* spp. (Abbott *et al.*, 2003; Krejčí *et al.* 2009*). Also, in contrast with the overwhelming majority of *Aeromonas* species, *A. trota* and few strains of other species are susceptible to the beta-lactam antibiotic ampicillin (Martin-Carnahan and Joseph, 2005*; Saavedra *et al.*, 2004; Janda and Abbott, 2010*). *Aeromonas* are facultatively anaerobic and chemoorganotrophic organisms. They produce acid and often acid with gas from distinct carbohydrates, especially from D-glucose. Usually, aeromonads are oxidase and catalase positive, reduce nitrates to nitrites and synthesise numerous hydrolytic enzymes which for several times are involved in pathogenic processes. Generally, they are resistant to the vibriostatic agent O/129, have β-galactosidase activity and do not hydrolyse urea or use arabitol, D-raffinose or D-amygdalin (<2% of strains tested). The mol % G+C DNA content varies between 57% and 63% (Martin-Carnahan and Joseph, 2005*).

1.1.2 Diversity and occurrence of Aeromonas species

Until now, the genus included 23 described species and two unnamed hybridisation groups (HG): *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. jandaei*, *A. veronii*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. popoffii*, *Aeromonas* sp. HG11, *Aeromonas* species Group 501 (HG13) (Martin-Carnahan and Joseph, 2005*), *A. molluscorum* (Miñana-Galbis *et al*., 2004*), *A. simiae* (Harf-Monteil *et al.*, 2004*), *A. bivalvium* (Miñana-Galbis *et al*., 2007*), *A. aquariorum* (Martínez-Murcia *et al*., 2008*), *A. tecta* (Demarta *et al.*, 2008*), *A. fluvialis* (Alperi *et al.*, 2010*), *A. piscicola* (Beaz-Hidalgo *et al.*, 2009*), *A. taiwanensis*, *A. sanarellii* (Alperi *et al.*, in press*). Very recently the species name *Aeromonas diversa* has been proposed to include the members of *Aeromonas* species Group 501 (Miñana-Galbis *et al.*, 2010a).

While some of these groups display certain homogeneity, the majority of *Aeromonas* spp. exhibit intrataxon variability and the prevalence of some taxa is quite

* See table 1.1 on page 10.

superior in relation to others. Nevertheless, the variability and incidence of each species may be biased by different circumstances intrinsic to the methodology applied by different studies among other features (Janda and Abbott, 2010*). The fact is that for some phylogenetic groups (*A. molluscorum*, *A. bivalvium*, *A. piscicola*, HG11 and *Aeromonas* species Group 501 – *Aeromonas diversa*) the number of specimens scrutinised is low as well as their geographic and source origins (Hickman-Brenner *et al.*, 1987*; Hickman-Brenner *et al.*, 1988*; Janda *et al.*, 1996*; Borrell *et al.*, 1998*; Miñana-Galbis *et al.*, 2004a*; Miñana-Galbis *et al.*, 2007*; Fontes *et al.*, 2010*; Miñana-Galbis *et al.*, 2010a; Janda and Abbott, 2010*) and sometimes taxa have been described based solely in one strain (*A. simiae*, *A. fluvialis*, *A. taiwanensis*, *A. sanarellii*) (Harf-Monteil *et al.*, 2004*; Beaz-Hidalgo *et al.*, 2009*; Alperi *et al.*, 2010*; in press*). It is obvious that these species are not extensively and well characterised and any deviation of the predicted phenotype or genotype may preclude the labelling of future (and previously) isolated specimens of these taxa (Janda and Abbott, 1998; Christensen *et al.*, 2001) and thus their report, if proper identification methods are not applied (discussed below). Also, some species (*A. eucrenophila*, *A. encheleia*, *A. sobria*, *A. jandaei*, *A. schubertii*, *A. trota*, *A. popoffii*) although extensively characterised when described (7 to 25 strains used in species delineation) have been rarely and/or poorly detected in the majority of studies independently of the source investigated (Popoff and Verón, 1976; Hickman-Brenner *et al.*, 1988*; Carnahan *et al.*, 1991a*; Carnahan *et al.*, 1991b*; Esteve *et al.*, 1995*; Huys *et al.*, 1997a*; Huys *et al.*, 1997b*; Borrell *et al.*, 1998*; Demarta *et al.*, 2000*; Soler *et al.*, 2002*; Awan *et al.*, 2009*; Nováková *et al.*, 2009*). In relation to the occurrence of *A. trota*, a bias is certainly introduced by the use of ampicillin in the isolation media in the great majority of surveys, constituting an outstanding example of how the methodological procedures may influence results (Huddleston *et al.*, 2007). For instance, Albert and colleagues (2000) have isolated in culture media without ampicillin high numbers of *A. trota* specimens from human clinical and non-clinical samples as also from environmental sources in contrast to the majority of reports. Nevertheless, by far the great majority of *Aeromonas* strains are resistant to this antimicrobial agent whereas many other microorganisms are not, thus its use helps in aeromonads recovering (Martin-Carnahan and Joseph, 2005*; Huddleston *et al.*, 2007). Other species, some of them recently described, (*A. allosaccharophila*, *A. aquariorum*, *A. tecta*) are probably more distributed than

* See table 1.1 on page 10.

expected but may go undetected (when using certain identification methods; discussed below) by close resemblance with other more occurring and well known species (Saavedra *et al.*, 2007*; Demarta *et al.*, 2008*; Figueras *et al.*, 2009*).

On the contrary, some species (*A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. veronii*) are well defined, extensively detected in different niches worldwide and characterised (Gray and Stickler, 1989*; Ali *et al.* 1996*; Borrell *et al.*, 1998*; Singh, 2000*; Soler *et al.*, 2002*; Castro-Escarpulli *et al.*, 2003*; Sen and Rodgers, 2004*; Saavedra *et al.*, 2007*) which, by inherence, confers them higher intrataxon variability. In fact, *A. hydrophila* is the phylogenetic group rendering more accounts and includes three subspecies (*A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *dhakensis* and *A. hydrophila* subsp. *ranae*) (Huys *et al.*, 2003), from which the taxonomic position of *A. hydrophila* subsp. *dhakensis* has recently been questioned by two different groups and will be discussed below (Martínez-Murcia *et al.*, 2009; Miñana-Galbis *et al.*, 2009). Furthermore, the *A. salmonicida* taxon includes psychrophilic and mesophilic non-motile specimens comprising five subspecies (*A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *smithia* and *A. salmonicida* subsp. *pectinolytica*) and also a group of mesophilic motile strains isolated from different environments including human clinical sources (Martin-Carnahan and Joseph, 2005*; Martínez-Murcia *et al.*, 2005). *A. veronii* includes two biovars (*A. veronii* bv. sobria and *A. veronii* bv. veronii) from which *A. veronii* bv. sobria has been continuously wrongly referred as *A. sobria* (Martin-Carnahan and Joseph, 2005*; Janda and Abbott, 2010*). Also, *A. media* strains form two different subgroupings (possibly two distinct subspecies of *A. media*) from which one phenotypically resembles *A. caviae* and the other contains the *A. media* type strain, which in turn include two groupings with striking phenotypic differences that could be considered distinct biovars of this supposed "subspecies" (Altwegg *et al.*, 1990*; Huys *et al.*, 1996). Indeed, these species present a higher variability and also a larger occurrence in relation to the remaining species, although, as stated above, reports may be biased by methodological procedures.

1.1.3 Distribution of Aeromonas species and routes of transmission

As mentioned, aeromonads are globally present in distinct ecological niches. Some phylogenetic groups are more predictable to be found in specific environments than others,

* See table 1.1 on page 10.

but it is almost certain that a particular species can be found in what can be considered an atypical niche for it.

Notwithstanding this universal distribution and quoting Janda and Abbott (2010*), "Today, the genus *Aeromonas* is considered to be almost synonymous with water and aquatic environment..." In fact aeromonads can be found in fresh, saline and estuarine waters, whether polluted or unpolluted (Soler *et al.*, 2002*; Henriques *et al.*, 2006a*; Huddleston *et al.*, 2006*). Also, they can be found in surface or groundwater (Massa *et al.* 2001), rivers, lakes and ponds (Borrell *et al.*, 1998*; Picão *et al.*, 2008a*), in mineral and thermal waters (Biscardi *et al.*, 2002) and even in public distribution systems (treated or untreated) and commercialised bottled water (Borrell *et al.*, 1998*; Koksal *et al.* 2007; Razzolini *et al.*, 2008*). These waters are in their majority used by humans and, in some cases, by other animals either for direct consumption (drinking, cooking, and bathing) or other activities (irrigation, therapeutic and recreational activities), constituting a route of contact between humans, other animals, produce and aeromonads (Borrell *et al.*, 1998*; Pianetti *et al.*, 2004; Scoaris *et al.*, 2008*). It is especially worrisome the high recovering of aeromonads in drinking water, prompting the inclusion of *A. hydrophila* in the Contaminant Candidate Lists of organisms which require future regulation under the Safe Drinking Water Act of the United States Environmental Protection Agency (EPA 1998; 2005) and the implementation in some countries of standards on *Aeromonas* to assess water quality (Gavriel *et al.* 1998; Legnani *et al.* 1998; Massa *et al.* 2001). Also, aeromonads were found in soil and sediments (Carter *et al.*; 1995; Huddleston *et al.* 2006*). Additionally, *Aeromonas* species were collected from sewage, wastewaters and sludge of distinct origins and in different stages of treatment whose final effluents or sludge can be used for distinct activities in agriculture and aquaculture (Moura *et al.*, 2007; Picão *et al.*, 2008b*; Rahman *et al.*, 2009; Al-Bahry *et al.*, 2009). Actually, the aquaculture environment, including water and fish, diseased or healthy, are rich reservoirs of aeromonads, who are responsible for massive economical losses all over the world, especially *A. salmonicida* (Singh and Sanyal, 1999*; Saavedra *et al.*, 2004; Akinbowale *et al.*, 2006*; Kozińska, 2007*; Reith *et al.*, 2008). Also, ornamental fish and aquarium water are niches for aeromonads (Martínez-Murcia *et al.*, 2008*).

In this way, aeromonads are also commonly recovered from animals other than humans. Among these and apart from fish, which are widely reported receptacles for

* See table 1.1 on page 10.

aeromonads, insects (Huys *et al.*, 2005), crustaceans (Vaseeharan *et al.*, 2005), molluscs (Evangelista-Barreto *et al.*, 2006*; Ottaviani *et al.* 2006*), amphibians (Pearson *et al.*, 2000*), reptiles (Turutoglu *et al.*, 2005), birds and mammals (Ceylan *et al.*, 2009) were also found to harbour *Aeromonas* species, both in healthy and disease states. Additionally, *A. veronii* and *A. jandaei* were found in symbiotic relations with different species of medicinal leeches used to control postoperative venous congestion (Laufer *et al.*, 2008*). It was suggested that the *A. veronii* specimens found in the gut of these animals have a dual life style (symbiont or pathogen) depending on the host (leech or human) (Silver *et al.*, 2007). Hence, as mentioned above, animals are part of the spectrum of *Aeromonas* species vectors through different niches. In fact, not only the contact with and consumption of uncooked contaminated animals, presents a risk of acquiring aeromonads, but also the animals dejects in the environment and the use of manure as fertiliser in agriculture pose potential avenues of contamination by these organisms.

Actually, as with water, the food chain is one of the putative main routes of humans acquired *Aeromonas* infections (Janda and Abbott, 2010*). *Aeromonas* species are largely recovered from an extraordinary array of food products as seafood, including fish, molluscs, and crustaceans (Neyts *et al.* 2000*; González-Rodríguez *et al.* 2002*), meat (Rodríguez-Calleja *et al.* 2006*), vegetables (Neyts *et al.* 2000*; McMahon and Wilson, 2001*), milk and milk products (Nahla, 2006), which are perfect *Aeromonas* transporters to humans and also other animals. In Figure 1.1 a schematic view of *Aeromonas* spp. routes of transmission is presented.

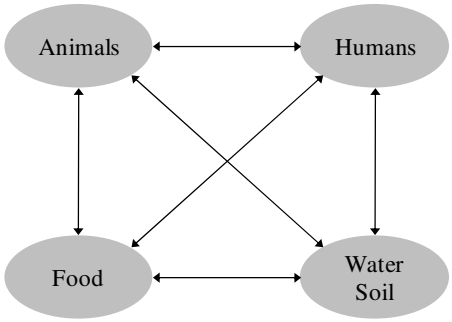

Figure 1.1 *Aeromonas* spp. avenues of dissemination among the distinct environments known to harbour aeromonads. Virtually, once present, any niche is a source of contamination by *Aeromonas* species.

Regularly, *Aeromonas* species are components of the microbial communities associated to humans (Figueras, 2005). They are recovered from multiple human samples (tissues, faeces, blood and other fluids) usually as causative agents of infection, associated or not with other microorganisms, but are also recovered from asymptomatic healthy

* See table 1.1 on page 10.

people (Demarta *et al.*, 2000*; Hua *et al.*, 2004*; Hiransuthikul *et al.*, 2005; Galindo *et al.*, 2006; von Graevenitz, 2007). The most clinically reported specimens are *A. caviae*, *A. veronii* and *A. hydrophila*, which account for more than 85% of health complications due to aeromonads, which mainly affect children, immunocompromised and the elderly but also healthy and immunocompetent people (Figueras, 2005; Janda and Abbott, 2010*). Other *Aeromonas* species and a biovar (*A. bestiarum*, *A. salmonicida*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. jandaei*, *A. veronii* bv. veronii, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. popoffii*, *A. diversa*, *A. aquariorum*, *A. tecta*, *A. taiwanensis* and *A. sanarellii*) have also been implied in human infections, or for one time at least, have been recovered from human clinical sources (Figueras *et al.*, 2005; Demarta *et al.*, 2008*; Figueras *et al.*, 2009*, Miñana-Galbis *et al.*, 2010a; Alperi *et al.*, in press*). On the other hand, species considered primarily environmental (only or majorly recovered from sources not related to human clinical environment) are *A. salmonicida*, *A. sobria*, *A. media*, *A. eucrenophila*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. bestiarum*, *A. popoffii*, *A. molluscorum*, *A. simiae*, *A. bivalvium*, *A. fluvialis* and *A. piscicola* (Janda and Abbott, 1998; Demarta *et al.*, 2000*; Miñana-Galbis *et al.*, 2002*; Valera and Esteve, 2002*; Miñana-Galbis *et al.*, 2007*; Beaz-Hidalgo *et al.*, 2009*; Alperi *et al.*, 2010*; Fontes *et al.*, 2010*).

Besides appearing as free living bacteria, aeromonads may form biofilms (Bomo *et al.*, 2004; Scoaris *et al*., 2008*; Chung and Okabe, 2009). Biofilms are organised microbial communities of frequently different species which have coordinated behaviour and functional heterogeneity. Biofilms are found in biotic and abiotic surfaces as plant and animal hosts and drinking water distribution systems. Specimens aggregated in biofilms are more resistant to antibiotics and disinfectants, host immune responses and other stressful conditions, exhibiting differential expression of genes as virulence genetic determinants (Hall-Stoodley and Stoodley, 2009; Nadell *et al.*, 2009). In this way, biofilms are hot-spots for aeromonads accumulation and persistence in biotic and abiotic surfaces and contamination. This is especially important in the case of drinking water distribution systems where *Aeromonas* species are highly prevalent (Khün *et al.*, 1997; Bomo *et al.*, 2004). An interesting finding was the ability of aeromonads to form biofilms in microbial fuel cells circuits, playing important roles in electric current generation (Chung and Okabe, 2009).

* See table 1.1 on page 10.

Table 1.1 displays a compilation of the sources from where each *Aeromonas* species was recovered as well as relevant traits considering their ecology. The distribution patterns are highly resumed and confined to some niches, probably not including all possible sources from where aeromonads were collected. Even though, an exhaustive search was done aiming the presentation of comprehensive and *bona fide* information.

Table 1.1 Isolation sources and relevant features of *Aeromonas* species[a].

***Aeromonas* species**	**Psp.[b]**	**Sources**	**Relevant traits**
A. hydrophila	Ah	Type strain (ATCC 7966) recovered from tin of milk with a fishy odor. Humans and other animals (diseased, healthy). Water (saline, fresh, polluted, unpolluted, treated, untreated); aquaculture environment. Food (meat, fish, molluscs, vegetables, milk and milk products). Sediments, slaughterhouse.	Type species of the genus. Complete genome of the type strain available. Three subspecies (intrataxon variability). Most detected species (biased by ID methods?). Important human pathogen (biased?).
A. bestiarum	Ah	Type strain (ATCC51108) recovered from diseased fish. Mainly from environmental and aquatic sources (fresh, saline; untreated, treated). Fish (diseased, healthy), food (fish, molluscs, meat, vegetables), different clinical human sources, household environment, sediments.	Occasional human clinical specimen.
A. salmonicida	Ah	Type strain (ATCC33658) recovered from salmon. Mainly from diseased fish (also healthy) and aquaculture environments. Food (fish, molluscs, vegetables, milk products); water (saline, fresh, untreated, treated, polluted, unpolluted). Slaughterhouse; human clinical sources.	Important fish pathogen. Complete genome of strain A449 available. Five subspecies (intrataxon variability). Occasional human clinical specimen.
A. caviae	Ac	Type strain (ATCC15468) recovered from guinea pig. Humans and other animals (diseased; healthy). Water (saline, fresh, polluted, unpolluted, treated, untreated); aquaculture environment. Food (meat, fish, molluscs, vegetables milk and milk products). Sediments, slaughterhouse.	Highly detected (biased by ID methods?). Important human pathogen.
A. media	Ac	Type strain (ATCC33907) recovered from fish farm effluent. Mainly from aquatic environment (fresh water; polluted, unpolluted); aquaculture environment. Fish (diseased; if in healthy, not specified), food (meat, fish, molluscs, milk products); human faeces. Activated sludge; slaughterhouse.	Occasional human clinical specimen. Two phenotypically distinct groups.
A. eucrenophila	Ac	Type strain (ATCC23309) recovered from freshwater fish. Mainly from freshwater and fish (diseased; if in healthy not specified). Food (meat, fish; molluscs), human (diseased); household environment.	Occasional human clinical specimen.
A. sobria	As	Type strain (ATCC43979) recovered from fish. Fish and water environment (fresh, saline); molluscs. Clinical human sources.	Difficult to predict the real *A. sobria* specimens reported in the literature.
A. jandaei	As	Type strain (ATCC49568) recovered from human patient with diarrhoea. Human clinical sources, animals (diseased fish; leeches); water (saline, fresh, treated, untreated).	Rare human pathogen. Modestly detected.
A. schubertii	As	Type strain (ATCC43700) recovered from a forehead abscess. Human and rarely animals (diseased); food (meat, fish, molluscs, vegetables); water (saline, fresh, treated, untreated).	Rare human pathogen. Modestly detected.

Table 1.1 Isolation sources and relevant features of *Aeromonas* species[a] (continued).

***Aeromonas* species**	**Psp.**	**Sources**	**Relevant traits**
A. veronii		Humans and other animals (diseased; healthy).	Two biovars.
		Water (saline, fresh, polluted, unpolluted, treated, untreated), food (meat, fish, molluscs, vegetables and milk products).	Most common *A. veronii* bv. sobria.
		Sediments, slaughterhouse, aquaculture environment.	
	NA	Type strain (ATCC35624 - *A. veronii* bv. veronii) recovered from spuctum of drowning victim.	Rare human pathogen.
	As	*A. veronii* bv. sobria (ATCC9071) isolated from diseased frog.	Important human pathogen.
A. trota	As	Type strain (ATCC49657) recovered from human faeces.	Rare human pathogen.
		Mainly from human clinical sources (diseased humans).	Susceptible to ampicillin.
		Food (meat, molluscs, vegetables); freshwater.	Modestly detected (sampling bias?).
A. allosaccharophila	NA	Type strain (CECT4199) recovered from diseased elver.	Occasional human clinical specimen
		Water (fresh, treated drinking water); fish (diseased); slaughterhouse (pig carcasses and equipment); human faeces.	
A. encheleia	NA	Type strain (ATCC51929) recovered from healthy juvenile freshwater eel.	
		Mainly from water (fresh, untreated, treated) and fish (diseased; healthy). Aquaculture environment.	Occasional human clinical specimen.
		Food (fish, molluscs); human faeces; sediments.	Modestly detected.
A. popoffii	NA	Type strain (LMG17541) recovered from drinking water production plant.	
		Mainly from water (usually fresh but also saline; treated, untreated).	Rare human pathogen.
		Human (diseased); fish; food (rabbit meat); household environment.	Modestly detected.
A. molluscorum	NA	Type strain (CECT5864) recovered from wedge-shells.	Rarely detected.
		Molluscs and estuarine waters (polluted).	
A. simiae	NA	Type strain (CIP107798) recovered from healthy monkey faeces.	Rarely detected.
		Animal faeces (monkey, pig).	
A. bivalvium	NA	Type strain (CECT7113) recovered from cockles.	Rarely detected
A. aquariorum	NA	Type strain (CECT7289) recovered from water of aquarium of ornamental fish.	Recently described species.
		Water (aquarium of ornamental fish); fish (commercialised ornamental fish); human clinical sources.	Rare human pathogen (biased by ID methods?).
A. tecta	NA	Type strain (CECT7082) recovered from stool of children with diarrhoea.	Recently described species.
		Human (diseased; healthy); water (treated, untreated); household environment; fish.	Possible phenotypic resemblance with *A. eucrenophila.*
			Rare human pathogen.
A. piscicola	NA	Type strain (CECT7443) recovered from diseased salmon.	Recently described species.
		Fish (diseased).	
A. fluvialis	NA	Type strain (CECT7401) recovered from river water.	Recently described species. Only one strain available
A. taiwanensis	NA	Type strain (CECT7403) recovered from a burn wound infection.	Recently described species. Only one strain available
A. sanarellii	NA	Type strain (CECT7402) recovered from a burn wound infection.	Recently described species. Only one strain available
A. diversa (Group 501)	NA	Reference strain (ATCC43946; CDC2478-85) recovered from human leg wound.	Rare human clinical specimen
Aeromonas sp. HG11	NA	Reference strain (LMG13075; CDC1306-83) recovered from ankle suture with no infection.	Rarely detected (biased by ID methods?).
		Human clinical sources; water (fresh).	Rare human clinical specimen

[a] References used to construct this table are not extensively cited here, but are presented in the text and marked with an asterisk (*).

[b] Phenospecies (the term will be clarified in section 1.2): Ah, *A. hydrophila*; Ac, *A. caviae*; As, *A. sobria*; NA, not applicable.

1.2 *Aeromonas* spp. identification and typing

1.2.1 Taxonomic history

Members of the genus *Aeromonas* were supposedly detected for the first time in drinking water by Zimmermann in 1890 and designated *Bacillus puntactus*. Later, these specimens were nominated *Aeromonas punctata* (Altwegg and Geiss, 1989). In 1891, Sanarelli isolated *Bacillus hydrophilus fuscus*. Other strains with similar phenotypic characteristics were isolated from water, diseased frogs and fish, comprising the latter known *Aeromonas hydrophila* group. Additionally, pathogenic strains were isolated from fish and assigned to species *Bacterium salmonicida* by Lehmann and Neumann in 1896; this species is currently recognised as *Aeromonas salmonicida*. In the following years, organisms at the present time belonging to the genus *Aeromonas* were classified in different genera: *Bacillus*, *Bacterium*, *Proteus*, *Pseudomonas* and several more. For an extended period of time, there was an increasing number of described species and scarce agreement about the nomenclature and classification of aeromonads (Farmer III *et al.*, 2006).

The genus *Aeromonas* was established by Kluyver and Van Niel in 1936 including pseudomonads that fermented carbohydrates with the production of carbon dioxide and hydrogen (Stanier and Adams, 1944). Since then, numerous species were assigned to the genus and several different frameworks for classifications based on physiological and biochemical characteristics were proposed. In fact, surrounded by controversy, the taxonomic history of this group is marked by several changes. Not only the assignment of each new species to the genus, but also the taxonomic positioning locus of the genus to already established families, raised problematic questions and controversy (Colwell *et al.*, 1986). *Aeromonas* strains after being included in the genus *Proteus* and *Pseudomonas* were for the first time classified as belonging to the genus *Aeromonas* in 1957 in the seventh edition of *Bergey's Manual of Determinative Bacteriology*, as Stanier had previously proposed in 1943 (Stanier, 1943; Altwegg and Geiss, 1989). In 1984, Popoff recognised four species within the genus in *Bergey's Manual of Systematic Bacteriology*, which was included in the family *Vibrionaceae*: *A. hydrophila*, *A. caviae*, *A. sobria* (the mesophilic and motile species) and *A. salmonicida* (the psychrophilic non-motile species)

(Martínez-Murcia *et al.*, 1992a; Martin-Carnahan and Joseph, 2005; Farmer III *et al.*, 2006).

In 1986 Colwell *et al.*, demonstrating molecular genetic evidences that *Aeromonas* form a phylogenetically distinct family of the so-called eubacteria, proposed the creation of the Family *Aeromonadaceae* (Colwell *et al.*, 1986). During the 1980s, based on DNA-DNA hybridisation studies carried out by several authors, a more refined phylogeny of the genus was reached. However, the incongruence between phenotypic and genotypic classifications improved the difficulty of species delineation (Huys *et al.*, 1994). Kuijper and colleagues in 1989 affirmed "there certainly is no doubt that our current ability to identify *Aeromonas* species is less than satisfactory" (Kuijper *et al.*, 1989). In the following years, diverse biochemical tests were regularly used for *Aeromonas* identification but genetically distinct hybridisation groups (HG) could not be unambiguously separated by phenotypic methods and a clear-cut differentiation between some *Aeromonas* species was not possible (Janda *et al.*, 1996; Borrell *et al.*, 1997). There was then, a need to make use of consistent traits for species discrimination and delineation (Martínez-Murcia *et al.*, 1992a). In spite of such discrepancies, several studies permitted improvements in biochemical species identification (Carnahan *et al.*, 1991c, Abbott *et al.*, 1992) and the use of DNA hybridisation studies enabled a better differentiation of specimens. Consequently the number of species expanded and by that time 12 species were described: *A. caviae, A. enteropelogenes, A. eucrenophila, A. hydrophila, A. ichthiosmia, A. jandaei, A. media, A. salmonicida, A. schubertii, A. sobria, A. trota,* and *A. veronii* (Martínez-Murcia *et al.*, 1992a).

With the advent of nucleic acid sequencing technology, prokaryote phylogenetic relationships could be ascertained more easily and reliably than was ever dreamed possible. rRNAs were the most useful and most used molecules to establish affiliations between organisms (Woese, 1987). The phylogenetic studies based on 16S rRNA gene sequence analysis were for the first time applied to study interrelationships of aeromonads by Martínez-Murcia and co-workers in 1992a. They concluded that although the overall percent sequence similarity in the genus is very high, all genospecies possessed characteristic 16S rRNA gene sequences. On the other hand, several inconsistencies were found between the 16S rRNA gene sequencing data and the DNA-DNA hybridisation analysis as, in some cases, results obtained with the two approaches did not match

(Martínez-Murcia *et al*., 1992a). In another study, the same group of investigators reported the failure of 16S rRNA gene sequences to discriminate between closely related species (Collins *et al.*, 1993). Nevertheless, the nucleotide sequence of 16s rRNA gene was (and still is) generally and validly accepted for phylogenetic purposes and several approaches based on this gene were taken to elucidate *Aeromonas* taxonomy (Figueras *et al.*, 2000; Martin-Carnahan and Joseph, 2005). Several other molecular methods were used to achieve this purpose, but some were too discriminative to have taxonomic value and others did not permit a definitive species differentiation. Moreover, results not always correlated well with DNA-DNA homology data (Huys *et al.*, 1996; Borrell *et al.*, 1997). Due to the improvement of technology, reclassification (Collins *et al.*, 1993), extended descriptions of known species (Hickman-Brenner *et al.*, 1987; Altwegg *et al.*, 1990) and reports of new ones (Carnahan *et al.*, 1991b; Martínez-Murcia *et al.*, 1992b; Esteve *et al.*, 1995; Ali *et al.*, 1996; Huys *et al.*, 1997a) was possible. By the year 2000, 14 named species and two unnamed genomic groups were comprised in the genus and latter included in the last edition of *Bergey's Manual of Systematic Bacteriology*: *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. jandaei*, *A. veronii* (*A. veronii* bv. veronii and *A. veronii* bv. sobria), *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. popoffii*, *Aeromonas* Enteric Group 501 and HG11 (Figueras *et al.*, 2000; Martin-Carnahan and Joseph, 2005). Taxonomically, *Aeromonas* species are included in the *Bacteria* domain, XIV Phylum *Proteobacteria*, Class III *Gammaproteobacteria*, Order XII *Aeromonadales*, Family I *Aeromonadaceae* and the Genus I *Aeromonas* (Brenner *et al.*, 2005).

Despite the fact that DNA reassociation remains the gold standard for species delineation, the development of extremely rapid 16S rRNA gene sequencing protocols and the continuing increase of the number of sequences deposited in databases, this approach became widely applied and several species were (and are still being) described based only in the phylogenetic information deduced from this marker (Stackebrandt and Goebel, 1994). But, as mentioned, soon it became evident that this molecular chronometer does not have enough resolution power to discriminate between closely related organisms belonging to some lineages (Woese, 1987; Martínez-Murcia *et al.*, 1992a; Collins *et al.*, 1993; Stackebrandt and Goebel, 1994). To overcome this problem and going along with the new advances in phylogeny and taxonomy, novel molecular markers were searched in order to

establish and elucidate the relationships within *Aeromonas* species. Polyphasic approaches analysed individually and/or in simultaneous different housekeeping genes aptitude to elucidate *Aeromonas* phylogeny which, in general, was proven to be excellent (Yáñez *et al.*, 2003; Soler *et al.*, 2004; Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009). However, a comprehensive multilocus sequence analysis involving concatenated sequences of several housekeeping genes for all species of the genus has not been published to date. Notwithstanding, new species descriptions have already been based in a multilocus phylogenetic analysis using different housekeeping genes (Alperi *et al.*, 2010; in press; Miñana-Galbis *et al.*, 2010a).

The controversy surrounding some taxa still exists and the validity of some species is still being questioned (Saavedra *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009). In fact, due to genetic closely relationships between distinct phylogenetic groups, reports of contradictory values of DNA relatedness and different isolates clustering and interspecies divergence values of distinct housekeeping genes, the debate has been exalted (Yáñez *et al.*, 2003; Soler *et al.*, 2004; Saavedra *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.* 2009).

Since *Bergey's Manual of Systematic Bacteriology* last edition (Martin-Carnahan and Joseph, 2005), 10 novel *Aeromonas* species have been described, namely, *A. molluscorum* (Miñana-Galbis *et al*., 2004), *A. simiae* (Harf-Monteil *et al.*, 2004), *A. bivalvium* (Miñana-Galbis *et al*., 2007), *A. aquariorum* (Martínez-Murcia *et al*., 2008), *A. tecta* (Demarta *et al.*, 2008), *A. fluvialis* (Alperi *et al.*, 2010), *A. piscicola* (Beaz-Hidalgo *et al.*, 2009), *A. taiwanensis*, *A. sanarellii* (Alperi *et al.*, in press) and the designation "*A. diversa"* has been proposed for the *Aeromonas* sp. Group 501 (Miñana-Galbis *et al.*, 2010a). *A. culicicola* and *A. sharmana* were shown not to be new *Aeromonas* species (Huys *et al.*, 2005; Martínez-Murcia *et al.*, 2007).

1.2.2 Phenotypic methods to type and identify aeromonads

As already mentioned *Aeromonas* present an intragenus phenotypic variability which precludes the straightforward identification of specimens. Over the years, scientists have made efforts to circumscribe this problem and numerous methodologies with species identification and typing purposes based on aeromonads biochemical characteristics were published (Martin-Carnahan and Joseph, 2005).

Identification schemes include a battery of biochemical tests in order to obtain a biochemical profile for each specimen analysed aiming its allocation to a determinate species (identification) or phenotypic group (typing). These identification methods were primarily designed based on clinically recovered strains but also applied to environmental aeromonads (Carnahan *et al.*, 1991c; Abbott *et al.*, 1992; Khün *et al.*, 1997; Janda *et al.*, 1996; Abbott *et al.*, 2003). In the majority of cases each species displays a typical biochemical profile, but an extensive subset of tests has to be applied to differentiate groups and not all specimens fall into one of the recognised groups because it has an unusual or atypical profile (Borrell *et al.*, 1997; Abbott *et al.*, 2003). Moreover, the continuous addition of novel species and thus of "new" phenotypic traits, increases the difficulty to identify organisms which can be further deepened when non standardised methodologies are applied. For some species a limited number of strains is known and thus their biochemical profile relies in few or in a unique strain, resulting in limited knowledge of the traits of such groups (Abbott *et al.*, 2003; Janda and Abbott, 2010). Additionally, as identifications schemes were based in biochemical profiles of clinical strains, when applied to environmental strains many specimens fall outside of the normalised profiles being inaccurately identified (Ørmen *et al.*, 2005; Nováková *et al.*, 2009). In this way, in the majority of studies and much in the clinical setting when phenotypic methods are applied to classify aeromonads, identification does not go beyond the phenospecies complex (Ko *et al.*, 1996; Ghenghesh *et al.*, 2001; Palú *et al.*, 2006; Wu *et al.*, 2007). The need to create such groupings arose from the difficulty to separate specimens with similar biochemical profiles but that are genotypically distinct, as demonstrated by different DNA hybridisation studies and more recent phylogenetic analysis (Huys *et al.*, 1994; Abbott *et al.*, 2003; Soler *et al.*, 2004). The *A. hydrophila* complex harbours *A. hydrophila*, *A. bestiarum* and *A. salmonicida*. The *A. caviae* complex includes *A. caviae*, *A. media* and *A. eucrenophila*. Additionally, the *A. sobria* complex includes *A. veronii* bv. sobria, *A. jandaei*, *A. schubertii* and *A. trota*. (Abbott *et al.*, 2003). Moreover, in several studies, the identification of isolates relies on commercial systems, which do not correctly identify specimens of this genus and often label them as *A. hydrophila* (Figueras, 2005; Janda and Abbott, 2010). In this way, the prevalence and distribution patterns of species may be biased by this limitative classification (Table 1.1). Actually, *A. hydrophila* is the most reported species responsible for human clinical cases although *A. caviae* and *A. veronii* bv. sobria are in

reality the more prevalent (Figueras, 2005). Also, *A. veronii* bv. sobria (phenotypically resembles *A. sobria* in some tests) continues to be wrongly referred as *A. sobria* in the literature, precluding the assessment of the real occurrence of both species (Table 1.1). In fact, the majority if not all the "*A. sobria*" specimens associated to clinical reports probably are *A. veronii* bv. sobria isolates (Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010).

Numerical taxonomy, based on biochemical tests results, was also used to cluster aeromonads in accordance to their species affiliation. Nevertheless results obtained were not in absolute accordance with the identification of isolates (Miñana-Galbis *et al.*, 2002; Valera and Esteve, 2002). Other phenotypic methods based on enzymes and other proteins, fatty acids or whole cells analysis were used to identify and type aeromonads (Martin-Carnahan and Joseph, 2005), including, gas-liquid chromatographic analysis of fatty acid methyl esters (FAME) (Huys *et al.*, 1994), multilocus enzyme electrophoresis (MLEE) (Miñana-Galbis *et al.*, 2004b), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (Donohue *et al.*, 2007) and serotyping (Janda *et al.*, 1996). Nevertheless, the taxonomic value of much of these techniques is arguable as they failed to identify some strains or disagreement with DNA-DNA hybridisation data was recorded, being more useful as strain typing methods (Huys *et al.* 1996; Borrell *et al.*, 1997).

Phenotypic methods are laborious, time-consuming and prone to variations from study to study; in the majority of cases, they do not accurately enable *Aeromonas* species identification (Janda *et al.*, 1996). Nevertheless, they provide information about biochemical and physiological properties of the isolates which are not accessible by the solely use of molecular methods, also offering a mean of evaluating microorganisms behaviour and genetic diversity in a large-scale manner (Singh *et al.*, 2006; Janda and Abbott, 2010). A remarkable example of the importance of phenotypic traits of the isolates is the susceptibility of *A. trota* specimens and rarely of other *Aeromonas* species strains to ampicillin (Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010), which is detectable by antibiotic susceptibility profiling.

1.2.3 Molecular methods to assess Aeromonas spp. diversity and identification

The application of molecular techniques to the study of the genus *Aeromonas* allowed a more comprehensive framework of the knowledge on these specimens, from the ecological to the taxonomical areas.

1.2.3.1 Molecular fingerprinting

Genotyping enables the discrimination and cataloguing of specimens by comparative genome analysis, allowing the assessment of the genomic variation among a bacterial population which can belong to the same species, habitat or host or different ones (van Belkum *et al.*, 2001). Genetic fingerprint techniques are important for several researches within microbiology, including epidemiology, phylogeny and evolution and population genetics. By accessing the structural and functional diversity below the species level (i.e., microdiversity), organisms dissemination may be tracked, genome diversification and evolutionary speciation may be detected and functional diversity of microbial communities and ultimately the ecosystem function as a whole, may be understood (Schloter *et al.*, 2000; van Belkum *et al.*, 2001). Different molecular typing methods are used for comparative genome analysis in order to establish subspecific genetic relationships between organisms of the same species and different ones, collected from different environments (e.g. hosts vs. water) or the same ecological niches (e.g. same vegetable sample or water distribution system) or geographic origins (Schloter *et al.*, 2000; van Belkum. *et al.*, 2001; Singh *et al.*, 2006). For each survey purpose the molecular fingerprinting method should be carefully chosen (Figueras *et al.*, 2006).

For the study of the above mentioned relationships among aeromonads, molecular fingerprinting methods included among others ribotyping (Altwegg *et al.*, 1991; Moyer *et al.*, 1992; Demarta *et al.*, 2000), pulsed field gel electrophoresis (PFGE) (Borchardt *et al.*, 2003), amplified fragment length polymorphism (AFLP) (Huys *et al.*, 1996), PCR based random amplified polymorphic DNA (RAPD-PCR) (Szczuka and Kaznowski, 2004; Aguilera-Arreola *et al.*, 2005; Saavedra *et al.*, 2007), repetitive element sequence-based PCR [rep-PCR (including BOX, ERIC and REP elements)] (Szczuka and Kaznowski, 2004; Aguilera-Arreola *et al.*, 2005; Tacão *et al.*, 2005a), restriction length fragment polymorphisms (RFLP) of 16S-23S intergenic spacer regions (Martínez-Murcia *et al.*, 2000) and PCR amplified fragments analysed on denaturing gradient gel electrophoresis

(PCR-DGGE) (Tacão *et al.*, 2005b; Calhau *et al.*, 2010). These studies demonstrated high genetic variability among aeromonads, which was independent of the source of isolation (Demarta *et al.*, 2000; Borchardt *et al.*, 2003, Szczuka and Kaznowski, 2004) with few exceptions (Aguilera-Arreola *et al.*, 2005). On the other hand, only rarely, investigators were able to establish an intra-species genetic close relationship between aeromonads collected from different sources (Altwegg *et al.*, 1991; Demarta *et al.*, 2000; Szczuka and Kaznowski, 2004; Saavedra *et al.*, 2007; Rahman *et al.,* 2009). Moreover, although the general belief that environmental sources (especially water) are the main vehicles of patients contamination with aeromonads, the overwhelming majority of studies were not able to identify specimens displaying the same fingerprints collected from epidemiologically linked sources (Moyer *et al.*, 1992; Martínez-Murcia *et al.*, 2000; Borchardt *et al.*, 2003; Szczuka and Kaznowski, 2004; Aguilera-Arreola *et al.*, 2005; von Graevenitz, 2007; Rahman *et al.,* 2009). Also important is the fact that not always molecular typing techniques are applied for *Aeromonas* sp. strains discrimination whether for epidemiological, ecological or taxonomical purposes (Figueras *et al.*, 2006; Akinbowale *et al.*, 2007b; von Graevenitz, 2007; Wu *et al.*, 2007; Pablos *et al.*, 2009), precluding the assessment of the real diversity and relationships between strains.

1.2.3.2 Phylogenetic analyses

As mentioned above, the taxonomy and phylogeny of the genus experienced a revolution by the application of genome based techniques to assess relationships among aeromonads. First, by DNA-DNA hybridisation studies and then, by 16S rRNA gene sequencing analysis, description of new phylogenetic groups and reclassification of the previously known were done, inciting a plethora of genetic studies on *Aeromonas* species and fuelled debates on their classification (Martínez-Murcia *et al.*, 1992a; Martin-Carnahan and Joseph, 2005; Saavedra *et al.*, 2006).

1.2.3.2.1 DNA-DNA hybridisation

DNA-DNA reassociation data reflects the overall similarity of strains in study. It is the gold standard for delineating species which comprises strains displaying DNA reassociation values of 70% or greater with ΔTm of 5ºC or less and presenting phenotypic characteristics in accordance with such grouping (Stackebrandt and Goebel, 1994;

Stackebrandt *et al.*, 2002). Early DNA-DNA binding studies recognised 8 and then 12 hybridisation groups among the four classically recognised *Aeromonas* phenospecies, constituting the so-called genospecies (genetically distinct species difficult to distinguish phenotypically). Rapidly the number of hybridisation groups expanded as new species were described (Huys *et al.* 1994; Yáñez *et al.*, 2003; Martin-Carnahan and Joseph, 2005). Currently, the assignment of a hybridisation group to a described species is outdated as due to the clarification of aeromonads phylogenetic relationships provided by additional phylogenetic methods, it becomes redundant (Janda and Abbott, 2010). Different methods are used to assess DNA relatedness among aeromonads and usually results obtained in different studies are in agreement, but striking discrepancies in relation to certain species were reported (Huys *et al.*, 1997b; Huys *et al.*, 2001; Martínez-Murcia *et al.*, 2005; Nhung *et al.*, 2007). Excluding reported species found to be latter synonyms of previously described species (Huys *et al.*, 2001; Huys *et al.*, 2005) these discordances are in general related to contentious taxonomic issues within the genus and will be further discussed below. The DNA-DNA hybridisation is a laborious and time-consuming method demanding highly experienced technicians and is not feasible in all laboratories. Moreover, as seen with aeromonads, it is prone to variability and non-uniformity between different methodologies and laboratories and it is not possible to develop a database to analyse and compare strains as it relies in the pairwise comparison of two genomes. Additionally, it is only applicable to culturable organisms (Gevers *et al.*, 2005; Martens *et al.*, 2008). In this way, in 2002, the ad hoc committee encouraged all scientists to "develop new methods to supplement or supplant DNA-DNA reassociation" (Stackebrandt *et al.*, 2002).

1.2.3.2.2 16S rRNA gene

Phylogenetic inferences based on this gene further supported the constitution of the family *Aeromonadaceae* and demonstrated that it is useful for genus assignment purposes (Martínez-Murcia *et al.*, 1992a). The intragenus phylogenetic relationships based on the 16S rRNA gene reveal that this is a tight group of species exhibiting 96.8-100% of sequence similarities and even though groupings correlate well with DNA-DNA binding results, some discrepancies were found. Also, 16S rRNA gene sequences lack resolution to split closely related species (Martínez-Murcia *et al.*, 1992a; Collins *et al.*, 1993; Saavedra *et al.*, 2006; Alperi *et al.*, in press). Moreover, multiple heterogeneous copies of the 16S

rRNA gene have been detected in different *Aeromonas* species strains. In fact, distinct intragenomic microheterogeneities were found particularly in variable regions of the gene containing nucleotidic signature regions that allow the majority of species to be identified and discriminated from the other species. Such mutations may affect proper identification of some strains and is an additional reason for the fact that the 16S rRNA gene is not the best suited to assess phylogenetic relationships between aeromonads (Morandi *et al.*, 2005; Alperi *et al.*, 2008). Nevertheless, the fast and easy classification of organisms based on 16S rRNA gene (including those non-culturable) and the enlarging database available and easily accessed prompted its use as a phylogenetic marker (Stackebrandt and Goebel, 1994). Several methods based on the 16S rRNA gene profiling and/or sequencing to identify *Aeromonas* species were developed, including probes targeting this taxonomic marker and RFLP (restriction fragment length polymorphisms) of the PCR amplified 16S rRNA gene (Borrell *et al.*, 1997; Figueras *et al.*, 2000; Soler *et al.* 2002) and are used in different surveys to classify *Aeromonas* species (Demarta *et al.*, 2000; Castro-Escarpulli *et al.*, 2003; Kozińska, 2007; Pérez-Valdespino *et al.*, 2009).

1.2.3.2.3 Housekeeping genes

Housekeeping genes are single copy genes, constitutively expressed and essential for cell maintenance which seldom undergo horizontal transfer. These protein encoding genes are thus under stabilising selection for conservation of metabolic functions and evolve relatively slow, constituting reliable molecular markers for inferring genetic interrelationships among bacteria (Urwin and Maiden, 2003). In fact, several housekeeping genes may represent whole genomes reliably for species assessment purposes, as they have the potential to predict whole genome relationships with satisfactory precision (Zeigler, 2003). On the other hand, protein encoding genes evolve faster than 16S rRNA genes as they present superior nucleotide substitution rates and thus have greater phylogenetic resolving power (Yamamoto and Harayama, 1996; Soler *et al.*, 2004; Martens *et al.*, 2008). Additionally, a multilocus sequence analysis (MLSA) using multiple housekeeping genes should provide a more robust phylogeny as the concomitant use of different *loci* provides cumulative reliable data of genomic relationships between bacteria from a particular group (Gevers *et al.*, 2005; Martens *et al.*, 2008). Also, the distortion to the phylogenetic analysis introduced by possible recombination events in a single gene should be avoided by the use

of concatenated sequences of powerful phylogenetic markers (Gevers *et al.*, 2005). The MLSA approach accurately establishes relationships between closely related individuals based on information easily stored and accessed in databases, therefore, constituting a reliable alternative to DNA-DNA hybridisation (Martens *et al.*, 2008). Moreover, in contrast with the DNA-DNA hybridisation and 16S rRNA gene sequencing, MLSA is capable of accurately establish relationships in different taxonomic levels, from the intraspecific to at least the genus level (Gevers *et al.*, 2005; Martens *et al.*, 2008). A minimum of five genes encoding metabolic functions was suggested for reliable definition of species (Stackebrandt *et al.*, 2002), nevertheless, the careful selection of genes to discriminate species may turn this number superior to the required for equalising or even surpassing the power of DNA reassociation in species assessment purposes (Zeigler, 2003).

The usefulness of distinct housekeeping genes to establish evolutionary relationships within *Aeromonas* has been appraised. Phylogenetic markers included *gyrB*, *rpoD*, *rpoB*, *dnaJ*, *recA* and *cpn60* [corresponding to the universal target (UT) region] genes. Table 1.2 exhibits relevant information concerning studies conducted.

In general, all genes exhibit sequence divergences between distinct *Aeromonas* species, demonstrating high resolving power for speciation and, in some cases, enabling intraspecies strains discrimination (Yáñez *et al.*, 2003; Soler *et al.*, 2004; Saavedra *et al.*, 2006; Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009). As shown in table 1.2, the nucleotide substitution rates differ from gene to gene and, for instance, *gyrB* displays a mean sequence divergence superior to *rpoB* and *cpn60* UT, similar to that of *rpoD* and minor than *dnaJ* (Soler *et al.*, 2004; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009). Smaller sequences of *rpoB*, *dnaJ*, *recA* and *cpn60* UT provided similar results as larger sequences of *gyrB* and *rpoD* (Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009), nevertheless by using sequences of approximately 500bp for *gyrB*, results obtained are the same (Martínez-Murcia *et al.*, 2008). On the other hand, although distinct molecular markers seem to be evolutionarily well synchronised, in some cases they show different mutational behaviours for closely related specimens. In fact, the placement of some strains differed according to analysis based in one or other gene (Soler *et al.*, 2004; Küpfer *et al.*, 2006; Sepe *et al.*, 2008). The *recA* gene based analysis provided inconsistent results in comparison with

previous analysis based on *gyrB* and *rpoB*, as some strains affiliated with different taxonomic groups (Sepe *et al.*, 2008). Unfortunately, no possible explanations were further scrutinised. Additionally, for instance, *gyrB* and *rpoD* showed different resolutions to discriminate certain *Aeromonas* species, being *rpoD* better to split *A. salmonicida* from *A. bestiarum* and *gyrB* more indicated to distinguish *Aeromonas* sp. HG11 from *A. encheleia* and *A. veronii* from *A. allosaccharophila* (Soler *et al.*, 2004). These incongruent results may derive from horizontal transfer and subsequent recombination events, but these findings demand further intensive phylogenetic studies based in additional molecular markers for such strains (Soler *et al.*, 2004; Martens *et al.*, 2008).

Table 1.2 Genes used to study phylogeny of *Aeromonas* species.

Gene	Gene product (function)	Length[a] (bp)	Nuc. substitution rates (%)[b]		Borderline or exceptional cases	Reference
			Intraspp.	Interspp.		
gyrB	B subunit of type IIA DNA topoisomerase (introduces negative supercoils in DNA molecule).	957	≤2.6	>3	*A. salmonicida*/*A. bestiarum*; *A. bestiarum*/*A. popoffii*; *A. encheleia*/*A.* sp. HG11	Yáñez *et al.*, 2003
		957	≤2.3	>3	*A. salmonicida*/*A. bestiarum*; *A. encheleia*/*A.* sp. HG11	Soler *et al.*, 2004
		1100	NP[c]	7.76[d]	*A. encheleia*/*A.* sp. HG11	Küpfer *et al.*, 2006
rpoD	Primary essential factor of σ^{70}-type σ factors (binds to and directs RNA polymerase to specific promoters of the transcription initiation).	813	≤2.6	>3	*A. encheleia*/*A.* sp. HG11; *A. veronii*/*A. allosaccharophila*	Soler *et al.*, 2004
rpoB	β subunit of RNA polymerase (responsible for the majority of RNA polymerase catalytic function).	558	NP	6.07[d]	*A. veronii*/*A. allosaccharophila*; *A. salmonicida*/*A. bestiarum*; *A. bestiarum*/*A. popoffii*; *A. encheleia*/*A.* sp. HG11	Küpfer *et al.*, 2006
dnaJ	Heat-shock protein 40 (involved in protein translation, folding, unfolding, translocation, degradation, primarily by stimulating the ATPase activity of chaperone proteins - Hsp70).	891	≤3.3	>5.2	*A. hydrophila* subsp. *dhakensis*/*A. hydrophila*; *A. veronii*/*A. allosaccharophila*; *A. encheleia*/*A.* sp. HG11	Nhung *et al.*, 2007
recA	RecA (DNA strand exchange related to recombinational DNA repair; different roles in SOS response).	272	NP	7.8[d]	*A. encheleia*/*A.* sp. HG11; *A. culicicola*/*A. veronii*	Sepe *et al.*, 2008
cpn60 (UT)	Type I chaperonin Cpn60, also Hsp60 or GroEL (protein folding; intercellular signalling molecules; modulation of immune response; virulence).	555	≤3.5	≥3.7	*A. encheleia*/*A.* sp. HG11; *A. hydrophila* subsp. *dhakensis*/*A. hydrophila*; *A. veronii*/*A. allosaccharophila*	Miñana-Galbis *et al.*, 2009

Functions, length of the fragments used, percentage of nucleotide substitutions and exceptional cases are detailed.

[a] Sequence length used for the study.

[b] Nucleotide substitution rates. Intraspp., intraspecies; interspp., interspecies.

[c] NP, not presented by the authors.

[d] Mean value.

Aeromonas species groupings obtained with each of the above mentioned housekeeping genes were consistent with previous phylogenetic analysis of the genus as determined by DNA-DNA hybridisation results and 16S rRNA gene sequence analysis (Yáñez *et al.*, 2003; Soler *et al.*, 2004; Saavedra *et al.*, 2006; Nhung *et al.*, 2007; Küpfer *et al.*, 2006; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009). Nevertheless, in relation to this molecular marker, nucleotide degeneracy was superior for all genes and phylogenetic analyses obtained were more refined in relation to DNA reassociation data conducting to an improvement of *Aeromonas* systematic (Martínez-Murcia *et al.*, 2005; Nhung *et al.*, 2007). In fact, phylogenies based on these housekeeping genes sequences clearly separated pairs of species as *A. trota*/*A. caviae* and *A. hydrophila*/*A. media* exhibiting one or three nucleotide differences in the 16S rRNA gene, correspondingly, and low levels of DNA relatedness. Also, *A. salmonicida*/*A. bestiarum* sometimes indistinguishable by 16S rRNA gene sequencing and exhibiting high levels of DNA reassociation (Martínez-Murcia *et al.*, 1992a; Martínez-Murcia *et al.*, 2005) may be discriminated by housekeeping genes analysis (Soler *et al.*, 2004; Martínez-Murcia *et al.*, 2005; Nhung *et al.*, 2007). In this way, contentious taxonomic issues ensuing from the lack of criteria for discriminating closely related species, incorrect strains grouping by 16S rRNA gene sequence analysis and incongruent DNA reassociation data are being supplanted by the application of phylogenetic analysis based on such housekeeping genes (Soler *et al.*, 2004; Nhung *et al.*, 2007; Miñana-Galbis *et al.*, 2009).

The use in concomitance of several housekeeping genes sequences to deduce phylogenetic relationships between aeromonads gives, as expected, a more reliable and robust analysis (Soler *et al.*, 2004; Küpfer *et al.*, 2006; Sepe *et al.*, 2008). The multilocus phylogenetic analysis (MLPA) approach is a promising field to abrogate the uncertainties remaining all over these years in relation to some phylogenetic groups (Alperi *et al.*, in press). Only very recently, three novel species (*A. fluvialis*, *A. taiwanensis* and *A. sanarellii*) have been described complying with the recommendation of the use of distinct *loci* for new species proposals, but a comprehensive MLPA of the genus is being expected (Alperi *et al.*, in press; 2010).

Apart from phylogenetic inferences the same housekeeping genes are also very useful for *Aeromonas* species identification although the scarce appliance of such methodology in the surveys conducted for assessment of *Aeromonas* species occurrence

and diversity. In fact, rarely detected species such as *A. allosaccharophila*, *A. simiae*, *A. eucrenophila* and recently described ones as *A. aquariorum* and *A. tecta*, which may go unrecognised under more popular species, have been identified in few investigations by using strain identification based on housekeeping genes sequencing (Saavedra *et al.*, 2007; Kozińska, 2007; Demarta *et al.*, 2008; Figueras *et al.*, 2009; Fontes *et al.*, 2010). These findings indicate that these species may be more abundant and distributed than apparently it seemed before and apart from providing additional data on ecology and diversity, they strengthen these species clusters (Saavedra *et al.*, 2006; 2007; Figueras *et al.*, 2009; Fontes *et al.*, 2010). Additionally, a pioneering study combining the use of PCR amplified fragments of housekeeping genes and DGGE (PCR-DGGE) for assessing diversity and *Aeromonas* spp. population dynamics has been developed and demonstrated to be a powerful method not only for aeromonads typing and identification purposes but also to evaluate diversity and dynamics of *Aeromonas* in complex bacterial communities (Tacão *et al.*, 2005b; Calhau *et al.*, 2010).

As mentioned before, the inclusion of higher number of strains in study enables a more reliable and robust phylogeny. For instance the low number of recognised *Aeromonas* sp. HG11 dictates the uncertain taxonomic status of this species (Soler *et al.*, 2004; Küpfer *et al.*, 2006); the inclusion of the newly identified strains of *A. allosaccharophila* provided data to support the original proposal of the species (Saavedra *et al.*, 2007) and the same happened with the recently identified *A. simiae* by Fontes and others (2010).

Other molecular methods were used for aeromonads identification and phylogeny assessment, as AFLP, RFLP and PFGE but as with others techniques mentioned before they were shown to be more useful for typing than for taxonomic purposes (Huys *et al.*, 1996; Borrell *et al.*, 1997; Martínez-Murcia, 1999; Martin-Carnahan *et al.*, 2005).

1.2.4 Contentious issues, borderline cases and other taxonomic comments

As mentioned above, several controversies still surround aeromonads phylogeny, due to the lack of definitive criteria to split closely related species, incongruent reported results and also mislabelling of strains.

A. salmonicida and *A. bestiarum* are validated distinct species which display closely related phylogenetic relationships. Phenotypically, it is very difficult to

unequivocally distinguish members of the two species (Abbott *et al.*, 2003; Martínez-Murcia *et al.*, 2005). Reported DNA reassociation values between strains of these groups were above the 70% threshold, not providing definitive clear-cut differentiation between them, constituting a case of lack of refinement of DNA pairing data for species delineation (Martínez-Murcia *et al.*, 2005). Additionally, the 16S rRNA gene sequences of *A. salmonicida* and *A. bestiarum* differ by only two nucleotides and for some strains of these species it is indistinguishable (Yáñez *et al.*, 2003; Martínez-Murcia *et al.*, 2005). Phylogenetic analyses based on the housekeeping genes mentioned before enabled a more striking distinction between these species, although for some genes the divergence values recorded situated in the boundaries of intra and interspecies delineations thresholds (Table 1.2). The *gyrB* and *rpoB* genes sequence divergences between strains of these two species (2.2-4.3% and 2.6%, respectively) may not provide conclusive information in relation to some strains as they confine between intra and interspecies distinction values (Soler *et al.*, 2004; Küpfer *et al.*, 2006). On the contrary, *rpoD*, *dnaJ*, *recA* and *cpn60* UT mutation rates (6.8-8.7%, 5.2-6.2%, 4.9% and ≥5.4%, correspondingly) enabled the separation of these species and emphasise the advantage of using distinct housekeeping genes for phylogenetic inferences (Soler *et al.*, 2004; Martínez-Murcia *et al.*, 2005; Saavedra *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009). *A popoffii* is also closely related to *A. salmonicida* and *A. bestiarum* on the basis of DNA pairing data, 16S rRNA gene and housekeeping genes sequencing analyses. Incongruent DNA reassociation results were reported by Huys *et al*. (1997a) and Nhung and colleagues (2007) which were higher for the latter. Nevertheless, global results clearly distinguish *A. popoffii* from the other two species (Soler *et al.*, 2004; Nhung *et al.*, 2007, Miñana-Galbis *et al.*, 2009).

The phylogenetic status of *A. allosaccharophila* as a distinct species of *A. veronii* is still a current controversy within aeromonads phylogeny. Although biochemically heterogeneous and in spite of the records of inconsistent phenotypic traits obtained by different researchers, *A. allosaccharophila* specimens are phenotypically distinguishable from other *Aeromonas* species (Valera and Esteve, 2002; Huys *et al.*, 2001). Also, on the basis of 16S rRNA gene sequence analysis, *A. allosaccharophila* is clearly distinctive from the remaining species of the genus (Martínez-Murcia *et al.*, 1992b). On the other hand, DNA pairing data results obtained in different studies were outstandingly contradictory, being comprised between 0% and 40% or in the borders or even superior to the 70%

threshold of species delineation (Huys *et al.*, 2001; Yáñez *et al.* 2003; Nhung *et al.*, 2007). The phylogenetic analysis based on the housekeeping genes mentioned above gave also incongruent information. While *gyrB* and *recA* sequences allow the distinction between strains of the two species (Yáñez *et al.* 2003; Soler *et al.*, 2004; Sepe *et al.*, 2008), *rpoD*, *rpoB*, *dnaJ* and *cpn60* UT sequence divergence values are bellow the interspecies thresholds or in the cavity between intra and interspecies cut values (Table 1.2), precluding the clear distinction of *A. veronii* and *A. allosaccharophila* strains (Soler *et al.*, 2004; Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Miñana-Galbis *et al.*, 2009). The uncertainty still surrounding *A. allosaccharophila* taxonomic place claims for additional DNA-DNA hybridisation and MLPA studies.

Another conflictory issue includes *Aeromonas eucrenophila*, *Aeromonas encheleia* and *Aeromonas* sp. HG11. *Aeromonas* sp. HG11 was the remaining of enteric group 77 which gave rise to *A. veronii* and includes three strains that were not genetically related to the latter (Hickman-Brenner *et al.*, 1987). *A. eucrenophila* and *A. encheleia* experienced further descriptions and strains reclassifications in which, on the basis of biochemical, FAME and whole cell proteins analysis, AFLP, RFLP and DNA pairing data, a group of *A. eucrenophila* strains and also *Aeromonas* sp. HG11 were included in *A. encheleia* group (Huys *et al.*, 1996; Huys *et al.*, 1997b). Currently, the problematic issue involves *A. encheleia* and *Aeromonas* sp. HG11. In fact, phenotypic and 16S rRNA gene based analysis distinguish strains from each species (Borrell *et al.*, 1997; Huys *et al.*, 1997b; Martínez-Murcia, 1999; Valera and Esteve, 2002). On the other hand, by comparing the different housekeeping genes for each group, sequence divergences of *gyrB* genes are borderline with the interspecies threshold (Yáñez *et al.* 2003, Soler *et al.*, 2004; Küpfer *et al.*, 2006) and analysing *rpoD*, *rpoB*, *dnaJ*, *recA*, *cpn60* UT genes divergences fall within the limits of intraspecies values, suggesting that these groups may constitute a single taxon (Soler *et al.*, 2004; Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Miñana-Galbis *et al.*, 2009). Also, in these studies few strains of these species were used what, as mentioned before, may preclude proper phylogenetic inferences. Additionally, discrepancies between reported DNA-DNA hybridisation values involving the same strains of these two species (lower than 37% and higher than 80%) promote the confusion surrounding these species (Huys *et al.*, 1997b; Martínez-Murcia, 1999; Nhung *et al.*, 2007). As with *A. allosaccharophila* and *A. veronii*, additional DNA-DNA hybridisation

and MLPA studies are needed to definitely clarify the relationship between these specimens.

The taxonomic status of *A. hydrophila* subsp. *dhakensis* has been also questioned. Albeit DNA pairing data positioned the *A. hydrophila* subsp. *dhakensis* strains in the *A. hydrophila* cluster, different evidences indicate that this is not a subspecies within the *A. hydrophila* group but a distinct *Aeromonas* species (Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Miñana-Galbis *et al.*, 2009) and Martínez-Murcia and co-workers (2009) suggested that those specimens are in fact *A. aquariorum* strains.

Several species were described and latter found to be synonyms of previously known species. *A. ichthiosmia* and *A. culicicola* were found to be latter subjective synonyms of *A. veronii* (Collins *et al.*, 1993; Huys *et al.*, 2001; Huys *et al.*, 2005), which was further supported by housekeeping genes based phylogenetic analysis (Küpfer *et al.*, 2006; Saavedra *et al.*, 2006; Nhung *et al.*, 2007; Miñana-Galbis *et al.*, 2009). On the contrary, a significant nucleotide divergence (4.7%) between *recA* sequences of *A. veronii* and *A. culicicola* was found (Sepe *et al.*, 2008) but the meaning of this remains unexplained.

A. enteropelogenes and *A. trota* were proposed and described in a short period of time. They are synonymous species and despite the most common usage of the name "*A. trota*", the former has priority as it was published and validated first (Janda and Abbott, 2010). A similar problem exists with *A. caviae* and *A. punctata*. These species are synonyms and share the same type strain whose code was wrongly assigned to the *A. punctata* specimen on the 1980 Approved List of Bacterial Names. Moreover the type strain of *A. punctata* should have been listed with the code which currently is the one for the type strain of *A. eucrenophila* (Martin-Carnahan and Joseph, 2005). In this way two different proposals to the Judicial Commission are needed to solve the resulting controversies. On one hand, by using the firstly given code to the *A. punctata*/*A. caviae* type strain this species would share the same type strain with *A. eucrenophila*. On the other hand, the name "*A. punctata*" has priority for the same reasons mentioned above for *A. enteropelogenes*, nevertheless as "*A. caviae*" is more commonly used, the proposal would be to legitimate this name (Janda and Abbott, 2010).

The incorrect labelling of strains is an additional factor for *Aeromonas* taxonomic controversies. Different reference strains are assigned to a species and belong to another

taxon as with some *A. allosaccharophila* strains primarily classified as *A. veronii* (Saavedra *et al.*, 2007) and the *A. hydrophila* ATCC7965 recently shown to belong to the *A. salmonicida* group (Miñana-Galbis *et al.*, 2010b). Also, strains from different species are given the same collection number as it happened with *Aeromonas* sp. HG11 ATCC35942 and the type strain of *A. allosaccharophila* (Janda and Abbott, 2010). The misidentification of strains used as references for different investigations as, in particular, phylogenetic history establishment, conducts to erroneous data which compromises the validity of such investigations, precluding the assessment of the real relationships among the specimens in study.

Apart from clarifying phylogenetic relationships among specimens and giving an insight into the *Aeromonas* species communities' diversity and structure, the use of molecular methods enabled the identification of genetic determinants potentially involved in pathogenic and antibiotic resistance processes.

1.3 Pathogenic and virulence traits of aeromonads

1.3.1 Aeromonas associated diseases

The spectrum of human infections due to *Aeromonas* spp. is vast. As mentioned before, although different species have been implicated in human health complications, *A. caviae*, *A. veronii* bv. sobria and *A. hydrophila* account for the most recovered species from the clinical setting (Janda and Abbott, 2010). They may act as primary or secondary agents of infection which may be intestinal or extraintestinal. Underlying diseases compromising the host immune status are frequently implicated in the acquisition of *Aeromonas* infections but healthy individuals are also susceptible (Figueras, 2005).

Gastroenteritis is the most common clinical presentation of *Aeromonas* infection. Symptomatic manifestations vary greatly, depending on the host, and range from a self-limiting to a chronicle diarrhoea and from mild to a more severe, invasive dysenteric form (Galindo *et al.*, 2006). Healthy international travellers, adults, children and also immunocompromised individuals, are all susceptible to acquire *Aeromonas* related gastroenteritis, sometimes proven to be mediated by contact or consumption of

contaminated food and water (Figueras, 2005; von Graevenitz, 2007; Janda and Abbott, 2010).

In the extraintestinal array of infections, skin and soft-tissue infections (SSTI) and bacteremia, which may evolve to sepsis, are the most recorded *Aeromonas* complications (Figueras, 2005). SSTI including wound and burn infections, may involve processes in cutaneous surfaces, as cellulitis and furunculosis, or severe tissue necrosis, as myonecrosis and ecthyma gangrenosum. These infections usually arise following abrasion, penetrating injuries or more severe traumas with exposure to environmental sources as water and soil (Figueras, 2005; Galindo *et al.*, 2006), as seen in the tsunami survivors in Thailand (Hiransuthikul *et al.*, 2005). Furthermore, nosocomial transmitted SSTI may occur (Figueras *et al.*, 2009; Janda and Abbott 2010). They can affect individuals with underlying diseases, more prone to deep seated infections, and also healthy ones (Figueras, 2005).

Bacteremia, which usually evolves to sepsis, is the third most occurring aeromonads infection and can be the primary or secondary complication, the last usually a sequel of a primary *Aeromonas* infection or colonisation (Figueras, 2005; Janda and Abbott, 2010). Characterised by its fulminant nature, the fatality rate ranges from 25% to 62.5% (Galindo *et al.*, 2006; Figueras, 2005). It affects mainly immunocompromised individuals but may also occur in healthy people by exposure to water and soil.

Other aeromonads induced human complications are hemolytic uremic syndrome, meningitis, peritonitis, respiratory tract infection, ocular infections, urinary tract infections and hepatobiliary or pancreatic infections. Usually the underlying medical complications associated to *Aeromonas* infections are malignancy, diabetes mellitus, gastrointestinal disorders, hepatobiliary diseases (cirrhosis) among others immunocompromising conditions (Figueras, 2005; Janda and Abbott, 2010).

In other animals, *Aeromonas* spp., commonly *A. hydrophila* and *A. salmonicida*, are responsible mainly for skin lesions, hemorrhagic diseases and sepsis in fish, amphibians, reptiles, birds and mammals (Martin-Carnahan and Joseph, 2005). These species are known for important economic losses in farm raised fish, mostly *A. salmonicida* the etiological agent of furunculosis in wild and cultivated fish (Reith *et al.*, 2008).

1.3.2 The virulence factors

Bacterial pathogens harbour different virulence determinants that depending on the host-pathogen interactions enable the bacteria to cause disease. The differential expression of genes encoding virulence determinants in different milieu as host and environmental sources is decisive for the intensity of the disease inflicted and, thus, the presence of such genes in environmental bacteria has to be considered dangerous (Chopra, 2008). These virulence factors enable the infectious agent to colonise, entry, establish, replicate, cause tissue damage, overcome host defences and spread within the host, eventually causing its death (Yu *et al.*, 2004). *Aeromonas* species produce an arsenal of virulence determinants, at least similar to known virulence factors in recognised human pathogens, which appear to play important roles in the wide spectrum of diseases associated to these organisms (Chopra, 2008). The virulence determinants that *Aeromonas* spp. possess are structural and cell associated features and extracellular factors.

1.3.2.1 Adhesins

Structural components as pili, flagella and other adhesion factors are important virulence determinants for initial stages of infection as they enable bacteria with motility and adhesion capacities facilitating the invasion and colonisation of the host (Galindo *et al.* 2006). Type I (Fim) and type IV pili (Tap, Flp, Msha), a single constitutive polar flagellum (Fla) and inducible lateral flagella (Laf) have been identified in *Aeromonas* species (Kirov *et al.*, 2000; Kirov *et al.*, 2004; Merino *et al.*, 2006; Boyd *et al.*, 2008; Dacanay *et al.*, 2010). These organelles are also important for biofilm formation and may be or not concomitantly present in the same strain (Kirov *et al.*, 2004). *A. hydrophila* ATCC 7966 possesses a polar flagellum but not lateral flagella (Seshadri *et al.*, 2006). *A. salmonicida* A449 harbours the complete set of genes for the two types of flagella, however the operons include disrupted genes involved in the production of the organelles, which is consistent with the non motility of *A. salmonicida* (Reith *et al.*, 2008).

Other structural adhesion factors are also involved in the protection of *Aeromonas* spp. from host defences. The S-layer, an outer protein coat which apart from the adhesion capacity helps the bacterium to evade serum killing has been identified in several *Aeromonas* species, being a major virulence factor for *A. salmonicida* strains (Esteve *et al.*, 2004; Dacanay *et al.*, 2010). This structure is present in *A. salmonicida* A449 and

apparently absent from *A. hydrophila* ATCC 7966 (Seshadri *et al.*, 2006; Reith *et al.*, 2008). Also, the lipopolysaccharide which is involved in the anchoring of the S-layer to cell surface, adherence to epithelial cells and resistance to serum killing by host cells is present in different species (Aguilar *et al.*, 1997) as well as a capsular polysaccharide, which has been implied in adherence and especially in invasion properties of *Aeromonas* spp. in addition to cell protection (Merino *et al.*, 1997).

1.3.2.2 Extracellular factors

Several extracellular molecules secreted by aeromonads have also been linked to their virulence. Extracellular enzymes as proteases, lipases and others are important for degradation of host cell components, contribute to cell nutrition and help evade host cell defences, thus, facilitating the invasiveness and establishment of infection (Pemberton *et al.*, 1997; Galindo *et al.*, 2006).

1.3.2.2.1 Proteases

Serine proteases and metalloproteases have been identified in different *Aeromonas* spp. and characterised (Cascón *et al.*, 2000; Esteve and Birbeck, 2004; Yu *et al.*, 2007). Generally, these enzymes have caseinolytic and elastolytic activities, but ASP from *A. sobria* (serine protease) has been shown to additionally degrade fibrinogen which may be related to weakened plasma clottability of *A. sobria* probably contributing to subsequent hemorrhagic tendencies in sepsis caused by strains of these species (Imamura *et al.*, 2008). Also, AsaP1 (caseinolytic metalloprotease) from *A. salmonicida* additionally triggers host immune responses (Arnadottir *et al*., 2009). Furthermore, serine proteases play a role in activating other virulence involved molecules (Chacón *et al.*, 2004; Yu *et al.*, 2007).

1.3.2.2.2 Lipases

Lipolytic and/or phospholipolytic genetic determinants are widely present in different *Aeromonas* spp. (Chacón *et al.*, 2003; Sen and Rodgers, 2004). The majority of the enzymes have been shown to have similarities in their amino acid sequences, although distinct physical properties and in some cases also different activities. In fact, the *lipH3* and *lip* genes code for lipases with no phospholipase activity (Anguita *et al.*, 1993; Chuang *et al.*, 1997), whereas *apl-1* and *pla* encoded lipases do have phospholipase activity

(Ingham and Pemberton, 1995; Merino *et al.*, 1999). The *plc* gene does not have significant similarity with the remaining genes but encodes a lipolytic protein with phospholipase activity (Merino *et al.*, 1999). Also, a glycerophospholipid cholesterol acyltransferase (GCAT) has been identified but it appears to have an indirect role rather than a major importance in aeromonads virulence (Vipond *et al.*, 1998).

1.3.2.2.3 Other extracellular lytic activities

Other lytic activities have been assigned to aeromonads, as collagenolytic, chitinolytic and amylolytic, to name but a few (Pemberton *et al.* 1997; Galindo *et al.* 2006). Several of these secreted enzymes are present in *A. salmonicida* A449 and in less number in *A. hydrophila* ATCC 7966 (Seshadri *et al.*, 2006; Reith *et al.*, 2008). Additionally, recently described ones are also present, as TagA in the former, which is involved in serum resistance and prevention of erythrocyte lysis, and enolase in the latter, which is a secreted and expressed protein in the bacterial surface able to bind to plasminogen, which may indicate a role in aid of dissemination of bacteria in the host (Seshadri *et al.*, 2006; Chopra, 2008; Reith *et al.*, 2008; Sha *et al.*, 2009).

1.3.2.2.4 Siderophores

Siderophores are iron chelator molecules important for the bacterial iron acquisition in low iron conditions, as within hosts (Seshadri *et al.*, 2006). Two distinct molecules were described in *Aeromonas* spp., amonabactin and enterobactin, which can be both present in the same strain or individually (Galindo *et al.*, 2006). Amonabactin seems to have influence on aeromonads virulence but not enterobactin, which is less distributed among the different *Aeromonas* species (Zywno *et al.*, 1992; Seshadri *et al.*, 2006). Genes involved in the production of siderophores are present in both *A. salmonicida* A449 and *A. hydrophila* ATCC 7966, including the earlier described and others not previously reported (Seshadri *et al.*, 2006; Reith *et al.*, 2008).

1.3.2.2.5 Toxins

The most studied extracellular factors involved in *Aeromonas* spp. virulence are molecules which display hemolytic, cytotoxic or cytotonic and enterotoxic activities. For many years an intense debate occurred around these molecules: on one hand, regarding if

the different characterised molecules from different strains of the same species and different ones were synonyms and, on the other hand, if to the same molecule only one or more functions were assigned (Asao *et al.*, 1986; Rose *et al.*, 1989; Fujii *et al.*, 1998; Buckley and Howard, 1999). In fact, the nomenclature of these molecules is confusing as also the specific functional characteristics of each molecule. It is due to the use of different methods in their characterisation, different specimens to achieve such characterisation and the assignment of different names to similar molecules or, in reverse, the equal designation of distinct molecules (Martin-Carnahan and Joseph, 2005; Balsalobre *et al.*, 2009a). Currently is known that these biological activities may reside in the same molecule or in different ones and different molecules may be present in the same *Aeromonas* strain (Chopra and Houston, 1999; Galindo *et al.*, 2006). Furthermore, closely related toxins found in different strains share similar functions and modes of action as also structural and functional differences, which may be related to adaptation to specific biological activities (Ferguson *et al.*, 1997).

Cytotoxic enterotoxins like aerolysin, Act and structurally and functionally related hemolysins are secreted through a type II secretion system (T2SS) and have been shown to be cytotoxic, enterotoxic and hemolytic (Chopra, 2008; Galindo *et al.*, 2006). Act and aerolysin form pores in eukaryotic cells; this has been assumed to be the main virulent mechanism of action for the latter, but the receptor for these toxins in cell membranes is different (Galindo *et al.*, 2006). Apart from these activities Act further induces lethality in mice when injected intravenously. Several studies by Chopra and colleagues on host response to Act demonstrated that it induces apoptosis and inflammatory responses in murine macrophages and colonic epithelial cells that are related to the cytotoxicity and enterotoxicity activities reported for this powerful toxin (Chopra *et al.*, 2009). The host responses to aerolysin have been poorly characterised (Galindo *et al.*, 2006). Other molecules genetically related to these toxins were studied by Epple and colleagues (2004) and Fujii *et al.* (1998; 2008) and appear to have similar ways to induce diarrhoea, although they act upon intestinal cells in a different manner: contrary to Act these molecules elicit enterotoxic responses without intestinal cell disruption, even though they are cytolytic.

Cytotonic enterotoxins have also been identified in several distinct *Aeromonas* sp. isolates, which cause intestinal fluid accumulation in different intestinal loop or infant mice assays and exhibit cytotonic activity (no degeneration of intestinal mucosa and

elongation of cells). Some of these toxins are heat-stable, as Ast from *A. hydrophila* SSU and others heat-labile, as Alt from the same strain. The relation of distinct heat labile or heat stable cytotoxins to one another is unknown but apparently their action is very similar unlike the cytotoxic enterotoxins (Chopra and Houston, 1999; Galindo *et al.*, 2006).

Aeromonas sp. induced diarrhoea was associated to the presence of the different enterotoxins alone or in combination in strains and some interaction was noticed between Act, Alt and Ast which could affect the severity of the disease (Chopra *et al.*, 2009). Additionally, the distinct families of enterotoxins are widely distributed among different *Aeromonas* species collected from different environments (Kingombe *et al.*, 1999; Chacón *et al.*, 2003; Wu *et al.*, 2007).

Other hemolytic proteins were detected in aeromonads, which do not have molecular relation to the previously mentioned and neither between them, although they do have nucleotidic sequence similarity with genes present in both *A. hydrophila* ATCC 7966 and *A. salmonicida* A449 complete genomes (Erova *et al.*, 2007; Singh *et al.*, 2009). In fact, the *A. hydrophila* ATCC 7966 genome includes an impressive array of genes coding for cytotoxic enterotoxins, cytotonic enterotoxins and other hemolysins (Seshadri *et al.*, 2006). Also, different genes encoding hemolytic and enterotoxic activities are present in lower number and diversity in *A. salmonicida* A449 (Reith *et al.*, 2008).

Shiga toxins encoding genes (*stx1* and *stx2*) have also been identified in *Aeromonas* species. These toxins are known to be important virulence factors in gastroenteritis, hemorrhagic colitis and hemolytic-uremic syndrome pathogenic processes of *E. coli* and *Shigella dysenteriae* (Alperi and Figueras, in press).

1.3.2.3 Secretion systems

Bacterial secretion systems enable the transport of proteins across the cell membrane(s), a basic cellular function that is also important for pathogenic bacteria interactions with eukaryotic host cells. In Gram-negative bacteria, these export systems were grouped according to the characteristics of the secretion apparatus (Gerlach and Hensel, 2007).

Aeromonads use type II secretion to export aerolysin, amylase, protease and other periplasmic proteins (Howard *et al.*, 2006; Reith *et al.*, 2008). This secretory system is present in *A. hydrophila* ATCC 7966 and *A. salmonicida* A449, in which is probably also

responsible for the secretion of VapA, the main constituent of the S-layer. Also, type I and Tat secretion pathways are present in the former (Seshadri *et al.*, 2006; Reith *et al.*, 2008).

Type III secretion systems (T3SS) or injectisomes are sophisticated apparatus which allow Gram-negative bacteria adhering to a cell membrane to deliver secreted proteins directly into the cytosol of the host cell (Cornelis, 2006). T3SS play a role in symbiotic relations of bacteria with their hosts, but in the great majority of cases, they contribute to pathogenic dealings between specimens and hosts (Ghosh, 2004; Silver *et al.* 2007). The secretion apparatus is made up of more than 20 components, from which a core of structural proteins is phylogenetically conserved among different species allowing the clustering of the different systems in seven distinct families (Cornelis, 2006). The Ysc T3SS from *Yersinia* spp. (Fig. 1.2) is the archetype of one of these families and the injectisomes described in *Aeromonas* spp. belong to this family (Yu *et al.*, 2004; Vilches *et al.*, 2004; Sha *et al.*, 2005; Cornelis, 2006). The basal structure of the injectisome is anchored in the bacterial inner membrane spanning through the peptidoglycan and outer membrane from where the needle complex projects ending in a translocation pore, when active (Cornelis, 2006). On the other hand, more than 100 effector proteins secreted and translocated by T3SS are known, thus varying among different bacteria and reflecting the adjustment required for the specific relations between the pathogens and hosts (Ghosh, 2004; Cornelis, 2006). Effector toxins induce profound changes in host cells and T3SS carrying pathogens cause different symptoms including diarrhoea, other infections and sepsis (Ebanks *et al.*, 2006).

T3SS found in distinct strains of *A. hydrophila* (Yu *et al.*, 2004; Sha *et al.*, 2005) *A. salmonicida* (Burr *et al.*, 2002; Ebanks *et al.*, 2006) and in *A. piscicola* AH-3, formerly *A. hydrophila* (Vilches *et al.*, 2004; Beaz-Hidalgo *et al.*, 2009), were characterised. In *A. hydrophila* and *A. piscicola*, the cluster of genes encoding this machinery is located in the chromosome; the AexT-like or AexU effector secreted and translocated by the T3SS was identified (Yu *et al.*, 2004; Vilches *et al.*, 2004; Sha *et al.*, 2005; Sha *et al.*, 2007; Vilches *et al.*, 2008).

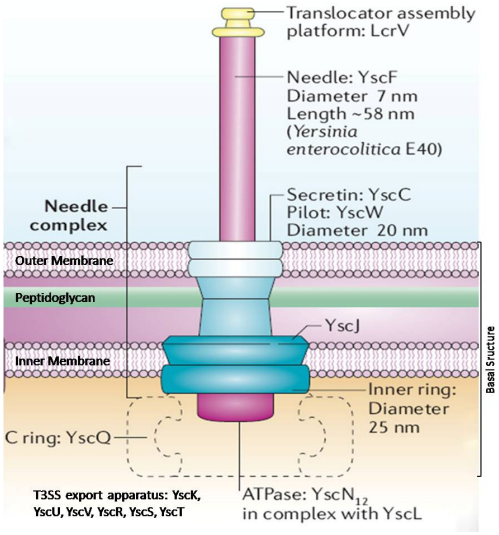

Figure 1.2 Schematic representation of the Ysc injectisome of *Yersinia* spp. (adapted from Cornelis, 2006). The structural protein YscV (homologue of AscV) is embedded in the inner membrane but its exact location has not been elucidated (Cornelis, 2006; Moraes *et al.,* 2008).

In *A. salmonicida* the T3SS is encoded in plasmids and five different effectors (AexT, AopP, AopH, AopO and Ati2), which in some cases are concomitantly present in the same strain, were found (Stuber *et al*., 2003a; Ebanks *et al.*, 2006; Fehr *et al.*, 2007; Reith *et al.*, 2008). AexT and AexU are the unique effectors found in aeromonads that are encoded in the chromosome (Sierra *et al.*, 2007; Reith *et al.*, 2008). The former is a bifunctional enzyme possessing ADP-ribosylating and GAP activities both leading to cytoskeleton actin filament depolymerisation and thus cytophatic effect in host cells. It has been hypothesised that this activity could play a role in the T3SS antiphagocytic activity (Fehr *et al.*, 2007). AexU is an ADP-ribosyltransferase toxin also displaying antiphagocytic activity and induces apoptosis in cells and mouse lethality (Sierra *et al.*, 2007). The AopP effector was shown to have the potential to regulate host inflammatory responses (Fehr *et al.*, 2006).

In general, the studies conducted point to an important and in some cases a major role for T3SS in the virulence of *Aeromonas* spp. (Vilches *et al.*, 2004; Yu *et al.*, 2004; Dacanay *et al.*, 2006). T3SS related genes appear to be distributed among different *Aeromonas* species (Chacón *et al.*, 2004; Wu *et al.*, 2007; Aguilera-Arreola *et al.*, 2007; Figueras *et al.*, 2009; Silver and Graf, 2009). In *A. salmonicida* A449 the T3SS operon is located on plasmid pAsa5 as well as three effectors (AopH, AopO, and Ati2) and, as mentioned, the *aexT* gene is encoded on the chromosome (Reith *et al.*, 2008). *A. hydrophila* ATCC 7966 does not have genes coding for T3SS apparatus and the *aexT*

gene is also missing (Seshadri *et al.*, 2006). Apart from evident interspecies differences in T3SS, there were evidences for genetic and regulatory differences in T3SS among different strains of the same species (Ebanks *et al.*, 2006).

Interestingly, the symbiont of the medicinal leech *Hirudo verbana*, *A. veronii*, also possesses an entire and functional T3SS (as well as AexT and AexU encoding genes) that possibly has a dual role for the lifestyle of *A. veronii* (Sha *et al.*, 2007; Silver *et al.*, 2007). The T3SS is critical for the colonisation of the leech by *A. veronii* additionally protecting the bacteria from phagocytosis by leech hemocytes (symbiotic relationship) and also important for the virulence of *A. veronii* against mammal macrophages and mice (pathogenic relationship). The fact that this specimen possesses *aexT* and *aexU* genes and a functional T3SS which exerts different effects in distinct animals suggests that the effectors can be differentially expressed or provoke different effects depending on host cells (Silver *et al.*, 2007; Silver and Graf, 2009). On the other hand, no *ascV* gene (homolog of *yscV*, encoding an inner-membrane structural component of the T3SS) was detected in the *A. jandaei* specimens also found to establish a symbiotic relation with leeches (Laufer *et al.*, 2008). Apart from the peculiarity of these findings, this is of paramount importance since *Aeromonas* species infections following leech treatment have been reported (Figueras, 2005).

The type IV secretion-like conjugative transfer machinery (which can be involved in both the conjugal DNA transfer between bacteria and the delivery of virulence factors from bacteria to their eukaryotic host) has been found in different *Aeromonas* sp. plasmids, but its role in the virulence of specimens has not been assessed (Rhodes *et al.*, 2004; Fricke *et al.*, 2009; Rangrez *et al.*, 2010). Nevertheless, some of these type IV secretion based conjugative plasmids carried antibiotic resistance genes (Rhodes *et al.*, 2004; Fricke *et al.*, 2009) which enhances the potential health hazard of strains.

Additionally, a type VI secretion system (T6SS) operon was identified and characterised in *A. hydrophila* and its role on cytotoxicity to host cells was reported (Suarez *et al.*, 2008). Furthermore, Hcp (hemolysin coregulated protein) and VgrG1 are toxin effectors secreted and translocated by T6SS directly into host cells, although Hcp was also found to be able to bind to macrophages cell membranes. The VgrG1 toxin has ADP ribosyltransferase activity which induces cytotoxicity and subsequent apoptosis of host cells and Hcp also induces apoptosis and has immunogenic potential (Suarez *et al.*,

2008; Suarez *et al.*, 2010). The genome of *A. hydrophila* ATCC 7966 harbours the predicted entire operon for T6SS and is highly similar to the one reported and mentioned above (Seshadri *et al.*, 2006; Suarez *et al.*, 2008), whereas in *A. salmonicida* A449 it is unlikely to be functional as some key genes coding for T6SS have interruptions or disruptions (Reith *et al.*, 2008).

1.3.2.4 Additional virulence traits

1.3.2.4.1 Quorum sensing

Quorum sensing (QS) is a mechanism of bacterial cell-to-cell communication in which gene expression is regulated in response to an expanding bacterial population. The target genes may be involved in virulence and biofilm formation, among other traits. In Gram-negative bacteria the LuxRI system is the archetype for the majority of QS systems described. In this system, when chemical compounds (autoinducers – N-acylhomoserine lactones) synthesised by the LuxI enzyme and secreted by the various bacteria reach a threshold concentration they bind to and activate a transcriptional regulator, LuxR, which then activates the regulation of gene expression. An additional QS pathway is the *luxS* based system that uses autoinducer-2 (AI-2), known to be an interspecies communication signal. Different QS systems may be present in the same strain (Jayaraman and Wood, 2008). In aeromonads, LuxR and LuxI homologs have been described in *A. salmonicida* (AsaR and AsaI) and LuxS has been additionally found in *A. hydrophila* (AhyR, AhyI, LuxS), as well as the production of N-acylhomoserine lactones (AHLs) (Swift *et al.*, 1997; Kozlova *et al.*, 2008). Also, genes coding for both QS pathways were identified in *A. salmonicida* A449 (Reith *et al.*, 2008) and *A. hydrophila* ATCC 7966 (Seshadri *et al.*, 2006). Additionally, it has been reported that LuxRI homologs are widely distributed among different species of the genus (Jangid *et al.*, 2007). Furthermore, LuxRI mediated QS has been shown to control extracellular proteolytic activity, biofilm formation, T6SS and to contribute for the virulence for fish and mice of *A. hydrophila* but did not affect swimming or swarming motility, neither Act activities. On the contrary, LuxS mediated QS was shown to negatively regulate (reduce) the overall virulence of *A. hydrophila* (Khajanchi *et al.*, 2009). In this way, the presence in aeromonads of a regulatory mechanism of virulence factors that responds to an expanding bacterial population like the

one needed for pathogenic interactions, further enhances the pathogenic potential of these specimens.

1.3.2.4.2 Biofilms

As mentioned before, specimens aggregated in biofilms are more resistant to antimicrobial agents and disinfectants, host immune responses and other stressful conditions and they exhibit differential expression of genes as virulence genetic determinants. By their nature, biofilms are important components of bacterial survival and thus a significant threat to animal and human well being, which is characteristically persistent and related to chronic infections (Hall-Stoodley and Stoodley, 2009; Naddel *et al.*, 2009). In addition, the QS signalling system plays a major role in the development, maintenance and organisation of these communities (Jayaraman and Wood, 2008). *Aeromonas* species are capable of forming biofilms in abiotic surfaces, being inclusively able to incorporate and persist in biofilms in drinking water distribution pipes, and pili and lateral and polar flagella seem to be important promoters (Béchet *et al.*, 2003; Gavín *et al.*, 2003; Bomo *et al.*, 2004; Kirov *et al,* 2004; Chung and Okabe, 2009). Moreover, QS has a regulatory role in biofilms development by *Aeromonas* spp. (Lynch *et al.*, 2002; Kozlova *et al.*, 2008; Khajanchi *et al.*, 2009). This feature may have an important impact in aeromonads pathogenicity in the way that chronicle infections by aeromonads as well as its persistence in chlorinated waters have been reported (Kühn *et al.*, 1997; Galindo *et al.*, 2006; Seshadri *et al.*, 2006), being tempting to speculate that may be due to the ability of these organisms to form biofilms.

1.3.2.4.3 Genes involved in regulation of pathogenic processes

Although not being directly involved in the infection process, some genes have a regulatory role in pathogenic processes. For instance, *dam* (DNA adenine methyltransferase), *gidA* (glucose inhibited division) and *fur* (ferric uptake regulator) genes were shown to have a regulatory role on different virulence factors and traits in *A. hydrophila* (Chopra *et al.*, 2009). Also, *acrV* and *axsE* were shown to be regulatory genes of *A. hydrophila* T3SS (Sha *et al.*, 2007).

1.3.3 Are Aeromonas spp. true pathogens?

Although important progress has been made in recognising *Aeromonas* spp. virulence determinants, their biological activities and the host immune response they evoke, no specific single virulence factor can be identified as primarily responsible for the disease state in the host (Chopra, 2008). The virulence of aeromonads is a multifactorial and complex feature, likely to result from different combinations of virulence determinants which lead to different clinical manifestations as seen for other known pathogens (Gavín *et al.*, 2003). Moreover, although some species are more isolated from clinical environments than others, these potential virulence factors seem to be strain and not species specific traits which further complicates the prediction of which potential virulence determinants are involved in aeromonads infections (Janda and Abbott, 2010).

In this way, the difficulty to establish these microorganisms as *bona fide* pathogens, particularly enteropathogens, still remains (Figueras *et al.*, 2007; Janda and Abbott, 2010). Actually, although the molecular armoury of virulence determinants detected in aeromonads (much identified by similarity with others from known pathogens), no clinical outbreaks were recorded and no animal model that would faithfully reproduce *Aeromonas* diarrhoea associated syndromes was found (Janda and Abbott, 2010). Also, rarely epidemiological linkages between isolates collected from patients and possible sources of transmission were established and the recovering of strains from asymptomatic carriers is not unusual (von Graevenitz, 2007). Even though, sporadic cases and small outbreaks references exist (von Graevenitz, 2007; Janda and Abbott, 2010) and even a curious case in which an healthy investigator accidentally ingested an *A. trota* strain developed diarrhoea and the same strain was collected from the individual faeces (Carnahan *et al.*, 1991b), eventually satisfying the unfulfilled Koch postulate of an animal model mimicking such infection (Figueras *et al.*, 2007). Actually, there is no doubt of the ability of certain strains to cause disease, only not the understanding of the mechanisms or routes of action (Janda and Abbott, 2010).

In addition, aeromonads display different resistance patterns, frequently multiresistance profiles, to a wide array of antibiotics and carry genetic elements encoding virulence factors and antibiotic resistance determinants prone to be horizontally transmitted, which further outstands their danger to human and animal populations.

1.4 Antibiotic resistance in *Aeromonas* spp.

The complete genome sequencing of *A. hydrophila* ATCC 7966 and *A. salmonicida* A449 revealed that both strains carry an array of genes to counteract antibacterial factors present in the environment, including several antibiotics used for human and other animal clinical treatment and toxic compounds (Seshadri *et al.*, 2006; Reith *et al.*, 2008). In fact, it was pointed out by authors that *A. hydrophila* ATCC 7966 carries distinct enzymes which may have bioremediative as well as industrial potential (Seshadri *et al.*, 2006) as also seen for *A. molluscorum*, formerly identified as *A. veronii* (Cruz *et al.*, 2007; unpublished results).

The genus *Aeromonas* is considered to have a typical antibiotic susceptibility profile (Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010). Classically, aeromonads are considered inherently resistant to ampicillin, with the exception of *A. trota* specimens (Janda and Abbott, 2010, Saavedra *et al.*, 2004). Nevertheless, several isolates from other *Aeromonas* species (*A. hydrophila*, *A. caviae*, *A. jandaei*, among others) have been shown to be susceptible to this antimicrobial agent (Goñi-Urriza *et al.*, 2000b; Saavedra *et al.*, 2004; Martin-Carnahan and Joseph, 2005; Koksal *et al.*, 2007; Janda and Abbott, 2010).

In general, *Aeromonas* species display higher resistance rates to penicillins, 1st and 2nd generation cephalosporins (narrow spectrum) and macrolides, whereas 3rd and 4th generation cephalosporins (extended spectrum), carbapenems, monobactams, aminoglycosides, phenicols, quinolones, tetracyclines and antifolates (especially the combination trimethoprim/sulfamethoxazole) are usually more effective against aeromonads (Kämpfer *et al.*, 1999; Vila *et al.*, 2003; Henriques *et al.*, 2006a; Palú *et al.*, 2006; Akinbowale *et al.*, 2007b). Nevertheless, studies concerning antimicrobial resistance in *Aeromonas* species not always corroborate the expected profiles as reported resistance rates to the same antibiotic vary and these values are increasing for some drugs (Ko *et al.*, 1996; Goñi-Urriza *et al.*, 2000b; Saavedra *et al.*, 2004; Akinbowale *et al.*, 2006; Jacobs and Chenia, 2007; Koksal *et al.*, 2007; Moura *et al.*, 2007; Scoaris *et al.*, 2008). For instance, as mentioned, 3rd and 4th generation cephalosporins are usually active against *Aeromonas* spp., being less effective to 0% to 6% of strains (Ko *et al.* 1996; Vila *et al.* 2003; Hiransuthikul *et al.*, 2005; Palú *et al.* 2006), although approximately 30% resistant

isolates to the former (Koksal *et al.*, 2007; Scoaris *et al.*, 2008) and 54% to the latter were registered (Saavedra *et al.*, 2004). Also, imipenem resistance rated within 0% and 10% (Kämpfer *et al.*, 1999; Palú *et al.*, 2006; Moura *et al.*, 2007; Ceylan *et al*., 2009) but Saavedra *et al.* (2004) reported resistance to this antimicrobial agent in 19% of strains and Balsalobre *et al.* (2009b) found 40% isolates displaying co-resistance to imipenem and meropenem. Resistance to chloramphenicol rated between 0% and 13% (Kämpfer *et al.*, 1999; Goñi-Urriza *et al.*, 2000a; Akinbowale *et al.*, 2006; Palú *et al.*, 2006; Rahman *et al.*, 2009), but 31% and 52% of resistant isolates were reported by Vila *et al*. (2003) and Scoaris *et al.* (2008), respectively. Additionally, some studies reflect the increasing aeromonads resistance to antifolates, especially trimethoprim or sulfamethoxazole alone (Ko *et al.*, 1996; Goñi-Urriza *et al.*, 2000b; Huddleston *et al.*, 2006; Scoaris *et al.*, 2008), tetracyclines (Ko *et al.*, 1996; Schmidt *et al.*, 2001b; Chang *et al.*, 2007; Jacobs and Chenia, 2007; Rahman *et al.*, 2009) and quinolones, especially 1st generation quinolones (Goñi-Urriza *et al.*, 2000a; 2000b; Rahman *et al.*, 2009; Alcaide *et al.*, 2010) which may be related to the use of such antimicrobial agents in the prevention and treatment of diseases in human and animals, including aquaculture and livestock production (Goñi-Urriza *et al.*, 2000a; Vila *et al.*, 2003; Henriques *et al.*, 2008).

The continuous case reports of health complications caused by *Aeromonas* sp. (Figueras, 2005) and the awareness that antibiotic resistance exists in these organisms prompted the establishment of consensus guidelines for testing aeromonads by the Clinical and Laboratory Standards Institute (CLSI, 2006). The document M-45A includes information and interpretive criteria for broth microdilution and disk diffusion susceptibility testing for *A. caviae, A. hydrophila, A. jandaei*, *A. schubertii,* and *A. veronii*, including the two biovars (CLSI, 2006).

In relation to species-specific profiles and taking into account the ampicillin susceptibility of *A. trota* specimens, antibiotic resistance patterns did not vary significantly between different *Aeromonas* species (Kämpfer *et al.*, 1999; Schmidt *et al.*, 2001b). Even though some studies indicate a species related variability in antibiotic susceptibility (Overman and Janda, 1998; Ko *et al.*, 1996), they were considered to be preliminary (Janda and Abbott, 2010). In fact, there are not extended studies focusing antibiotic resistance including all, or at least, the majority of *Aeromonas* species described. Instead, as mentioned before, surveys majorly rely on one or all of the three most common *Aeromonas*

phenospecies or do not assess isolates phylogenetic groups at all (Goñi-Urriza *et al.*, 2000a; Saavedra *et al.*, 2004; Palú *et al.*, 2006; Rahman *et al.*, 2009), precluding the establishment of such correlation.

Also, in general, there are no evident differences between profiles exhibited by clinical and non-clinical isolates (Kämpfer *et al.*, 1999; Jacobs and Chenia, 2007), although Palú and collaborators (2006) found that clinical strains have shown resistance to a wider variety of antimicrobial agents than food isolated specimens. Given the higher antibiotic selective pressure at which clinical isolates are exposed in relation to non-clinical isolates it would be expectable a higher level of antibiotic resistance, but further studies are needed on this subject. Actually, at least in relation to certain antibiotics, resistance rates seem to vary according to specific environmental selective pressures. In fact, aquaculture environments and their surroundings (Schmidt *et al.*, 2001b; Saavedra *et al.*, 2004; Akinbowale *et al.*, 2007b; Rahman *et al.*, 2009), waste water treatment plants (Moura *et al.*, 2007; Rahman *et al.*, 2009) and polluted water environments (Goñi-Urriza *et al.*, 2000a; 2000b; Henriques *et al.*, 2006a) seem to be a source of unusual or higher drug resistant aeromonads. It is evident the anthropogenic influence in the higher recovering of resistant aeromonads in these environments certainly due to the use of antibiotics in different human activities (Goñi-Urriza *et al.*, 2000a; Schmidt *et al.*, 2001b), which selects resistant strains harbouring antibiotic resistance genetic determinants able to disseminate throughout distinct niches. In reality, several genetic elements involved in *Aeromonas* sp. antibiotic resistance have been described.

1.4.1 Aeromonas antibiotic resistance mechanisms

1.4.1.1 Beta-lactams

The most popular mechanism of antibacterial resistance among aeromonads is the production of three chromosomally encoded beta-lactamases, which have been described and identified in different *Aeromonas* species. These enzymes are the main responsible for beta-lactam resistance among *Aeromonas*. They may be or not concomitantly present in the same strain and their coordinate expression is induced by the presence of beta-lactam antibiotics (Alksne and Rasmussen, 1997; Walsh *et al.*, 1997; Avison *et al.*, 2004). Although minor differences in relation to substrate specificities among distinct enzymes belonging to the same group exists, closely related molecules share similar biochemical

profiles and can be grouped in cephalosporinases (Bush-Jacoby group 1 or Ambler's class C), penicillinases/oxacillinases (Bush-Jacoby group 2d or Ambler's class D) and metallo-beta-lactamases (Bush-Jacoby group 3 or Ambler's class B) (Hayes *et al.*, 1994; Walsh *et al.*, 1995; 1997; Bush and Jacoby, 2010).

Distinct names have been given to each of these enzymes, generally in accordance to the species name from which it has been purified. The class C cephalosporinases (e.g., CepH, CepS, AsbA1, ASA-3, TRU-1) are related to the AmpC family of beta-lactamases and mainly hydrolyse narrow-spectrum cephalosporins, being resistant or less susceptible to beta-lactamase inhibitors than the OXA-type enzymes (Hayes *et al.*, 1994; Walsh *et al.*, 1995; Alksen and Rasmussen, 1997; Avison *et al.*, 2000; De Luca *et al.*, 2010). The family C-2 of plasmid borne AmpC beta-lactamases (CMY, FOX, MOX) found in many *Enterobacteriaceae* appears to derive from *Aeromonas* sp. chromosomal AmpC (Poole, 2004).

Class D beta-lactamases with penicillinase activity (e.g., AmpH, AmpS, AsbB1 or OXA-12, ASA-2) hydrolyse preferentially penicillins. Particularly in some cases, act mainly upon oxacillin or cloxacillin and are susceptible to beta-lactamase inhibitors as clavulanic acid (Hayes *et al.*, 1994; Walsh *et al.*, 1995; Alksen and Rasmussen, 1997; Avison *et al.*, 2000).

The aeromonads class B carbapenemases (e.g., CphA, ImiS, AsbM1, ASA-1) are specific carbapenem hydrolysing enzymes (especially imipenem) and as with other metallo-beta-lactamases (MBL) are resistant to serine beta-lactamases inhibitors being susceptible to metal ion chelators as EDTA (Hayes *et al.*, 1994; Alksen and Rasmussen, 1997; Walsh *et al.*, 1998; Avison *et al.*, 2000). One of the most problematic issues concerning these enzymes is the difficulty to routinely detect them *in vitro* (Janda and Abbott, 2010). Moreover, available commercial tests do not detect the aeromonads MBL (Balsalobre *et al.*, 2009b). In fact, *Aeromonas* strains carrying the *cphA* type gene frequently display the susceptible phenotype to imipenem *in vitro* conditions (Rossolini *et al.*, 1995; Walsh *et al.*, 1997; Balsalobre *et al.*, 2009b). It has been shown that the increase on the inoculum size reduces the susceptibility of MBL producing aeromonads to imipenem, indicating that *in vivo* conditions (higher microbial population) favour the development of resistance to that antimicrobial agent. In this way, from a clinical point of view, all *Aeromonas* sp. strains harbouring *cphA*-like genes should be considered

imipenem resistant (Rossolini *et al.*, 1995). It is not known why *cphA*-like harbouring aeromonads do not express the carbapenems resistant phenotype. As mentioned above, the expression of the genes encoding the three beta-lactamases is controlled by the same mechanism which is induced by beta-lactams (Walsh *et al.*, 1997). This coordinate expression involves a complex induction mechanism which is not fully understood (Alksen and Rasmusen, 1997; Avison *et al.*, 2004). Table 1.3 depicts the typical chromosomal beta-lactamases found in distinct *Aeromonas* species.

Table 1.3 Chromosomal beta-lactamases, according to Bush-Jacoby groups, usually found in *Aeromonas* sp.

Bush-Jacoby Group (2010)/Ambler's class	Name	Species	Reference
Group 1/Class C	CepH	*A. hydrophila*	Avison *et al.,* 2000
	CepS	*A. veronii* bv. sobria	Walsh *et al.,* 1995; 1998
	AsbA1	*A, jandaei*	Alksen and Rasmussen, 1997
	ASA-3	*A. salmonicida*	Hayes *et al.,* 1994
	TRU-1	*A. trota*	De Luca *et al.,* 2010
Group 2d/Class D	AmpH	*A. hydrophila*	Avison *et al.,* 2000
	AmpS	*A. veronii* bv. sobria	Walsh *et al.*, 1995; 1998
	AsbB1 (OXA-12)	*A, jandaei*	Alksen and Rasmussen, 1997
	ASA-2	*A. salmonicida*	Hayes *et al.,* 1994
Group 3a/Class B	CphA	*A. hydrophila*	Avison *et al.,* 2000
	ImiS	*A. veronii* bv. sobria	Walsh *et al.,* 1995; 1998
	AsbM1	*A, jandaei*	Alksen and Rasmussen, 1997
	ASA-1	*A. salmonicida*	Hayes *et al.,* 1994

All these *Aeromonas* chromosomal beta-lactamases exhibit a narrow hydrolytic spectrum of activity as each enzyme specifically acts upon a restrict group of beta-lactams, penicillins (penicillinases), 1st and 2nd generation cephalosporins (cephalosporinases) and carbapenems (carbapenemases) (Walsh *et al.*, 1997; Avison *et al.*, 2000). In this way, aeromonads have a potent armoury to evade beta-lactams activity which is induced by their presence, thus the use of such antibacterial agents in clinical settings should be avoided in the treatment of *Aeromonas* sp. infections (Goñi-Urriza *et al.*, 2000a; Koksal *et al.*, 2007).

All three types of beta-lactamases have been found and characterised from *A. hydrophila* (Avison *et al.*, 2000), *A. veronii* bv. sobria (Walsh *et al.*, 1995; 1998), *A. jandaei* (Alksne and Rasmussen, 1997) and *A. salmonicida* (Hayes *et al.*, 1994), but they can be found in other *Aeromonas* species (Janda and Abbott, 2010). Also, it has been suggested that there is a species related distribution of *cphA*-like genes since it has been majorly detected in *A. hydrophila*, *A. veronii*, *A. jandaei* but not or rarely in *A. caviae*,

A. trota and *A. schubertii* (Rossolini *et al.*, 1995; Walsh *et al.*, 1997). Additionally, the cephalosporinase TRU-1 from *A. trota* has been genetically and biochemically characterised being the sole beta-lactamase detected in different strains of this species, consistent with the unique susceptibility pattern of *A. trota* (De Luca *et al.*, 2010).

Other *bla* genes have been identified in *Aeromonas* species strains (Table 1.4). An *A. hydrophila* clinical isolate was found to harbour a $bla_{\text{AER-1}}$ coding for a carbecillin hydrolysing beta-lactamase, in a mobilisable genetic element in addition to other antibiotic resistance genes (ARG). It was probably chromosomally inserted and could be transferred and expressed in *E. coli* (Hedges *et al.*, 1985). Also, an *A. caviae* clinical isolate contained a bla_{TEM} closely related to $bla_{\text{TEM-12}}$ in terms of nucleotide sequence however no activity against ceftazidime was detected. It was located in a self-transferable plasmid which could be transferred by conjugation to *E. coli*. This *bla* gene was not expressed in the parental strain but it was so in the transconjugants (Sayeed *et al.*, 1996). Although rarely, extended-spectrum beta-lactamases (ESBLs) have also been detected in *Aeromonas* sp. Plasmid encoded $bla_{\text{TEM-24}}$ genes have been identified in one *A. caviae* and one *A. hydrophila* strain both collected in the clinical setting (Marchandin *et al.*, 2003; Fosse *et al.*, 2004). Also, an environmental *A. media* strain resistant to most beta-lactams except cephamycins and carbapenems was found to harbour a $bla_{\text{PER-1}}$ gene as part of a Tn*1213* composite transposon encoded in a conjugative plasmid, additionally containing aminoglycosides resistance genes (Picão *et al.*, 2008b).Very recently a new $bla_{\text{PER-6}}$ with low carbapenemase activity has been identified in an *A. allosaccharophila* riverine strain and located in the chromosome (Girlich *et al.*, 2010). Also, a new plasmid encoded AmpC beta-lactamase, MOX-4, was found in a conjugative plasmid of an *A. caviae* clinical isolate. This strain also harboured a CTX-M-3 ESBL and a TEM-1 (Ye *et al.*, in press).

Even though, *cphA*-like genes are the mostly detected MBL encoding genes, acquired MBL have been recently detected in *Aeromonas* sp. (Table 1.4). One *A. caviae* clinical strain was found to harbour a $bla_{\text{IMP-19}}$ inserted on a class 1 integron located on a plasmid which could not be transferred by conjugation. Interestingly, this MBL conferred reduced susceptibility to carbapenems, high resistance to cephalosporins and cefoxitin. Results of distinct susceptibility tests were not concordant in relation to imipenem resistance profile (Neuwirth *et al.*, 2007). Also, a $bla_{\text{VIM-4}}$ inserted on a class 1 integron was detected in an *A. hydrophila* clinical strain resistant to penicillins, cephalosporins and

imipenem. As with other aeromonads MBL, the detection of this enzyme was not achieved by using commercial Etest (Libisch *et al.*, 2008).

Table 1.4 Other beta-lactamases found in *Aeromonas* sp. and its corresponding Bush-Jacoby group.

Bush-Jacoby (2010) /Ambler's class	Name	Organism	Source	Genomic location	Reference
Group 1/Class C	MOX-4	*A. caviae*[b]	Clinical (human)	Conjugative plasmid	Ye *et al.,* in press
Group 1/Class C	CMY-2	*A. salmonicida*	Aquaculture	Conjugative plasmid	McIntosh *et al.*, 2008
Group 2/Class A	~TEM-12[a]	*A. caviae*	Clinical (human)	Conjugative plasmid	Sayeed *et al.,* 1996
Group 2b/Class A	TEM-1	*Aeromonas sp*	Estuary	ND[c]	Henriques *et al.*, 2006a
Group 2b/Class A	TEM-1	*A. caviae*[b]	Clinical (human)	Probably chromosomal	Ye *et al.*, in press
Group 2b/Class A	SHV-1	*A. media*	Estuary	ND	Henriques *et al.*, 2006a
Group 2be/Class A	TEM-24	*A. caviae*	Clinical (human)	Conjugative plasmid	Marchandin e*t al.,* 2003
Group 2be/Class A	TEM-24	*A. hydrophila*	Clinical (human)	Conjugative plasmid	Fosse *et al.,* 2004
Group 2be/Class A	PER-1	*A. media*	Activated sludge	Tn1213 composite transposon on conjugative plasmid	Picão *et al.*, 2008b
Group 2be/Class A	PER-3	*A. caviae*	NS	Complex class 1 integron	Integrall[e]
Group 2be/Class A	PER-6	*A. allosaccharophila*	River	Chromosome	Girlich *et al*., 2010
Group 2be/Class A	CTX-M-2	*A. hydrophila*	NS	NS	Radice *et al.,* 2002
Group 2be/Class A	CTX-M-3	*A. caviae*[b]	Clinical (human)	Probably chromosomal	Ye *et al.*, in press
Group 2c/Class A	AER-1	*A. hydrophila*	Clinical (human)	MGE probably inserted on chromosome	Hedges *et al.,* 1985
Group 2c/Class A	PSE-1	*A. veronii*	Aquaculture	Integron (class or other genomic location NS[d])	Jacobs and Chenia, 2007
Group 2c/Class A	PSE-1	*A. encheleia*	Aquaculture	Integron (class or other genomic location NS)	Jacobs and Chenia, 2007
Group 2c/Class A	PSE-1	*A. hydrophila*	Aquaculture	Integron (class or other genomic location NS)	Jacobs and Chenia, 2007
Group 2d/Class D	OXA-1	*A. allosaccharophila*	Lake	Class 1 integron (plasmid)	Picão *et al.*, 2008a
Group 2d/Class D	OXA-2	*Aeromonas sp.*	Estuary	Class 1 integron (other genomic location NS)	Henriques *et al.*, 2006a
Group 2d/Class D	OXA-2	*A. hydrophila*	Estuary; foodborne-outbreak	Class 1 integron (other genomic location NS)	Henriques *et al.,* 2006a; Chang *et al.*, 2007
Group 2d/Class D	OXA-2	*A. caviae*	Clinical (human)	Class 1 integron (probably chromosomal)	Pérez-Valdespino *et al.,* 2009
Group 2d/Class D	OXA-2	*A. salmonicida*	Aquaculture	Integron (class or other genomic location NS)	Jacobs and Chenia, 2007
Group 2d/Class D	OXA-21	*A. hydrophila*	Clinical (human)	Class 1 integron (other genomic location NS)	Lee *et al.*, 2008
Group 2d/Class D	OXA-10	*A. hydrophila*	Fish	Class 1 integron (other genomic location NS)	Integrall
Group 2d/Class D	OXA-10	*A. caviae*	NS	Class 1 integron (other genomic location NS)	Integrall
Group 2d/Class D	OXA-10	*A. veronii*	Cattle	Class 1 integron (other genomic location NS)	Barlow *et al.*, 2008
Group 3a/Class B	IMP-19	*A. caviae*	Clinical (human)	Class 1 integron in non-conjugative plasmid	Neurwith *et al.*, 2007
Group 3a/Class B	VIM-4	*A. hydrophila*	Clinical (human)	Class 1 integron (other genomic location NS)	Libisch *et al.*, 2008

Source of isolation of strains and genomic location of genes are also displayed.
[a] Closely related to bla_{TEM-12} but with a different spectrum of activity.
[b] The same *A. caviae* strain harbours the three beta-lactamases indicated.
[c] ND, not determined.
[d] NS, not specified.
[e] http://integrall.bio.ua.pt (last accessed in 6.05.2010)

Additionally, *bla*$_{OXA}$, *bla*$_{PER}$, *bla*$_{PSE-1}$, *bla*$_{CMY}$, other *bla*$_{TEM}$ and *bla*$_{SHV}$ genes have been detected in *Aeromonas* sp. (Table 1.4) collected from different sources in some cases as gene cassettes inserted on class 1 integrons (Henriques *et al.*, 2006a; Jacobs and Chenia, 2007; Barlow *et al.*, 2008; McIntosh *et al.*, 2008; Picão *et al.*, 2008a; Pérez-Valdespino *et al.*, 2009; Ye *et al.*, in press).

1.4.1.2 Tetracyclines

Tetracyclines tolerant aeromonads have been increasingly recovered from different environments probably owing to the extensive use of these antibiotics in human and veterinary medicine and agriculture (Akinbowale *et al.*, 2007a; Balassiano *et al.*, 2007). Indeed, *Aeromonas* species harbouring tetracycline resistance determinants have been recovered from clinical and food sources and from aquatic environments frequently related to strong anthropogenic pressure as the aquaculture environment, where the use of tetracyclines is extensive (Nawaz *et al.*, 2006; Balassiano *et al.*, 2007; Jacobs and Chenia, 2007; Henriques *et al.*, 2008).

The Tet determinants found among aeromonads include genes coding for efflux pumps [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(31) and *tet*(Y)], ribosomal protection proteins encoding genes [*tet*(M)] and genes coding for enzymatic inactivation [*tet*(34)] (http://faculty.washington.edu/marilynr/tetweb2.pdf; last accessed in 11.02.2010). *tet*(A) and *tet*(E) are the most common tetracycline resistance genes among strains (Agersø *et al.*, 2007; Balassiano *et al.*, 2007; Jacobs and Chenia, 2007; Henriques *et al.*, 2008). Nevertheless, Akinbowale and colleagues (2007a) found *tet*(M) and *tet*(E) to be the most frequently found Tet determinants among aeromonads from aquaculture sources and Nawaz *et al.* (2006) identified predominantly *tet*(E) and *tet*(B) within *A. veronii* fish isolates. More than one Tet determinant may be found in the same strain (Agersø *et al.*, 2007; Jacobs and Chenia, 2007; Balassiano *et al.*, 2007) but it does not appear to have any correlation with the resistance level of strains (Akinbowale *et al.*, 2007a). Several studies report the isolation of tetracyclines resistant *Aeromonas* sp. isolates not harbouring any of the well-recognised Tet determinants among the genus (Jacobs and Chenia, 2007; Balassiano *et al.*, 2007), indicating that other *tet* genes are involved in the antibiotic resistance and that surveys should explore the presence of less encountered determinants (Akinbowale *et al.*, 2007a). On the other hand, a problem may arise when strains

harbouring *tet* genes go undetected, if not scrutinised for their presence, by presenting a non-resistant phenotype. In fact, the determinants may be carried on mobile genetic elements (MGE) often carrying additional ARG and/or over-expressed when subjected to different selective pressures (Henriques *et al.*, 2008).

Actually, Tet determinants in *Aeromonas* sp. may be encoded in the chromosome (Balassiano *et al.*, 2007; Henriques *et al.*, 2008) or associated to plasmids and transposons (Schmidt *et al.*, 2001a; Rhodes *et al.*, 2004; Agersø *et al.*, 2007; Akinbowale *et al.*, 2007a; Balassiano *et al.*, 2007; Gordon *et al.*, 2008). Frequently these are conjugative elements carrying additional ARG which may be gene cassettes inserted on integrons that are concomitantly transferred to other specimens (Schmidt *et al.*, 2001a; Rhodes *et al.*, 2004; Agersø *et al.*, 2007; Akinbowale *et al.*, 2007a). Moreover, IncU tetracycline resistance plasmids from which many carry a *tet*(A) in a Tn*1721* like transposon and class 1 integrons with additional ARGs are common among *Aeromonas* species strains, especially *A. salmonicida* (Rhodes *et al.*, 2004). Recently a novel tetracycline repressor protein coding gene associated to a *tet*(Y) gene [*tetR*(Y)] has been found in a plasmid of an *A. bestiarum* strain collected from sediment of a river, additionally carrying florfenicol, streptomycin and sulphonamide resistance genes (Gordon *et al.*, 2008). Table 1.5 displays Tet determinants found in distinct *Aeromonas* species.

Table 1.5 Tetracycline resistance determinants found in distinct *Aeromonas* sp., genomic location of genes and source of isolation of strains.[a]

***tet* gene**	**Genomic location**	**Species**	**Source**
tet (A)	Transposons on conjugative plasmids (eg.: pFBAOT6), conjugative and non-conjugative plasmids, chromosome	*A. salmonicida, A. hydrophila, A. caviae, A. veronii, A. encheleia* and other non identified *Aeromonas* sp.	Aquaculture, estuary, hospital effluent, food products, human clinical sources
tet (B)	NS[b]	*A. hydrophila, A. encheleia, A. veronii*	Aquaculture
tet (C)	Mobilisable plasmid (eg.: pRAS3), conjugative and non-conjugative plamids, chromosome	*A. salmonicida, A. hydrophila, A. veronii*	Aquaculture, estuary
tet (D)	Conjugative and non-conjugative plasmids, chromosome	*A. hydrophila, A. veronii* and other non identified *Aeromonas* sp.	Aquaculture
tet (E)	Conjugative and non-conjugative plasmids, chromosome	*A. hydrophila,A. salmonicida, A. bestiarum, A. caviae, A. veronii, A. encheleia* and other non identified *Aeromonas* sp.	Aquaculture, food products, human clinical sources
tet (Y)	Non-conjugative plasmid	*A. bestiarum*	River sediments
tet (M)	Conjugative and non-conjugative plasmids, chromosome (probably)	*A. hydrophila, A. veronii* and other non identified *Aeromonas* sp.	Aquaculture
tet (31)	Conjugative plasmid (pRAS2)	*A. salmonicida*	NS
tet (34)	NS	NS	NS

[a] References: L'Abée-Lund and Sørum (2000; 2002); Schmidt *et al.* (2000; 2001b), Rhodes *et al.*, 2004; Nawaz *et al.*, 2006; Agersø *et al.*, 2007; Akinbowale *et al.*, 2007a; Balassiano *et al.*, 2007; Jacobs and Chenia, 2007; Gordon *et al.*, 2008; Henriques *et al.*, 2008; http://faculty.washington.edu/marilynr/tetweb2.pdf (last accessed in 11.02.2010).
[b] NS, not specified

1.4.1.3 Quinolones

Aeromonads are usually susceptible to quinolones which have been continuously pointed out as drugs of choice for treatment of *Aeromonas* sp. infections (Ko *et al.*, 1996; Koksal *et al.*, 2007; Alcaide *et al.*, 2010). However, growing resistance to these antibacterial agents, especially to first generation quinolones, has been reported (Goñi-Urriza *et al.*, 2000a; 2000b; Rahman *et al.*, 2009). This increase has been related to selective pressure of resistant isolates caused by the use of quinolones in human and veterinary medicine and their high persistence in the environment (Cattoir *et al.*, 2008; Alcaide *et al.*, 2010).

The identified mechanisms involved in quinolones resistance among aeromonads are either chromosomally or plasmid encoded. The former include mutations in the quinolones targets of DNA gyrase (*gyrA* gene) and/or of topoisomerase II (*parC* gene) and is the mechanism of quinolones resistance primarily found among aeromonads (Alcaide *et al.*, 2010). Changes in the quinolones resistance determining regions (QRDRs) of *gyrA* were determined to be the most common among *Aeromonas* species. Although double mutations in the two genes have been related to higher quinolones resistance, it was not always evident within aeromonads (Arias *et al.*, in press; Alcaide *et al.*, 2010). Also, the mutations detected on both genes were homogeneous regardless of origin and species affiliation as mutations in QRDRs of *gyrA* and *parC* genes of *A. hydrophila*, *A. sobria*, *A. caviae*, *A. salmonicida*, *A. media*, *A. veronii* and *A. popoffii* collected from human, water and fish were identical. Apart from QRDRs mutations, the overexpression of efflux pumps is another chromosomally encoded mechanism which has been mentioned to contribute to quinolones resistance among *Aeromonas* species, nevertheless results not always corroborate this involvement, pleading for further attention (Alcaide *et al.*, 2010).

Previously, plasmid-mediated quinolones resistance determinants, *qnr* genes, have only been reported in *Enterobacteriaceae* and in *Vibrionaceae*; nevertheless, they have been recently found in different *Aeromonas* species, recovered from river (Cattoir *et al.*, 2008), lake (Picão *et al.*, 2008a), and clinical environment (Sánchez-Cespedes *et al.*, 2008; Arias *et al.*, in press). In all cases, a *qnrS2* gene was identified and successfully transferred to *E. coli*, but only once by conjugation (Arias *et al.*, in press). The *A. allosaccharophila* plasmid harbouring a *qnrS2* gene also contained a class 1 integron with four resistance markers, namely, penicillins, aminoglycosides, chloramphenicol and rifampicin resistance

genes (Picão *et al.*, 2008a). The genetic context of these genes was very similar and the majority was part of a mobile insertion cassette element (a transposon-like structure) and/or inserted in an open reading frame coding for a zinc metalloprotease (Cattoir *et al.*, 2008; Picão *et al.*, 2008a; Sánchez-Cespedes *et al.*, 2008).

These findings demonstrate that *Aeromonas* species are important dissemination vehicles of plasmid borne quinolones resistance, previously confined to *Enterobacteriaceae*, in different environments, acting in some cases as silent reservoirs of these resistance markers (Picão *et al.*, 2008a).

1.4.2 Mobile genetic elements in aeromonads

From the exposed in this section and in section 1.3 of this chapter, it is notable the wide range of genetic structures present in *Aeromonas* species genomes prone to jump from cell to cell dragging a panoply of genetic determinants not only of antibiotic resistance, but also of virulence.

A striking aspect clearly differentiating *A. hydrophila* ATCC 7966 and *A. salmonicida* A449 genomes is the presence of five plasmids in the last and the presence of a variety of IS elements and other structures presumably acquired through horizontal gene transfer, which are absent from *A. hydrophila* ATCC 7966 genome (Seshadri *et al.*, 2006; Reith *et al.*, 2008). In fact, *A. salmonicida* A449 carries three cryptic and two large plasmids from which, one harbours the T3SS genes and the other contains a Tn*21* composite transposon that carries genes coding for resistance to mercury as well as a class 1 integron with streptomycin, quaternary ammonia compounds, sulphonamides and chloramphenicol resistance gene cassettes (Reith *et al.*, 2008). Additionally, a *tet*(A) gene is present on this plasmid. Also, numerous IS (88 IS) were found in *A. salmonicida* A449 which have contributed to the large numbers of pseudogenes (170 pseudogenes) present in this genome, in contrast to *A. hydrophila* ATCC 7966 which, as mentioned, does not harbour any IS and presents 7 pseudogenes (Seshadri *et al.*, 2006; Reith *et al.*, 2008).

1.4.2.1 Integrons

Integrons may carry and express an impressive array of ARGs and although not mobile themselves they are frequently associated to conjugative plasmids and other transposable elements (Moura *et al.*, 2007). Apart from beta-lactams, tetracyclines and

quinolones *Aeromonas* species present resistance to other antibiotics as aminoglycosides, sulphonamides, phenicols, trimethoprim and trimethoprim/sulfamethoxazole which is frequently encoded in gene cassettes present in integrons (Chang *et al.*, 2007; Barlow *et al.*, 2008; Lee *et al.*, 2008). Class 1 integrons are the most detected, whereas class 2 integrons have been rarely found and any other class of integrons was identified among aeromonads (Henriques *et al.*, 2006a; Moura *et al.*, 2007; Barlow *et al.*, 2008; Lee *et al.*, 2008). Even though, Jacobs and Chenia (2007) reported the detection of *intI3* gene among aeromonads collected from aquaculture environment, but no further characterisation of these putative integrons has been made.

Gene cassettes inserted in different arrangements on class 1 integrons were mainly *aadA* and *aacA* (aminoglycosides resistance genes), *dfrA* and *dfrB* (trimethoprim resistance genes), *catB* and *cml* (chloramphenicol resistance genes) but also bla_{OXA} and bla_{PSE} (beta-lactams resistance genes), *catA* (chloramphenicol resistance gene), *arr* (rifampicin resistance gene), and *ereA2* (erythromycin resistance gene), as well as open reading frames of unknown function, *orfC*, *orfF* and *orfD* (Henriques *et al.*, 2006a; Chang *et al.*, 2007; Jacobs and Chenia, 2007; Moura *et al.*, 2007; Barlow *et al.*, 2008; Lee *et al.*, 2008; Pérez-Valdespino *et al.*, 2009). Also, as mentioned, the unique $bla_{\text{IMP-19}}$ and $bla_{\text{VIM-4}}$ MBL found in *Aeromonas* sp. were gene cassettes inserted in class 1 integrons additionally carrying an *aacA4* gene (Neuwirth *et al.*, 2007; Libisch *et al.*, 2008). These site-specific recombination structures were present in different *Aeromonas* species as *A. hydrophila*, *A. veronii*, *A. caviae*, *A. bestiarum*, *A. salmonicida*, *A. encheleia* and *A. allosaccharophila* collected from estuarine and lake waters (Henriques *et al.*, 2006a; Picão *et al.*, 2008a), wastewater treatment plants (Moura *et al.*, 2007), aquaculture environments and fish (Schmidt *et al.*, 2001a; 2001b; Jacobs and Chenia, 2007), food and animals for food production (Chang *et al.*, 2007; Barlow *et al.*, 2004; 2008) and from clinical settings (Lee *et al.*, 2008; Pérez-Valdespino *et al.*, 2009). Class 2 integrons with cassette arrays *estx-sat2-aadA1* (Moura *et al.*, 2007) and *dfrA1-sat2-aadA1* (Barlow *et al.*, 2008) were found in *Aeromonas* sp. strains and any other characterised class 2 integron of *Aeromonas* sp. was found.

In spite of gene cassettes found not always explain the phenotype exhibited by strains, possibly because resistance mechanisms were encoded in other genetic structures or by weak expression of gene cassettes (Moura *et al.*, 2007; Pérez-Valdespino *et al.*, 2009), for several times the resistance phenotypes of aeromonads reflect the presence of

these genetic elements and multiresistance profiles are common among *Aeromonas* species carrying integrons (Schmidt *et al.*, 2001b; Moura *et al.*, 2007; Chang *et al.*, 2007; Pérez-Valdespino *et al.*, 2009). Moreover, Chang and colleagues (2007) found that the frequency of resistance to the majority of antibiotics tested was significantly higher among integron positive isolates compared to integron negative isolates and multiresistance profiles were more common among the former.

In this way, integrons have an important counterpart in *Aeromonas* sp. antibiotic resistance and in their role as reservoirs of ARG (Chang *et al.*, 2007; Lee *et al.*, 2008). The considerable frequent location of integrons on conjugative or mobilisable plasmids in aeromonads extends this role and implies them as agents of transfer and dissemination of resistance determinants among different bacterial populations in distinct environments (Schmidt *et al.*, 2001a; 2001b; Moura *et al.*, 2007). Table 1.6 depicts integrons and gene cassette arrangements found in *Aeromonas* species.

1.4.2.2 Resistance plasmids

From the exposed above it is obvious the frequent occurrence of plasmids mediating antibiotic resistance, commonly to several drugs, among *Aeromonas* species. Distinct IncU and IncA/C type plasmids and others, whose incompatibility group was not specified, have been found and their genetic content has been characterised (Rhodes *et al.*, 2004; Gordon *et al.*, 2008; McIntosh *et al.*, 2008; Fricke *et al.*, 2009).

The IncU type plasmids mentioned before are very common among *A. salmonicida* strains and distribute through different environments and hosts. In aeromonads they are closely related, sharing a common backbone. The ARG integrated as gene cassettes in class 1 integrons associated to these elements may vary and include *aadA1*, *aadA2*, *sul1*, *sul2*, *dfrA16*, *dfrB3* and *catA2* genes. The tetracycline resistance IncU plasmid pFBAOT6, from an *A. caviae* isolated from hospital effluent has been completely sequenced and its genetic load encompasses a complete class 1 integron, carrying an *aadA2* gene cassette, and transposable elements which may constitute a novel Tn*1721* flanked composite transposon harbouring a *tet*(A) gene (Rhodes *et al.*, 2004). It was shown that the aeromonads IncU type plasmids are highly promiscuous as they could be conjugatively transferred between distinct bacterial organisms (L'Abée-Lund and Sørum, 2002; Bello-López *et al.*, 2010).

Table 1.6 Gene cassettes arrays present in integrons found in distinct *Aeromonas* sp.[a]

intI	Cassette array	Genomic Location	Species	Source of isolation
I	*aac(6')-Ib-cr-bla*$_{OXA-1}$*-catB3-arr-3*	Plasmid	*A. allosaccharophila*	Lake
I	*aacA3-bla*$_{OXA-21}$*-catB3*	NS/ND[c]	*A. hydrophila, A. veronii*	Clinical (human)
I	*aacA4-bla*$_{VIM-4}$	NS/ND	*A. hydrophila*	Clinical (human)
I	*aadA1*	Chromosomal, conjugative plasmid, other NS/ND	*A. hydrophila, A. veronii, A. caviae, A. salmonicida, A. media, Aeromonas* sp.	WWTP[d], cattle, aquaculture, estuary, foodborne-outbreak, clinical (human)
I	*aadA1/aacA4-nit1-catB3-qacEΔ1-sul1- ISCR1-bla*$_{PER-3}$[b]	NS/ND	*A. caviae*	NS
I	*aadA1-aacA3*	NS/ND	*A. hydrophila*	Clinical (human)
I	*aadA1-bla*$_{OXA-2}$	NS/ND	*A. hydrophila*	Estuary
I	*aadA2*	Chromosomal, conjugative plasmid (pFBAOT6), other NS/ND	*A. hydrophila, A. veronii, A. caviae, A. salmonicida, A. media, Aeromonas* sp.	WWTP, cattle, aquaculture, estuary, clinical (human), hospital effluent
I	*aadA2-cmlA1-aadA1*	NS/ND	*A. sobria*	Clinical (human)
I	*aadA2-qacEΔ1-sul1-ISCR1-catA2-qacEΔ1-sul1-orf5-orf6*[b]	Conjugative plasmids (pRA3; pAr-32)	*A. hydrophila, A. salmonicida*	Fish
I	*aadA4a*	NS/ND	*A. hydrophila*	Clinical (human)
I	*aadA7*	Conjugative plasmid (pSN254)	*A. salmonicida*	Aquaculture
I	*arr-2-aacA4-drfA1-orfC*	NS/ND	*A. hydrophila*	Clinical (human)
I	*arr-2-dfrA1-orfC*	NS/ND	*A. caviae*	NS
I	*arr-3-aacA4*	Conjugative plasmid	*A. hydrophila*	Clinical (human)
I	*arr-3-aacA4-bla*$_{OXA-10}$*-aadA1*	NS/ND	*A. hydrophila*	Fish
I	*arr-3-aacA4-dfrA1*	NS/ND	*A. hydrophila*	Clinical (human)
I	*bla*$_{OXA-2}$*-aadA1-bla*$_{OXA-2}$*-orfD*	NS/ND	*A. caviae*	Clinical (human)
I	*bla*$_{OXA-2}$*-orfD*	NS/ND	*A. hydrophila, Aeromonas sp.*	Estuary, foodborne-outbreak
I	*bla*$_{OXA-10}$*-aacA4-aadA1*	NS/ND	*A. caviae*	NS
I	*catB3-aadA1*	NS/ND	*A. hydrophila, A. veronii, A. caviae, A. sobria*	Poultry, Clinical (human)
I	*catB3-aadA2*	NS/ND	*A. hydrophila*	Fish
I	*catB8-aadA1*	NS/ND	*A. hydrophila, A. caviae, A. sobria*	Cattle, estuary, foodborne-outbreak, clinical (human)

Table1.6 Gene cassettes arrays present in integrons found in distinct *Aeromonas* sp.[a] (continued).

intI	Cassette array	Genomic Location	Species	Source of isolation
I	*catB8-aadA17*	NS/ND	*A. media*	WWTP
I	*cmlA5-bla*$_{OXA-10}$*-aadA1*	NS/ND	*A. veronii*	Cattle
I	*dfrA1-aadA1*	Chromosomal, conjugative plasmid, other NS/ND	*A. hydrophila, A. salmonicida, A. sobria , A. bestiarum, A. allosaccharophila, Aeromonas* sp.	WWTP, aquaculture, fish
I	*dfrA1-aadA10*	NS/ND	*A. hydrophila*	NS
I	*dfrA1-aadA1-catB2*	Conjugative plasmid, other NS/ND	*A. hydrophila*	Aquaculture
I	*dfrA1-aadA1-orfF-catB2*	Conjugative plasmid, other NS/ND	*A. hydrophila, A. veronii, A. bestiarum*	Aquaculture
I	*dfrA1-cmlA5*	NS/ND	*A. caviae*	NS
I	*dfrA5-aacA4-catB3*	NS/ND	*A. sobria*	Clinical (human)
I	*dfrA5-ereA2*	Non-conjugative plasmid	*A. veronii*	Clinical (human)
I	*dfrA12*	NS/ND	*A. media*	Estuary
I	*dfrA12-aadA2*	Plasmid, other NS/ND	*A. hydrophila, A. caviae*	Estuary, clinical (human)
I	*dfrA12-orfF*	NS/ND	*A. hydrophila*	Fish
I	*dfrA12-orfF-aadA2*	NS/ND	*A. hydrophila, A. veronii, A. caviae, A. media*	WWTP, foodborne-outbreak, clinical (human), aquatic animal, ornamental fish carriage water Animal faeces
I	*dfrA12-orfF-aadA2*(*-qacΔE-sul1-orf5Δ178-tnpA-mphR-mrx-mphA*)	Tn21-like transposon (probably in a plasmid)	*A. hydrophila*	
I	*dfrA12-tnp-aadA2*	NS/ND	*A. hydrophila*	Fish
I	*dfrA14-aadA6/aadA10*	NS/ND	*A. hydrophila*	Fish
I	*dfrA15-cmlA4-aadA2*	NS/ND	*A. caviae*	Clinical (human)
I	*dfrA16*	Conjugative plasmids (e.g. pRAS1), other NS/ND	*A. salmonicida*	Aquaculture, fish, other NS
I	*dfrA17*	NS/ND	*A. sobria*	Clinical (human)
I	*dfrA28-orfV*	NS/ND	*A. hydrophila*	Fish
I	*dfrB1-aadA1*	Conjugative plasmid	*A. hydrophila*	Aquaculture
I	*dfrB1-aadA1-catB2*	NS/ND	*A. sobria*	Fish
I	*dfrB3*	Conjugative plasmid, other NS/ND	*A. salmonicida*	Aquaculture, other NS

Table 1.6 Gene cassettes arrays present in integrons found in distinct *Aeromonas* sp.[a] (continued).

intI	Cassette array	Genomic Location	Species	Source of isolation
I	*dfrB3-aadA1*	NS/ND	*A. sobria*	Fish
I	*dfrB4-catB3-aadA1*	NS/ND	*A. hydrophila, A. veronii, A. sobria, A. encheleia*	Cattle, foodborne-outbreak, clinical (human), fish
I	*IS1066*	NS/ND	*A. caviae*	Cattle
I	*orf-aadA5*	NS/ND	*A. hydrophila*	Fish
I	*orf-cat-aadA1*	Plasmid (pAsa4)	*A. salmonicida*	Fish
I	*qacE2-orfD*	NS/ND	*A. hydrophila, A. veronii, A. caviae, A. salmonicida*	Aquatic animal, foodborne-outbreak, other NS
I	*tnpA-aacA4-bla*$_{IMP-19}$	Non-conjugative plasmid	*A. caviae*	Clinical (human)
II	*estX-sat2-aadA1*	Chromosomal	*Aeromonas sp.*	WWTP
II	*dfrA1-sat2-aadA1*	NS/ND	*A. veronii*	Cattle

When available, genomic location of integrons and source of isolation of specimens are indicated.
[a] Based on information collected from: Schmidt *et al.* (2001a; 2001b); Barlow *et al.* (2004; 2008); Rhodes *et al.*, 2004; Henriques *et al.*, 2006a; Poole *et al.*, 2006; Chang *et al.*, 2007; Moura *et al.*, 2007; Neurwith *et al.*, 2007; Lee *et al.*, 2008; Libisch *et al.*, 2008; McIntosh *et al.*, 2008; Picão *et al.*, 2008a; Pérez-Valdespino *et al.*, 2009; Integrall [http://integrall.bio.ua.pt (last accessed in 6.05.2010)].
[b] Complex class 1 integron.
[c] NS/ND, not specified or not determined by authors.
[d] WWTP, wastewater treatment plant.

Moreover, *in vivo* transfer of pRAS1 from *A. salmonicida* to *A. hydrophila* in infected fish has been accomplished indicating that successful plasmid transfer between these two important fish pathogens can occur in natural conditions (Bello-López *et al.*, 2010).

The conjugative plasmid pRA1 of *A. hydrophila* is the reference plasmid of the IncA/C type plasmid family. Due to the ability of these plasmids to spread across distinct taxonomic groups recovered from environmental and clinical sources, they are regarded as a considerable health threat. These plasmids have a type IV secretion like conjugative transfer system. Comparing to other IncA/C plasmids, pRA1 has a restricted resistance spectrum limited to sulphonamides and tetracycline. It has been suggested that this finding reflects a possible earlier stage of multidrug resistance plasmid evolution, since it has been recovered in 1971 (Fricke *et al.*, 2009).

In contrast, the IncA/C plasmid of *A. salmonicida* strains from aquaculture was found to confer resistance to ampicillin, florfenicol, oxytetracycline, streptomycin, sulphonamides and $HgCl_2$ (McIntosh *et al.*, 2008). Sequence analysis revealed the presence of a bla_{CMY-2} (ESBL), a class 1 integron with an *aadA7* gene cassette, an array of *floR*, *tetA/tetR, sul2 and strA/strB* genes and an entire *mer* operon coding for resistance to mercury. Additionally, a multidrug efflux pump coding gene (*sugE*) was identified. The conjugative transfer of this plasmid to distinct bacterial species was successful. Furthermore, it was noted that the mercury resistance, as with the remaining ARGs, could be used as a selective marker for this plasmid (McIntosh *et al.*, 2008).

The association of heavy-metal and antibiotic resistance in the same plasmid is recurrent and the concomitant presence of distinct resistance markers co-selects for both types of resistance. In this way, polluted environments with high heavy-metal concentrations are important hot spots for indirect selection and spread of ARG (McIntosh *et al.*, 2008; Zhang *et al.*, 2009). Actually, Huddleston and colleagues (2006) found arsenite (metalloid) and mercury resistant *Aeromonas* sp. and located this resistance on plasmids, from which the one harbouring mercury resistance was conjugative.

Whilst some of the *Aeromonas* sp. plasmids are conjugative, some, as the pRAS3 of *A. salmonicida*, are not capable of self-transmission, nevertheless can be mobilised with conjugative plasmids co-existing in the same strain (L'Abée-Lund and Sørum, 2002).

Structures like integrons, transposons, insertion sequences (IS) and, although less frequently reported, insertion sequence common region (IS*CR*) which act to assemble and re-assort ARGs on bacterial plasmids are present in aeromonads R-plasmids, conferring these MGE the ability of accumulating transferable multiple ARG and participate in the flow of DNA transfer between bacteria of distinct ecological niches (L'Abée-Lund and Sørum, 2002; Rhodes *et al.*, 2004; Gordon *et al.*, 2008; McIntosh *et al.*, 2008). Moreover, genes that are not commonly found in *Aeromonas* sp. and sometimes only detected in certain bacterial species, as the mentioned above *floR* (Gordon *et al.*, 2008) and *qnrS2* genes (Picão *et al.*, 2008a), are encoded on these MGE further demonstrating the power plasmids have to accomplish and perpetuate ARG mobility in nature.

1.4.2.3 Virulence plasmids

The role of plasmids in *Aeromonas* species pathogenicity has been poorly scrutinised. The best known form of plasmid mediated virulence in aeromonads is the secretion of toxic effectors by means of T3SS machinery encoded in *A. salmonicida* strains plasmids (Stuber *et al.*, 2003a; Reith *et al.*, 2008). Recently, Majumdar and colleagues (2009) reported the presence of a 21kb plasmid in *A. hydrophila* which plays a role in macrophage apoptosis and systemic spread of the specimen in mice. Nevertheless, the genetic bases of the factors responsible for the observed cytotoxicity were not investigated as neither if the plasmid is itself a cytotoxic factor or has a regulatory role in cytotoxic factors release.

In resume, *Aeromonas* species act as reservoirs and disseminators of antibiotic resistance and potentially of other harmful traits in distinct environments, as, as mentioned, they are widely distributed in different ecological niches. Given the fact that acquisition of *Aeromonas* related diseases is considered to be primarily due to exposure to contaminated water, soil and food, environmental aeromonads harbouring ARG are not only putative transmission vehicles of such determinants to pathogenic bacteria but are also potential sources of severe clinical complications and treatment failures.

2. Scope of this thesis

2.1 The use of untreated water in Portugal

Water is an essential good for the well being of all living organisms. Waterborne infections caused by pathogens are very common worldwide and they are especially prevalent in non-developed areas (Ashbolt, 2004). Actually, in rural areas, the water supply systems are subjected to different quality adulterants as faecal contaminants, pesticides and other chemical pollutants (Ashbolt, 2004; Badach *et al.*, 2007). The role of water as a contamination route is well known, so it is of paramount importance the supplying of safe drinking water to all (WHO, 2006).

Portugal has an historical use of untreated water for several human activities that include direct or indirect consumption, agriculture, livestock farming and recreational activities. In fact, mostly in rural areas, water is frequently obtained from particular springs or underground aquifers, by means of wells, drilled wells, fountains, etc. Representing powerful niches for microorganisms, parasites and human health harmful substances, these waters are not usually subjected to regular monitoring of their quality.

The Decree-Law 243/2001 (D.L. 243/2001 from September 5) regulates the quality of water for human consumption in Portugal, transposing the Drinking Water Directive of the European Union, Council Directive 98/83/EC, into national law. The objective is to "protect human health from the adverse effects of any contamination of water intended for human consumption by ensuring that it is wholesome and clean" (C.D. 98/83/EC; D.L. 243/2001). It is noteworthy that they do not apply to natural mineral waters and waters that have medicinal use, which have their own regulations. One of the exemptions permitted refers to "water intended for human consumption from an individual supply providing less than 10 m^3 a day as an average or serving fewer than 50 persons, unless the water is supplied as part of a commercial or public activity" (C.D. 98/83/EC; D.L. 243/2001). The obligatory quality standards to ensure that the water intended for human consumption is wholesome and clean are set out in this decree-law annex I and include rigorous limits on the concentrations of microorganisms and chemicals permitted. The regulation does not include standards on *Aeromonas* to assess water quality, despite the potential health hazard that these microorganisms represent.

In 2005, the European Court of Justice ruled that the drinking water in Portugal did not respect the directive mentioned above, since it failed to acquiesce with several

microbiological and chemical parameters. In 2008, Portugal received from the European Commission a final warning in drinking water, since the latest monitoring report showed that drinking water in many areas of Portugal still failed to fulfil the microbiological limits set out in the Court's judgement. It was considered unacceptable that Portugal had not yet guaranteed the safety of drinking water throughout the country (European Commission [updated 2008]).

Thus, regarding the overall information analysed above, one may speculate on two issues, i) in the case of particular and not monitored waters frequently used for human direct consumption, bath, irrigation in agriculture and other human activities, the risk of consumption of deteriorated water is very high; ii) the presence of putative pathogens as *Aeromonas* are known to be, is not assessed and is allowed.

2.2 The Portuguese smallholding farms

As in all industrialised countries, the importance of agriculture in Portugal has been decreasing in the last years, although it remains high comparing to the average values registered in the Economic Union (COM (2003) 359/F).

The agricultural activity in Portugal is characterised for being mainly managed by families with smallholding farms. In 2005, autonomous single producers which use exclusively or uniquely family labour force on their agricultural holdings, constituted more than 90% of the producers in agriculture. Also, ¾ of the agricultural holdings had less than 5 hectares corresponding to only 11.4% of the utilised agricultural area (INE, 2007).

Fresh produce is considered a possible source of foodborne outbreaks caused by several pathogens. It can become contaminated in any point of the food chain production, but the first period of contamination is the field. In fact, the likely sources of contamination during initial processing are contamination from animals (manure and wild animal faeces), farm workers, spraying fungicides and herbicides and irrigation water. This contamination may be on and in the plants, i.e., the pathogens may survive in the leaf surface but may also reach the interior of the plants (Lynch *et al*., 2009). In less favoured areas, where farm holdings are not specialised and rudimental techniques are used, the probability of the cultures to become and remain contaminated with pathogens is high.

The rural areas where the agricultural related samples scrutinised on this study were collected, mirror the agricultural activity in Portugal. In fact, small agricultural holdings belonging to autonomous single producers, that together with their families manage the business, were selected. The majority of the products cultivated are for self-consumption or for selling in small village markets but also to bigger retailers. Commonly, the use of natural fertilisers is adopted and the water used in the agricultural and livestock activities originates from private aquifers or springs that, as mentioned above, are not treated nor regularly examined for their quality.

2.3 Aims of this dissertation

Why studying *Aeromonas*? Aeromonads are distributed among manifold environments with very different characteristics denoting the complexity and functional capacity of the genus. In fact, this genus is characterised by high diversity not only at the interspecies but also at the intraspecies level. Moreover, these microorganisms harbour genetic and physiological traits (antimicrobial resistance and pathogenicity, to name but a few) that, for example, enable them to survive under selective pressures.

Over the last years, the scientific literature has been invaded by studies, reports and reviews regarding the genus *Aeromonas*. These studies enclose a wide range of areas that involve occurrence among several environments, phylogenetic and taxonomic diversity and functional variety, including different features of their role in animal and human infections, antibiotic resistance and complex physiological relationships. Besides the interest on global microbial diversity, this concernment arises from the ancient knowledge of the *Aeromonas* spp. risk to human and animal health.

Although few studies reporting the occurrence, genetic traits and antibiotic resistance of aeromonads from different environments in Portugal exist (Saavedra *et al.*, 2004; Tacão *et al.*, 2005a; 2005b; Henriques *et al.*, 2006a; Moura *et al.*, 2007; Fontes *et al.*, 2010), no specific study focusing phylogenetic and functional diversity of *Aeromonas* species collected from water and agricultural sources in this country was published.

Taking into account the information compiled in this dissertation introduction and items 2.1 and 2.2 of this chapter, the diversified study here presented intended to establish a well characterised *Aeromonas* collection from different sources directly or indirectly for

human and/or animal use in Portugal in order to contribute to the overall knowledge on these organisms but also to shed a light on the aeromonads communities in Portugal. Additionally, the information extracted from this study may stimulate the awareness on the ecological, human and animal negative impact that *Aeromonas* spp. presence putatively represents.

Regarding each chapter within this study, specific objectives were:

- Chapter 3 – assess the genetic and phylogenetic diversity of *Aeromonas* species among untreated waters from different sources in several regions from Portugal, by RAPD fingerprinting and *gyrB* sequencing analysis.
- Chapter 4 – determine *Aeromonas* species genetic and phylogenetic diversity in agricultural correlated sources (irrigation, drinking and cleaning water, produce, faeces and raw milk) from various farms and assess the possibility of water being the contamination route between those sources. RAPD analysis and *gyrB* gene sequencing methods were applied.
- Chapter 5 – establish the antibiotic susceptibility patterns and appraise the genetic bases of antimicrobial resistance among the bacterial strains, including screening for the presence of genes encoding antibiotic resistance (*bla* and *tet* genes) as well as the occurrence and characterisation of class 1, class 2 and class 3 integrons.
- Chapter 6 – prospect for lipolytic and proteolytic extracellular activities and screen for the presence of genes coding for putative virulence factors as type III secretion system (T3SS), aerolysin-like and lipase proteins among *Aeromonas* spp. strains.
- Chapter 7 – characterise genetically and phenotypically the three different phylogenetic groups of isolates found that are distinct from the *Aeromonas* species described until now.

3. Occurrence and phylogenetic diversity of *Aeromonas* spp. in untreated water sources

Abstract

Aeromonads are known to be ubiquitous in several habitats, mainly in aquatic environments. In this study, mineral waters and untreated waters from fountains, wells, drilled wells and mines, used by the surrounding populations, were collected in different regions of Portugal, in order to assess *Aeromonas* spp. occurrence and phylogenetic diversity. Molecular typing of the isolates was performed by using random amplification of polymorphic DNA (RAPD) and 80 out of 206 isolates exhibited different fingerprints. There was remarkable genotypic diversity, since distantly related patterns were recovered. Additionally, strains with the same RAPD profile were present in several sampling sites. Also, strains recovered from the same sampling site at different times displayed similar RAPD profiles and different ones. On the basis of *gyrB* sequence alignments it was observed that the strains clustered in 14 phylogenetic groups with 68 *gyrB* gene different sequences: *A. hydrophila, A. bestiarum*, *A. media*, *A. tecta*, *A. veronii*, *A. salmonicida*, *A. eucrenophila, A. caviae, A.* sp. HG11*, A. popoffii, A. encheleia* and *A. allosaccharophila* and a group of 6 isolates displayed molecular variants of the *gyr*B gene significantly different from all known *Aeromonas* spp., indicating that they may constitute representatives of two non-described species.

Keywords: *Aeromonas* spp.; untreated water; diversity; phylogeny

3.1 Introduction

Aeromonas species are motile or non-motile, psychrophilic and mesophilic gram-negative rods that form a monophyletic group in the γ-3 subgroup of the class Proteobacteria, the so-called *Aeromonadaceae* Family (Martínez-Murcia *et al.*, 1992a; Martin-Carnahan and Joseph, 2005).

Until February 2010, the genus comprises the following species, including one unnamed DNA homology group (HG): *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (biovars sobria and veronii), *A. jandaei*, *A.* sp. HG11, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia*, *A. popoffii* (Martin-Carnahan and Joseph, 2005) *A. molluscorum* (Miñana-Galbis *et al*., 2004), *A. simiae* (Harf-Monteil *et al.*, 2004), *A. bivalvium* (Miñana-Galbis *et al*., 2007), *A. aquariorum* (Martínez-Murcia *et al*., 2008), *A. tecta* (Demarta *et al.*, 2008), *A. fluvialis* (Alperi *et al.*, 2010), *A. piscicola* (Beaz-Hidalgo *et al.*, 2009), *A. taiwanensis*, *A. sanarellii* (Alperi *et al.*, in press) and *A. diversa* (formerly Enteric group 501) (Miñana-Galbis *et al.*, 2010a). *A. ichthiosmia*, *A. enteropelogenes* and *A. culicicola* were found to be synonyms of *A. veronii*, *A. trota* and *A. veronii*, respectively (Demarta *et al*., 2008; Alperi *et al.*, in press) and according to Martínez-Murcia and co-workers (2007), the proposed species

A. sharmana is not a member of the genus. The taxonomy of the genus is a topic of continual change and controversy (Tacão *et al.*, 2005b; Saavedra *et al.*, 2006; Alperi *et al.*, 2010). In fact, new species are often described and reclassification and extended description of the existing ones is frequent (Tacão *et al.*, 2005a; Saavedra *et al.*, 2006; Demarta *et al.*, 2008). In order to assess *Aeromonas* genotypic diversity several molecular fingerprinting methods were established and applied (Borchardt *et al.*, 2003; Szczuka and Kaznowski, 2004; Tacão *et al.*, 2005a; 2005b) and numerous sequence-based phylogenetic analysis, targeting several different genes, were performed (Martínez-Murcia *et al.*, 1992a; Yánez *et al.*, 2003; Soler *et al.*, 2004; Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Miñana-Galbis *et al.*, 2009; Alperi *et al.*, in press). The housekeeping gene *gyrB*, which encodes the B-subunit of DNA gyrase, proved to be an excellent molecular marker for phylogenetic inference in this genus (Yánez *et al.*, 2003; Soler *et al.,* 2004; Küpfer *et al.*, 2006).

Widespread on various habitats, *Aeromonas* have been isolated from soil, food, patient and healthy humans, animals (Figueras, 2005; Martínez-Murcia *et al.*, 2008) and mainly from aquatic environments (Kühn *et al.*, 1997; Borrell *et al.*, 1998; Ghenghesh *et al.*, 2001; Massa *et al.*, 2001; Di Bari *et al.*, 2007). Specifically, several studies reported the occurrence, prevalence and large diversity of these organisms in different treated or untreated water sources, enhancing the potential health hazard that the presence of such microorganisms in water used for human activities may represent (Kühn *et al.*, 1997; Legnani *et al.*, 1998; Massa *et al.*, 2001; Di Bari *et al.*, 2007; Koksal *et al.*, 2007). In fact, *Aeromonas* species display resistance to diverse groups of antibiotics by means of different mechanisms (Schmidt *et al.*, 2001a; Saavedra *et al.* 2004; Jacobs and Chenia, 2007; Koksal *et al.*, 2007). Moreover, a number of genetic determinants of virulence were described in the genus (Kühn *et al.*, 1997; Chopra *et al.*, 2009), being putatively responsible for a panoply of animal diseases (including skin lesions and septicaemia) and localized and systemic infections in humans (including gastroenteritis, septicaemia, meningitis, wound infections, ocular infections, respiratory tract diseases) (Figueras, 2005; von Graevenitz, 2007; Martínez-Murcia *et al.*, 2008). Exposure to contaminated water, food and soil are the main risks of acquiring *Aeromonas* associated infections (Figueras, 2005). Actually, aeromonads were the most recovered agents from skin and soft-tissue infections among tsunami survivors in Southern Thailand and most of these people had been exposed to the floodwaters (Hiransuthikul *et al.*, 2005). However, the role of contaminated drinking water

consumption in *Aeromonas* spp. acquired infections remains unclear since close genetic relatedness of clinical and environmental *Aeromonas* isolates was not undoubtedly described (von Graevenitz, 2007; Janda and Abbott, 2010). *A. hydrophila* was included in the Contaminant Candidate Lists of organisms which require future regulation under the Safe Drinking Water Act of the United States Environmental Protection Agency (EPA, 1998; 2005) and standards on *Aeromonas* to assess water quality were implemented in some countries (Gavriel *et al.*, 1998; Legnani *et al.*, 1998; Massa *et al.*, 2001). Nevertheless, in spite of the increasing number of clinical cases associating *Aeromonas* spp. to complications in human health, limited awareness exists about the severe infections they may generate (Figueras, 2005).

Investigations on occurrence and diversity of members of this genus are important in order to know which are the sources and routes of transmission to understand the significance of waters of public use as reservoirs of aeromonads.

In Portugal there is still a large number of people using untreated water for direct consumption and other human activities. In the present study, and to our knowledge for the first time, *Aeromonas* occurrence and diversity in untreated water (UnW) samples from different sources in several regions from Portugal were investigated.

3.2. Material and Methods

3.2.1 Sampling

Untreated drinking water (DW) samples were collected in autoclaved bottles, from fountains, wells, drilled wells and mines, between June and December of 2004, May, June, August and October of 2005 and January of 2007. A total of 93 samples were obtained. Sampling sites were located in North (Districts of Viana do Castelo, Vila Real, Oporto), Centre [Districts of Aveiro, Lisbon, Viseu (Vouzela), Leiria (Pombal)] and South [District of Faro (Lagos)] of Portugal (Fig. 3.1). In some cases, samples were recovered at the same sampling site at different times (Vila Real, sampling sites 47, 48 and 52 in December 2004, July 2005 and January 2007; Lagos, sampling sites 43, 44 and 45 in December 2004 and May 2005). Mineral waters (MW) were collected from two water springs in the Centre. Two samples were collected in the same water spring (WS1 sampling sites 1 and 2) and the other in a different one (WS2 sampling site 3). Samples were examined within 5 h.

Figure 3.1 Map of continental Portugal with sampling districts and municipalities (indicated by arrows).

Aeromonads were isolated by the membrane filter technique. Briefly, 100 ml of each sample were filtered through 0.45 µm pore size cellulose ester filter (Pall Life Sciences, USA), membranes were placed in glutamate starch phenol-red agar (GSP) (Merck, Germany), supplemented with 50 µg/ml of ampicillin (Merck, Germany) and plates were incubated at 30 ºC overnight. Yellow colonies in GSP medium were selected and pure cultures were obtained by repeated isolation of individual colonies in the same medium. Pure cultures were stored in 17% glycerol at -80ºC.

3.2.2 DNA extraction

A single colony was resuspended in 100 µl TE buffer and 200 µl chelex 20% (Bio-Rad, USA) were added; the mixture was vortexed. Cycles of 10 min at 95 ºC followed by 10 min at -20 ºC were repeated three times. The mixtures were centrifuged for 5 min at 13000 rpm in a 5415D – Eppendorf (Germany) centrifuge. The supernatant was transferred to another tube and stored at -20 ºC until use.

3.2.3 Genotyping

Random amplification of polymorphic DNA (RAPD) PCR was performed using the primer OPA 16 (Lockhart *et al.*, 1997). The reaction mixtures (25 µl) contained 1x PCR buffer (buffer with $MgCl_2$), 400 µM dNTPs, 4 µM of primer OPA16, 1 U of Taq ultratools DNA polymerase (Biotools B&M Labs, S.A., Spain) and 100 – 200 ng of genomic DNA. PCR amplifications were carried out in a PTC-100^{TM} Peltier Thermal Cycler (MJ Research, INC, USA) as follows: initial denaturation (94 ºC for 4 min), 40 cycles of denaturation (94 ºC for 1 min), annealing (36 ºC for 1 min) and extension (72 ºC for 1 min) and a final extension (72 ºC for 5 min). Amplicons were separated by electrophoresis in a 1.3% agarose gel-TAE buffer stained with ethidium bromide at 60 V for 1 h 40 min. Results were observed on a UV transilluminator and images were acquired with the Gel DocMega camera system 5.01 (Biosystematica, UK). Genetic profiles were visually analysed by intra-gel patterns comparison and isolates representative of each RAPD pattern were selected for phylogenetic analysis. Isolates displaying the same gene sequence in phylogenetic analysis were again submitted to RAPD fingerprinting in order to obtain a definitive discrimination of the isolates.

3.2.4 Phylogenetic analysis

The determination of phylogenetic relationships among isolates as well as the taxonomic position of each one relative to described *Aeromonas* spp. was based on *gyrB* gene partial sequences. PCR amplification of the *gyrB* gene was performed with primers gyrB3F and gyrB14R (Yáñez *et al.*, 2003). The final reaction mixture (50 µl) contained 1x PCR buffer (buffer with $MgCl_2$), 200 µM dNTPs, 0.2 µM of each primer, 1 U of Taq DNA polymerase (Biotools B&M Labs, S.A., Spain) and 50 – 100 ng of genomic DNA. PCR conditions applied were as follows: initial denaturation at 94 ºC for 3 min, 35 cycles of denaturation at 94 ºC for 15 s, annealing at 55 ºC for 30 s and extension at 72 ºC for 45 s, and a final extension at 72 ºC for 3 min. Amplified products were analysed on a 1% agarose gel-TAE buffer stained with ethidium bromide and visualized as described above. Amplicons were purified with QIAquick PCR purification kit (QIAGEN, Germany), following the manufacturer's instructions. Sequencing analysis using primer gyrB3F (Yañez *et al.*, 2003) were performed as previously described (Saavedra *et al.*, 2006), using

the BigDye Terminator V3.1 cycle sequencing kit and the ABI Prism® 3100 *Avant* Genetic Analyzer (Applied Biosystems, USA).

Sequences were analysed with the Chromas 1.45 software. Alignments of *gyrB* partial sequences of the isolates collected in this study and from all *Aeromonas* species present in the Molecular Diagnostics Center (MDC), Orihuela, Spain, culture collection including type strains, were obtained using the CLUSTALX program (Larkin *et al.*, 2007). Phylogenetic tree was produced by the neighbor-joining method (Saitou and Nei, 1987) with the Kimura's 2-parameter method (Kimura, 1980), using the MEGA4 program (Tamura *et al.*, 2007).

3.3 Results

3.3.1 Aeromonas isolation from water samples

Water samples were collected from different, either private or public, sources and had in common the fact of being untreated. Most of them were not regularly subjected to monitoring for quality assessment purposes. In all cases the water was used for drinking and other human activities.

A total of 93 untreated drinking water samples were collected from 86 sampling sites (Fig. 3.1). 184 presumptive aeromonads were isolated from 30 samples. Table 3.1 exhibits the geographic distribution and numbers of samples and isolates collected from these sources.

Regarding the sampling sites that were sampled in different occasions, recovery of *Aeromonas* spp. was not always achieved (4 cases). For example, in sampling site 45, in Lagos, *Aeromonas* spp. were recovered in December 2004 but a sample collected at the same site in May 2005 failed to display typical *Aeromonas* colonies.

Table 3.1 Total numbers of isolates and drinking water samples collected from fountains, wells, drilled wells and mines in different regions of Portugal.

	North	**Centre**	**South**
Sampling sites	61	22	3
Samples collected	65	22	6
Samples with *Aeromonas* spp.	14	12	4
Isolates collected	70	103	11

In the Centre of Portugal, 3 mineral water samples were collected from 3 sampling sites and 22 isolates presumptively members of the genus *Aeromonas* were obtained.

Physical and chemical characteristics of these waters are on Table 3.2. The mineral water from sampling sites 1 and 2 is hypothermal chloride bicarbonate and contains 903.6 mg/l of dissolved minerals. They were clinically used either by oral consumption or bathing. It was indicated in the treatments of dermatosis, gastrointestinal tract and female genital diseases and rheumatism. The mineral water from sampling site 3 collected in a different water spring, is sulphuric and has the same medical purpose of mineral water from water spring 1.

Table 3.2 Physical and chemical characteristics of mineral waters.

Sampling Site	Turbidity	Temperature (ºC)	HCO_3 (ml)	Conductivity (µs/cm)	pH	Hydrogeochemical type
Water spring 1 (sampling sites 1 and 2)	clear	24.6	1.1	1148	6	Chloride
Water spring 2 (sampling site 3)	slightly turbid	17.3	1.8	1338	5.5	Sulphurous

3.3.2 Genotyping

A total of 206 isolates was submitted to RAPD analysis, resulting in 80 different patterns and for each, one representative strain was chosen. 31 patterns were displayed uniquely by one isolate.

The same profile was occasionally obtained from isolates from different sampling sites (10 cases) and from the same sampling site, on different sampling dates (1 case). For instance, isolate DW.126 (representative strain A.126) from sampling site 62 in Porto has the same RAPD profile of isolate DW.A1/2 (representative strain A.126) collected in sampling site 84 in Póvoa de Varzim. Also, isolate DW.096A (representative strain A.096A) has the same RAPD profile of isolates represented by DW.96/2_1 (representative strain A.096A), collected in sampling site 43 in December 2004 and May 2005, respectively. On the other hand, up to 9 different genetic fingerprints were collected from the same sampling site. Sample 136 collected in sampling site 79 in the district of Lisbon harboured 25 isolates with 9 different RAPD profiles, represented by strains A.135/10, A.136/3, A.136/4, A.136/5, A.136/12, A.136/13, A. 136/15, A.136/24, and A.136/26. In Figures 3.2 and 3.3 examples of different RAPD-PCR results are shown.

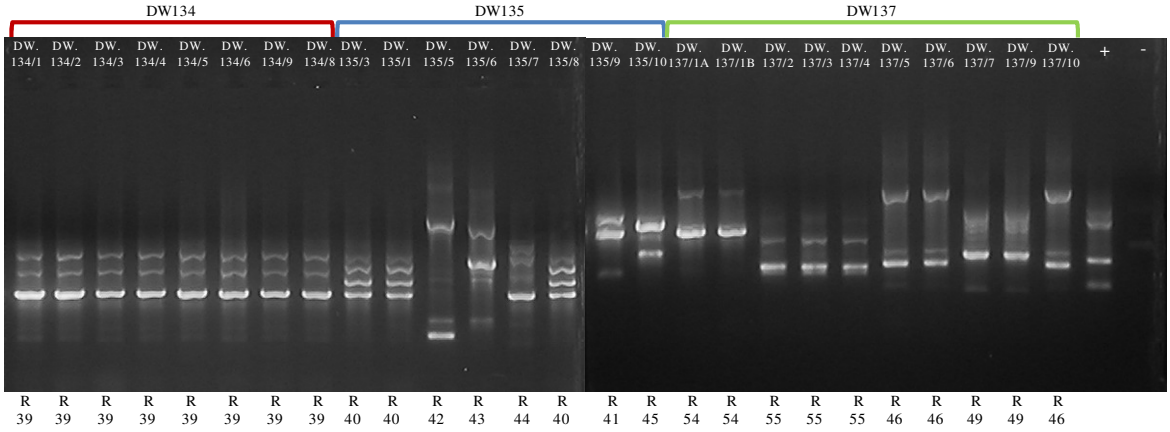

Figure 3.2 Examples of genotyping results by RAPD-PCR obtained for isolates from the same sample.

Legend to figure:
+, positive control; –, negative control.
All isolates (8) recovered from sample DW134 displayed the same RAPD profile (R39). Among 9 isolates from sample DW135 6 distinct profiles were recorded (R40, R41, R42, R43, R44 and R45). Within the 10 isolates collected from sample DW137, four different RAPD profiles were detected (R46, R49, R54 and R55).

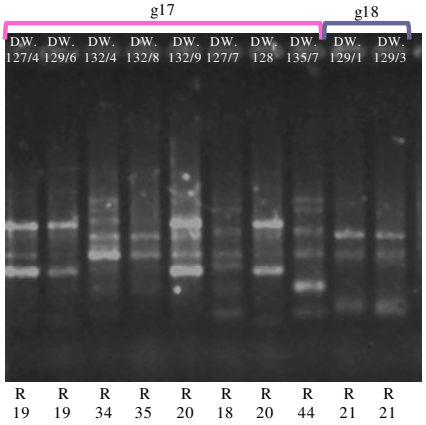

Figure 3.3 Examples of RAPD patterns of isolates displaying identical *gyrB* gene partial sequences.

Legend to figure:
gyrB gene partial sequence g17 (*A. bestiarum*) was exhibited by 18 isolates, among which 6 different RAPD profiles were detected (R18, R19, R20, R34, R35, R44). *gyrB* gene partial sequence g18 was exhibited by four *A. media* isolates (two shown in figure) displaying the same RAPD profile (R21).

3.3.3 Identification and phylogenetic analysis

A representative of each RAPD profile was selected for sequencing analysis of *gyrB* gene. Partial sequencing of this gene resulted in an approximately 500 bp high quality nucleotide sequence. The unrooted phylogenetic tree was constructed by using the *gyrB* gene partial sequence of the isolates and strains from MDC culture collection, including type strains (Fig. 3.4).

The clustering of strains was consistent with all species of the genus described to date as the well characterised strains from MDC culture collection grouped accordingly to their species affiliation, except the unique strain of the newly described species *A. sanarellii* which has grouped with the *A. caviae* phylogenetic cluster.

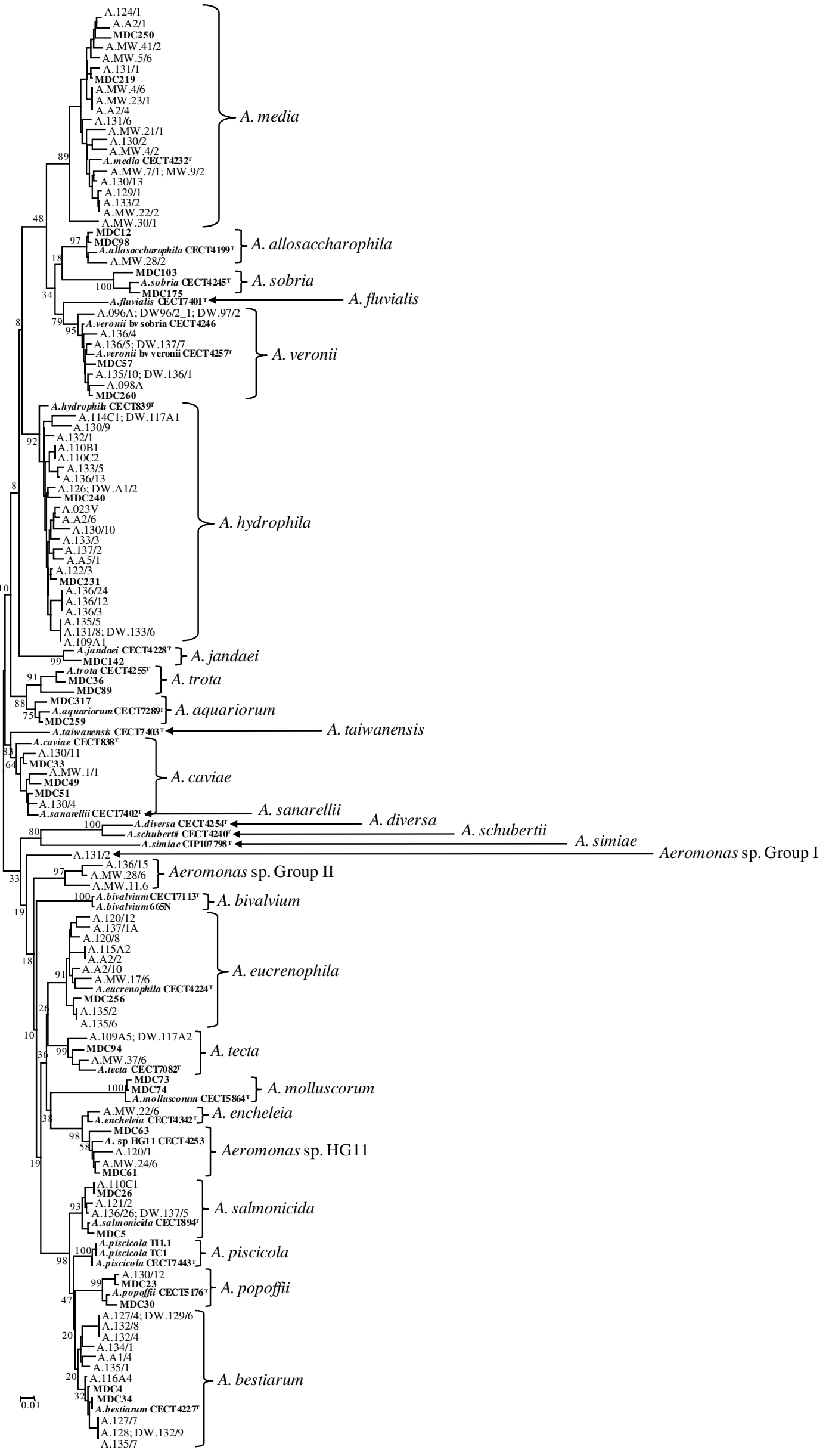

Figure 3.4 Unrooted phylogenetic tree based on *gyrB* gene sequences, demonstrating the relationships of the strains collected (A.xxx) to reference and other representative strains from MDC (Orihuela, Spain) *Aeromonas* culture collection (**MDCxxx**).

Legend to figure:
Strains collected from mineral water samples are signalized with MW. Isolates displaying the same RAPD profile recovered from different sampling sites and also from the same sampling site at different dates are shown side by side with the respective representative strain. Phylogenetic groups displaying different *gyrB* gene sequences different from the all known are indicated (*Aeromonas.* sp. Group I and *Aeromonas.* sp. Group II). The tree was constructed by the neighbor-joining method with the Kimura's 2-parameter method. Bootstrap values based on 1000 replicates are shown. Scale bar, represents 0.01 substitutions per site.

Sixty-eight different *gyrB* gene partial sequences were obtained. Up to 6 different species were found in the same sample (MW sample 3 collected in water spring 2). By comparison of the nucleotidic sequences, members of *A. hydrophila* were preponderant with 56 isolates corresponding to approximately 27%. Other isolates were identified as *A. bestiarum* (35 isolates; 17%), *A. media* (34 isolates; approximately 17%), *A. tecta* (20 isolates; approximately 10%), *A. veronii* (19 isolates; approximately 9%), *A. salmonicida* (15 isolates; approximately 7%), *A. eucrenophila* (12 isolates; approximately 6%), *A. caviae* (4 isolates; approximately 2%), *Aeromonas* sp. HG11 (2 isolates; 1%), *A. popoffii* (1 isolate; 0.5%), *A. encheleia* (1 isolate; 0.5%) and *A. allosaccharophila* (1 isolate; 0.5%). Tables 3.3 and 3.4 exhibit the distribution (number of isolates and representative strains of each RAPD profile) of *Aeromonas* identified species collected from drinking and mineral waters, respectively.

Table 3.3 Number of isolates and representative strains (in brackets) of each species collected from untreated water in different regions from Portugal.

Zone / Species	North	Centre	South
A. hydrophila	20 (9)	36 (12)	-
A. bestiarum	10 (4)	25 (6)	-
A. media	7 (2)	14 (7)	-
A. tecta	19 (1)	-	-
A. veronii	1 (1)	7 (2)	11 (2)
A. salmonicida	3 (2)	12 (1)	-
A. eucrenophila	9 (7)	2 (1)	-
Aeromonas sp. Group II	-	1 (1)	-
A. caviae	-	3 (2)	-
A. HG11	1 (1)	-	-
Aeromonas sp. Group I	-	2 (1)	-
A. popoffii	-	1 (1)	-

Table 3.4 Number of isolates and representative strains (in brackets) of each species collected in MW samples in the different sampling sites.

Sampling Site / Species	WS1.SS1	WS1.SS2	WS2.SS3
A. media	6 (4)	5 (3)	2 (2)
A. tecta	-	-	1 (1)
A. eucrenophila	-	-	1 (1)
Aeromonas spp. Group II	-	-	3 (2)
A. caviae	1 (1)	-	-
A. HG11	-	-	1 (1)
A. encheleia	-	-	1 (1)
A. allosaccharophila	-	1 (1)	-

A group of isolates displayed a molecular variant of *gyrB* gene different from any other species known so far, grouping in two different clusters and forming well defined and robust branches. *Aeromonas* sp. Group I, represented by strain A.131/2, is composed

by two isolates displaying similar RAPD profiles collected from the same untreated water sample. *Aeromonas* sp. Group II is constituted by four isolates, one of which collected from an untreated water sample and three others from the same mineral water sample. These isolates displayed three different RAPD profiles represented by strains A.136/15, A.11/6 and A.28/6 (Fig. 3.4).

Edited *gyrB* sequences of these isolates were compared to the GenBank nucleotide data library using the BLAST software at The National Center of Biotechonology Information website (http://www.ncbi.nlm.nih.gov/) in order to examine their unknown phylogenetic affiliation. Group I was most closely related to *Aeromonas eucrenophila* (accession number AY101813, 94% of identity). Group II was also most closely related to *Aeromonas eucrenophila* (AM116969 and AM116970, 93% of identity).

3.4. Discussion

This study aimed to investigate the occurrence and diversity of *Aeromonas* spp., a recognised pathogen, in untreated drinking and mineral waters used for human and animal consumption from several regions of Portugal

A total of 96 untreated water samples, including mineral waters, were collected and 206 *Aeromonas* spp. isolates were recovered from 33 samples. Fingerprinting analysis revealed 80 different genetic profiles that clustered in 14 phylogenetic groups corresponding to 68 *gyrB* gene different sequences. These results demonstrate the frequent occurrence and considerable diversity of *Aeromonas* spp. in untreated waters used for human or animal consumption in Portugal. It is remarkable the large aeromonads phylogenetic diversity in mineral water samples, since 17 out of 22 isolates showed different genotypes and 16 divergent *gyrB* nucleotidic sequences that clustered in 8 groups were recovered.

In sampling site 43 (Lagos), isolate DW.096A was recovered in December 2004 and in May 2005 eight isolates displaying the same genotypic profile as isolate DW.096A were collected (all isolates are represented by strain A.096A). These results indicate that these specimens probably are the same clone and that it may persist on time. In sampling site 44 (Lagos) there was no recovering of *Aeromonas* spp. in December 2004, although in May 2005 isolate DW.97/2 (represented by strain A.096A) was recovered and displayed a

genetic fingerprint similar to those collected in sampling site 43. This suggests, on one hand that *Aeromonas* spp. population in this site is highly dynamic and, on the other hand, that there is a geographical distribution of the same clone. In sampling site 45, isolate DW.098A (strain A.098A) was collected in December 2004 but none was recovered in May 2005. All these strains clustered in the *A. veronii* group (Fig. 3.4).

In the District of Vila Real, isolate DW.114C1 (*A. hydrophila* strain A.114C1) was collected in December 2004 in sampling site 52 and in July 2005, isolates DW.120/1 (*A.* sp. HG11 strain A.120/1), DW.120/8 (*A. eucrenophila* strain A.120/8) and DW.120/12 (*A. eucrenophila* strain A.120/12) were recovered from the same site. All these strains displayed different genetic profiles and clustered in different phylogenetic groups (Fig. 3.4). In the same district, in December 2004, isolate DW.109A1 (*A. hydrophila* strain A.109A1) and 19 isolates represented by strain A.109A5 (*A. tecta*) (Fig. 3.4), were recovered in sampling site 47. Neither in July 2005 nor in January 2007 *Aeromonas* spp. were detected. In the same way, in sampling site 48 in the same district, isolate DW.110B1 (*A. hydrophila* strain A.110B1), 2 isolates represented by A.110C1 (*A. salmonicida*) and other two represented by A.110C2 (*A. hydrophila*) (Fig. 3.4) were isolated and no recovering was achieved in July 2005. These results indicate a great fluctuation in the occurrence of *Aeromonas* spp. in these locations.

In 10 occasions, specimens pertaining to the same genotype, thus probably of clonal origin, were isolated from different sampling sites and, in some cases, distantly situated (four cases). For example, isolate DW.135/10 (representative strain A.135/10) isolated in sampling site 78 in the District of Porto is genotypically similar to isolate DW.136/1 (represented by strain A.135/10) recovered in sampling site 79 in the District of Lisbon. Also, isolates displaying the same genotypic profile were collected from the same sampling site, suggesting multiple isolations of the same strain. In addition, isolates with different genotypes were recovered. For example, isolates MW.17/6 (strain A.17/6) and MW.24/6 (strain A.24/6) were both isolated from MW sampling site 3 and exhibited different RAPD profiles, indicating that they are not closely related, as it was proved by sequencing analysis, since they clustered in *A. eucrenophila* group and *A.* HG11 cluster, correspondingly. Also, strains displaying different genotypes but similar *gyrB* gene sequences were isolated from the same sampling site or from different ones. For instance, strain A.115A2 isolated from sampling site 53 in the District of Vila Real and A.A2/2

recovered from sampling site 85 in District of Porto are *A. hydrophila* displaying the same molecular variant of the *gyrB* gene and belong to different genotypes. The results here presented demonstrate that there is a great phylogenetic variability in some sampling sites and that the same clone may be present in distantly related locations.

Several studies focusing on the diversity of *Aeromonas* spp. in drinking water, including drinking water supplies and bottled water (Kühn *et al.*, 1997; Gavriel *et al.*, 1998; Legnani *et al.*, 1998; Koksal *et al.*, 2007) have been reported. Additionally, some studies concerning untreated water used for human activities and from sources like fountains, mines, wells and drilled wells have also been reported (Ghenghesh *et al.*, 2001; Massa *et al.*, 2001; Borchardt *et al.*, 2003; Di Bari *et al.* 2007). In Portugal, there is still a significant number of people using water from untreated sources for consumption. Since members of this genus are considered potential waterborne pathogens and contaminated water may be the source of outbreaks of disease (WHO, 2006) recommended international standards for drinking water should take in to account the presence of these organisms in water.

In this study, *A. hydrophila* was the most frequently recovered species, followed by *A. bestiarum* and *A. media*. Also, *A. tecta*, *A. veronii*, *A. salmonicida*, *A. eucrenophila, A. caviae, Aeromonas* spp. HG11*, A. popoffii, A. encheleia*, *A. allosaccharophila* and two groups of unidentified *Aeromonas* species were collected. Aeromonads isolated from similar water sources in other studies were most frequently identified as *A. hydrophila, A. allosaccharophila, A. jandaei, Aeromonas* spp. HG2, *A trota, A. sobria* and *Aeromonas spp.* (Di Bari *et al.*, 2007), *A. hydrophila* and *A. caviae* (Borchardt *et al.*, 2003), *A. hydrophila*, *A. caviae* and *A. salmonicida* (Ghenghesh *et al.*, 2001) and *A. hydrophila*, *A. salmonicida* and *A. caviae* (Massa *et al.*, 2001). Borrell *et al.* (1998) found *A. bestiarum*, *A. hydrophila*, *A. media*, *A. veronii*, *A. salmonicida* and *A. caviae* in untreated drinking waters and by the phenotypic methods applied to identify the specimens could not ascertain the taxonomic position of 20 isolates (Borrell *et al.,* 1998). Also, Koksal and colleagues (2007) isolated *A. hydrophila*, *A. sobria*, *A. caviae*, *A. salmonicida*, *A. veronii* and *A. jandaei* from tap water and domestic water tanks. When compared to previously reported studies from similar sources, *Aeromonas* spp. phylogenetic diversity is greater in the samples analysed during this study. Moreover, the identification methods applied in those studies were biochemical tests that are not always reliable and often

limitative. Whereas, in this study, molecular techniques, that are more accurate and yield a definitive identification, were used. Thus, the real diversity in the samples analysed was achieved. In fact, we were able not only to assess the microdiversity within our samples, by characterising isolates at the intraspecies level, but also to identify undoubtedly each of the different strains by taking advantage of the use of a powerful tool to assess phylogeny in the genus *Aeromonas*. The *gyrB* gene has been extensively used for phylogenetic studies (Soler *et al.*, 2004; Küpfer *et al.*, 2006; Saavedra *et al.*, 2006) but limitedly for assessing occurrence and diversity of aeromonads in different environments (Saavedra *et al.*, 2007; Fontes *et al.*, 2010). In our opinion, studies of occurrence and diversity of aeromonads should always make use of molecular techniques in order to avoid precluding the real significance of each phylogenetic group in the environment. Figueras (2005) also emphasized the need for an accurate species identification and assessment of the real significance of each species in the clinical field in order to support knowledge and understanding of *Aeromonas* epidemiology.

Nevertheless, contrary to what was expected, based on *gyrB* gene phylogenetic analysis the unique *A. sanarellii* strain grouped in the *A. caviae* cluster. As shown by *rpoD* gene sequence analysis and multi-locus phylogenetic analysis (MLPA) this strain is closely related to *A. caviae* although constituting an independent branch in the genus (Alperi *et al.*, in press). In our study, the *gyrB* gene has shown not to be sufficiently discriminatory to separate these two distinct phylogenetic lines. Previously, Yáñez *et al.* (2003) and Soler and colleagues (2004) have also verified that some strains of closely related species (*A. salmonicida* and *A. bestiarum*) could not be differentiated by using phylogenetic information based solely in the *gyrB* gene. Unfortunately, when describing *A. sanarellii*, the authors did not give any information on the *gyrB* based relationships of this specimen and the remaining *Aeromonas* species (Alperi *et al.*, in press), thus it is not possible to compare results. Additionally, the description of this novel species was based in the characterisation of a single strain, an increasing tendency that, as stated by Christensen *et al.* in 2001, "is insufficient to represent variability without *a priori* knowledge of population structure" and thus may contribute to biased results in organisms classification and taxonomic studies (Christensen *et al.*, 2001; Felis and Dellaglio, 2007; Janda and Abbott, 2010). In conclusion, *A. sanarellii* and *A. caviae* might have clustered together due to bias introduced by their close phylogenetic relationship and use of a single strain of

A. sanarellii. In this view, the affiliation of the three *A. caviae* strains (four isolates) could be dubious, but as the clustering of these strains in different evolutionary trees is consistent with this affiliation (data not shown) we considered them as members of the *A. caviae* phylogenetic group.

Among human clinical *Aeromonas* isolates the most prevalent species are *A. hydrophila*, *A. caviae* and *A. veronii* biotype sobria which have been involved in a wide array of extraintestinal and systemic infections. But, also *A. media*, *A. jandaei*, *A. bestiarum*, *A. salmonicida*, *A. sobria*, *A. eucrenophila*, *A. schubertii*, *A. encheleia*, *A. allosaccharophila*, *A. trota* and *A. popoffii* were implied in human health complications (Figueras, 2005). In 2003, Borchardt and collaborators (2003) suggested that human enteropathogenic strains are rare in drinking water. However, members of almost of all these phylogenetic groups were isolated from our samples, in fact, *A. hydrophila* was the most occurring species. Studies about virulence determinants and antimicrobial resistance of these isolates are presented in chapters 5 and 6 of this thesis.

Concerning the new phylogenetic lines, to the best of our knowledge, specimens displaying molecular variants of the *gyrB* gene like these were not previously detected. *Aeromonas* sp. Group I is formed by 2 isolates displaying the same genotype represented in Fig. 3.4 by A.131/2 and the *Aeromonas* sp. Group II aggregates 4 isolates with 3 different genotypic profiles, strains A.136/15, A.11/6 and A.28/6 in Fig. 3.4. Specimens of the latter group were also found in vegetables in another investigation conducted by us (Chapter 4 of this dissertation). With the perspective of a possible taxonomic proposal, further studies are needed on these taxa. Extended information about these groups is given in chapter 7 of this thesis.

The present work highlights the occurrence and wide diversity of *Aeromonas* spp. in untreated water for human and animal direct or indirect consumption in Portugal, revealing that non-described species and potential virulent ones are present, emphasizing the need for standards on *Aeromonas* in drinking and recreational waters.

4. Diversity of *Aeromonas* spp. in agricultural correlated sources: is it water a contamination route?

Abstract

The occurrence and phylogenetic diversity of *Aeromonas* spp. in different agricultural sources and assessment of the possibility of water being the contamination route were analysed in this study. Irrigation waters and produce, cows' drinking water and faeces, udder's cleaning water before milking, milk and cleaning water of the milking equipment were sampled. 277 aeromonads were recovered from which 91 displayed different RAPD fingerprints. No genetic correlation was found between *Aeromonas* spp. collected from vegetables and irrigation waters. Close genetic individuals were collected from cows' drinking water and faeces. Isolates recovered from udder's cleaning water and milk displayed the same genetic profile. On the basis of *gyrB* gene sequencing, isolates clustered in nine phylogenetic groups: *A. media*, *A. salmonicida*, *A. hydrophila*, *A. caviae*, *A. eucrenophila*, *A. bestiarum*, *A. allosaccharophila* and a group of isolates displayed molecular variants of the *gyr*B gene different from all known *Aeromonas* spp., indicating that they may constitute representatives of two non-described species.

There is a frequent occurrence and a considerable diversity of *Aeromonas* spp. in agricultural sources in Portugal. Occasionally, water might have been the contamination route.

Keywords: *Aeromonas* spp., diversity, phylogeny, vegetables, faeces, milk, water, contamination route.

4.1 Introduction

The Family *Aeromonadaceae* is a monophyletic group in the γ-3 subgroup of the class Proteobacteria that comprises the genus *Aeromonas*. The members of this genus are motile or non motile, psychrophilic and mesophilic gram-negative rods (Martínez-Murcia *et al.* 1992a; Martin-Carnahan and Joseph, 2005).

One of the issues of public concern regarding *Aeromonas* spp. is their potential pathogenicity to human and animals, since they possess many virulence determinants (Callister and Agger, 1987; Chopra *et al.*, 2009). Several reports indicate a wide range of animal diseases and different systemic infections in humans due to *Aeromonas* spp. (Figueras, 2005; Janda and Abbott, 2010). Also, the patterns of antibiotic resistance displayed by these organisms increase their potential health hazard (Saavedra *et al.*, 2004; Palú *et al.*, 2006; Ceylan *et al.*, 2009). Consequently, their broad distribution on different habitats is a problematic question. In fact, aeromonads are considered natural inhabitants of aquatic environments but they are also widespread in different kinds of food, soil and in healthy and patient animals and humans (Borrell *et al.*, 1998; Neyts *et al.*, 2000; Massa *et al.*, 2001; Figueras, 2005).

In relation to food, *Aeromonas* spp. have been isolated from produce, dairy products and different types of animal origin (Neyts *et al.*, 2000; McMahon and Wilson, 2001; Nahla, 2006). The handling and processing of food products are major causes of food bacterial contamination (McMahon and Wilson, 2001; Verdier-Metz *et al.*, 2009; Lynch *et al.*, 2009). Irrigation water and the use of manure as fertiliser containing *Aeromonas* spp. can be sources of contamination of vegetables (McMahon and Wilson 2001; Pianetti *et al.*, 2004; Lynch *et al.*, 2009). In the same way, the contaminated water used for cleaning milking equipments and for udder preparation before the milking process, is an important source of contamination (Nahla, 2006; Verdier-Metz *et al.*, 2009). In this way, produce and milk can become contaminated with *Aeromonas* species and act as possible vehicles for dissemination of foodborne *Aeromonas* gastroenteritis (Callister and Agger, 1987; Neyts *et al.*, 2000; McMahon and Wilson, 2001; Nahla, 2006). Also, the consumption and exposure to *Aeromonas* contaminated water represent a human and animal risk of acquiring *Aeromonas* species infections (Figueras 2005; Janda and Abbott, 2010). Currently, the importance of *Aeromonas* sp. in foodborne and waterborne disease outbreaks is not well established (von Graevenitz, 2007). However, these organisms harbour many of the virulence determinants that are associated with the pathogenicity of other well known agents of gastroenteritis (Chopra *et al.*, 2009). Two reasons for the difficulty of establishing *Aeromonas* as a pathogen are, on one hand, the genetic misrelation between environmental and clinical specimens and, on the other hand, the lack of use of molecular methods in almost all studies that would able the establishment of those connections (von Graevenitz, 2007; Janda and Abbott, 2010).

Several molecular fingerprinting methods were established and applied to assess *Aeromonas* genotypic diversity (Szczuka and Kaznowski, 2004; Tacão *et al.*, 2005a; 2005b) and numerous phylogenetic analysis using different genes, were performed (Martínez-Murcia *et al.*, 1992a; Yañez *et al.*, 2003; Soler *et al.*, 2004; Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Miñana-Galbis *et al.*, 2009; Alperi *et al.*, in press). The *housekeeping* gene *gyrB*, which encodes the B-subunit of DNA gyrase, proved to be an excellent molecular marker for phylogenetic inference in this genus (Yañez *et al.*, 2003; Soler *et al.*, 2004; Küpfer *et al.*, 2006). Until February 2010, the genus comprised 24 species including one unnamed DNA homology group (HG), although *A. culicicola* was considered a latter synonym of *A. veronii* (Alperi *et al.*, 2010; in press; Miñana-Galbis *et al.,* 2010a). In fact,

the taxonomy of the genus arises controversy and is continually changing because new species are often described and reclassification and extended description of the existing ones is common (Tacão *et al.*, 2005a; 2005b; Saavedra *et al.*, 2006).

In this study, and to the best of our knowledge for the first time, we investigated the occurrence and diversity of *Aeromonas* species in different agricultural correlated sources and the possibility of water being the route of contamination between those sources.

4.2 Material and methods

4.2.1 Sampling

Sampling scheme was designed to investigate the occurrence and diversity of *Aeromonas* species in different agricultural correlated sources and to assess the possibility of water being the transmission vehicle. In 10 farms, irrigation waters and cultivated vegetables were collected. Sampling sites consisted in eight smallholdings, whose raised products are in some cases only for self consumption and in others also for sale in small village farmers' markets; two farms with bigger holdings and a higher commercial perspective that sell to supermarkets and groceries were also included. These sites are located in two different regions of Portugal, North and Centre (Fig. 4.1), where an historical agriculture practice exists. It is noteworthy that the irrigation water used in nine sites is supplied by private aquifers and usually not subjected to microbiological quality assessment or treatment.

In one stable, cow's drinking water, faeces, udder's cleaning water before milking, milk and the last cleaning water of milking equipment were collected.

4.2.2 Aeromonads isolation

Water samples (irrigation waters, cow's drinking water and cleaning water of the milking equipment and udder) were collected in autoclaved bottles and presumptive aeromonads isolated by the membrane filter technique as described in chapter 3.

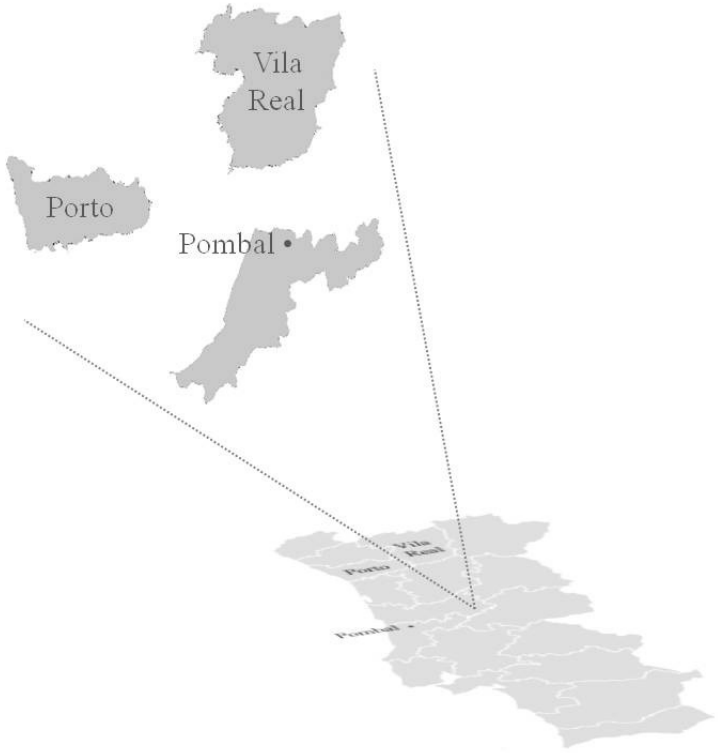

Figure 4.1 Sampling sites were located in the Districts of Porto and Vila Real and in the Municipality of Pombal (District of Leiria).

Vegetable samples were collected directly from soil, stored in individual stomacher bags and chilled until laboratory examination, within five hours. These samples were treated in two different ways. In the first method, 25 g of each sample were added to 225 ml of buffered peptone water (BPW), pummelled in a stomacher Lab Blender (IUL, Spain) for 90 s and the homogenate was incubated at 30 ºC overnight. One millilitre of the overnight pre-enriched homogenate was ten-fold diluted in sterilised water and filtered as described (chapter 3). Membranes were placed in GSP, supplemented with 50 µg/ml of ampicillin and plates were incubated at 30 ºC overnight. In the second methodology, 25 g of each sample were washed by shaking with 200 ml of sterilized distilled water for five minutes. The mixture was filtered as mentioned and membranes were placed in 250 ml of BPW and incubated at 30 ºC overnight. A loop of the overnight pre-enriched mixture was streaked on three GSP agar plates supplemented with 50 µg/ml of ampicillin (A, B and C) and incubated overnight at 30 ºC.

Faecal samples were collected immediately after defecation using sterilised spatula and placed into sterile tubes. Samples were transported directly to the laboratory and analysed. 25 g of each sample were placed aseptically into stomacher bags with 225 ml of

BPW and pummelled for 90 s in a stomacher. The homogenate was incubated at 30 ºC overnight. A loop of the overnight pre-enriched homogenate was streaked on three GSP agar plates supplemented with 50 µg/ml of ampicillin (A, B and C) and incubated overnight at 30 ºC.

During the milking process, one milk sample was collected aseptically to a sterilised tube and three methods were used in order to recover *Aeromonas* spp. isolates. The first one consisted in the direct inoculation of a GSP plate supplemented with 50 µg/ml of ampicillin with a loop of milk. In the second one, 2 ml of milk were added to 40 ml of BPW, followed by incubation at 30 ºC overnight and streak of the overnight pre-enriched mixture on three plates of GSP supplemented with 50 µg/ml of ampicillin. Plates A, B and C were incubated at 30 ºC overnight. In the third process, two millilitres of milk were added to 100 ml of sterilised distilled water and filtered as told before. Membranes were placed in 250 ml of BPW and incubated at 30 ºC overnight. Three plates of GSP supplemented with 50 µg/ml of ampicillin were streaked with a loop of the overnight pre-enriched mixture and incubated at 30 ºC overnight.

In all cases, typical *Aeromonas* yellow colonies in GSP medium were selected and pure cultures were obtained by repeated isolation of individual colonies in the same medium. Isolates were stored in 17% glycerol at -80 ºC.

4.2.3 Typing and phylogenetic analysis

DNA extraction was achieved as described in chapter 3. For genotyping, random amplification of polymorphic DNA (RAPD) PCR was performed as shown in chapter 3. In order to settle phylogenetic relationships between isolates and establish their taxonomic positions in relation to described *Aeromonas* spp., a phylogenetic study based on *gyrB* partial sequence was applied as explained in chapter 3.

4.3 Results

4.3.1 Sampling and isolates recovering

A total of 37 samples, including water (13 samples), vegetables (21 samples), faeces (two samples) and raw milk (one sample), were collected from 11 sampling sites

and 277 presumptive *Aeromonas* spp. were isolated from 23 samples. Details concerning sampling sites, samples and isolates recovering are given in Table 4.1.

For two times, *Aeromonas* spp. were recovered from water samples and not from the vegetables (sampling site 7) or faeces (sampling site 11) collected in the same site. Also, in sampling sites 1, 2, 3, 4 and 6 specimens were isolated from vegetables and not from the irrigation water sample collected in the same place.

There were no differences in the number of aeromonads obtained by the different isolation methods. Nevertheless, some procedures were more laborious and time-consuming than others. In relation to vegetables, the first procedure was less laborious and time-consuming and in relation to milk samples procedures, it was easier and faster the direct inoculation of the culture media with a loop full of milk.

4.3.2 Fingerprinting analysis

The 277 isolates recovered were subjected to RAPD analysis and 91 distinct profiles were detected. 39 patterns were displayed uniquely by one isolate. Up to 9 different genotypes were found in the same sample (VL12 and VL18).

Additionally, for seven times the same fingerprint was detected in different samples of the same kind of source collected in the same sampling site. For example, in sampling site 1, isolates represented by strain A.L1-2 recovered from sample VL1 and isolates V.L2-1 (strain A.L1-2) and V.L3-12 (strain A.L1-2) recovered from samples VL2 and VL3, respectively, displayed the same genotype, R79.

In two occasions, isolates displaying the same RAPD profile were recovered from samples from different sources collected in the same sampling site. Isolate CW.AV-11 (strain A.AV-11) from cow's drinking water and four isolates represented by CF.V2-3 (strain A.AV-11) from cow's faeces had identical RAPD profiles. Also, isolates represented by strain A.ALU-1 collected from udder's cleaning water displayed the same fingerprint as those represented by isolate M.L-1 (strain A.ALU-1) recovered from milk.

Figures 4.2, 4.3, 4.4 and 4.5 display examples of different results obtained in RAPD-PCR analysis.

Table 4.1 Details concerning sampling sites, samples and isolates recovering.

Sampling Site	Type of farm/ Kind of use[a]	Sample	*Aeromonas* spp.[b]
1 Póvoa de Varzim (AS.SS1) District of Porto	Smallholding	Well water (IWA0)	-
	Small market sales	Lettuce (VL1)	+
		Lettuce (VL2)	+
		Lettuce (VL3)	+
2 Póvoa de Varzim (AS.SS2) District of Porto	Smallholding	Well water (IWA1)	-
	Street sales	Lettuce (VL4)	-
		Lettuce (VL5)	-
		Parsley (VL6)	-
		Lettuce (VL7)	+
3 Gondomar (AS.SS3) District of Porto	Smallholding	Well water (IWA2)	-
	Self consumption	Celery (VL8)	+
		Parsley (VL9)	+
4 Gondomar (AS.SS4) District of Porto	Smallholding	Well water (IWA3)	-
	Self consumption	Lettuce (VL10)	+
5 Pombal (AS.SS5) District of Leiria	Smallholding	Well water(IWA4)	+
	Small market sales	Water cress (VL11)	+
		Lettuce (VL12)	+
6 Pombal (AS.SS6) District of Leiria	Smallholding	Well water (IWA6)	-
	Small market sales	Lettuce (VL13)	-
		Parsley (VL14)	+
7 Pombal (AS.SS7) District of Leiria	Greenhouse	Well water (IWA7)	+
	Supermarkets sales	Lettuce (VL15)	+
		Parsley (VL16)	-
8 Pombal (AS.SS8) District of Leiria	Greenhouse	Drilled well water (IWA8)	-
	Supermarkets sales	Lettuce (VL17)	-
9 Pombal (AS.SS9) District of Leiria	Smallholding	Drilled well water (IWA9)	+
	Self consumption	Water cress (VL18)	+
		Parsley (VL19)	+
		Lettuce (VL20)	+
10 Pombal (AS.SS10) District of Leiria	Smallholding	Well water (IWA10)	+
	Small market sales	Lettuce (VL21)	+

Table 4.1 Details concerning sampling sites, samples and isolates recovering (continued).

Sampling Site	Type of farm Kind of use[a]	Sample	*Aeromonas* spp.[b]
Stable (SE)	NA	Cow's drinking water (CWAV)	+
District of Vila Real	NA	Udder's cleaning water (UWALU)	+
		Milking equipment cleaning water (EWALS)	-
		Cow 1 faeces (CFV1)	-
		Cow 2 faeces (CFV2)	+
		Milk (ML)	+

[a] NA, Not applicable.
[b] Presence (+) or absence (-) of *Aeromonas* species.

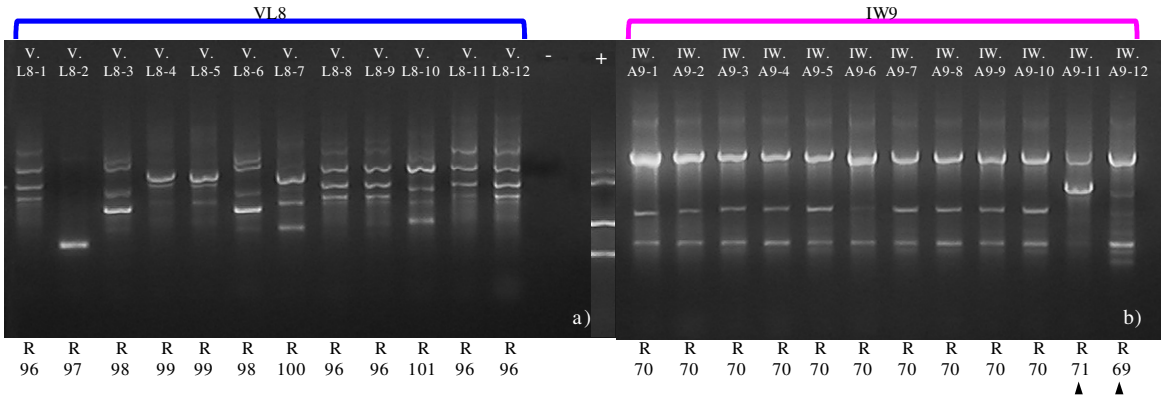

Figure 4.2 Examples of RAPD-fingerprints of isolates recovered from a) vegetables and b) irrigation waters.

Legend to figure:
+, positive control; –, negative control.
Isolates recovered from sample VL8 collected in AS.SS3. Within this sample, six distinct RAPD profiles were identified (R96, R97, R98, R99, R100 and R101). Isolates collected from IWA9 recovered in AS.SS9. Three different RAPD profiles were exhibited by the 12 isolates recovered from this sample (R69, R70 and R71), from which two are unique (▲).

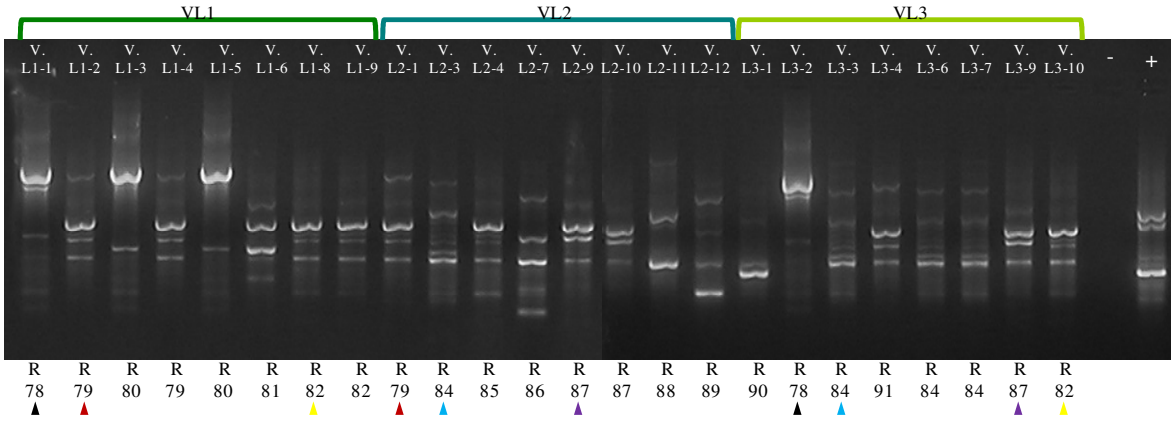

Figure 4.3 Diversity of RAPD patterns present in three distinct samples collected in the same sampling site.

Legend to figure:
+, positive control; –, negative control.
Samples VL1, VL2 and VL3 were collected in AS.SS1. Isolates exhibiting the same RAPD profile were present in distinct samples (R78 ▲, R79 ▲, R82 ▲, R84 ▲ and R87 ▲).

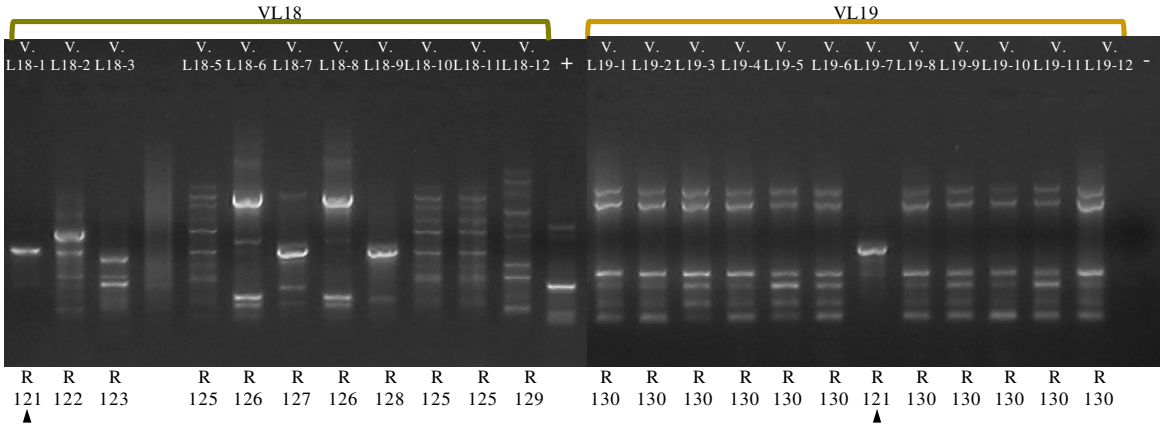

Figure 4.4 Examples of RAPD profiles present in two samples from the same sampling site.

Legend to figure:
+, positive control; –, negative control.
Samples were collected from AS.SS9. VL18 harboured a great genetic diversity: 9 distinct strains among 12 isolates (fingerprints from 11 isolates are shown). In contrast, in sample VL19, RAPD pattern R130 was dominant and the unique distinct fingerprint was similar to one found in sample VL18 (R121 ▲).

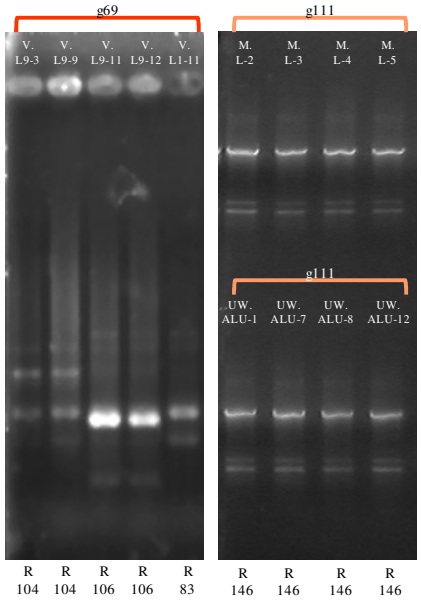

Figure 4.5 Examples of genotypes displayed by isolates harbouring the same *gyrB* gene partial sequence.

Legend to figure:
gyrB gene partial sequence g69 was exhibited by five *A. media* specimens collected from two distinct vegetable samples (VL1 and VL9) exhibiting three different RAPD patterns. *gyrB* gene partial sequence g111 was exhibited by all *A. caviae* isolates collected from milk (M.L-x; 18 specimens) and udder's cleaning water before milking (UW.ALU-x; 12 isolates), all exhibiting the same RAPD fingerprint (R146).

4.3.3 Identification and phylogeny

Strains representing distinct RAPD profiles were selected for *gyrB* gene analysis and partial sequencing of this gene resulted in a 500 bp high quality nucleotide sequence. 69 *gyrB* different sequences were obtained and an unrooted phylogenetic tree was constructed by using these *gyrB* gene partial sequences and those from strains from MDC *Aeromonas* culture collection, including type strains (Fig. 4.6). The clustering of strains was consistent with all species of the genus described to date as the well characterised strains from MDC culture collection grouped accordingly to their species affiliation, with

the exception of the unique *A. sanarellii*, as already seen in chapter 3. Up to five different species were found in the same sample (IWA10). Isolates affiliated to *A. media* (111 isolates; 40%), *A. salmonicida* (43 isolates; approximately 16%), *A. hydrophila* (34 isolates; approximately 12%), *A. caviae* (30 isolates; approximately 11%), *A. eucrenophila* (18 isolates; approximately 7%), *A. bestiarum* (11 isolates; 4%) and *A. allosaccharophila* (10 isolates; approximately 4%). *A. media* was the most frequently recovered species. A group of isolates displayed molecular variants of *gyrB* considerably different from all known species, clustering in two distinct groups, forming well defined and robust branches. Eight isolates (3%) clustered in the *Aeromonas* sp. Group II identified in chapter 3. These isolates exhibited two different genotypes: one represented by strain A.L8-3, displayed by two isolates collected from the same sample, the other, displayed by six isolates recovered from the same sample in a different sampling site from the others and represented by strain A.L10-4. The other cluster, *Aeromonas* spp. Group III, is formed by 12 isolates (4%) collected from the same vegetable sample, displaying the same RAPD profile, represented in the phylogenetic tree by strain A.L15-1. Edited *gyrB* sequences of these isolates were screened against the GenBank nucleotide data library using the BLAST software (http://www.ncbi.nlm.nih.gov/) in order to examine their unknown phylogenetic affiliation. Group II was most closely related to *A. eucrenophila* (AM116970 and MJ868378, 93% of identity) and Group III was most closely related to *A. hydrophila* (accession number DQ665888, 93% of identity). Table 4.2 highlights the *Aeromonas* species found in each source.

Table 4.2 Number of isolates and representative strains (in brackets) of *Aeromonas* sp. collected from each source.

Species	**Sources[a]**					
	IW	**V**	**CW**	**CF**	**UW**	**M**
A. media	12 (4)	88 (38)	11 (5)	-	-	-
A. salmonicida	-	30 (5)	1 (1)	12 (6)	-	-
A. hydrophila	21 (7)	13 (4)	-	-	-	-
A. caviae	-	-	-	-	12 (1)	18 (1)
A. eucrenophila	4 (3)	14 (8)	-	-	-	-
A. sp. Group I	-	12 (1)	-	-	-	-
A. bestiarum	6 (2)	5 (1)	-	-	-	-
A. allosaccharophila	4 (1)	6 (3)	-	-	-	-
A. sp. Group II	-	8 (2)	-	-	-	-

[a] IW, Irrigation waters; V, Vegetables; CW, Cows' drinking water; CF, Cows' faeces; UW, Udder's cleaning water before milking; M, Milk

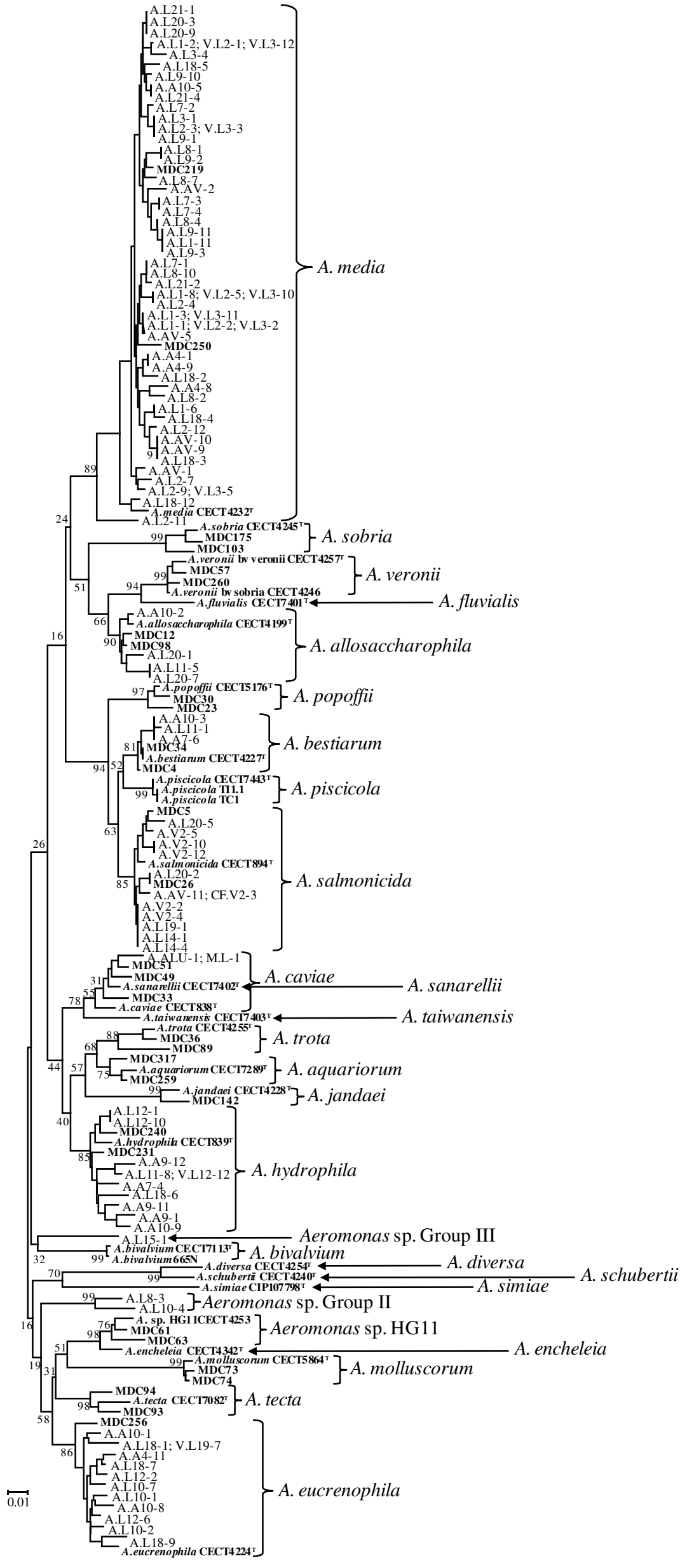

Figure 4.6 Unrooted phylogenetic tree based on *gyrB* gene sequences, demonstrating the relationships of the strains collected (A.xxx) to reference and other representative strains from MDC (Orihuela, Spain) *Aeromonas* culture collection (**MDCxxx**).

Legend to figure:
Isolates displaying the same RAPD profile recovered from different samples are displayed side by side with the respective representative strain. Phylogenetic groups displaying *gyrB* gene sequences different from the all known are indicated (*Aeromonas.* sp. Group II and *Aeromonas.* sp. Group III). The tree was constructed by the neighbor-joining method with the Kimura's 2-parameter method. Bootstrap values based on 1000 replicates are shown. Scale bar, represents 0.01 substitutions per site.

4.4 Discussion

The goal of this study was to assess *Aeromonas* species diversity in agricultural correlated sources (irrigation, drinking and cleaning water, produce, faeces and raw milk) from various farms and assess the possibility of water being the contamination route of those sources.

A total of 37 samples were collected and 277 *Aeromonas* spp. were recovered from 23 samples. 91 different molecular fingerprints were recovered and clustered in nine phylogenetic groups revealing 69 *gyrB* gene different sequences (Table 4.2 and Fig. 4.6).

A. media, A. salmonicida, *A. hydrophila*, *A. eucrenophila*, *A. bestiarum* *A. allosaccharophila* and members of two non described phylogenetic groups were detected in vegetables samples. In 1987, Callister and Agger reported the presence of *A. hydrophila*, *A. caviae* and others non identified *Aeromonas* species from grocery store produce and suggested that these organisms are ubiquitous in this kind of products. Also, McMahon and Wilson (2001) recovered members of *A. schubertii*, *A. hydrophila*, *A. trota*, *A. caviae* and *A. veronii* bv veronii species from organic vegetables, indicating that these organisms form part of the natural flora of the samples analysed. Neyts and co-workers (2000) accounted *A. media* (HG5) and *A. caviae* (HG4) as the most recovered species from vegetables collected in retail shops and supermarkets, reporting also the presence of *A. salmonicida* (HG3) and *A. bestiarum* (HG2) in these kinds of samples. Members of the phenospecies complexes *A. hydrophila*, *A. caviae* and *A. sobria* were also detected in vegetables by Castilho *et al.* (2009). Some studies focused also in the detection of virulence factors (Callister and Agger, 1987; Castilho *et al.*, 2009) and others in the incidence of resistance to antibiotics (Palú *et al.*, 2006) of *Aeromonas* species recovered from different vegetables. All of them highlighted the widespread distribution of aeromonads in produce and the possibility of it being an important vehicle of transmission of the potential food pathogen *Aeromonas* sp. Our findings are in agreement with the large dissemination of *Aeromonas* species in produce and emphasize the great diversity they may harbour as 176 isolates were recovered, from which 62 displayed different RAPD patterns.

Few studies about the diversity of aeromonads in animal faeces exist. Gray and Stickler (1989) reported the presence of *A. hydrophila*, *A. caviae* and *A. sobria* specimens

from cow faeces and *A. caviae* and *A. hydrophila* from pig faeces, indicating that aeromonads are minor components of the faecal flora of such animals. Also, *A. caviae* and *A. veronii* bv sobria were recovered from cattle faeces (Barlow *et al.*, 2004) and *A. simiae* from monkey faeces (Harf-Monteil *et al.*, 2004). Ceylan and colleagues (2009) isolated *A. caviae* and *A. sobria* from cattle and sheep faeces and *A. hydrophila* from horse faeces, reporting low occurrence of aeromonads in these animals faecal samples. In this, study, from two cows' faecal samples only one harboured aeromonads that were assigned to *A. salmonicida*. To our knowledge, this species was detected before in fish faeces (Gustafson *et al.*, 1992) and we didn't find reports on its isolation from other faecal sources. Our results are in agreement with the low occurrence and diversity of aeromonads in healthy animal faecal samples. Nevertheless we sampled only two animals what is a low number for inferring a sustained conclusion.

In previous studies, *A. hydrophila*, *A. sobria* and *A. caviae* were collected with different frequencies from raw milk samples (Melas *et al.*, 1999; Yucel *et al.*, 2005; Nahla, 2006). Also, Borrell *et al.* (1998) isolated *A. caviae*, *A.* caviae/*A. media*, *A. salmonicida*, *A. veronii* bv. sobria and *A. media* from milk. In this study, one raw milk sample was collected in a stable with milk production. *A. caviae* was the only species recovered from that sample and all 18 isolates displayed the same genetic profile, represented by A.ALU-1 in the phylogenetic tree (Fig. 4.6). The low occurrence and diversity of aeromonads in milk reported on this study might have been due to the fact that only one sample from one sampling site was investigated. Nevertheless, the hygiene in milking practices may be the reason for the low rate of aeromonads recovering. Actually, the last cleaning water of the milking equipment failed to display aeromonads.

It is well known the important role that water plays in bacterial contamination of produce and different kinds of foods, animals and humans (Neyts *et al.*, 2000; WHO, 2006; Lynch *et al.*, 2009). In addition, water may act as the contamination route between those sources (Borrell *et al.*, 1998; McMahon and Wilson, 2001). As *Aeromonas* species are widespread in untreated and treated water from different sources and are ubiquitous in several other environments (Kühn *et al.*, 1997; Neyts *et al.*, 2000; Massa *et al.*, 2001; Figueras, 2005), it is expected that water works as an aeromonads avenue of contamination through the food chain. *Aeromonas* phenospecies *A. caviae*, *A. sobria* and *A. hydrophila* with cytotoxic activity and adhesiveness ability were isolated from irrigation waters in

Italy, demonstrating the possible risk of irrigation waters to human health and the need for bacteriological monitoring of these sources (Pianetti *et al.*, 2004). We consider that it is of paramount importance that apart for faecal contaminators, the standards of water quality assessment include *Aeromonas* species. In this study, as assessed by RAPD analyses, no genotypic close relations were found between *Aeromonas* isolates from vegetables and those found in irrigation waters from the same sampling site. It is possible that the vegetables contamination was not driven by the irrigation water, but, beside the fact that colonies were randomly selected, this might have been due to the multiplicity of specimen occurrence and not all colonies have been checked. On the contrary, isolate CW.AV-11 (strain A.AV-11) recovered from cows' drinking water and four isolates collected from one of the cows faeces (represented by isolate CF.V2-3 in Fig. 4.6) displayed the same RAPD profile. Gray and Stickler (1989) hypothesised that during their study cattle acquired their faecal aeromonads from drinking water, based on the fact that the higher isolation of faecal aeromonads coincided with the higher occurrence of *Aeromonas* species in animals' drinking water. In our case we have molecular evidences that the isolates recovered from both samples are genetically very similar if not the same. Thus, one may speculate that the isolates recovered from the faeces may have had their primary origin in drinking water, corroborating Gray's and Stickler's (1989) hypothesis. In the same way, the isolates recovered from udder's cleaning water before milking (represented by strain A.ALU-1) belong to the same genotype of the isolates collected from milk (represented by isolate M.L-1 in Fig. 4.6). These results indicate that the isolates collected from milk may have had been transmitted by the udder's cleaning water. No *Aeromonas* species were isolated from the last cleaning water of the milking equipment. This is an indication that the cleaning of the equipment is efficient and that the contamination that occurs is prior to the milking process.

With exception to sites 7 and 8, all sampling sites were smallholding farms where usually natural fertilisers are used and no chemical treatments are employed, thus the potential for the produce to become and remain contaminated with enteric pathogens is increased. It is also important to underline that in these sites the water used is untreated and, apart from irrigation, it is also used for human and animal consumption, what is a very common fact in several regions of Portugal. Only in sampling site 8 there was no *Aeromonas* spp. recovering, both from water and vegetable samples. This farm is a big

holding where periodical water analyses are made and all the factors that may affect production are controlled. In that way, one may speculate that for this reason there was no detection of *Aeromonas* species.

As reported by Figueras (2005) the clinical *Aeromonas* species most frequently isolated from humans are *A. hydrophila*, *A. caviae* and *A. veronii* biotype sobria which have been involved in a broad range of infections. Additionally, *A. media*, *A. jandaei*, *A. bestiarum*, *A. salmonicida*, *A. sobria*, *A. eucrenophila*, *A. schubertii*, *A. encheleia*, *A. allosaccharophila*, *A. trota* and *A. popoffii* were associated to human health problems (Figueras, 2005). *A. hydrophila* is considered a foodborne pathogen (WHO, 2007). In this study, apart from *Aeromonas* sp. Group II and *Aeromonas* sp. Group III, isolates recovered belonged to one of these phylogenetic lines (Table 4.2 and Fig. 4.6). In spite of the fact that the isolates here recovered were not proved yet to be virulent, it is a reality that potentially pathogenic individuals exist in the samples analysed. Moreover, human, produce and animals are in contact with those sources. On chapters 5 and 6 studies on the antibiotic resistance and the occurrence of virulence genetic determinants among isolates are presented.

The identification schemes used in the majority of the studies are phenotypic techniques that sometimes do not provide a reliable data on the occurrence and significance of *Aeromonas* different taxa on food, water or other sources. In fact, in several studies isolates were biochemically identified and affiliated to the phenospecies complexes *A. hydrophila*, *A. caviae* or *A. sobria* (Gray and Stickler, 1989; Palú *et al.* 2006; Castilho *et al.*, 2009) that comprises *A. hydrophila*, *A. bestiarum* and *A. salmonicida*, *A. caviae*, *A. media* and *A. eucrenophila*, *A. veronii* bv sobria, *A. jandaei*, *A. schubertii* and *A. trota*, respectively (Abbott *et al.*, 2003). Only Neyts and colleagues (2000) reported the isolation of *A. media* (HG5) and *A. salmonicida* (HG3) from vegetables and we could not find any other reports on the recovering of these species or of *A. eucrenophila* and *A. allosaccharophila* from this kind of samples. Also, *A. salmonicida* that is a recognised animal pathogen (Reith *et al.*, 2008) was not previously identified in animal faecal samples besides fish (Gustafson *et al.*, 1992), to the best of our knowledge. Also, for the first time and to our knowledge, members of the species *A. allosaccharophila* were recovered from vegetables. Until now their presence only had been reported in diseased elvers, human faeces, pig carcasses (Saavedra *et al.*, 2007), fish (Kozińska, 2007), lake and river water

(Picão *et al.*, 2008a; Krejčí *et al.*, 2009) and treated drinking water (Razzolini *et al.*, 2008). Our results reveal other possible ecological niches for these specimens. Actually, in this study we used molecular techniques that are more accurate and yielded a definitive identification of all isolates recovered, providing the real diversity present in samples analysed. Molecular techniques that provide reliable genotyping and phylogenetic affiliation should always be used in diversity studies as they are important tools to establish genetic relationships between isolates recovered from different sources and to assess their real diversity. Moreover, as *Aeromonas* is a very heterogeneous genus that arise controversies in different levels as taxonomy and pathogenicity, it is essential that studies concerning these microorganisms include this kind of procedures that afford trustworthy data.

Nonetheless, contrary to what was expected but similar to results obtained in chapter 3 of this dissertation, based on *gyrB* gene phylogenetic analysis the unique *A. sanarellii* strain clustered in the *A. caviae* grouping. The *rpoD* and MLPA have shown that this is a closely related species to *A. caviae*, still constituting an independent line within the genus (Alperi *et al.*, in press). In this study as also in the one presented in chapter 3 of this thesis, the *gyrB* gene did not enable the unquestionable discrimination of these two species, as has also been shown to happen with some strains of closely related species (Yánez *et al.*, 2003; Soler *et al.*, 2004). Even though, the authors did not give any information on the *gyrB* based relationships of this specimen and the remaining *Aeromonas* species (Alperi *et al.*, in press), precluding a comparison of results. Additionally, the description of this novel species was based in the characterisation of a single strain, a trend presenting potential pitfalls for taxonomic purposes and leading to biased results in organisms classification and taxonomic studies (Christensen *et al.*, 2001; Felis and Dellaglio, 2007; Janda and Abbott, 2010). In conclusion, these two distinct species might have clustered together due to bias introduced by their close phylogenetic relationship and use of a single strain of *A. sanarellii*. From this point of view, the affiliation of the *A. caviae* strain (representing 30 isolates) collected from milk and udder's cleaning water could be dubious, but as the clustering of this strain in different evolutionary trees is consistent with this affiliation (data not shown) we considered it as a member of the *A. caviae* phylogenetic group.

Regarding the new phylogenetic branches, the designated *Aeromonas* sp. Group II harbours eight isolates in which two display the same RAPD profile and are represented by A.L8-3 and six belong to the same genotype, different from the other one, represented by A.L10-4 (Fig. 4.6). Specimens of this phylogenetic branch were also detected in untreated and mineral waters (chapter 3). *Aeromonas* sp. Group III is composed by 12 isolates displaying the same genotype and is represented in the phylogenetic tree by A.L15-1. These isolates' *gyrB* gene significantly different molecular variant indicates that they may constitute representatives of two non-described species. Further studies are needed on these organisms in order to carry out a taxonomical purpose. On chapter 7 further information regarding these phylogenetic lines are discussed.

There is a common occurrence and substantial diversity of *Aeromonas* spp. in different agricultural sources in Portugal, from which, some are for animal and human consumption. In some cases, water may have been the contamination route of different sources.

5. Antibiotic resistance traits and integrons characterisation

Abstract

In this work, antibiotic resistance traits of a collection of 169 different *Aeromonas* strains characterised in previous chapters of this thesis were scrutinised. Antibiotic susceptibility profiles were established and strains were screened for the presence of *bla* and *tet* genes and also for class 1, class 2 and class 3 integrase genes, using PCR amplification with primers targeted for conserved sequences and hybridisation methodologies using as probes internal fragments of the genes. Further characterisation of *bla* genes and of the variable regions of integrons (often associated to resistance characteristics) was done by sequencing analysis.

As expected, the aeromonads collection was less susceptible to penicillins, the first generation cephalosporin and erythromycin. Few strains resistant to imipenem, tetracycline, aminoglycosides, fluoroquinolone and trimethoprim/sulfamethoxazole were identified. All were sensitive to third and fourth generation cephalosporins, aztreonam and few displayed decreased susceptibility to chloramphenicol. 44% of strains were resistant to five or more antibiotics. Thirty-six encoding sequences of CphA, one of VIM and TEM and 22 of *tet* (A), (E), (C) or (D) genes were found and no OXA-, SHV-, IMP-types or *tet* (B) were identified. Variable regions of 8 class 1 integrons and one class 2 integron were characterised; class 3 integron structures were not found. Multiresistant *Aeromonas* species carrying distinct genetic traits of antibiotic resistance were present in a wide range of sources which may be used for human or animal consumption. Taking into account that *Aeromonas* strains may be pathogenic, these specimens are not only putative disseminators of drug resistance throughout different chains but also potential causes of severe clinical complications and treatment failures.

Keywords: antibiotic resistance, integrons, beta-lactamases, tetracycline resistance determinants, untreated water, vegetables, faeces, milk

5.1 Introduction

An important threat to human or animal well being is the resistance to antimicrobial agents, an increasing problem due to the abusive use of antibiotics in medicine, aquaculture and livestock. Well known human or animal pathogens as well as bacteria usually present in natural environments harbour intrinsic or acquired mechanisms enabling them to evade the action of antibiotics (Henriques *et al.*, 2006a; Libisch *et al.*, 2008). Often, these mechanisms are encoded on mobile genetic elements as integrons, plasmids or transposons that contribute to the spread of antibiotic resistance genes (ARG) through different specimens and environments (Moura *et al.*, 2007; Barlow *et al.*, 2008). In fact, bacteria harbouring ARG may come into contact with humans and animals through different vehicles with water playing an important role as source of contamination (Zhang

et al., 2009). Given these reasons it is of paramount importance to investigate the panoply of antibiotic resistance traits residing in non clinical environments (Henriques *et al.*, 2006b; Zhang *et al.*, 2009).

Aeromonas species are present in a wide range of aquatic and clinical environments and also foodstuff (Palú *et al.*, 2006). They are well known for their role in numerous human and animal health complications worldwide. In fact, due to the increasing number of putatively virulent strains isolated and to the phenotypic characteristics of antibiotic resistance *Aeromonas* display, these organisms constitute an issue of concern in human and veterinary medicine (Lee *et al.*, 2008; Scoaris *et al.*, 2008; Ceylan *et al.*, 2009). Water and food are established as the main vectors of aeromonads transmission to humans and animals (Kämpfer *et al.*, 1999; Palú *et al.*, 2006; Jacobs and Chenia, 2007).

Antibiotic resistance traits of *Aeromonas* spp. were studied in different environments as estuarine (Henriques *et al.*, 2006a; Henriques *et al.*, 2008), rivers (Goñi-Urriza *et al.*, 2000a; Gordon *et al.*, 2008), wastewater treatment plants (Moura *et al.*, 2007; Rahman *et al.*, 2009), drinking water (Ghenghesh *et al.*, 2001; Koksal *et al.*, 2007; Scoaris *et al.*, 2008), aquacultures (Schmidt *et al.*, 2001b; Nawaz *et al.*, 2006), food (Palú *et al.*, 2006; Chang *et al.*, 2007) and clinical (Vila *et al.*, 2003; Lee *et al.*, 2008). These investigations reveal that among aeromonads it is possible not only to find resistance to different classes of antibiotics but also strains that display multiresistance phenotypes (Ko *et al.*, 1996; Akinbowale *et al.*, 2006; Koksal *et al.*, 2007). Higher resistance rates were noticed against β-lactams (penicillins and first generations cephalosporins) probably associated with the production of coordinately induced and chromosomally encoded beta-lactamases of distinct families they may harbour (Walsh *et al.*, 1997; Avison *et al.*, 2004). In fact, *bla* genes and several other ARG contributing for the multiresistance phenotypes reported were detected in aeromonads. These determinants were encoded in the chromosome and/or in mobile genetic elements (Henriques *et al.*, 2006a; Akinbowale *et al.*, 2007a; Moura *et al.*, 2007; Picão *et al.*, 2008a; Balsalobre *et al.*, 2009b). For example, aminoglycosides, chloramphenicol and trimethoprim resistance determinants were found especially in class 1 but also in class 2 integrons, some of them located in conjugative plasmids (Schmidt *et al.*, 2001b; Jacobs and Chenia, 2007; Moura *et al.*, 2007; Lee *et al.*, 2008). Tetracycline resistance genes were also largely detected among aeromonads (Nawaz *et al.*, 2006; Akinbowale *et al.*, 2007a), and may be encoded in the chromosome

(Schmidt *et al.*, 2001b; Henriques *et al.*, 2008) and/or in plasmids and transposons (Schmidt *et al.*, 2001b; Rhodes *et al.*, 2004; Agersø *et al.*, 2007). Several other resistance markers that pose serious concern in the medical field such as bla_{IMP}, bla_{VIM} and extended-spectrum beta-lactamases were found among aeromonads (Neuwirth *et al.*, 2007; Libisch *et al.*, 2008; Ye *et al.*, in press).

In this context and in order to anticipate the risks presented by aeromonads, it is essential to study *Aeromonas* spp. diversity, to identify their contamination routes and investigate the genetic determinants they possess that negatively impact humans and animals. The aim of this study was to scrutinise the antibiotic resistance phenotypic and genetic traits of *Aeromonas* strains collected from different sources in Portugal, which include untreated water (drinking and mineral water), agricultural sources (irrigation water and vegetables) and stable environment sources (cows' drinking water and faeces, udder's cleaning water and milk). In this way, a contribution to the risk assessment of animal and human populations in contact with these sources is given.

5.2 Material and methods

5.2.1 Bacterial isolates

In this study, 169 different *Aeromonas* strains characterised in Chapters 3 and 4 of this dissertation were included. For PCR and hybridisation studies concerning the detection of β-lactamases, positive controls were the same used by Henriques *et al.* (2006a), except that the positive control for *cphA/imiS* marker was *Aeromonas hydrophila* strain G.I10.28 (Henriques *et al.*, 2006a). To investigate the presence of tetracycline resistance determinants, positive controls were as in Henriques and colleagues (2008). Negative controls were used in all experiments. Control strains for integrase and integron screening and characterisation were as in Moura *et al.* (2007).

5.2.2 Antibiotic susceptibility testing

Antibiotic resistance profiles were established by the disc diffusion method in agar Mueller-Hinton (Merck, Germany) according to the CLSI guidelines. The antimicrobial agents tested were amoxicillin – AMX (10µg), amoxicillin/clavulanic acid – AMC (30µg), ticarcillin – TIC (75µg), ticarcillin/clavulanic acid – TIM (85µg), cefalotin – CEF (30µg),

ceftazidime – CAZ (30µg), cefepime – FEP (30µg), aztreonam – ATM (30µg), imipenem – IPM (10µg), gentamicin – GEN (10µg), kanamycin – KAN (30µg), tobramycin – TOB (10µg), netilmicin – NET (30µg), tetracycline – TET (30µg), chloramphenicol – CHL (30µg), erythromycin – ERY (15µg), trimethoprim/sulfamethoxazole – SxT (25µg) and ciprofloxacin – CIP (5µg) (Oxoid, UK). Streptomycin – STR (10µg) was tested in strains harbouring aminoglycosides resistance genes encoded in class 1 integrons.

Interpretation of results was done based on CLSI documents M45 for AMC, CEF, FEP, CAZ, IPM, ATM, GEN, TET, CIP, SxT and CHL and M100 for AMX, TIC, TIM, ERY, KAN, TOB and NET. *E. coli* strain ATCC25922 was used as quality control in antimicrobial susceptibility testing.

5.2.3 PCR screening for antibiotic resistance genes

The presence of DNA sequences presumptively representing β-lactamase encoding genes and tetracycline resistance genes was investigated by PCR in the 169 strains. Nucleotide composition of the primers, annealing temperatures and predicted amplicons sizes are listed in Table 5.1.

PCR reaction mixtures contained 1x PCR buffer (buffer with $(NH4)_2SO_4$), 3mM $MgCl_2$, 80µM dNTPs (Bioron, Germany), 0.3µM of each primer, 0.02U Taq polymerase (Fermentas Life Sciences, Lithuania) and 100 – 200ng of total DNA in a final volume of 25µl. For the screening of *bla* genes, PCR amplification was as described in Henriques *et al.* (2006a). PCR amplification of *tet* genes was as follows: initial denaturation (94ºC for 2 min), 29 cycles of denaturation (94ºC for 20 s), annealing (52ºC for 10 s) and extension (65ºC for 45 s) and one cycle of denaturation (94ºC for 20 s), annealing (52ºC for 10s) and extension (65ºC for 4 min). Positive and negative controls were included in all reactions. All PCR reactions were done in MyCyclerTM thermal cycler (Bio-Rad, USA). PCR products were separated by electrophoresis in a 1.5% agarose gel-TAE buffer stained with ethidium bromide and observed by UV transillumination.

Isolates displaying positive results for each PCR reaction and those who gave a negative result for the imipenem resistance determinants in study but displayed an imipenem resistance phenotype were further analysed by Southern blot hybridisation.

Table 5.1 Primers used in this study.

Primer pair	Target	Sequence (5' – 3')	Tan (ºC)[a]	Amplicon size (bp)	Reference
TEM_F	bla_{TEM}	AAA GAT GCT GAA GAT CA	44	425	Speldooren *et al.*, 1998
TEM_R		TTT GGT ATG GCT TCA TTC			
SHV_F	bla_{SHV}	GCG AAA GCC AGC TGT CGG GC	62	304	Henriques *et al.*,2006a
SHV_R		GAT TGG CGG CGC TGT TAT CGC			
OXA-A_F	bla_{OXA-A}	ACA CAA TAC ATA TCA ACT TCG C	53	814	Ouellette *et al.*, 1987
OXA-A_R		AGT GTG TTT AGA ATG GTG ATC			
OXA-B_F	bla_{OXA-B}	CAA GCC AAA GGC ACG ATA GTT G	56	561	Henriques *et al.*,2006b
OXA-B_R		CTC AAC CCA TCC TAC CCA CC			
OXA-C_F	bla_{OXA-C}	CGT GCT TTG TAA AAG TAG CAG	53	652	Huovinen *et al.*, 1988
OXA-C_R		CAT GAT TTT GGT GGG AAT GG			
AER_F	*cphA/imiS*	GCC TTG ATC AGC GCT TCG TAG TG	60	670	Henriques *et al.*,2006a
AER_R		GCG GGG ATG TCG CTG ACG CAG			
IMP_F	bla_{IMP}	GAA TAG AGT GGA TTA ATT CTC	55	232	Henriques *et al.*,2006b
IMP_R		GGT TTA AYA AAA CAA CCA CC			
VIM_F	bla_{VIM}	GAT GGT GTT TGG TCG CAT ATC G	58	475	Henriques *et al.*,2006a
VIM_R		GCC ACG TTC CCC GCA GAC G			
TetA_F	*tet* (A)	GCT ACA TCC TGC TTG CCT TC	52	211	Nawaz *et al.*, 2006
TetA_R		GCA TAG ATC GCC GTG AAG AG			
TetB_F	*tet* (B)	TCA TTG CCG ATA CCA CCT CAG	52	391	Nawaz *et al.*, 2006
TetB_R		CCA ACC ATC ATG CTA TTC CAT CC			
TetC_F	*tet* (C)	CTG CTC GCT TCG CTA CTT G	52	897	Nawaz *et al.*, 2006
TetC_R		GCC TAC AAT CCA TGC CAA CC			
TetD_F	*tet* (D)	TGT GCT GTG GAT GTT GTA TCT C	52	844	Nawaz *et al.*, 2006
TetD_R		CAG TGC CGT GCC AAT CAG			
TetE_F	*tet* (E)	ATG AAC CGC ACT GTG ATG ATG	52	744	Nawaz *et al.*, 2006
TetE_R		ACC GAC CAT TAC GCC ATC C			
intI1F	*intI1*	CCT CCC GCA CGA TGA TC	55	280	Kraft *et al.*, 1986
intI1R		TCC ACG CAT CGT CAG GC			
intI2F	*intI2*	TTA TTG CTG GGA TTA GGC	52	233	Goldstein *et al.,* 2001
intI2R		ACG GCT ACC CTC TGT TAT C			
intI3F	*intI3*	AGT GGG TGG CGA ATG AGT G	50	600	Goldstein *et al.,* 2001
intI3R		TGT TCT TGT ATC GGC AGG TG			
5'-CS	Class 1 integron variable region	GGC ATC CAA GCA GCA AG	58.5	Variable	Lévesque *et al.,* 1995
3'-CS		AAG CAG ACT TGA CCT GA			
hep74	Class 2 integron variable region	CGGGATCCCGGACGGCATGCACGATTTGTA	60	Variable	White *et al.*, 2001
hep51		GAT GCC ATC GCA AGT ACG AG			
A2-4F	*aadA1*	CCA AAT CTG GCA AAA GGG T	55		This study

[a] Annealing temperature

5.2.4 Southern blot hybridisation

Amplicons obtained using primers targeting antibiotic resistance genes were confirmed by Southern blot hybridisation as described by Henriques *et al.* (2006b). Briefly, PCR products were ran in 1.5% agarose gel, vacuum transferred to positively charged nylon membranes (Hybond N+; Amersham, Germany) for 150min and cross-linked under UV irradiation for 5min. Positive and negative controls were included on each gel. DNA from strains used as positive controls was used as template to obtain digoxigenin-labelled specific probes for each gene. PCR reactions were performed as described above, using DIG-DNA Labelling Mix (Roche Molecular Biochemicals, USA) instead of DNTPs. Hybridisation occurred overnight under stringent conditions (42ºC in 50% formamide buffer) and detection was performed using DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals, USA) following manufacturer's instructions.

Genomic DNA from strains displaying imipenem resistance and negative results in PCR detection of *cphA*/*imiS*, bla_{VIM} and bla_{IMP} was digested with restriction enzyme *Sal*I (Fermentas Life Sciences, Lithuania). Digestion products were separated by agarose gel electrophoresis, transferred to nylon membranes and hybridised to the *cphA*/*imiS* probe as described above.

5.2.5 Sequencing analysis

Amplicons obtained using primers targeting *bla* genes were confirmed by DNA sequencing. For that they were purified with JETQUICK PCR purification spin kit (Genomed, Germany) and sequenced with the primers used in PCR reactions. Sequence similarity searches were done with the BLAST program (http://www.ncbi.nlm.nih.gov/). Nucleotide sequences were translated to amino acid sequences with the ExPASy translate tool available online (http://us.expasy.org/tools/dna.html). Sequence alignments were done with the CLUSTALX program (Larkin *et al.*, 2007).

5.2.6 Integrase screening by dot-blot hybridisation

The strains were screened for the presence of class 1, class 2 and class 3 integrase genes by dot-blot hybridisation. Genomic DNA of integrase positive controls was used as template in PCR reactions with primers listed in Table 5.1 to obtain DIG labelled specific probes for each *intI* gene, as described above. Strains were cultured in Tryptic Soy Agar

(Merck, Germany) and cell suspensions of each strain were individually prepared by boiling a fresh colony in 20µl of distilled water for 15min at 100ºC. After denaturation with 0.5M of NaOH at 50ºC for 5min, suspensions were equilibrated in 20X SSC. The DNA was transferred onto positively charged nylon membranes (Hybond N+; Amersham, Germany) and subsequent hybridisation and detection of positive signals was as described in Moura *et al.* (2007). Positive and negative controls were used in all procedures to ensure the specificity of hybridisation. Strains positive by dot-blot hybridisation were subsequently confirmed by PCR amplification.

5.2.7 Integron characterisation

Characterisation of integrons was done by sequence analysis of their variable regions. Therefore, genomic DNA of confirmed *intI1* positive isolates was submitted to PCR with primer sets targeting integrons variable regions (Table 5.1), as described elsewhere (Moura *et al.*, 2007). The PCR amplification of class 2 integron variable region was done using the Extensor HI-Fidelity PCR Master Mix (ABgene, United Kingdom) and amplification was as follows: initial denaturation (94ºC for 5min), 35 cycles of denaturation (95 ºC for 30s), annealing (55ºC for 30s) and extension (68ºC for 3min) and a final extension (68ºC for 10min). The band with the expected size was extracted from the agarose gel using the Silica Bead DNA extraction KIT (Fermentas Life Sciences, Lithuania) according to the manufacturer instructions.

PCR products and gel excised band were subsequently purified as described above and sequenced in both strands with the primers used in PCR amplification. For the variable region of the class 2 integron, primer walking using primer A2-4F was additionally applied (Table 5.1). Sequence similarity searches were done within the INTEGRALL database (http://integrall.bio.ua.pt/) and the BLAST program.

5.3 Results

5.3.1 Antibiotic resistance profiles

For all strains, patterns of resistance against 18 antibiotics belonging to different groups were established. β-lactams included penicillins (aminopenicillins and carboxypenicillins), cephalosporins (1st, 3rd and 4th generation), a monobactam and a

carbapenem. Susceptibility to aminoglycosides, tetracyclines, fluoroquinolones, macrolides, chloramphenicol and the combination trimethoprim/sulfamethoxazole was also tested. Susceptibility to streptomycin was investigated in strains harbouring class 1 integrons with *aadA* gene cassettes.

Resistance to at least two antimicrobial agents was recorded for 98% of strains and 44% of strains was multiresistant (resistant to five or more antibiotics). Amoxicillin was the less effective antimicrobial agent as 97.6% of the strains were resistant, followed by cefalotin (89.9%) and ticarcillin (79.9%) and no strains susceptible to erythromycin were collected. All strains were sensitive to ceftazidime, cefepime and aztreonam. Figure 5.1 displays the antibiotic susceptibility profile of the aeromonads collection and Figure 5.2 shows the profiles obtained for strains from the different environments.

Four strains displayed a remarkable resistance phenotype as they were resistant to 9 or 8 antimicrobial agents and additionally had reduced susceptibility to one, two or three different antibiotics: strains A.096A (*A. veronii*), A.131/2 (*Aeromonas* sp. Group I) and A.131/8 (*A. hydrophila*) collected from drinking water samples and strain A.V2-5 (*A. salmonicida*) collected from a cow's faeces sample (Table 5.2).

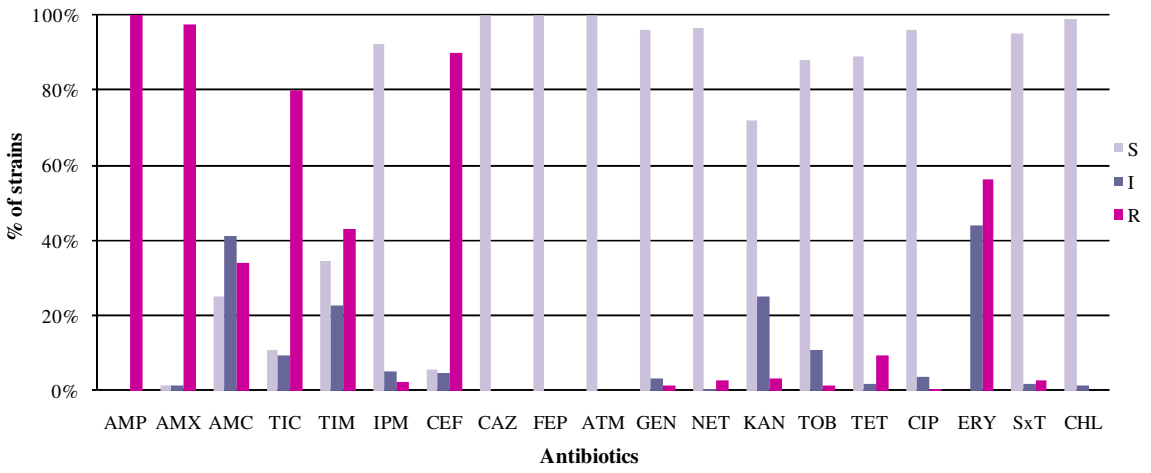

Figure 5.1 Antimicrobial susceptibility profile of the aeromonads collection.

Legend to figure:
S, susceptible; I, intermediary resistance profile; R, resistant. Vertical axis values refer to percentage of strains S, I, R to each antibiotic (horizontal axis).

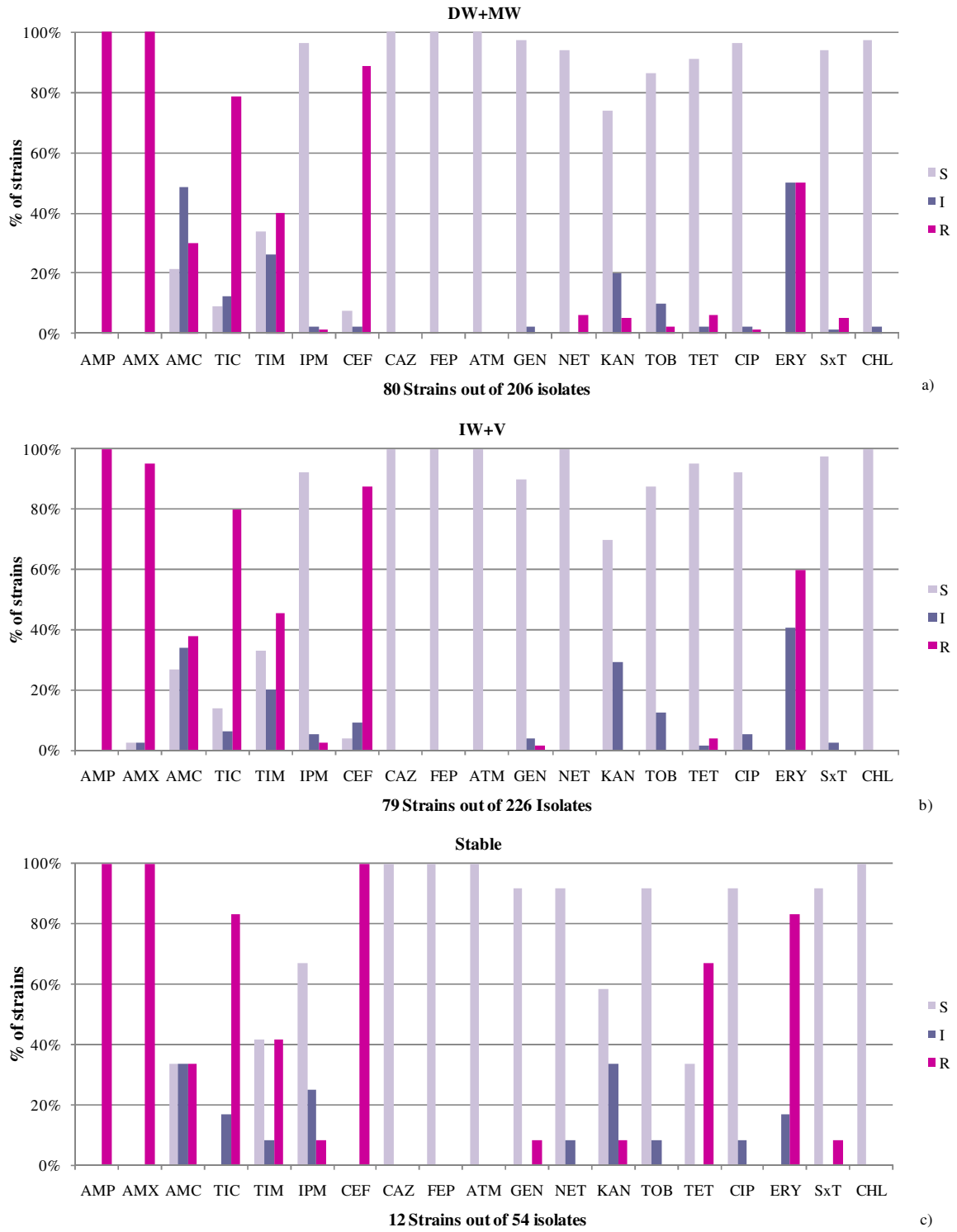

Figure 5.2 Antibiotic susceptibility profiles of *Aeromonas* strains collected from each of the different environments studied.

Legend to figure:
Number of collected strains/isolates from each environment is indicated.
a) Untreated waters (DW, drinking water; MW, water). b) Agricultural sources (IW, irrigation water; V, vegetables). c) Stable environment (Cows drinking water and faeces; udders cleaning water and milk). S, susceptible; I, intermediary resistance phenotype; R, resistant. Vertical axis values refer to percentage of strains S, I, R to each antibiotic (horizontal axis).

5.3.2 Genetic bases of antibiotic resistance

The presence of sequences of β-lactamase genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA}$, *cphA*/*imiS*, *bla*$_{IMP}$ and *bla*$_{VIM}$) and tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D) and *tet*(E) was analysed for all strains by PCR. Positive results were confirmed by southern-blot hybridisation with DIG-labelled probes specific for each gene. Figures 5.3 and 5.4 present examples of typical results obtained by PCR amplification with different primer pairs and confirmation by hybridisation. Table 5.2 summarizes the phenotypic and genotypic results. In table 5.3 the ARG profiles distributed by phylogenetic group are presented and table 5.4 depicts the ARG profiles in relation to the type of source.

The antibiotic resistance genetic determinants studied were present in 49 strains. Thirty-seven strains (22%) carried one of the *bla* genes tested. The most occurring one was *cphA*/*imiS* (36 strains), followed by *bla*$_{TEM}$ (one strain) and *bla*$_{VIM}$ (one strain). Only *A. hydrophila* strain A.A9-11 carried more than one *bla* gene simultaneously (*cphA*/*imiS* and *bla*$_{VIM}$). There was no evidence for the presence of genes encoding OXA, SHV and IMP β-lactamases.

Three strains (A.136/26, A.A7-6 and A.V2-4) were resistant to imipenem but apparently did not display any of the genetic determinants of imipenem resistance studied by PCR. Southern blots of genomic DNA from these strains hydrolysed with *Sal*I were hybridised to a *cphA*/*imiS* probe. All gave a positive result, indicating that they harbour sequences representing *cphA*/*imiS* – like genes (Fig. 5.3b).

For strain A.A9-11, a weak amplification product was obtained using primers targeting the *bla*$_{VIM}$ gene and the same happened with the hybridisation signal (Fig. 5.3d). Although several attempts were made and different strategies were used, it was not possible to obtain a PCR product in sufficient amount for sequencing.

Concerning *tet* genes, 22 strains (13%) carried one of the determinants studied. *tet* (E) was the predominant tetracycline resistance gene, being detected in 12 strains (7%). *tet* (A) was present in eight strains (4.7%) and *tet* (C) and *tet* (D) were identified in one strain each (0.6%). *tet* (B) was not found among the bacterial strains. Also, there was no detection of different *tet* genes in the same strain.

In 10 cases *bla* and *tet* genes were present in the same strain. *cphA*/*imiS* and *tet* (A) were found in four strains from drinking water and vegetables (*A. hydrophila* strains A.023V and A.L12-10, *A. bestiarum* strain A.L11-1, and *A. salmonicida* strain A.L14-1),

bla$_{TEM}$ and *tet* (A) were detected in *A. veronii* strain A.096A collected from drinking water and *cphA*/*imiS* and *tet* (E) were present in five strains of drinking water and cows' faeces (*A. hydrophila* strains A.114C1, A.131/8, and A.133/3, *A. eucrenophila* strain A.135/6 and *A. salmonicida* strain A.V2-4).

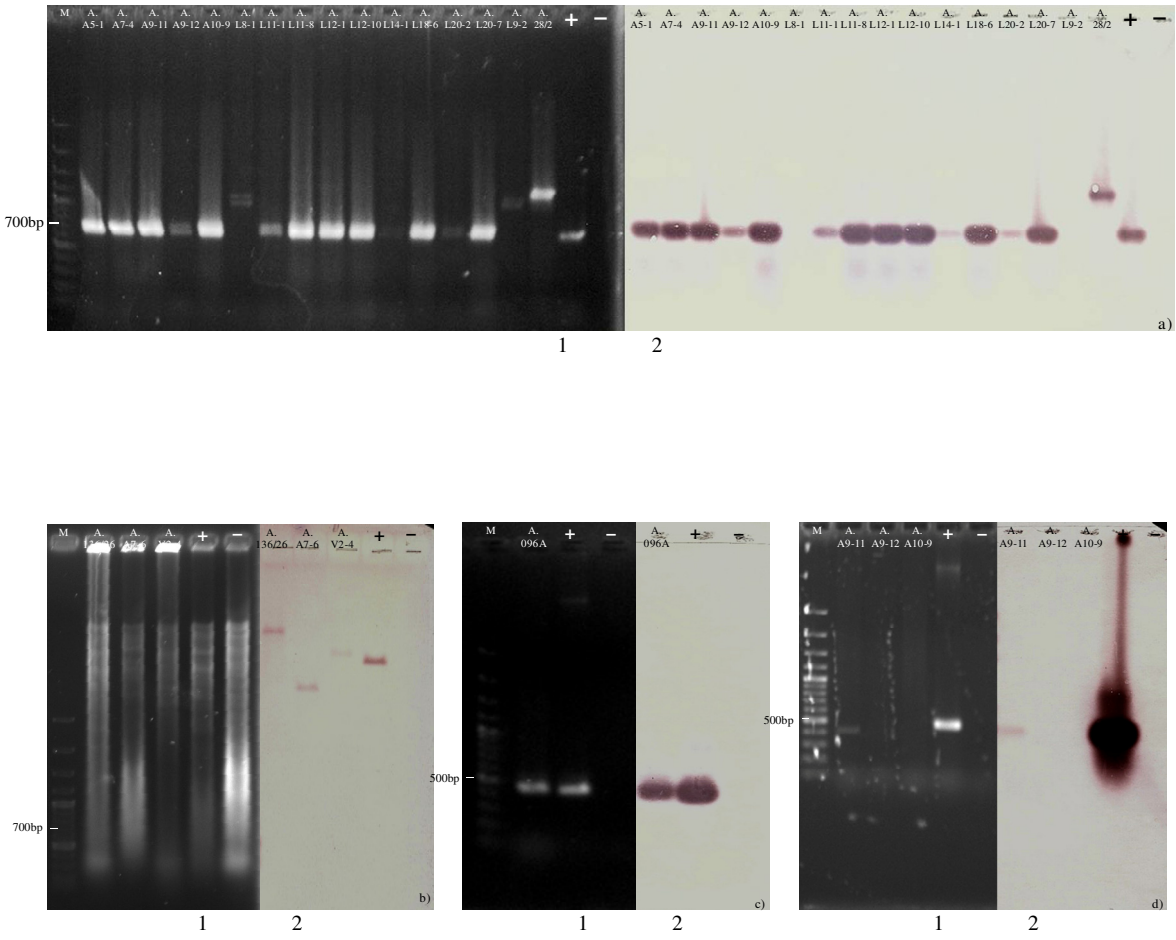

Figure 5.3 Examples of results obtained in PCR (1) and Southern-blot hybridisation (2) screening of *bla* genes.

Legend to figure:
a) *cphA*/*imiS* screening; b) restriction analyses with *Sal*I and subsequently hybridisation with *cphA*/*imiS* probe; c) *bla*$_{TEM}$ screening; d) *bla*$_{VIM}$ screening.
M, Gene Ruler^{TM} 100bp Plus DNA Ladder (Fermentas Life Sciences, Lithuania). + and – indicate appropriate positive and negative controls for each assay, respectively.

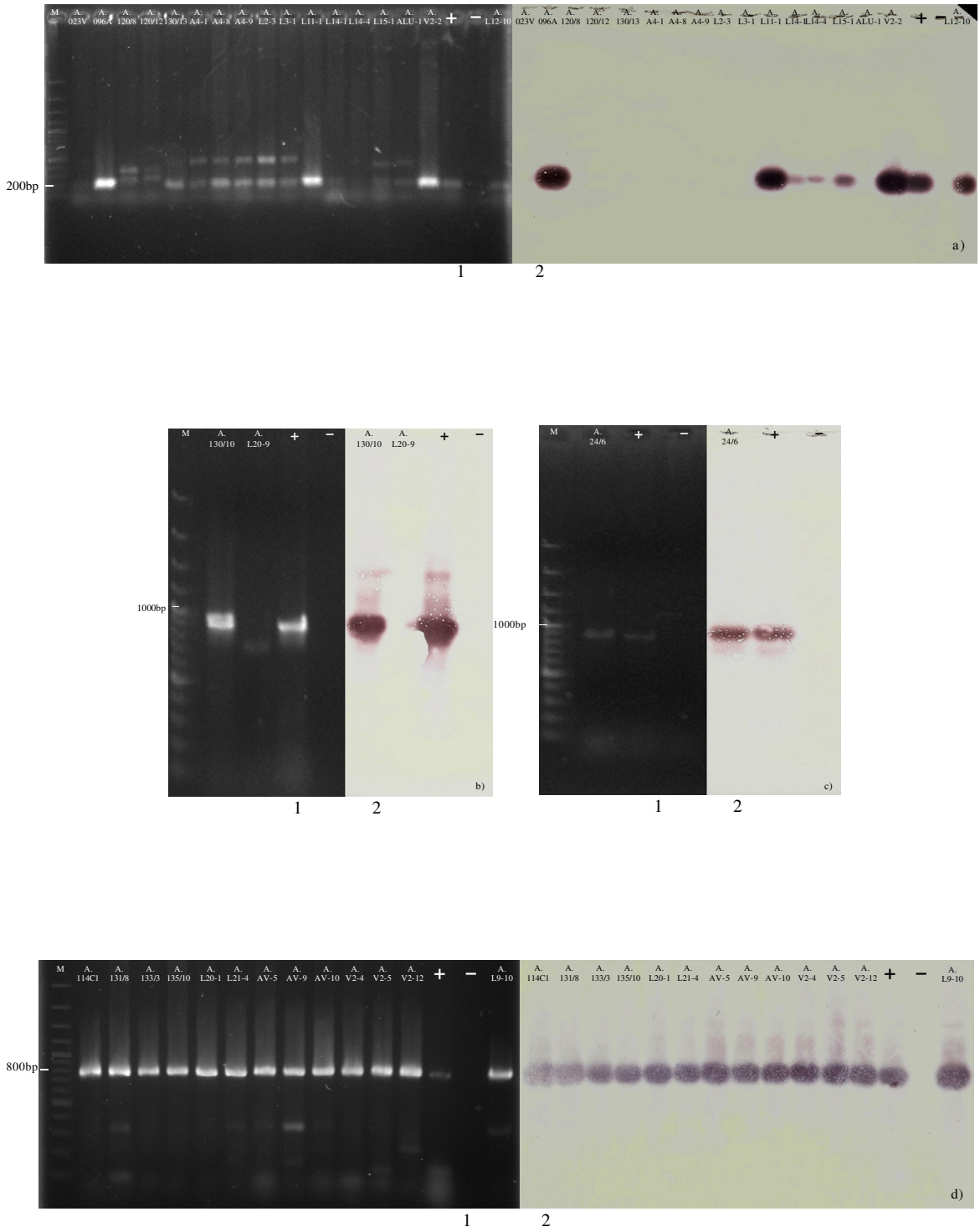

Figure 5.4 Examples of results obtained in PCR (1) and Southern-blot hybridisation (2) screening of tetracycline resistance genes.

Legend to figure:
Screening of: a) *tet*(A); b) *tet*(C); c) t*et*(D); d) *tet*(E).
M, Gene Ruler^{TM} 100bp Plus DNA Ladder (Fermentas Life Sciences, Lithuania). + and – indicate appropriate positive and negative controls for each assay, respectively.

Table 5.2 Characterisation of *Aeromonas* sp. strains collected from different sources in Portugal in what concerns isolation source, resistance phenotype, *bla* and *tet* genes content and integron characterisation.

Strain	Phylogenetic affiliation	Source[a]	N[b]	Resistance Phenotype	*bla* genes content			*tet* genes content				*intI*	Gene cassette array
					TEM	VIM	*cphA*	A	C	D	E		
A.28/2	*A. allosaccharophila*	MW	1	AMX	-	-	+	-	-	-	-	-	-
A.24/6	*A.* sp. HG11	MW	1	AMX, TIC, TIM, CEF	-	-	-	-	-	+	-	-	-
A.135/1	*A. bestiarum*	DW	3	AMX, TIC, CEF, ERY	-	-	+	-	-	-	-	-	-
A.130/4	*A. caviae*	DW	2	AMX, TIC, CEF, STR	-	-	-	-	-	-	-	I	*aadA2*
A.135/2	*A. eucrenophila*	DW	2	AMX, TIC, CEF	-	-	+	-	-	-	-	-	-
A.135/6	*A. eucrenophila*	DW	1	AMX, TIC, CEF, KAN, ERY	-	-	+	-	-	-	+	I	*dfrA12 - orfF - aadA2*
A.133/2	*A. media*	DW	1	AMX, TIC, TIM, CEF, STR	-	-	-	-	-	-	-	I	*aadA2*
A.A2/4	*A. media*	DW	3	AMX, TIM, CEF, ERY	-	-	-	-	-	-	-	II	*dfrA1-sat2-aadA1*
A.136/26	*A. salmonicida*	DW	12	AMX, AMC, TIC, TIM, IPM, CEF	-	-	+	-	-	-	-	-	-
A.096A	*A. veronii*	DW	10	AMX, AMC, TIC, TIM, TET, CIP, ERY, SxT, STR	+	-	-	+	-	-	-	I	*dfrA12 - orfF - aadA2*
A.098A	*A. veronii*	DW	1	AMX, AMC TIC, TIM	-	-	+	-	-	-	-	-	-
A.135/10	*A. veronii*	DW	2	AMX, TIC, CEF	-	-	+	-	-	-	-	-	-
A.131/2[c]	*Aeromonas* sp. Group I	DW	2	AMX, AMC, TIC, CEF, KAN, TET, ERY, SxT	-	-	-	-	-	-	-	-	-
A.023V	*A. hydrophila*	DW	1	AMX, TIC, TIM, CEF	-	-	+	+	-	-	-	-	-
A.110B1	*A. hydrophila*	DW	1	AMX, TIC, CEF, ERY	-	-	+	-	-	-	-	-	-
A.110C2	*A. hydrophila*	DW	2	AMX, AMC, TIC, TIM, CEF, ERY	-	-	+	-	-	-	-	-	-
A.114C1	*A. hydrophila*	DW	5	AMX, CEF, TET	-	-	+	-	-	-	+	-	-
A.130/9	*A. hydrophila*	DW	1	AMX, TIC, TIM, CEF, ERY	-	-	+	-	-	-	-	-	-
A.130/10	*A. hydrophila*	DW	1	AMX, TIC, CEF, STR	-	-	-	-	+	-	-	I	*catB8 - aadA1*
A.131/8	*A. hydrophila*	DW	2	AMX, TIC, CEF, KAN, TET, ERY, SxT, STR	-	-	+	-	-	-	+	I	*dfrA12 - orfF - aadA2*
A.133/3	*A. hydrophila*	DW	2	AMX, TIC, CEF, TET, ERY, SxT	-	-	+	-	-	-	+	I	*dfrA12 - orfF - aadA2*
A.133/5	*A. hydrophila*	DW	1	AMX, TIC	-	-	+	-	-	-	-	-	-
A.135/5	*A. hydrophila*	DW	1	AMX, CEF, ERY	-	-	+	-	-	-	-	-	-
A.136/12	*A. hydrophila*	DW	1	AMX, TIC, TIM, CEF, ERY	-	-	+	-	-	-	-	-	-

Table 5.2 Characterisation of *Aeromonas* sp. strains collected from different sources in Portugal in what concerns isolation source, resistance phenotype, *bla* and *tet* genes content and integron characterisation (continued).

Strain	Phylogenetic affiliation	Source[a]	N[b]	Resistance Phenotype	*bla* genes content			*tet* genes content				*intI*	Gene cassette array
					TEM	VIM	*cphA*	A	C	D	E		
A.136/13	*A. hydrophila*	DW	1	AMX, AMC, TIC, CEF, ERY	-	-	+	-	-	-	-	-	-
A.137/2	*A. hydrophila*	DW	3	AMX, CEF, ERY	-	-	+	-	-	-	-	-	-
A.A2/6	*A. hydrophila*	DW	1	AMX, AMC, TIC, TIM, CEF, ERY	-	-	+	-	-	-	-	-	-
A.126	*A. hydrophila*	DW; IW	9	AMX, ERY	-	-	+	-	-	-	-	-	-
A.A5/1	*A. hydrophila*	DW; IW	16	AMX, AMC, TIC, TIM, CEF, ERY	-	-	+	-	-	-	-	-	-
A.A7-6	*A. bestiarum*	IW	3	AMX, AMC, TIC, IPM, CEF, ERY	-	-	+	-	-	-	-	-	-
A.A7-4	*A. hydrophila*	IW	1	AMX, AMC, TIC, TIM, CEF, ERY	-	-	+	-	-	-	-	-	-
A.A9-11	*A. hydrophila*	IW	1	AMX, AMC, TIC, TIM, CEF, ERY	-	+	+	-	-	-	-	-	-
A.A9-12	*A. hydrophila*	IW	1	AMX, AMC, TIC, CEF, ERY	-	-	+	-	-	-	-	-	-
A.A10-9	*A. hydrophila*	IW	1	AMX, TIC, CEF, ERY	-	-	+	-	-	-	-	-	-
A.L20-1	*A. allosaccharophila*	V	2	AMX, CEF, TET	-	-	-	-	-	-	+	-	-
A.L20-7	*A. allosaccharophila*	V	1	AMX, CEF	-	-	+	-	-	-	-	-	-
A.L11-1	*A. bestiarum*	V	5	AMX, TIC, CEF, TET	-	-	+	+	-	-	-	-	-
A.L11-8	*A. hydrophila*	V	5	AMX, ERY	-	-	+	-	-	-	-	-	-
A.L12-1	*A. hydrophila*	V	5	AMX, AMC, TIC, CEF, ERY	-	-	+	-	-	-	-	-	-
A.L12-10	*A. hydrophila*	V	1	AMX, TIC, CEF, ERY	-	-	+	+	-	-	-	-	-
A.L18-6	*A. hydrophila*	V	2	AMX, TIC, CEF, ERY	-	-	+	-	-	-	-	-	-
A.L21-4	*A. media*	V	9	AMX, TIC, TIM, TET, ERY	-	-	-	-	-	-	+	-	-
A.L14-1	*A. salmonicida*	V	9	AMX, TIC, IPM, CEF	-	-	+	+	-	-	-	-	-
A.L14-4	*A. salmonicida*	V	3	AMX, TIC, CEF	-	-	-	+	-	-	-	-	-
A.L20-2	*A. salmonicida*	V	5	CEF	-	-	+	-	-	-	-	-	-
A.L15-1	*A.* sp. Group III	V	12	CEF, ERY	-	-	-	+	-	-	-	-	-
A.V2-2	*A. salmonicida*	CF	3	AMX, TIC, CEF, TET, ERY, STR	-	-	-	+	-	-	-	I	*aadA2*
A.V2-4	*A. salmonicida*	CF	2	AMX, IPM, CEF, TET, ERY	-	-	+	-	-	-	+	-	-

Table 5.2 Characterisation of *Aeromonas* sp. strains collected from different sources in Portugal in what concerns isolation source, resistance phenotype, *bla* and *tet* genes content and integron characterisation (continued).

Strain	Phylogenetic affiliation	Source[a]	N[b]	Resistance Phenotype	*bla* genes content			*tet* genes content				*intI*	Gene cassette array
					TEM	VIM	*cphA*	A	C	D	E		
A.V2-5	*A. salmonicida*	CF	1	AMX, TIC, CEF, GEN, KAN, TET, ERY, SXT	-	-	-	-	-	-	+	-	-
A.V2-12	*A .salmonicida*	CF	1	AMX, TIC, CEF, TET, ERY	-	-	-	-	-	-	+	-	-
A.AV-5	*A. media*	CW	2	AMX, AMC, TIC, TIM, CEF, TET, ERY	-	-	-	-	-	-	+	-	-
A.AV-9	*A. media*	CW	1	AMX, AMC, TIC, TIM, CEF, TET, ERY	-	-	-	-	-	-	+	-	-
A.AV-10	*A. media*	CW	1	AMX, AMC, TIC, TIM, CEF, TET	-	-	-	-	-	-	+	-	-

Strains positive for one of the genetic resistance traits studied or a remarkable resistance phenotype were included in this table.
[a] CF, cow faeces; CW, cows drinking water; MW, untreated mineral water; DW, untreated drinking water; IW, untreated irrigation water; V, vegetables.
[b] Number of isolates collected of each strain.
[c] None of the investigated antibiotic resistance genetic trait was detected in this strain, although it displayed a noteworthy resistance phenotype.

Table 5.3 Combinations of antibiotic resistance genes among the different *Aeromonas* species recovered strains.

ARG patterns[a] / Species[b]	*tet*(A)	*tet*(A) + *cphA/imiS*	*tet*(A) + bla_{TEM}	*tet*(C)	*tet*(D)	*tet*(E)	*tet*(E) + *cphA/imiS*	*cphA/imiS*	bla_{VIM} + *cphA/imiS*
A. media (65)	-	-	-	-	-	4	-	-	-
A. hydrophila (30)	-	2	-	1	-	-	3	17	1
A. eucrenophila (20)	-	-	-	-	-	-	1	1	-
A. salmonicida (14)	2	1	-	-	-	2	1	2	-
A. bestiarum (13)	-	1	-	-	-	-	-	2	-
A. veronii (5)	-	-	1	-	-	-	-	2	-
Aeromonas sp. Group II (5)	-	-	-	-	-	-	-	-	-
A. allosaccharophila (5)	-	-	-	-	-	1	-	2	-
A. caviae (4)	-	-	-	-	-	-	-	-	-
A. tecta (2)	-	-	-	-	-	-	-	-	-
A. sp. HG11 (2)	-	-	-	-	1	-	-	-	-
A. popoffii (1)	-	-	-	-	-	-	-	-	-
A. encheleia (1)	-	-	-	-	-	-	-	-	-
Aeromonas sp. Group I (1)	-	-	-	-	-	-	-	-	-
Aeromonas sp. Group III (1)	1	-	-	-	-	-	-	-	-

Table 5.3 (continued)
The number of strains of each phylogenetic group displaying each pattern is shown.
[a] Combinations of antibiotic resistance genes detected.
[b] In brackets, the number of strains collected from each phylogenetic group.

Table 5.4 Combinations of ARG present in strains collected from the different sources scrutinised.

ARG[a] combinations	Sources (N.)[b]		
	UnW (80)	AS (79)	SE (12)
tet (A)	-	2	1
tet (A) + *cphA*/*imiS*	1	3	-
tet (A) + bla_{TEM}	1	-	-
tet (C)	1	-	-
tet (D)	1	-	-
tet (E)	-	2	5
tet (E) + *cphA*/*imiS*	4	-	1
cphA/*imiS*	17	11	-
bla_{VIM} + *cphA*/*imiS*	-	1	-

[a] ARG, Antibiotic resistance genes.
[b] Types of sources scrutinised and number of strains isolated from each source (in brackets). UnW, untreated water sources (namely, untreated drinking and mineral waters samples); AS, agricultural sources (including vegetables and untreated irrigation waters samples); SE, stable environment sources (specifically, cow's drinking water and faeces and udder's cleaning water before milking and milk).

5.3.3 cphA/imiS sequencing analysis

As mentioned before, a total of 36 strains gave positive signals in PCR and/or hybridisation experiments with *cphA*/*imiS* specific primers and/or probes. From 31 strains a *cphA*/*imiS* fragment was amplified and sequenced.

Altogether, 25 different sequence types were obtained that resulted in 25 different amino acid deduced sequences. Similarity between nucleotide sequences varied from 90% and 100%, while similarities within amino acid deduced sequences ranged between 92% and 100%. At the nucleotide level, none of the sequences shared 100% similarity with previously reported *cphA*/*imiS* present in the GenBank database. At the amino acid level, one deduced sequence shared 100% similarity with a known CphA β-lactamase (accession number CAD69003), while the remaining sequences were divergent, exhibiting silent mutations or amino acid substitutions never reported before.

In five cases the amplification products displayed a size different from the expected (~1kb); that happened with *A. hydrophila* strains A.131/8 and A.135/5, *A. media* strains A.L8-1 and A.L9-2 and *A. allosaccharophila* strain A.28/2. All these strains were susceptible to imipenem.

The nucleotide sequences of the amplicons obtained from strains *A. hydrophila* A.131/8 and A.135/5 and from *A. allosaccharophila* A.28/2 were determined and analysed with the following results:

i) For *A. allosaccharophila* A.28/2 amplicon, a sequence of 1098bp was obtained. The first 175bp displayed 96% of similarity with genes coding for tranposases of IS elements of transposon present in plasmid pFBAOT6 of *A. caviae* (accession number CR376602) and 84% of similarity with genes coding for tranposases of IS elements inserted in plasmids pAsa6 of *A. salmonicida* (accession number AM258965) and pAsa5 of *A. salmonicida* A449 (accession number CP000646). The sequence between nucleotide 176 and 1098 demonstrated 96% of sequence similarity to *cphA4* of *A. allosaccharophila* (accession number AY227050) and 94% to *cephA3* gene of *A. veronii* bv. sobria (accession number AY112998). Figure 5.5 displays a schematic view of the sequence.

ii) For strains A.131/8 and A.135/5, the amplified fragments had a length of 895bp and shared the same sequence. Similarity searches showed that the sequence between nucleotides 292 and 895 displayed 96% similarity with *cphA* of *A. hydrophila* (accession number X57102). For the first 291bp no significant similarity was found using the "highly similar sequences (megablast)" tool of BLAST program. Using the "somewhat similar sequences (blastn)" tool, 76.8% of similarity was found with 271bp out of 801bp of the *Pseudomonas mendocina* flagellar assembly (accession number CP000680) among other distinct genes. Figure 5.5 exhibits a schematic view of the coding capabilities supported by the nucleotide sequences of the amplicon obtained from these strains.

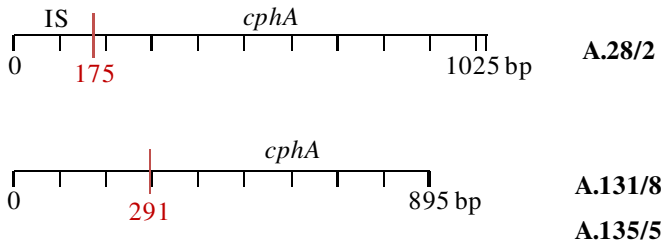

Figure 5.5 Schematic views of the nucleotide sequences of the amplicons obtained from strains A.28/2, A.131/8 and A135/5 in the PCR assay with primers AER_F and AER_R.

5.3.4 Integrase screening and integrons characterisation

In order to assess the existence of class 1, class 2 and class 3 integrons among our *Aeromonas* collection, strains were screened by dot-blot hybridisation for the corresponding integrase coding genes (*intI1*, *intI2*, *intI3*). Further confirmation of positive

results was achieved by PCR using specific primers. As outlined in Table 5.2, *intI1* gene was detected in eight strains (4.7%) and *intI2* gene was found in one strain (0.6%). *intI3*-carrying bacteria were not detected as neither more than one integrase coding gene in the same strain.

Characterisation of nine integrons was done by PCR amplification and subsequent sequence analyses of the variable regions using specific primers. DNA inserted in the variable regions of the eight class 1 integrons was amplified and ranged between ~1kb and 1.9kb. For the single class 2 integron detected, the variable region was approximately ~ 2kb in length (Fig. 5.6).

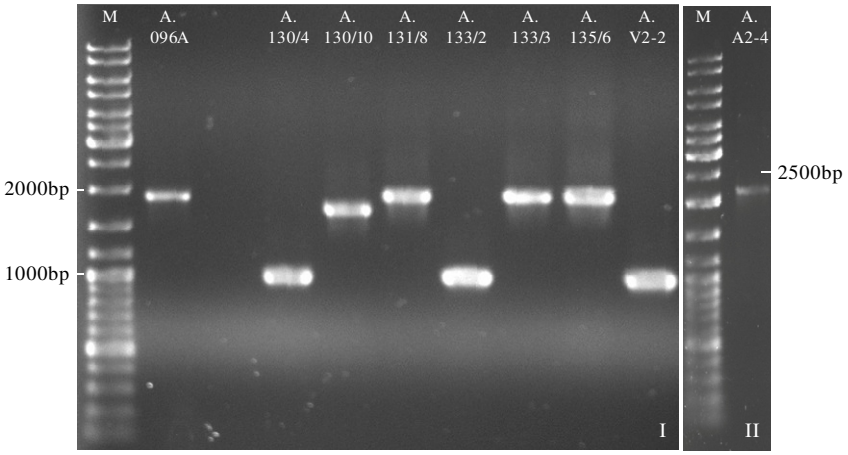

Figure 5.6 PCR products obtained from the amplification of integrons variable regions.
Legend to figure:
I, variable regions of class 1 integrons; II, variable region of the class 2 integron. M, Gene Ruler^{TM} DNA Ladder Mix (Fermentas Life Sciences, Lithuania).

Sequencing of both strands of the PCR products obtained from the variable region of class 1 integron confirmed the presence of three different arrays and five different gene cassettes (Table 5.2). The distinct cassette arrangements were: *dfrA12 – orfF – aadA2* (four strains); *aadA2* (three strains); *catB8 – aadA1* (one strain). The most occurring gene cassette was *aadA2* (seven times), coding for aminoglycosides adenyltransferase that confers resistance to streptomycin and spectinomycin; *dfrA12*, encoding dihydrofolate reductase which confers resistance to trimethoprim, and *orfF*, encoding a protein with unknown function, were both detected four times; finally, *aadA1*, with similar functions of *aadA2*, and *catB8* (coding for chloramphenicol acetyltransferase that confers resistance to chloramphenicol) were present in one cassette array.

Sequencing of the genetic content of the class 2 integron variable region allowed the identification of an array of three gene cassettes: *dfrA1 – sat2 – aadA1*. *dfrA1* encodes

a dihydrofolate reductase which confers resistance to trimethoprim and *sat2* codes for a streptothricin acetyltransferase, conferring resistance to streptothricin.

Susceptibility to streptomycin of strains harbouring class 1 integrons containing *aadA1* or *aadA2* genes was analysed. As it is shown in Table 5.2, five strains were resistant and two (A.133/3 and A.135/6) displayed reduced susceptibility to this antimicrobial agent.

All nucleotide sequences of the gene cassettes identified displayed 100% similarity with the previously reported and were submitted to the INTEGRALL and GenBank databases.

5.4 Discussion

In this study, antibiotic susceptibility patterns and genetic bases of antimicrobial resistance among the 169 bacterial strains were assessed.

Generally, the genus *Aeromonas* is referred as a homogeneous group in what concerns antimicrobial resistance profiles (Goñi-Urriza *et al.*, 2000a; Lee *et al.*, 2008). In accordance with previously reported results, in this work, higher resistance rates were recorded for penicillins and the narrow spectrum cephalosporin, while tetracycline, imipenem, aminoglycosides, trimethoprim/sulfamethoxazole, fluoroquinolone and, especially, extended spectrum cephalosporins, aztreonam and chloramphenicol, were more effective against strains (Kämpfer *et al.*, 1999; Goñi-Urriza *et al.*, 2000a; Henriques *et al.*, 2006a; Palú *et al.*, 2006; Akinbowale *et al.*, 2007b). Nevertheless, rates of resistance to tetracycline, chloramphenicol and /or trimethoprim/sulfamethoxazole recorded in this work were lower than those reported in studies done in clinic (Ko *et al.*, 1996; Vila *et al.*, 2003; Chang *et al.*, 2007), aquacultures and livestock production (Akinbowale *et al.*, 2006; Jacobs and Chenia, 2007; Ceylan *et al.*, 2009), fluvial and estuarine polluted environments (Goñi-Urriza *et al.*, 2000a; 2000b; Henriques *et al.*, 2006a) and wastewater treatment plants (Moura *et al.*, 2007; Rahman *et al.*, 2009). Additionally, among *Aeromonas* strains isolated from tap water and domestic water in Istanbul (Koksal *et al.*, 2007) and drinking water samples from distinct origins (Scoaris *et al.*, 2008), higher resistance rates to trimethoprim/sulfamethoxazole and chloramphenicol were reported, respectively, and in both studies 3rd generation cephalosporins and aztreonam resistance rates were superior when compared to our results. On the other hand, antibiotic resistance values obtained by

Ghenghesh and colleagues (2001) for aeromonads collected from untreated well water in an urban area were similar or lower than ours.

It is generally recognised that isolates recovered from clinical, farming (aquaculture, agriculture, livestock) and polluted environments are exposed to a higher selective pressure than those collected from natural non-polluted environments, thus displaying higher resistances to antimicrobial agents (Goñi-Urriza *et al.*, 2000b; Henriques *et al.*, 2006a). In this work, strains were isolated from untreated drinking water and mineral water samples taken either from pristine springs in mountains as from wells, fountains and mines mainly in rural but also in urban areas. Agricultural sources and stable samples were also taken in rural areas. Therefore, the selective pressure to which our samples were submitted is different, but for the majority is lower than samples inspected in other studies as aquacultures (Schmidt *et al.*, 2001b), wastewaters (Moura *et al.*, 2007), polluted waters (Henriques *et al.*, 2006a) or clinical environments (Vila *et al.*, 2003). In fact, resistance rates to tetracyclines and antifolates among aeromonads is notably higher in those environments, probably related to the use of such antimicrobial agents in the prevention and treatment of diseases in human and animals (Goñi-Urriza *et al.*, 2000a; Vila *et al.*, 2003; Henriques *et al.*, 2008).

In relation to aminoglycosides, the susceptibility to gentamicin and a slight less effectiveness of tobramycin against aeromonads (approximately 3% and 11% of strains displaying the intermediary resistance profile, respectively) has been also detected previously (Ko *et al.*, 1996; Goñi-Urriza *et al.*, 2000a). Commonly, the most recommended first therapeutic options for *Aeromonas* infections are fluoroquinolones (Ko *et al.*, 1996; Koksal *et al.*, 2007) which is further enhanced by our results.

Multiresistance phenotypes were registered in approximately 44% of strains (74 out of 169). Given the multiplicity of criteria adopted and type and number of antimicrobials tested in the investigations previously performed it is difficult to compare numbers of multiresistant strains collected by other authors. Nevertheless, Scoaris *et al*. (2008) and Jacobs and Chenia (2007) found 57% (12 out of 21) and 51% (19 out of 37) of aeromonads collected from drinking water and aquacultures, correspondingly, resistant to five or more antimicrobial agents. Also, multiple antibiotic resistance strains were scarce (3.4%) among riverine *Aeromonas* spp. (Goñi-Urriza *et al.*, 2000b) as within isolates collected from lettuce (Palú *et al.*, 2006).

We are aware that the addition of ampicillin to the isolation medium could have promoted the selection of penicillins resistant isolates, as for several times the mechanisms of resistance to these antibiotics are the same (Henriques *et al.*, 2006a). Nevertheless, as the majority of specimens of this genus is resistant to ampicillin and known to be able to produce coordinately inducible penicillinases (Bush-Jacoby group 2d or Ambler's class D), cephalosporinases (Bush-Jacoby group 1 or Ambler's class C) and metallo-beta-lactamases (Bush-Jacoby group 3 or Ambler's class B) (Walsh *et al.*, 1997; Avison *et al.*, 2004), the methodology was not expected to introduce relevant biases. In fact, in relation to penicillins and cefalotin our results were similar to others formerly reported that did not use ampicillin in the culture media (Ko *et al.*, 1996; Goñi-Urriza *et al.*, 2000a; Scoaris *et al.*, 2008; Jacobs and Chenia, 2007).

The association of β-lactamases inhibitor clavulanic acid with penicillins reflected an accentuated decrease in resistance to these antibiotics (AMX – 97.6%, AMC – 33.9%; TIC – 79.9%, TIM – 43%), that was more pronounced with amoxicillin. Also, cefalotin was one of the less efficient drugs against strains (79.9% resistant strains). These results are in agreement with the statement above, indicating that the penicillins and cephalosporins resistance is probably due to the action of the inducible penicillinases susceptible to clavulanic acid and cephalosporinases.

Identical susceptibility patterns were generally displayed by strains of different species (data not shown) and obtained from different sources (Fig. 5.2). Nevertheless, some discrepancies may be pointed out. For instance, 8 out of 16 tetracycline resistant strains were collected from the stable, representing a high number in this environment. Tetracycline is a commonly used drug in veterinary medicine (Akinbowale *et al.*, 2007a), which may justify the high level of resistance in this group of strains.

Strains included in the species *A. salmonicida* (14 strains), *A. media* (65 strains), *A. hydrophila* (30 strains), *A. veronii* (five strains), *A. bestiarum* (13 strains) and *Aeromonas* sp. Group I (one strain) displayed resistance to a broader number of antibiotics (9 to 12 drugs). *A. salmonicida* was the species where less susceptibility to imipenem was found: three out of four strains were resistant and four out of nine strains displayed reduced susceptibility to this antibiotic. Resistance to imipenem in non clinical strains supposedly not subjected to selective pressure by the use of such drug is a worrying trait as this is a last-resort antimicrobial agent used in the clinical environment (Henriques *et al.*, 2006b).

Other studies also reported that no significant differences were detected between different species, although clinical strains generally show higher resistance rates than environmental strains (Kämpfer *et al.*, 1999; Palú *et al.*, 2006).

We are aware that giving the nature and aim of this investigation, some species are only represented by one strain and different numbers of strains of the same species were collected from distinct sources. Nevertheless, the results presented mirror the antibiotic resistance profiles of the aeromonads collected in the environments studied.

The aeromonads collection was scrutinised for the presence of *tet* and *bla* genes. Beta-lactamases encoding genes were more occurring than *tet* determinants as 38 and 22 encoding sequences of each gene family were identified among 22% and 13% of strains, correspondingly. In this work, according with other studies, the occurrence of *tet*(B), *tet*(C) and *tet*(D) was nil or very low (Schmidt *et al.*, 2001b; Jacobs and Chenia, 2007; Henriques *et al.*, 2008) and *tet*(E) was the dominant gene found (Nawaz *et al.*, 2006; Henriques *et al.*, 2008; Agersø *et al.*, 2007). In relation to *bla* genes, *cphA*/*imiS* was the most detected. Only one bla_{VIM} was identified and a TEM-1 β-lactamase encoding gene was detected in *A. veronii*. Given the fact that $bla_{\text{TEM-1}}$ genes are typically plasmid encoded (Bush and Jacoby, 2010), this result may indicate the presence of a plasmid in this strain.

We could not find bla_{SHV}, bla_{OXA} or bla_{IMP} among strains. Few *bla* genes have been reported in aeromonads other than the genes codifying for the mentioned chromosomal penicillinases, cephalosporinases and carbapenemases. SHV, TEM and OXA narrow-spectrum enzymes have been rarely encountered in these specimens (Henriques *et al.*, 2006a; Picão *et al.*, 2008b) as well as extended-spectrum β-lactamases (Picão *et al.*, 2008b). VIM and IMP metallo-beta-lactamases acquired enzymes were identified in aeromonads in only one occasion (Neuwirth *et al.*, 2007; Libisch *et al.*, 2008). Thus our results are in accordance with the previously reported. The bla_{VIM} gene detected in the *A. hydrophila* strain could not be characterised as no sequencing analysis was possible although the weak but consistent positive results in PCR and hybridisation studies (Fig. 5.3d). This may be explained by a sequence divergence in the bla_{VIM} gene as also suggested by an uncommon resistance profile exhibited by this strain, which in opposition to other bla_{VIM} carrying strains, was susceptible to ceftazidime, cefepime and exhibited reduced susceptibility to imipenem. In fact, Gram-negative strains producing VIM metallo-beta-lactamases are usually resistant to ceftazidime, cefepime and imipenem (Libisch *et*

al., 2008). On the other hand, this strain also possesses a *cphA*/*imiS* gene, thus the intermediary resistance phenotype to imipenem may be due to the presence of such determinant.

The analysis of *cphA*/*imiS* sequences indicate that there is a high molecular diversity among this gene family, as 25 out of 31 sequences were unique, sharing less than 99% of similarity with the ones formerly reported. There are few studies concerning *cphA/imiS* diversity and distribution among this genus (Walsh *et al.*, 1997; Balsalobre *et al.*, 2009b) and, to the best of our knowledge, none done in a phylogenetically diverse aeromonads collection like the one in study. Although the detection of this gene was not high (36 out of 169 strains), probably due to the high genetic variability, it was notable its considerable distribution among different species (n=6), consistent with other studies as either the negative detection results in *A. caviae* and high occurrence in *A. hydrophila* (Walsh *et al.*, 1997; Balsalobre *et al.*, 2009b).

From the untreated water samples (DW and MW) a superior number of strains harbouring ARG was isolated (28 strains) comparing with the agricultural sources (19 strains). Also, the wider variety of ARG was found in strains from untreated waters (Table 5.4). Additionally, in the stable environment seven out of 12 strains carried one of the ARG exploited, illustrating once again the wide presence of antibiotic resistance traits in this environment. The detection of a higher number of resistance determinants in strains collected from untreated water is consistent with the higher resistance rates determined for these strains. Thus, a cautious relation between the quantitative and qualitative resistance profiles recorded and the ARG detected may be draw, especially in the stable environment. In fact, all strains collected from stable samples that carried a Tet determinant were resistant to tetracycline; also the unique strain collected from this site harbouring a CphA-like coding sequence displayed resistance to imipenem.

However, frequently the genotypic and phenotypic data did not correlate. On one hand, some strains carried one of the genes studied but did not express the resistance phenotype expected. Namely, eight out of 22 strains carrying a *tet* gene were susceptible to tetracycline. Also, 29 out of 36 specimens harbouring a CphA-like encoding sequence were susceptible to imipenem and three organisms containing this gene exhibited reduced susceptibility. These specimens act as silent reservoirs of ARG, as only by specific analysis the genes were detected. In relation to *cphA/imiS* genes, it has already been

reported that despite the presence of such genetic determinant not all strains displayed carbapenemase activity. The *cphA/imiS* gene expression mechanism was not entirely clarified, but it is known that it depends on the derepression of the gene, induced by the presence of beta-lactam antibiotics. For several times, *in vitro* conditions influence the enzymatic activity and strains remain susceptible even if they are carbapenemase producing (Walsh *et al.*, 1997; Balsalobre *et al.*, 2009b).

The three strains harbouring a *cphA/imiS* gene apparently truncated were susceptible to imipenem. Upstream of *cphA/imiS* gene of the strain A.28/6, was a 175bp sequence sharing similarity with *tnpA* gene from transposons associated with plasmids found in different *Aeromonas* sp. strains (accession numbers CR376602, AM258965 and CP000646). Plasmid content of this strain was assessed and no positive result was obtained (data not shown). Whereas this genetic region is really part of a transposase and if it is functional, is not known. The association of the *cphA/imiS* gene with mobile genetic elements could obviously present an emerging danger. Moreover, this gene codifies for carbapenem resistance which, as mentioned above, are last-resort antibiotics. In this case, we did not record carbapenemase activity with the methodology applied, but, as mentioned above, it does not mean that this strain, in other conditions, is not able to produce this enzyme. In relation to strains A.131/8 and A.135/5, no significant similarity was found between the 291bp upstream of the *cphA/imiS* gene and any other gene in the database. Further studies are needed to elucidate the genetic context of these structures and their putative role in antibiotic resistance. However, IS elements have been shown to interrupt genes in other aeromonads, apparently contributing to the formation of pseudogenes (Reith *et al.*, 2008). In this way, we cannot discard the possibility of these being pseudogenes formed by the insertion of such sequence lengths in the bacteria genomes.

On the other hand, strains were resistant or displayed the intermediary resistance phenotype to a certain antibiotic, but none of the studied genes was found in the organism, indicating that some other genetic encoded mechanisms different from the ones in study may be involved or that the genetic variations of genes may be sufficient, at least in the zone of the primers annealing, to preclude their identification.

Six strains displaying reduced susceptibility to imipenem did not seem to contain any of the studied genetic determinants of resistance to this antibiotic. Also, two strains were resistant and three other displayed the intermediary resistance phenotype to

tetracycline and any of the *tet* determinants in study was detected. Additionally, two strains had reduced susceptibility to chloramphenicol, one of them carrying an integron but no gene cassette coding for resistance to chloramphenicol was present. Furthermore, three strains were resistant to trimethoprim/sulfamethoxazole and two others displayed reduced susceptibility to that antibiotic. Although one of them harboured a class 1 integron, no gene cassette coding for resistance to trimethoprim/sulfamethoxazole was present. Additionally, 66 strains were resistant or displayed the intermediary susceptibility profile to aminoglycosides. Although a genetic basis for resistance to aminoglycosides, chloramphenicol or trimethoprim/sulfamethoxazole was not accounted for in this study.

The presence of class 1, class 2 and class 3 integrases was screened among the *Aeromonas* strains and corresponding integrons' variable regions were characterised. Eight *intI1* and one *intI2* carrying strains were detected and no *intI3* was found. Formerly, the integrase gene found among aeromonads was predominantly *intI1* but also *intI2* genes were found (Henriques *et al.*, 2006a; Moura *et al.*, 2007; Barlow *et al.*, 2008; Lee *et al.*, 2008) and there is only one report of detection of *intI3* among *Aeromonas* species (Jacobs and Chenia, 2007). In this study, approximately 5% of strains carried class 1 integrons with inserted gene cassettes which is much lower than the previously reported values that ranged from 11% to 35% approximately (Schmidt *et al.*, 2001b; Henriques *et al.*, 2006a; Chang *et al.*, 2007; Lee *et al.*, 2008; Pérez-Valdespino *et al.*, 2009). As mentioned above, this may be due to the environments from which our strains were collected that were subjected to lower selective pressures. The gene cassettes inserted in the variable regions of class 1 integrons (*aadA2*, *dfrA12*, *orfF*, *aadA1* and *catB8*) as well as cassettes arrangements are usually associated with these structures in aeromonads (Henriques *et al.*, 2006a; Chang *et al.*, 2007; Jacobs and Chenia, 2007; Moura *et al.*, 2007; Barlow *et al.*, 2008; Lee *et al.*, 2008), but the array *dfrA12-orfF-aadA2* was for the first time detected in the species *A. eucrenophila*. On the other hand, the occurrence of class 2 integrons recorded in this study (one out of 169 strains – 0.6%) is well within the numbers reported for *Aeromonas* sp. which have been rarely detected (Chang *et al.*, 2007; Moura *et al.*, 2007; Barlow *et al.*, 2008; Lee *et al.*, 2008).

One integron was identified in a strain isolated from cow faeces while the others were all present in DW strains. Thus, the environments from where wider variety of antibiotic resistance was recorded were also the environments from where integrons

carrying strains were isolated. Furthermore, *A. salmonicida*, *A. media, A. hydrophila* and *A. veronii* were found to be resistant to a broader number of antibiotics, again corresponding to the presence of integrons in strains. All strains harbouring integrons were resistant to five or more antibiotics (Table 5.2) and displayed the intermediary resistance phenotype to one to four different antibiotics additionally (data not shown). Also, two of the most resistant strains collected (*A. veronii* strain A.096A and *A. hydrophila* strain A. 131/8) harboured a class 1 integron (Table 5.2). Nevertheless, in certain cases, the gene cassette array of each class 1 integron identified did not explain the resistance phenotype of the strain (Table 5.2). Two strains carrying *aadA2* gene displayed the intermediary resistance phenotype to streptomycin. Also, strains harbouring *catB8* (n=1) and *dfrA12* (n=1) were susceptible to chloramphenicol and trimethoprim/sulfamethoxazole, respectively. This may be explained by the presence of weak integron promoters (Moura *et al.*, 2007). Even though, in the integron carrying a *catB8* gene cassette, an *aadA1* gene was also present, and this strain was resistant to streptomycin. *catB8* was the gene closer to the integron promoter, thus the phenotype of resistance to chloramphenicol was expected. Nevertheless, the resistance to streptomycin may be encoded in other genetic elements and not due to the gene cassette inserted on this integron, a statement valid to the other resistance phenotypes recorded. In fact, although in several studies integrons represent important antibiotic resistance platforms within strains (Chang *et al.*, 2007; Moura *et al.*, 2007) in our aeromonads collection this was not verified and dominant resistance phenotypes were related to resistance mechanisms encoded in other genetic contexts, especially the probable production of chromosomal penicillinases and cephalosporinases.

Our results demonstrate that multiresistant strains harbouring genetic traits of antibiotic resistance, sometimes putatively transferable, are present in different sources which in the majority are for human and animal consumption. Moreover, the gene *cphA/imiS* has a notable genetic diversity and thus a putative flexibility to adapt and evolve under different conditions. That may constitute an additional concern which may be enhanced if an association of this genetic determinant with mobile genetic elements occurs. This is the first study reporting the characterisation of antibiotic resistance in aeromonads in Portugal. This group frequently occurs in untreated waters and food products raised in smallholdings farms. In general, these sources are consumed by animals and humans and may act as vehicles of contamination by these organisms.

6. Contribution to virulence:

physiological features and genetic determinants

Abstract

Aeromonas spp. isolated from untreated waters, agricultural sources and from different types of samples collected in a stable were scrutinised for the presence of putative phenotypic and genetic traits of virulence known to be associated with pathogenic specimens of this genus. Potentially pathogenic features as the production of extracellular lipases and proteases and the presence of genes coding for putative virulence factors as aerolysin and related toxins, lipase proteins and type III secretion system component *ascV* and effector toxin, *aexT*, was evaluated in 169 *Aeromonas* strains. Also, the amplicon obtained by PCR of the aerolysin related genes of strains as well as those obtained *in silico* for sequences of these genes present in the GenBank database were further characterised by *Hpa*II restriction analysis. Extracellular proteolytic and lipolytic activities were exhibited by 96% and 100% of strains, respectively. Aerolysin related genes were detected in 43% of strains and two distinct *Hpa*II restriction patterns were obtained which corresponded with the previously reported (Kingombe *et al.*, 1999). In general, the restriction analysis done with the GenBank database sequences also yielded the same two major patterns. The *ascV* and *aexT* genes were found in 16% and 3% of strains, in some cases, concomitantly in the same specimen. Potential virulence genetic determinants are present in *Aeromonas* spp. isolates found in sources which in their majority are for human and animal consumption. Previously poorly scrutinised species and probably new not yet described species were found to harbour distinct putative virulence features.

Keywords: aerolysin; *act*; hemolysins; T3SS; *aexT*; *ascV*; untreated water; vegetables; faeces; milk.

6.1 Introduction

One of the most interesting traits of *Aeromonas* species, and probably the one that more prompts their relevance, is the ability that these organisms have in causing disease in such different animals as fish, reptiles and mammals, including humans (Seshadri *et al.*, 2006). In humans, *Aeromonas* spp. are regularly responsible for intestinal and extra intestinal infections as gastroenteritis, bacteremia, skin and soft tissue infections, to name but a few. Actually, aeromonads have often been implicated as a causative agent of diarrhoea (Figueras, 2005). Furthermore, these were the most recovered specimens from skin and soft tissues infections among tsunami victims in Thailand (Hiransuthikul *et al.*, 2005). The risk of acquiring health complications due to *Aeromonas* arises from the contact with, or consumption of, contaminated water and food (Castro-Escarpulli *et al.*, 2003; Sen and Rodgers, 2004).

The broad host range of aeromonads and their wide distribution in different habitats such as treated and untreated water, soil and foodstuff, demonstrates their great

adaptability to environmental changes (Sen and Rodgers, 2004; Galindo *et al.*, 2006). The genetic machinery of *Aeromonas* spp. includes determinants that play a vital role in ecology, survival and pathogenicity of these microorganisms. The assortment of virulence factors *Aeromonas* possess are part of this potent molecular armoury and enables them to colonise different hosts, invade and establish within the host cells (Galindo *et al.*, 2006).

Numerous extracellular proteins secreted by aeromonads that may be involved in virulence capabilities were identified and characterised from which, lipases, proteases, enterotoxic toxins and hemolysins are the best known (Galindo *et al.*, 2006). Genes encoding lipolytic and/or phospholipolytic activities are extensively present in *Aeromonas* spp. (Chacón *et al.*, 2003; Sen and Rodgers, 2004). *lipH3* and *lip* genes code for lipases with no phospholipase activity (Anguita *et al.*, 1993; Chuang *et al.*, 1997), whereas *apl-1* and *pla* encoded lipases do have phospholipase activity (Ingham and Pemberton, 1995; Merino *et al.*, 1999). The cytotoxic enterotoxins aerolysin, Act and other genetically close related hemolysins share similar functions and modes of action, although they present some structural and functional differences (Galindo *et al.*, 2006). The majority of these molecules display cytotoxic, enterotoxic and hemolytic activities from which the best characterised are aerolysin and Act. The last, apart from the mentioned biological activities, has shown to be lethal to mice when injected intravenously and to induce apoptosis and inflammatory response in murine macrophages and human intestinal epithelial cells. Other enterotoxic toxins are the cytotonic enterotoxins as Alt and Ast that do not cause degeneration of the intestinal tissue (Chopra *et al.*, 2009). Other hemolysins exist that do not share any homology with the aerolysin and Act family of toxins (Erova *et al.*, 2007; Singh *et al.*, 2009).

Type III secretion systems (T3SS) are Gram-negative bacteria devices to inject proteins (effectors) across bacterial and eukaryotic cell membranes directly into the cytosol of host cells. The secretion apparatus, also known as injectisome, is composed by a core of proteins that are conserved in sequence among different species. The basal structure of the machinery is anchored in the inner membrane spanning the peptidoglycan and the outer membrane, from where the needle device protrudes ending in a translocation pore when active. On the contrary, a large number of effector proteins have been reported, varying across bacteria as a result of adaptation to the type of association with the host. In their majority, effectors are toxins that alter the host's intracellular signalling pathways and

disrupt the cytoskeleton (Ghosh, 2004; Cornelis, 2006). In *Aeromonas* spp., T3SS have been found in different strains of *A. salmonicida* (Burr *et al.*, 2002; Ebanks *et al.*, 2006) and *A. hydrophila* (Yu *et al.*, 2004; Sha *et al.*, 2005) and characterised. Also, the T3SS of *A. piscicola* AH-3, formerly known as *A. hydrophila* has been described (Vilches *et al.*, 2004; Beaz-Hidalgo *et al.*, 2009). In *A. salmonicida*, the encoding genetic locus is located on plasmids and five effector toxins (AexT, AopP, AopH, AopO and Ati2) were identified (Stuber *et al.*, 2003a; Ebanks *et al.*, 2006; Fehr *et al.*, 2007; Reith *et al.*, 2008). In contrast, the T3SS genes in *A. hydrophila* and *A. piscicola* are encoded in the chromosome and AexT-like or AexU was found as effector toxin (Yu *et al.*, 2004; Vilches *et al.*, 2004; Sha *et al.*, 2005; Sha *et al.*, 2007; Vilches *et al.*, 2008). In some cases, more than one gene encoding different effector toxins were concomitantly found in the same strain (Dacanay *et al.*, 2006; Fehr *et al.*, 2006; Silver and Graf 2009). The *ascV* gene encodes an inner membrane protein of the secretion apparatus and was established as a suitable marker for the presence of T3SS (Stuber *et al.*, 2003b). The *aexT* gene, reported to be encoded in the chromosome, encodes AexT, a bifunctional protein translocated by the T3SS targeting the cytoskeleton (Fehr *et al.*, 2007).

T3SS genes and related effector toxins were also detected in few other *Aeromonas* spp. revealing that this powerful virulence machine may be distributed in the genus, nevertheless, in their majority, these studies focused in species or the phenospecies complex *A. hydrophila*, *A. veronii*/*A. sobria* and *A.caviae* (Burr *et al.*, 2005; Vilches *et al.*, 2004; Chacón *et al.*, 2004; Wu *et al.*, 2007; Aguilera-Arreola *et al.*, 2007; Figueras *et al.*, 2009; Silver and Graf, 2009). In fact, although more than 20 species have been identified in *Aeromonas* (Alperi *et al.*, in press), most studies regarding the screening for putative virulence factors were restricted to one or all three species/phenospecies complex mentioned (Wu *et al.*, 2007; Castilho *et al.*, 2009; Pablos *et al.*, 2009), precluding the assessment of the true incidence of these determinants in each species of the genus (Chacón *et al.*, 2003). In reality, these are the most prevalent species in clinical samples. However, more frequent implication of members of these species/complexes in health complications may be a consequence of misidentification owing to the phenotypic methods applied for identification purposes, artificially increasing the relevance of these species in virulence and pathogenicity studies (Figueras, 2005). Actually, recently it has been shown that *A. aquariorum*, a recently described species, presents a combination of putative

virulence factors and its clinical relevance has been pointed out; this indicates that this species may be globally distributed but possibly unrecognised under *A. hydrophila* or *A. caviae* (Figueras *et al.*, 2009).

In this study, a collection of phylogenetically well characterised *Aeromonas* spp. was screened for the presence of genetic determinants known to be virulence factors and physiological features potentially contributing to pathogenicity were assessed. Strains were obtained from diversified sources mostly related to human and animal consumption.

6.2 Material and Methods

6.2.1 Bacterial strains

In a previous work, 483 *Aeromonas* isolates were obtained from untreated waters (drinking water and mineral water), agricultural sources (untreated irrigation water and vegetables from smallholding farms) and from a stable (cows' drinking water and faeces, udder cleaning water and milk). After genetic characterisation, the 483 isolates were shown to represent 169 strains that were deeply characterised and used in this study. Depending on the type of gene studied (presented below), control strains used in experiments were *A. hydrophila* CECT839^T, *A. salmonicida* CECT894^T and an *A. veronii* strain containing *aexT* and *ascV* genes.

6.2.2 Extracellular lipolytic activity

Procedure was done as in Pavlov *et al.* (2004) with few modifications. Briefly, strains were streaked over the surface of Trypticase soy agar (Scharlau, Spain) supplemented with 1% of Tween 20 (v/v) (Calbiochem, Merck, Germany) as substrate. The formation of a precipitate around the inoculum was considered as a positive result.

6.2.3 Extracellular proteolytic activity

For the evaluation of extracellular protease production, strains were streaked over the surface of TSA (Merck, Germany) supplemented with 1% skim milk (w/v). A clear zone around the inoculum was taken as positive result (Pavlov *et al.*, 2004).

6.2.4 DNA amplification and visualisation

All PCR experiments were done in MyCycler™ thermal cycler (Bio-Rad, USA). Controls were included in all experiments. Primers were synthesised by STAB Vida (Portugal). *Taq* polymerase was from Fermentas Life Sciences (Lithuania) and dNTPs were from Bioron (Germany). PCR products were electrophoresed in 1.5% agarose gel using TAE buffer. After electrophoresis, gels were stained with ethidium bromide and visualised under UV transillumination.

6.2.5 Presence of genes coding for lipolytic enzymes

The presence of genes encoding lipolytic enzymes was investigated using PCR amplification and primers reported by Sen and Rodgers (2004). Primer set LipF and LipR targets conserved regions of genes *pla*, *lipH3*, *lip* and *apl-1* encoding lipolytic and/or phospholipolytic enzymes (Sen and Rodgers, 2004). Reaction mixtures of 25µl contained 1x PCR buffer (buffer with $(NH_4)_2SO_4$), 3mM $MgCl_2$, 80µM dNTPs, 0.3µM of each primer, 0.02U *Taq* polymerase and 100 – 200ng of total DNA. *A. hydrophila* CECT839^T was used as positive control.

6.2.6 Presence of genes coding for aerolysin-related toxins

The methodology of Kingombe *et al.* (1999) was used to detect and characterise the genes for aerolysin-related toxins present in our strains. For that, the primer pair AHCF1-AHCR1, targeting conserved regions common to the aerolysin, hemolysin and *act* genes was used as described by Kingombe and colleagues (1999). Amplification reactions were performed in a volume of 25µl containing 1x PCR buffer (buffer with $(NH_4)_2SO_4$), 2.5mM $MgCl_2$, 32µM dNTPs, 0.4µM of each primer, 0.04U *Taq* polymerase and 100 – 200ng of total DNA. The presence of a 232bp PCR product was recorded as a positive result.

According to Kingombe *et al.* (1999) hydrolysis of the amplicons with *Hpa*II generates three different patterns that in most cases are species related. For that reason, PCR products were subsequently digested with *Hpa*II (Fermentas Life Sciences, Lithuania) according to the manufacturer instructions and the resulting fragments were separated by vertical electrophoresis in 10% bisacrylamide gel (Bio-Rad, USA) for 30 min at 20V followed by 300min at 100V. Gels were stained with ethidium bromide and visualised as described previously. *A. hydrophila* CECT839^T was used as positive control.

6.2.7 Phylogenetic analysis of aerolysin-related toxins gene fragments

Amplicons exhibiting different restriction patterns were chosen for sequencing analysis. Products were purified with the JETQUICK PCR purification spin kit (Genomed, Germany) and sequencing was performed in both strands with the primers used in PCR reactions.

Additionally, an *in silico* approach was used to perform a phylogenetic analysis of aerolysin, hemolysin and *act* genes of *Aeromonas* species present in the GenBank database together with the sequences obtained by us. For that, nucleotide sequences available under the name of "aerolysin", "Act", "hemolysin" and "*gene name*-like" were aligned with the CLUSTALX 2.0.9 program and all sequences containing the 232bp region putatively amplified by the primer pair AHCF1-AHCR1 were chosen. Those with up to 3 base pair mismatches with the primers' sequence were also included. The 232bp fragment was used to construct phylogenetic trees by the neighbor-joining method (Saitou and Nei, 1987) with the Kimura's 2-parameter method (Kimura, 1980) using MEGA4 (Tamura *et al.*, 2007). Also, the virtual digestion patterns of the 232bp fragment from each gene were obtained using the Restriction Mapper tool available online (http://restrictionmapper.org/).

6.2.8 Detection of genes related to type three secretion system

To assess the presence of T3SS and related secreted toxins among strains, screening of *ascV* and *aexT* genes was performed. In all experiments, one *A. veronii* strain containing *aexT* and *ascV* genes and *A. hydrophila* CECT839^T were used as positive and negative controls, respectively.

For the detection of AscV encoding gene, PCR conditions and primers used were as in Wu *et al.* (2007) and for *aexT* were as in Braun *et al.* (2002). Reaction mixtures of 25µl contained 1x PCR buffer (buffer with $(NH_4)_2SO_4$), 2mM $MgCl_2$, 80µM dNTPs, 0.3µM of each primer, 0.04U Taq polymerase and 100 – 200ng of total DNA.

PCR positive results were confirmed by Southern-blot hybridisation. DNA from strains used as positive control served as template to obtain intragenic digoxigenin-labelled probes specific for each gene. PCR reactions were performed as described before, using DIG-DNA Labelling Mix (Roche Molecular Biochemicals, USA) instead of dNTPs. Genomic DNA from strains that were positive in PCR reactions for at least one of the two genes was digested with *Sal*I restriction enzyme (Fermentas Life Sciences, Lithuania) in

accordance with the manufacturer instructions. Digestion products were separated in 1.5% agarose gel, vacuum transferred for 150min to positively charged nylon membranes (Hybond N+; Amersham, Germany) and cross-linked under UV irradiation for 5min. Hybridisation occurred overnight under stringent conditions, at 42ºC in 50% formamide buffer. For confirmation of *aexT* gene sequences, hybridisation was also performed at low stringent conditions: 50ºC in 0% formamide buffer as described above and *A. salmonicida* CECT894^T was included as a further positive control. In both cases detection was performed using DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals, USA) following instructions provided by the manufacturer.

6.3 Results

6.3.1 General overview

Both phenotypic and genotypic characteristics related to virulence were determined for all strains. A compilation of the results obtained is presented in Table 6.1.

In what concerns phenotypic characteristics, the capability of producing extracellular lipases and proteases was tested in the 169 *Aeromonas* sp. strains. All strains (100%) displayed lipolytic activity but for proteolytic activity under the conditions here used, 7 strains performed negatively. This means that 162 strains (96%) exhibited proteolytic activity (Table 6.1). The 7 negative strains for proteolytic activity were: one *A. veronii* strain, one *A popoffii* strain, one *A. eucrenophila* strain, two *A. caviae* strains, all collected from UnW sources (drinking water – DW) and two *A. media* strains recovered from AS (one collected from irrigation water – IW – and the other isolated from a vegetable sample – V).

Molecular methods were used to evaluate the presence of known DNA sequences corresponding to genes coding for lipases, aerolysin-like toxins and genes related to T3SS. Out of 169 strains, the genome of 165 (98%) carried at least one of the sequences studied. Only four *A. media* strains did not harbour any of the genetic determinants investigated.

Table 6.1 Number of strains of each species harbouring every putative genetic determinant and displaying each of the extracellular enzymatic activities studied.

***Aeromonas* sp. (n)**[a]	**Genes coding for lipolytic activity**	**Aerolysin-related genes**		***aexT***	***ascV***	**Lip Actv.**[b]	**Prot. Actv.**[c]
		AER-A	**AER-B**				
A. media (65)	60	1	4	-	-	65	63
A. hydrophila (30)	30	12	2	-	4	30	30
A. eucrenophila (20)	19	1	15	-	1	20	19
A. salmonicida (14)	13	14	-	1	1	14	14
A. bestiarum (13)	13	12	-	2	10	13	13
A. veronii (5)	2	1	4	2	3	5	4
A. allosaccharophila (5)	-	-	-	-	5	5	5
A. sp. Group II (5)	5	1	-	-	2	5	5
A. caviae (4)	4	-	-	-	-	4	2
A. sp. HG11 (2)	2	-	2	-	-	2	2
A. tecta (2)	2	-	1	-	1	2	2
A. popoffii (1)	1	1	-	-	-	1	-
A. encheleia (1)	1	-	-	-	-	1	1
A. sp. Group I (1)	1	-	-	-	-	1	1
A. sp. Group III (1)	1	-	1	-	-	1	1
Total (169)	154	43	29	5	27	169	162
		72					

[a] Number of strains of each species scrutinised.
[b] Extracellular lipolytic activity.
[c] Extracellular proteolytic activity.

6.3.2 Markers of lipase genes

The genetic bases of lipolytic activity scrutinised were present in 154 strains (91%). Strains performing negative in PCR amplification of the lipase genes studied belonged to *A. salmonicida* (one strain), *A. eucrenophila* (one strain), *A. veronii* (three strains), *A. media* (five strains) and *A. allosaccharophila* (five strains) phylogenetic groups. The last, was the unique species which none of the strains collected contained the studied genes coding for lipolytic proteins.

6.3.3 Presence of sequences coding for aerolysin-related enzymes

The 232bp fragment of genes encoding aerolysin-related toxins was detected in 72 strains (43%), with higher occurrence among UnW recovered strains (60% of UnW isolated strains), followed by the SE specimens (50% of SE recovered strains) and AS isolated strains (24% of AS collected strains). All strains belonging to *A. allosaccharophila*, *A. caviae*, *A. encheleia* and *Aeromonas* sp. Group I species were negative in this test (Table 6.1).

The digestion with *Hpa*II of the 232bp amplicons from each strain disclosed two different patterns, AER-A and AER-B (Fig. 6.1). Digestion pattern AER-A contained three restriction fragments of 18bp, 66bp and 148bp and was found in 43 strains. The digestion pattern AER-B was identified in 29 strains and included two restriction fragments of 66bp

and 166bp. These results were confirmed by DNA sequence analysis and virtual digestion of the sequences using Restriction Mapper software. A schematic representation of restriction patterns is displayed in Figure 6.2. The pattern AER-A was exclusively or mostly found among *A. hydrophila*, *A. salmonicida*, *A. bestiarum*, *Aeromonas* sp. Group II and *A. popoffii*. On the other hand, pattern AER-B was solely or predominantly found in *A. media*, *A. eucrenophila*, *A. veronii*, *Aeromonas* sp. HG11, *A. tecta* and *Aeromonas* sp. Group III (Table 6.1). No relevant differences were found in the number of strains displaying one or the other pattern in relation to the source of isolation.

From the GenBank database, 68 aerolysin-related toxins encoding gene sequences were downloaded and each of the 232bp segments delimited by the sequences of primer pair AHCF1-AHCR1 was virtually digested with *Hpa*II. Six different patterns were obtained, including both AER-A and AER-B patterns.

A phylogenetic tree of the representatives of all alleles is shown in Figure 6.3. Sequences clustered in two main groups, from which one corresponds to the clustering of sequences exhibiting pattern AER-A and the other to the grouping of virtual amplicons exhibiting AER-B, although few exceptions. AER-A is typical of strains identified as *A. hydrophila*, while AER-B was common among *A. veronii*, *A. sobria* but also some *A. hydrophila* specimens.

6.3.4 Genes related to type three secretion system

Strains were scrutinised for the presence of DNA sequences of genes related to T3SS. PCR assay for *ascV* sequences resulted in multiband patterns in several strains. At the contrary, only DNA of a few strains resulted in a positive band for *aexT* sequences (Fig. 6.4). The results were confirmed by Southern-blot hybridisation of *Sal*I genomic digests, using DIG-labelled probes for each gene (Fig. 6.5).

The *ascV* marker was detected in 27 strains belonging to *A. hydrophila*, *A. veronii*, *A. allosaccharophila*, *A. salmonicida*, *A. bestiarum*, *A. eucrenophila*, *A. tecta* and *Aeromonas* sp. Group II phylogenetic groups (Table 6.1). This marker was more often found among strains collected from UnW sources (24% of UnW strains), followed by AS samples (9% of AS strains) and SE samples (8% of SE strains, corresponding to one strain).

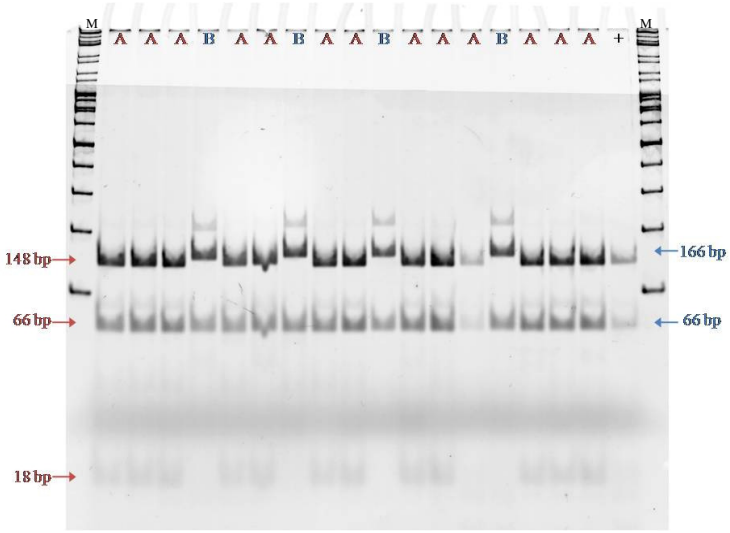

Figure 6.1 Example of a bisacrylamide gel resulting from the vertical electrophoresis of the digested 232bp AHCF1-AHCR1 amplicons of the strains collected.

Legend to figure:
A, pattern AER-A; B, pattern AER-B; +, positive control (CECT839^T); M, DNA ladder GeneRuler™ 100 bp Plus (Fermentas Life Sciences, Lithuania).
The different fragments resulting from the digestion with *Hpa*II are signalised by arrows. The red arrows indicate the fragments composing pattern AER-A and the blue arrows point to the fragments constituting pattern AER-B.

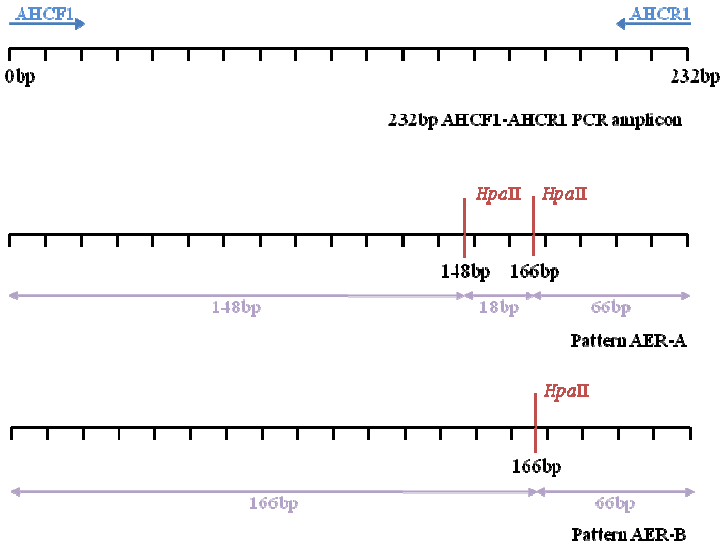

Figure 6.2 Schematic restriction maps of the 232bp AHCF1-AHCR1 PCR amplicons obtained from the digestion assay with *Hpa*II enzyme.

Legend to figure:
The 232bp amplicon is represented as well as the positions of the primers annealing and recognition sites by *Hpa*II. Pattern AER-A encloses three different fragments resulting from the recognition and digestion on three points by *Hpa*II. Pattern AER-B includes two distinct fragments resulting from the recognition and digestion on one point by *Hpa*II.

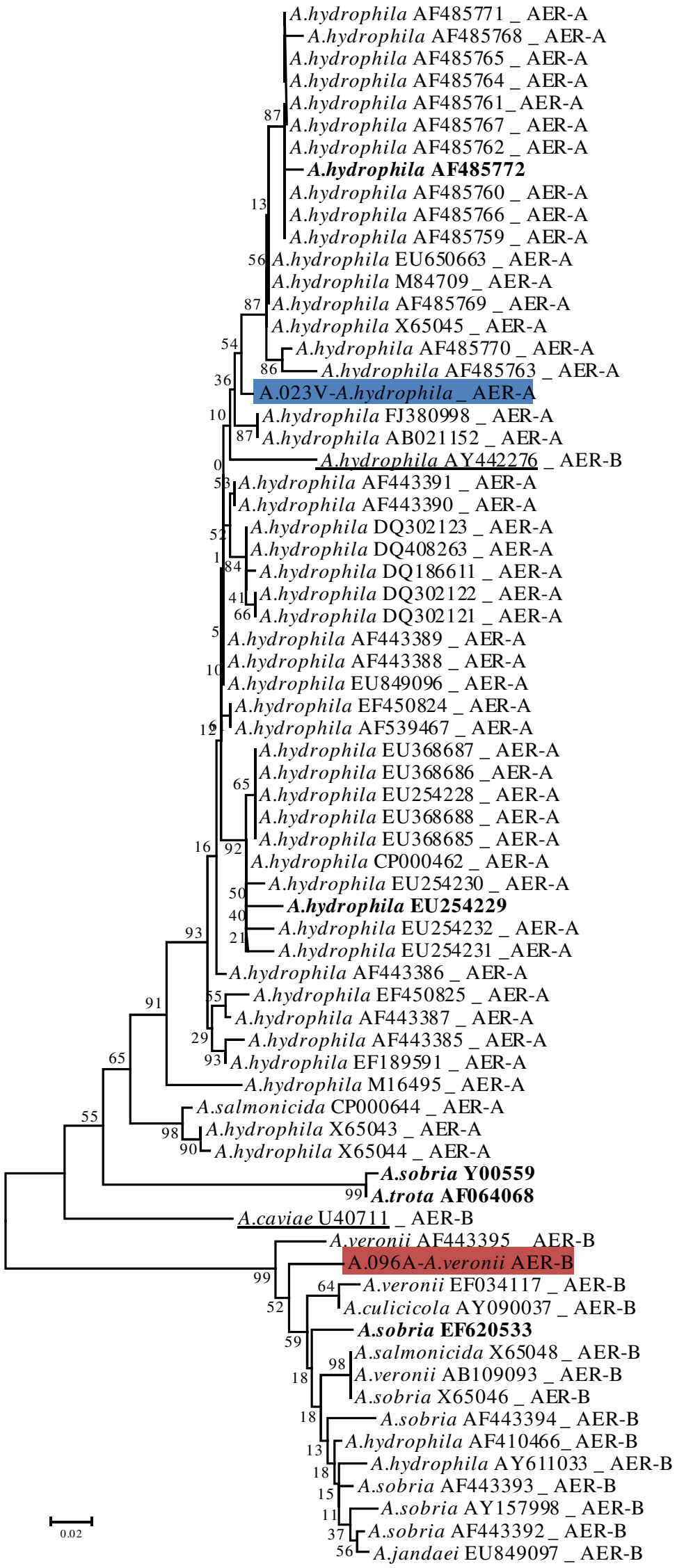

Figure 6.3 Phylogenetic tree based on the 232bp AHCF1-AHCR1 segment of the aerolysin, *act* and hemolysin related genes of *Aeromonas* spp. present in the GenBank database and representatives of each restriction pattern collected in this study.

Legend to figure:
Nucleotide sequences included in the database under the names "aerolysin", "*act*", "hemolysin" and "*gene name*-like" harbouring the entire 232bp segment were chosen.
The *Hpa*II restriction pattern of each sequence is shown side by side with the *Aeromonas* sp. and respective accession number.
Highlighted in blue (A.023V) and red (A.096A) are the strains collected in this study representing strains displaying pattern AER-A and AER-B, respectively.
Strains showing patterns AER-A or AER-B that clustered in a different group from the majority of strains displaying the same pattern are underlined.
In bold are the strains exhibiting other patterns than AER-A or AER-B.
The tree was constructed by the neighbor-joining method with the Kimura's 2-parameter method. Bootstrap values based on 1000 replicates are shown. Scale bar represents 0.02 substitutions per site.

Two *A. veronii* strains were positive for *aexT*. Additionally, on the hybridisation assays performed with the *aexT* gene probe at low stringent conditions, two strains of *A. bestiarum* and one strain of *A. salmonicida* gave a weak but distinguishable positive signal, indicating that these strains harbour *aexT*-like gene sequences (Fig. 6.5b2). All these strains were collected from UnW sources, namely from drinking water (DW) samples. The negative control strain, *A. hydrophila* CECT839^T, did not give a signal in any of the assays performed (Fig. 6.5).

Four specimens were positive for both *ascV* and *aexT* (two *A. bestiarum* and two *A. veronii* strains all collected from DW samples). One strain (*A. salmonicida* collected from a DW sample) harboured an *aexT*-like gene, as assessed by the low stringent screening, while no detection of the *ascV* gene was obtained.

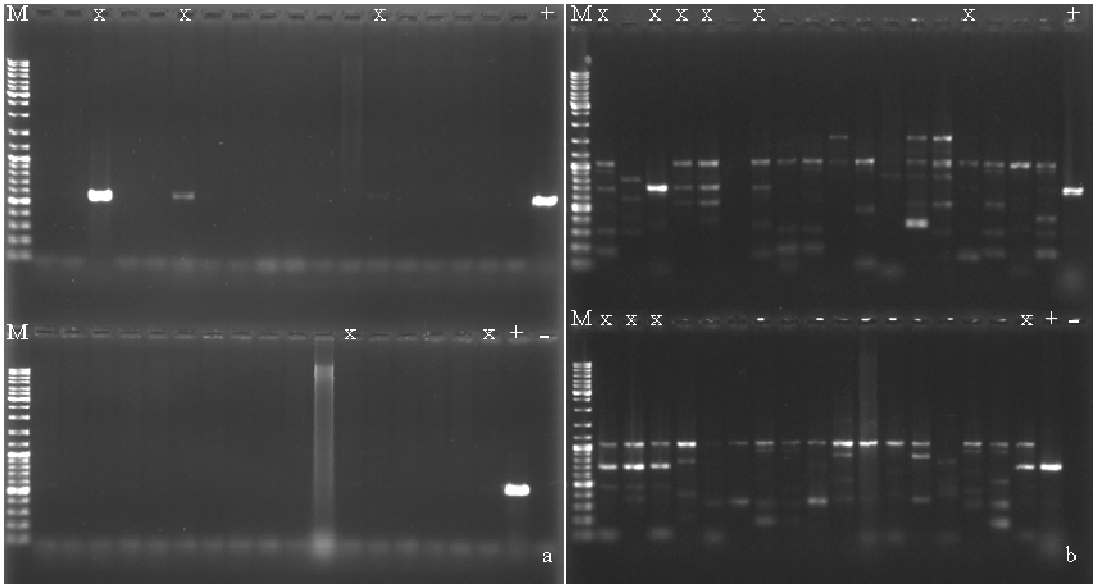

Figure 6.4 Examples of results from the PCR assays for the detection of *aexT* (a) and *ascV* (b) genes.

Legend to figure:
Lanes M, Gene Ruler^{TM} DNA Ladder Mix (Fermentas Life Sciences, Lithuania); Lanes X, strains selected for hybridisation studies; Lanes +, positive control (*A. veronii*); Lanes -, negative control (*A. hydrophila* CECT839^T),

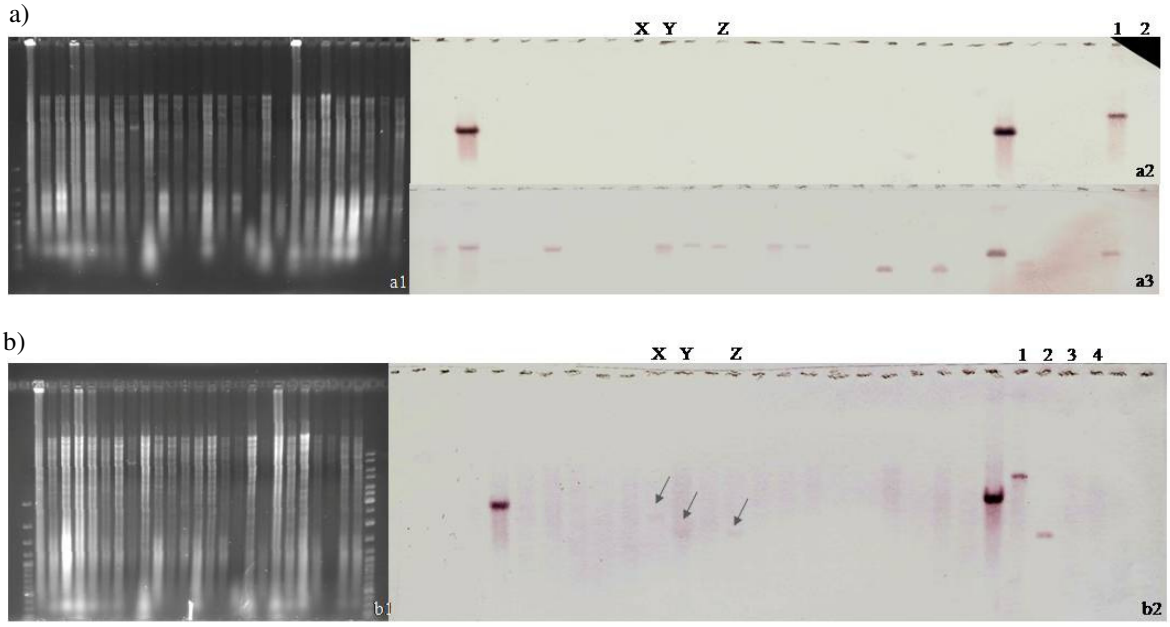

Figure 6.5 Examples of results obtained for the detection of *aexT* and *ascV* genes by Southern-blot hybridisation of digested DNA with *Sal*I restriction enzyme with DIG labelled probes for each gene.

Legend to figure:
a) Hybridisation performed under high stringent conditions (42ºC and 50% formamide buffer): a1, Total DNA digestion with *Sal*I; a2, Hybridisation of Southern-blotted *Sal*I digested DNA with *aexT* gene probe; a3, Hybridisation of Southern-blotted *Sal*I digested DNA with *ascV* gene probe. Lane 1, positive control (*A. veronii*); Lane 2, negative control (*A. hydrophila* CECT839^T).
b) Hybridisation performed under low stringent conditions (50ºC and 0% formamide buffer): b1, Digestion with *Sal*I enzyme of total DNA; b2, Southern-blot hybridisation of digested DNA with *aexT* gene DIG-labelled probe. The arrows in lanes X, Y and Z indicate positive signals not detected in the high stringent condition assay, as shown in lanes X, Y and Z in Figure 6.5a2. Lane 1, *A. veronii* positive control strain; Lane 2, *A. salmonicida* CECT894^T positive control strain; Lanes 3 and 4, *A. hydrophila* CECT839^T negative control strain.

6.3.5 Associations between virulence factors

In Tables 6.2 and 6.3 are highlighted the distinct combinations of putative virulence factors recorded among strains of the different phylogenetic groups recovered and in relation to the type of sources scrutinised.

Ten different combinations of one (three patterns), two (three patterns), three (three patterns) or four (one pattern) putative virulence genetic determinants were recorded. Lipolytic activity encoding genes, *ascV* and aerolysin-related genes were the unique found solely in a given strain, being the first the most occurring pattern among species. The combination of lipolytic activity genetic determinants and aerolysin-related encoding genes was the second most recorded and the pattern "lipolytic activity encoding genes/aerolysin related genes/*ascV*" was the third most frequently recovered combination. The unique obvious specific association of one gene to another was that *aexT* always appeared in combination with aerolysin related genes in different combinations with other genes.

Table 6.2 Combinations of putative virulence factors detected among the different *Aeromonas* phylogenetic groups recovered strains.

***Aeromonas* sp. (N)**[a]	**Putative Virulence Factors Combinations**[b]										
	Lip	**Lip/Aer**	**Lip/Aer/*ascV***	**Lip/*ascV***	***ascV***	**Aer**	**Lip/Aer/*aexT*/*ascV***	**Aer/*aexT*/*ascV***	**Lip/Aer/*aexT***	**Aer/*ascV***	**No genes detected**
A. media (65)	56	4	-	-	-	1	-	-	-	-	4
A. hydrophila (30)	14	12	2	2	-	-	-	-	-	-	-
A. eucrenophila (20)	4	14	1	-	-	1	-	-	-	-	-
A. salmonicida (14)	-	12	-	-	-	-	-	-	1	1	-
A. bestiarum (13)	1	2	8	-	-	-	2	-	-	-	-
A. veronii (5)	-	1	1	-	-	1	-	2	-	-	-
A. allosaccharophila (5)	-	-	-	-	5	-	-	-	-	-	-
A. sp Group II (5)	2	1	-	2	-	-	-	-	-	-	-
A. caviae (4)	4	-	-	-	-	-	-	-	-	-	-
A. sp. HG11 (2)	-	2	-	-	-	-	-	-	-	-	-
A. tecta (2)	-	1	-	1	-	-	-	-	-	-	-
A. popoffii (1)	-	1	-	-	-	-	-	-	-	-	-
A. encheleia (1)	1	-	-	-	-	-	-	-	-	-	-
A. sp Group I (1)	1	-	-	-	-	-	-	-	-	-	-
A. sp Group III (1)	-	1	-	-	-	-	-	-	-	-	-
Total (169)	83	51	12	5	5	3	2	2	1	1	4

The number of strains harbouring each combination is shown.

[a] Total number of strains collected from each phylogenetic group.

[b] Different combinations of putative virulence factors detected. Lip, lipolytic activity encoding genes; Aer, aerolysin related genes.

The species that displayed a higher number of distinct arrangements of genes were the *A. hydrophila*, *A. eucrenophila*, *A. bestiarum* and *A. veronii* groups with four different combinations each. The unique obvious association of a given pattern with a given species was for the *ascV* gene alone that was only displayed by the *A. allosaccharophila* strains. On the other hand, the *A. caviae* and the *A. allosaccharophila* species were the only groups whose strains displayed a unique pattern, the lipolytic activity genetic determinants by the former and *ascV* gene by the last.

Nine different combinations were detected in UnW sources collected strains, six in AS and three different combinations were identified among the SE recovered specimens. Three distinct arrangements were uniquely found among the UnW recovered strains and one combination of genes was only detected in the SE isolated specimens. Each of these patterns was exhibited by strains belonging to a unique species, for instance the arrangement "aerolysin related genes/*aexT*/*ascV*" was solely displayed by two *A. veronii* strains collected from UnW sources (DW sample). In fact, this observation corresponded to an association of strains of a species with a combination of genes in a given source and was observed for four times. Nevertheless, the patterns displayed by strains of each species were independent of the type of source.

Table 6.3 Combinations of putative virulence factors detected among *Aeromonas* spp. strains collected in relation to the type of source scrutinised.

Putative Virulence Factors Combinations[a]	**Sources (N.)**[b]		
	UnW (80)	**AS (79)**	**SE (12)**
Lip	26	52	6
Lip/Aer	31	16	5
Lip/Aer/*ascV*	10	2	-
Lip/*ascV*	4	1	-
ascV	1	4	-
Aer	2	1	-
Lip/Aer/*aexT*/*ascV*	2	-	-
Aer/*aexT*/*ascV*	2	-	-
Lip/Aer/*aexT*	1	-	-
Aer/*ascV*	-	-	1
No detected genes	1	3	-

The number of strains harbouring each combination is presented.

[a] Different combinations of virulence factors obtained. Lip, lipolytic activity encoding genes. Aer, aerolysin related genes.

[b] Types of sources investigated in this study and number of strains isolated from each source (in brackets). UnW, untreated water sources (namely, untreated drinking and mineral waters samples); AS, agricultural sources (including vegetables and untreated irrigation waters samples); SE, stable environment sources (specifically, cow's drinking water and faeces and udder's cleaning water before milking and milk).

6.4 Discussion

As important and recognised human and animal pathogens, *Aeromonas* species harbour an arsenal of virulence factors which enables them with a multifactorial ability of causing health complications (Chopra *et al.*, 2009). Nevertheless, not all strains provoke infections thus it is important to identify the ones that may be implicated in such processes. The screening of genetic determinants and phenotypic characteristics putatively associated with virulence is a straightforward approach to recognise these specimens and identify the sources posing health threats (Chacón *et al.*, 2003; Sen and Rodgers, 2004).

Aeromonas spp. can be hemolytic, enterotoxic, and cytotoxic or cytotonic. For many years it has been argued whether or not all of these biological activities reside in the same molecule or in different structures (Fujii *et al.*, 1998). It has been shown that in some cases the same molecule was able to provoke all the effects associated to these activities and in others, different activities were encoded in different genes. They were classified as enterotoxic cytotoxins, cytotonic enterotoxins and hemolysins (Chopra and Houston, 1999). In this report, we have focused on the screening of aerolysin, Act and related hemolysins.

The high occurrence of aerolysin like toxins in *A. salmonicida*, *A. bestiarum* and *A. veronii* strains was also recorded by other authors, but the detection of these genes in almost all of the few *A. encheleia* strains tested is in disagreement with our findings (Castro-Escarpulli *et al.*, 2003; Chacón *et al.*, 2003; Sen and Rodgers, 2004). The occurrence in *A. hydrophila* strains is very variable, but the majority of studies reports considerable occurrence of these genes in strains of this species (Chacón *et al.*, 2003; Wu *et al.*, 2007; Aguilera-Arreola *et al.*, 2007; Balsalobre *et al.*, 2009a).

In relation to *A. eucrenophila*, *A. allosaccharophila*, *A. popoffii* and *Aeromonas* sp. HG11, few studies concern the detection of putative virulence factors among these phylogenetic groups. In accordance with our results, Chacón *et al.* (2003) reported the presence of aerolysin related toxins in the unique *A. eucrenophila* and *Aeromonas* sp. HG11 strains collected by them. On the contrary, González-Rodriguez *et al.* (2002) did not find *A. eucrenophila* positive strains. The study conducted by Soler and colleagues (2002) and the one by Chacón *et al.* (2003) reported the occurrence of these genes in 88% of the

A. popoffii strains tested. The solely *A. allosaccharophila* strain examined by Chacón *et al.* (2003) did not yield positive result in their survey which is concordant with our results.

Previous studies have already reported the low occurrence of aerolysin related toxins in *A. caviae* and *A. media* species (Sen and Rodgers, 2004; Wu *et al.*, 2007; Castilho *et al.*, 2009; Pablos *et al*., 2009). However, several investigators using different primers sets targeting the same genes, accounted the significant or high occurrence of these genetic determinants in these species or the phenospecies complex containing *A. media* and *A. caviae* specimens (*A. caviae* complex) (González-Rodriguez *et al.,* 2002; Chacón *et al.,* 2003; Aguilera-Arreola *et al.,* 2007; Castilho *et al.*, 2009). In fact, it has been suggested that the occurrence of this family of genes may be underestimated in *A. media* and/or *A. caviae* probably due to the failure of some molecular techniques used to detect these genes in those specimens (Kingombe *et al.*, 1999; Castilho *et al.*, 2009).

Restriction analysis of the amplicon obtained by PCR with primers AHCF1 and AHCR1 produced two patterns which corresponded to patterns PCR-RFLP1 (AER-B) and PCR-RFLP2 (AER-A) obtained by Kingombe *et al.* (1999). We found that *A. hydrophila*, *A. salmonicida* and *A. bestiarum* strains displayed uniquely or majorly pattern AER-A, whereas *A. eucrenophila*, *A. veronii* and the two *Aeromonas* sp. HG11 specimens exhibited solely or mostly pattern AER-B; these results agree well with those of Kingombe and colleagues (1999). The study of Kingombe's group did not include *A. popoffii* and *A. allosaccharophila*; also, *A. tecta* and the three probably new phylogenetic lines isolated in this study were not available at that time.

When sequences deposited in databases were analysed *in silico*, six restriction patterns of the 232bp amplicon of aerolysin-like genes were obtained. Pattern AER-A was exhibited by 48 strains and the sequences grouped together in the same cluster of the phylogenetic tree (Fig. 6.3). Pattern AER-B was present on 15 strains and its sequences, with two exceptions (AY442276 and U40711), clustered together and apart from the one constituted by sequences displaying pattern AER-A. Five strains exhibited four other distinct restriction patterns that appeared dispersed in the two main clusters of the phylogenetic tree (accession numbers AF485772, EU254229, Y00559, AF064068 and EF620533) which none corresponded to the PCR-FLP3 reported by Kingombe and colleagues (1999). The 232bp fragments derived from the aerolysin related genes of *A. hydrophila* under the accession number AY442276 and *A. caviae* accession number

U40711 displayed the AER-B pattern, although, their nucleotide sequences are more closely related to sequences displaying pattern AER-A as shown in Figure 6.3. These results must be looked carefully because they may be the consequence of point mutations or sequencing errors.

Kingombe *et al.* (1999) suggested that the different patterns may correlate with different biological activities, with the different sequences coding for aerolysins-hemolysins, cytolytic enterotoxins or cytotonic enterotoxins. Most sequences in GenBank correspond to proteins whose biological activity has not been characterised. Even though, by analysing reports made by the distinct groups studying some of these toxins a difference is perceptible. The *act* gene from strain *A. hydrophila* SSU (accession number M84709) displaying pattern AER-A was found to encode a toxin that is lethal to mice when injected intravenously, hemolytic, cytotoxic and enterotoxic, inducing severe intestinal tissue damage (Galindo *et al.*, 2006). On the other hand, the hemolysins from *A. hydrophila* strain Sb (accession number AY611033) characterised by Epple and colleagues (2004) and *A. sobria* strain 357 (accession number AY157998) studied by Fujii *et al.* (1998) exhibiting pattern AER-B were found to be hemolytic, cytotoxic and enterotoxic without evidence for epithelial cell damage or mucosal inflammation. Thus, these are closely related molecules with similar biological functions that probably induce diarrhoea by the same pathways (Fujii *et al.*, 2008) but act upon intestinal cells in different manners: while Act, exhibiting pattern AER-A, causes intestinal tissue damage, the toxins described by Epple *et al.* (2004) and Fujii *et al.* (1998), displaying pattern AER-B elicit enterotoxic responses without injuring intestinal cells. Whether or not this difference correlates with the segregation of genes encoding these toxins into two major patterns (AER-A and AER-B) is not possible to state and it would only be possible by the characterisation (biological and molecular) of all the toxins described using the same methodology.

Therefore, aeromonads produce numerous similar toxins, encoded by related but different genes, with functional and molecular differences that may be associated to specific biological activities (Ferguson *et al.*, 1997; Chopra and Houston, 1999; Galindo *et al.*, 2006). This may be the result of adaptations of the isolates to different hosts and/or environments as deduced for *A. salmonicida* A449 (Reith *et al.*, 2008). Moreover, the recent publications of the *A. hydrophila* ATCC7966 and *A. salmonicida* A449 complete genomes further demonstrated that genes encoding different related toxins are present in

the same specimen (Seshadri *et al.*, 2006; Reith *et al.* 2008). The synthesis of more than one toxin may result in a synergistic effect of the different molecules on the pathogenicity of strains (Erova *et al.*, 2007; Singh *et al.*, 2009).

We further investigated the occurrence of genes related to the T3SS. The *ascV* gene encodes an inner membrane protein of the basal structure of the apparatus belonging to a highly conserved family of proteins found in every known T3SS, thus suitable to assess the presence of this secretory system among strains (Stuber *et al.*, 2003b). However, it is arguable that the presence of this gene in a strain implies the presence of the entire locus coding for the complete and functional secretory system. Nevertheless, as genes coding for different T3SS components occur concomitantly in the same strains, it is plausible the deduction of the presence of the whole system by the presence of one of the conserved structural components (Chacón *et al.*, 2004; Vilches *et al.*, 2004; Silver and Graf, 2009). The *aexT* gene codes for an effector toxin secreted by and translocated through the T3SS. It has been shown to interfere with important biological pathways of eukaryotic cells, to be implicated in the inhibition of phagocytosis and to induce morphological changes in fish cells including cell rounding and subsequent lysis (Sha *et al.*, 2007). Thus, AexT constitutes a potentially important virulence factor for *Aeromonas* spp..

Little is known about the occurrence of *ascV* and *aexT* genes or other T3SS genes or effectors among aeromonads. In fact, very few specimens of the genus were investigated for the presence of these genetic determinants and the majority of the phylogenetic groups had no representative strains included in the studies conducted. Earlier surveys reported the moderate to high occurrence of these genes among *A. hydrophila*, *A. caviae* and *A. veronii* species or the phenospecies complexes, mostly in *A. hydrophila* and *A. veronii* strains (Chacón *et al.*, 2004; Vilches *et al.*, 2004; Wu *et al.*, 2007; Silver and Graf, 2009). In addition, they were detected in almost all *A. salmonicida* strains studied (Braun *et al.*, 2002; Burr *et al.*, 2005). Only one to three strains of *A. media*, *A. bestiarum*, *A. eucrenophila*, *A. encheleia* (Braun *et al.*, 2002; Burr *et al.*, 2005) and *A. allosaccharophila* strains (Silver and Graf, 2009) were prospected and except for *A. bestiarum*, all harboured one or the other gene. Also, *A. piscicola* AH-3 harbours the entire operon for T3SS (Vilches *et al.*, 2004). In addition, the occurrence of T3SS genes comparing environmental or clinical specimens was variable (Vilches *et al.*, 2004; Yu *et al.*, 2004; Aguilera-Arreola *et al.*, 2005).

In this study, the presence of the *ascV* gene among strains was low (16%). Even though, it was present in strains of several different species (*A. bestiarum*, *A. allosaccharophila*, *A. hydrophila*, *A. veronii*, *Aeromonas* sp. Group II, *A. salmonicida*, *A. eucrenophila* and *A. tecta*), being more often detected in *A. bestiarum* (10 out of 14 strains), *A. allosaccharophila* (five out of five strains) and *A. veronii* (three out of five strains). Although we have no references of the occurrence of *ascV* in *A. bestiarum* isolates and only one for *A. allosaccharophila* (Silver and Graf, 2009), it is remarkable that almost all our strains of these species carry this gene. In fact, this was the unique putative virulence genetic determinant found in *A. allosaccharophila* strains from the ones prospected (Table 6.1).

The *aexT* gene was detected in two *A. veronii* strains (both collected from DW samples) under stringent conditions (42ºC and 50% formamide buffer) of hybridisation. When the hybridisation was performed under low stringent conditions (50ºC and 0% formamide), one *A. salmonicida* and two *A. bestiarum* strains (all isolated from DW samples) also displayed positive result (Table 6.1).

We decided to perform the surveying of *aexT* at both high and low stringent conditions as recently it was suggested that the occurrence of T3SS genes, including *aexT*, could be underestimated in the genus due to sequence divergence of this gene in distinct species (Silver and Graf, 2009). In fact, three strains that were negative in hybridisation under high stringent conditions performed positive for *aexT*-like sequences at low stringent conditions.

In contrast with other studies that reported a higher occurrence of the AexT encoding gene, inclusively, concomitantly with T3SS apparatus encoding genes (Chacón *et al.*, 2004; Aguilera-Arreola *et al.*, 2005; Wu *et al.*, 2007; Vilches *et al.*, 2008; Silver and Graf, 2009), we recorded a very low detection of *aexT* among our strains (3%). Moreover, only four strains contained simultaneously the *aexT* and *ascV* genes (two *A. bestiarum* and two *A. veronii* strains all collected from DW samples). Other effector toxins secreted and translocated through the T3SS have been identified in *Aeromonas*, namely, AexU, AopP, AopH, AopO and Ati2 (Sierra *et al.*, 2007; Reith *et al*., 2008) and many others are known in other genus (Sha *et al.*, 2005; Cornelis, 2006). In this way, a search for other effectors would be important. Additionally, the fact that *aexT*-like genes were detected in three strains only by low stringent conditions of hybridisation indicates that similar unknown

genes are present or that this gene may go undetected as already mentioned above. On the other hand, as seen in other studies (Burr and Frey, 2007; Vilches *et al*., 2008), one strain harboured the *aexT* but not the *ascV* gene.

Interestingly, only one *A. salmonicida* specimen carried *ascV* and other the *aexT* gene. Other studies that screened fish causing disease isolates showed the occurrence of these genes to be higher (Burr *et al.*, 2005; Burr and Frey, 2007) than in our environmental isolates. One may speculate that this difference in strains from the same species may correlate to the adaptation of specimens to their ecological niche. On the other hand, as the T3SS in *A. salmonicida* has been reported to be localised on plasmids and in strain JF2267 the pASvirA is thermolabile (Stuber *et al.*, 2003a; Reith *et al.*, 2008), it is not discardable the possibility of loss of plasmids during investigation procedures, not only regarding *A. salmonicida* specimens but also the other strains of other phylogenetic groups. The T3SS locus in *A. hydrophila* and *A. piscicola* have all been reported to be encoded in the chromosome (Yu *et al.*, 2004; Vilches *et al.*, 2004; Sha *et al.*, 2005), but nothing is known about the localisation of T3SS locus in other species of the genus.

We have also looked for genetic determinants coding for enzymes with lipolytic and phospholipase C activity in our aeromonads collection. The genetic determinants of these activities were detected in 91% of strains which correlated well with extracellular lipolytic activity, which was found in all strains. Even though, these findings also indicate that other genetic basis of extracellular lipolytic activity is present in strains performing negative in lipases PCR assay and exhibiting this physiological feature. Additionally, extracellular proteolytic activity was prospected and detected in 96% of the strains (Table 6.1). The lipases and proteases secreted by *Aeromonas* spp. are associated with their pathogenicity by the fact that they enhance their invasion through the host tissues and help in infection establishment by destroying cell components, overcoming host defences and providing nutrients for cell proliferation (Pemberton *et al.*, 1997; Galindo *et al.*, 2006). These properties are not solely capable of causing disease (Merino *et al.*, 1999), but are an important contribution to invasion and proliferation in host tissues.

Strains harbouring aerolysin related genes were more often collected from UnW and SE (60% and 50% of positive strains recovered from each source, correspondingly) and low occurrence was detected in AS recovered strains (24% of positive strains). Additionally, strains harbouring *ascV* were collected from all the environments prospected

(untreated waters, agricultural sources and stable) being more frequently isolated from untreated water samples (60% of positive strains). The *aexT* gene was solely detected among untreated waters specimens isolated specifically from drinking water samples. Ten different combinations of putative virulence factors were recorded and some obvious associations of gene arrangements vs. species, gene arrangements vs. source and gene arrangements vs. species vs. source were recorded. A higher number of strains carrying the genes prospected and more different combinations were detected in the UnW sources, thus being the source with more strains putatively virulent and posing a higher risk to human health and animal well being (Tables 6.2 and 6.3).

In conclusion, *Aeromonas* spp. strains isolated from different sources (untreated water and food products) related to direct or indirect human and animal consumption carry genetic determinants associated with known powerful virulence mechanisms present in pathogenic bacteria. Moreover, one recently described and poorly known species (*A. tecta*) and three potential new ones also carry these genetic determinants. Additionally, the presence of these putative virulence factors was detected in species poorly scrutinised before, extending the array of *Aeromonas* species that potentially may pose health threats. The potential multifactorial virulence pattern displayed by these strains poses a significant and relevant risk to humans and animals.

7. Polyphasic study of new phylogenetic lines in the genus *Aeromonas*

Abstract

During a study on diversity of *Aeromonas* species collected from different sources in Portugal, from which the majority are for human and/or animal consumption, a group of isolates was identified which displayed *gyrB* gene sequences different from those previously known. These isolates clustered in three distinct groups, *Aeromonas* sp. Group I (one strain from two isolates), *Aeromonas* sp. Group II (five strains within 12 isolates) and *Aeromonas* sp. Group III (one strain out of 12 isolates). A preliminary polyphasic study based on phenotypic and DNA sequence data of these specimens demonstrated that they may be representatives of three novel phylogenetic branches within *Aeromonas* species. Further studies are needed to provide formal species proposals.

Keywords: *Aeromonas*; new species.

7.1 Introduction

The genus *Aeromonas* is the type genus of the family *Aeromonadaceae* within the order *Aeromonadales* of the class *Gammaproteobacteria* and includes Gram-negative facultatively anaerobic rods, oxidase and catalase positive, which are resistant to the vibriostatic agent O/129 (Martin-Carnahan and Joseph, 2005). Aeromonads are found in different types of aquatic environments (Massa *et al.*, 2001; Biscardi *et al.*, 2002; Henriques *et al.*, 2006a; Moura *et al.*, 2007; Razzolini *et al.*, 2008), are also present in soil (Huddleston *et al.*, 2006), in different kinds of food products (Neyts *et al.*, 2000) and in distinct organic substrates of animal origin (Chang *et al.*, 2007; Ceylan *et al.*, 2009; Fontes *et al.*, 2010). Moreover, aeromonads are responsible for health complications in invertebrates and vertebrates (Janda and Abbott, 2010). Specifically, in fish, *Aeromonas* spp. are causative agents of furunculosis (Seshadri *et al.*, 2006; Reith *et al.*, 2008) and in humans they are associated with a wide array of clinical presentations, not only as an opportunistic pathogen but also as the primary cause of infection (Figueras *et al.*, 2005).

To date, 23 species were described: *A. hydrophila, A. bestiarum, A. salmonicida, A. caviae, A. media, A. eucrenophila, A. sobria, A. jandaei, A. veronii, A. schubertii, A. trota, A. allosaccharophila, A. encheleia, A. popoffii* (Martin-Carnahan and Joseph, 2005), *A. molluscorum* (Miñana-Galbis *et al.*, 2004), *A. simiae* (Harf-Monteil *et al.*, 2004), *A. bivalvium* (Miñana-Galbis *et al.*, 2007), *A. aquariorum* (Martínez-Murcia *et al.*, 2008), *A. tecta* (Demarta *et al.*, 2008), *A. fluvialis* (Alperi *et al.*, 2010), *A. piscicola* (Beaz-Hidalgo *et al.*, 2009), *A. taiwanensis, A. sanarellii* (Alperi *et al.*, in press). Additionally, there is

still an unnamed hybridisation group (HG11) whose taxonomic position still raises debates (Soler *et al.*, 2004; Nhung *et al.*, 2007) and the designation *A. diversa* has been proposed for the formerly known Group 501 (Miñana-Galbis *et al.*, 2010a).

The number of described species is growing rapidly and reclassifications of some species have been done leading to a complex taxonomic record of the genus (Saavedra *et al.*, 2006; Nhung *et al.*, 2007). These controversies arise on one hand by the great variability of specimens within the genus and, on the other hand, by the close phylogenetic relation between some species which difficult the determination of the affiliation of new strains (Alperi *et al.*, 2010).

For the majority of *Aeromonas* species the establishment of a characteristic phenotypic profile involves the analysis of a large number of traits. Only by doing this it is possible to achieve a final identification and a clear cut species differentiation, using phenotypic characteristics. Moreover, intraspecies phenotypic variability exists leading to additional species identification errors (Abbott *et al.*, 2003). Additionally, phenotypic and genotypic identification are not always in agreement (Martínez-Murcia *et al.*, 1992a; Janda and Abbott, 2010).

The sequence of the 16S rRNA gene is very similar between the different species of the genus, varying from 96.7% to 100% (Martínez-Murcia *et al.*, 1992a; Alperi *et al.*, 2010). Moreover, the genome of each strain contains several copies of this gene (10 in *A. hydrophila* ATCC 7966; Seshadri *et al.*, 2006) and in some strains intragenomic heterogeneities were identified in the V3 and V6 regions (according to the *E. coli* system; Brosius *et al.*, 1978), precisely the regions that present characteristic signature nucleotides for most species (Morandi *et al.*, 2005; Alperi *et al.*, 2008). Thus, the 16S rRNA gene does not allow the discrimination of a few closely related species hampering the proper classification of aeromonads (Martínez-Murcia *et al.*, 1992a; Collins *et al.*, 1993; Alperi *et al.*, in press).

Several housekeeping genes have been shown to be excellent molecular chronometers, both individually and in concomitance, to assess phylogenetic interrelationships of *Aeromonas* at the species level, generally permitting a final and accurate classification of each specimen analysed (Yañez *et al.*, 2003; Soler *et al.*, 2004; Küpfer *et al.*, 2006; Nhung *et al.*, 2007; Sepe *et al.*, 2008; Alperi *et al.*, 2010; in press). The *gyrB* and *rpoD* genes, in particular, have been successfully used by several

investigators to provide a robust phylogenetic frame useful on *Aeromonas* species identification (Yáñez *et al.*, 2003; Soler *et al.*, 2004; Saavedra *et al.*, 2006). Very recently, the multilocus phylogenetic analysis (MLPA) based on the analysis of five housekeeping genes as recommended by Stackebrandt *et al.* (2002) to define species, has been applied to phylogenetically allocate three new specimens (*A. fluvialis*, *A. taiwanensis* and *A. sanarellii*) by Alperi *et al.* (2010; in press).

As reported in previous chapters, during the course of this study, 7 bacterial strains belonging to the genus *Aeromonas* were found to display molecular variants of the *gyrB* gene that were different from those previously described. In order to clarify the taxonomic position of these strains, we performed a polyphasic approach involving phylogenetic analysis based on *gyrB*, *rpoD* and 16S rRNA genes sequences and some biochemical tests. Although this was a preliminary study and additional work is needed, the present results indicate that they represent new phylogenetic lineages within the genus *Aeromonas*.

7.2 Material and methods

7.2.1 Strains used in this study

The bacterial isolates (26) were obtained and genotyped by RAPD methodology as described in previous chapters. Those displaying the same RAPD profile were considered highly similar if not the same strain. Consequently, a single isolate was chosen for further studies. Table 7.1 depicts strains, sampling sites and sources of isolation.

Table 7.1 Strains selected in this study including sampling site and source of isolation.

Strain (N.)[a]	Sampling site[b]	Source[c]
A.131/2 (2)	SS73	UnDW (Public drinking fountain)
A.136/15 (1)	SS79	UnDW (Public drinking fountain)
A.L8-3 (2)	AS.SS4	Celery
A.L10-4 (6)	AS.SS5	Lettuce
A.MW.11/6 (2)	WS2.SS3	MW
A.MW.28/6 (1)	WS2.SS3	MW
A.L15-1 (12)	AS.SS7	Lettuce

[a] In brackets are presented the number of isolates represented by each strain.
[b] Sampling sites SS73 and SS79 were located in the district of Aveiro and Lisbon, respectively. Sampling sites AS.SS4 and AS.SS5 were in the district of Porto and sampling site AS.SS7 was in the district of Leiria (Chapters 3 and 4).
[c] UnDW, untreated drinking water; MW, mineral water.

7.2.3 Phylogenetic analysis

Detailed phylogenetic analysis included molecular data on the sequences of *gyrB* (obtained in chapters 3 and 4), *rpoD* and 16S rRNA genes. PCR conditions and primers used for the amplification of *rpoD* and 16S rRNA genes were as described previously (Martínez-Murcia *et al.*, 1992b; Soler *et al.*, 2004). Sequencing of the amplified products by using the appropriate primers for each gene and subsequent sequence analysis were done as explained in chapters 3 and 4 of this dissertation.

Partial sequences of each gene were individually aligned with *gyrB*, *rpoD* or 16S rRNA gene sequences from type and reference *Aeromonas* sp. strains from the culture collection of the Molecular Diagnostics Center (MDC), Orihuela, Spain, and unrooted phylogenetic trees were constructed as described in chapters 3 and 4 of this dissertation.

To obtain the phylogenetic tree based on the combination of sequences of *gyrB* and *rpoD* genes, the sequences of both genes from each strain were concatenated in the same order (*gyrB-rpoD*) to form a single nucleotide sequence as described for the MLPA approach (Alperi *et al.*, 2010; in press). To construct this phylogenetic tree, the same methodology mentioned before was applied.

7.2.4 Phenotypic analysis

A preliminary biochemical characterisation of the strains was achieved by using the API® 20NE and API® 20E commercial kits (Biomérieux, France) according to the manufacturer instructions. Additionally, standard methodology was used to test catalase and oxidase activities.

7.3 Results

7.3.1 Phylogenetic study based on gyrB gene sequence

The nucleotide sequences comprised between positions 473 and 1078 of *gyrB* gene were obtained for the 7 strains (*E. coli* numbering, based on *E. coli* strain K-12 *gyrB* gene sequence accession number X04341; Adachi *et al.*, 1987). The sequence alignment obtained with CLUSTALX program, revealed that there is an insertion triplet (ATG) in the *gyrB* partial sequences of all strains composing Group II in position 921 (nucleotides 922, 923, 924) according to *E. coli gyrB* numbering. This insertion is also present in the same

position of the *gyrB* gene partial sequences of *A. molluscorum* (ATG), *A. simiae* (ATG), *A. schubertii* (ACC) and *A. diversa* (ACC) strains used in this study (data not shown).

As seen in the *gyrB* based evolutionary tree (Figure 7.1), the 7 newly isolated strains formed three independent lines constituting well defined and distinct branches in the phylogenetic tree. Group I was solely formed by strain A.131/2, Group II included strains A.136/15, A.L8-3, A.L10-4, A.MW.11/6 and A.MW.28.6 and Group III contained strain A.L15-1.

Groups I and III formed independent branches, being relatively related to each other, displaying 93% of *gyrB* gene similarity (corresponding to 37 nucleotide differences). The main cluster formed by these two strains is independent from the deepest branch of the tree formed by *A. simiae*, *A. schubertii* and *A. diversa* as well as from the main cluster containing the remaining phylogenetic groups. Based on the obtained *gyrB* gene similarities, the described *Aeromonas* species most closely related to Group I were *A. aquariorum*, *A. taiwanensis*, *A. eucrenophila* and *A. tecta* (92% similarity). Similarly, *A. hydrophila*, *A. aquariorum*, *A. taiwanensis*, *A. bivalvium*, *A. bestiarum* and *A. eucrenophila* were the known *Aeromonas* species closest relatives of Group III (92% of similarity).

Group II formed an independent and robust branch in the phylogenetic tree comprising two strains, A.L10-4 and A.MW.11/6, related at 97% of *gyrB* sequence similarity and three strains, A.136/15, A.L8-3 and A.MW28/6, with sequence similarities of 97% and 98%. Overall *gyrB* sequence similarities within Group II varied between 95% and 98% (corresponding to 29 to 7 nucleotide differences). The *Aeromonas* species closest relatives of this group were *A. eucrenophila* (92% – 93% of similarity) and *A. tecta* (92% of similarity). Group II branched within a main cluster of species containing *A. bestiarum*, *A. salmonicida*, *A. piscicola*, *A. popoffii*, *Aeromonas* sp. HG11, *A. encheleia*, *A. molluscorum* and *A. bivalvium*.

Similarities of *gyrB* partial sequences of the 7 strains with other *Aeromonas* species varied between 87% and 93% (data not shown).

Table 7.2 exhibits *gyrB* partial sequence similarities and nucleotide differences between each of the 7 strains.

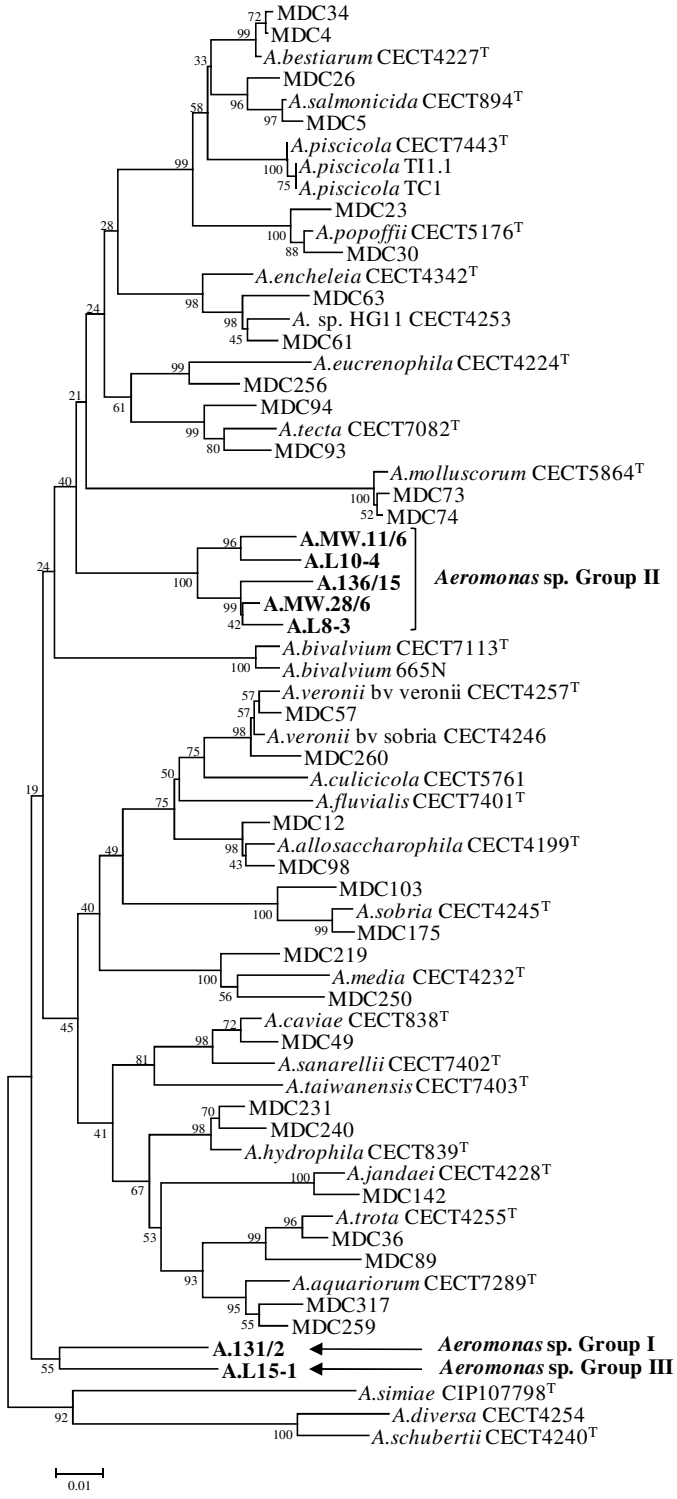

Figure 7.1 Unrooted phylogenetic tree based on the *gyrB* gene partial sequences of the 7 strains in study (in bold) and *Aeromonas* sp. strains of the MDC (Orihuela, Spain) culture collection, including type strains.

Legend to figure:
Strains of all known *Aeromonas* sp. are included. *Aeromonas* strains Groups I, II and III are indicated.
The tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) with the Kimura-2 parameter model (Kimura, 1980) with the MEGA4 program (Tamura *et al.,* 2007). Bootstrap values based on 1000 replicates are shown next to the nodes. The bar represents 0.01 base substitutions per site.

Table 7.2 Number of nucleotide differences (lower left) and sequence similarity rates (%; upper right) of the *gyrB* gene sequences of the 7 strains in study.

Strain	A.131/2	A.136/15	A.L8-3	A.L10-4	A.MW.11/6	A.MW.28/6	A.L15-1
A.131/2		91	92	91	90	92	93
A.136/15	56		97	96	95	98	91
A.L8-3	49	14		96	96	98	92
A.L10-4	56	21	21		97	97	91
A.MW.11/6	59	29	21	14		96	90
A.MW.28/6	47	11	7	18	22		92
A.L15-1	37	54	49	57	59	48	

7.3.2 Phylogenetic study based on rpoD gene

The nucleotide sequences comprised between positions 411 and 1042 of *rpoD* gene were obtained for the 7 strains (*E. coli* numbering, based on *E. coli* strain K-12 *rpoD* gene sequence accession number U00096, locus b3067; Burton *et al.*, 1981). Each strain displayed an exclusive *rpoD* partial gene sequence. By analysing the alignment of all *rpoD* partial sequences, a deletion of a triplet was observed in A.L15-1 in position 616-6620 (nucleotides 617, 618, 619) according to *E. coli rpoD* gene, which was not recorded for any other specimen (data not shown).

As seen in the unrooted phylogenetic tree based on *rpoD* partial sequences (Figure 7.2), the 7 strains formed three independent and well defined lines. Group I includes strain A.131/2, Group II is formed by strains A.136/15, A.L8-3, A.L10-4, A.MW.11/6 and A.MW.28.6 and Group III contained strain A.L15-1. This grouping was in accordance with the groups formed in the *gyrB* gene based tree.

Groups I and III formed similar to what has been seen in the *gyrB* phylogenetic tree, nevertheless grouped with the main cluster containing the majority of *Aeromonas* species. Between Groups I and III, *rpoD* sequence similarity was 93% (corresponding to 48 nucleotide differences). On the basis of *rpoD* gene partial sequence similarities with the described *Aeromonas* species, Group I was most closely related to *A. media* and *A. eucrenophila* (approximately 92% of similarity) and Group III to *A. media* (approximately 93% of similarity), but species as *A. tecta* and *Aeromonas* sp. HG11 had similar values, being Groups I and III neighbours in the phylogenetic tree.

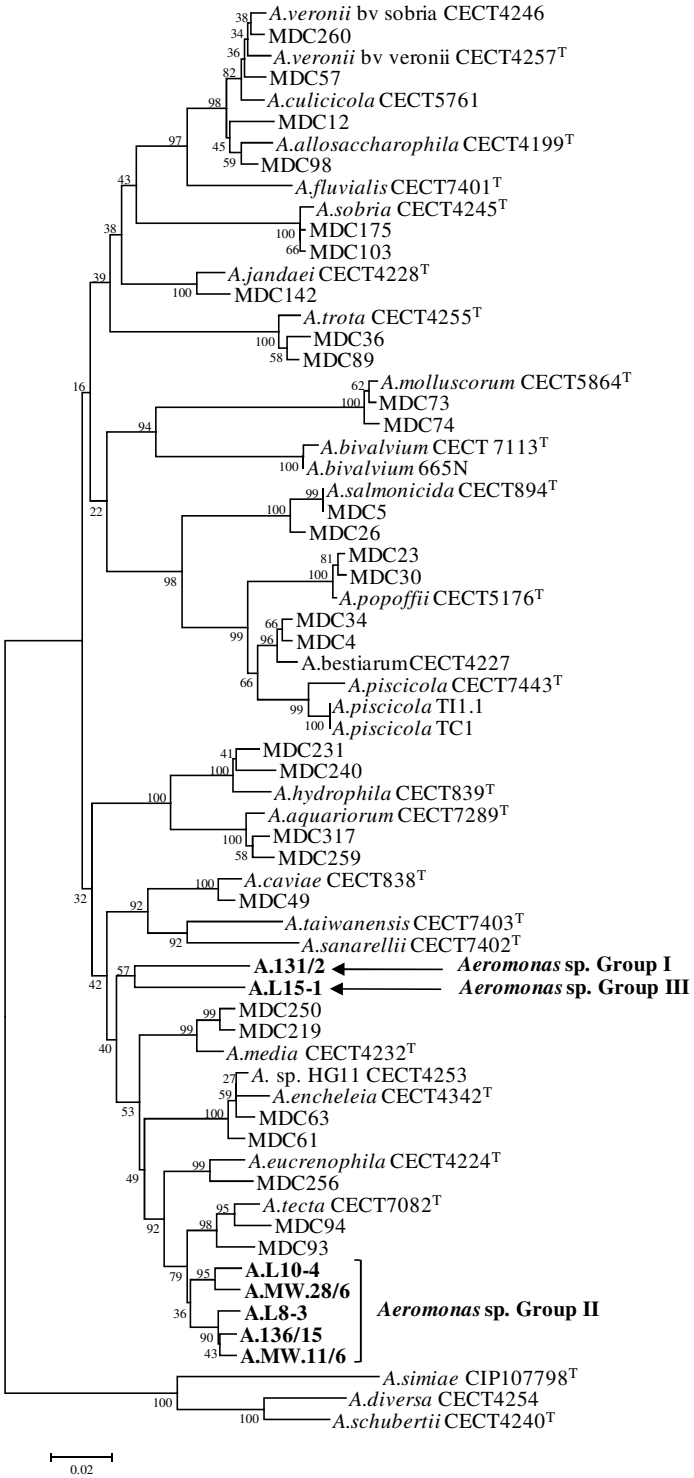

Figure 7.2 Unrooted evolutionary tree exhibiting the relationships of the 7 strains in study (in bold) and the *Aeromonas* sp. strains of the MDC (Orihuela, Spain) culture collection, including type strains, based on the *rpoD* gene partial sequences.

Legend to figure:
Strains of all known *Aeromonas* sp. are included. *Aeromonas* strains. Groups I, II and III are indicated. The tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) with the Kimura-2 parameter model (Kimura, 1980) with the MEGA4 program (Tamura *et al.,* 2007). Bootstrap values based on 1000 replicates are shown next to the nodes. The bar represents 0.02 base substitutions per site.

In contrast to the *gyrB* based phylogenetic tree, Group II grouped highly related to the species *A. tecta*, yet still constituting an independent and distinct branch. Other neighbour species on the tree were *A. eucrenophila* and *A. media*. Two subgroups formed within the major Group II, but distinct from subgroups resulting from *gyrB* analysis: one is composed by strains A.L10-4 and A.MW.28/6 (98% of *rpoD* partial sequence similarity) and the other by A.L8-3, A.136/15 and A.MW.11/6 (98% of *rpoD* partial sequence similarity).

Similarities of *rpoD* partial sequences of the 7 strains with other *Aeromonas* species varied between 83% and 96% (data not shown).

Table 7.3 displays data concerning the number of nucleotide differences between *rpoD* sequences for the 7 strains and the corresponding percentage of similarity.

Table 7.3 Number of nucleotide differences (lower left) and sequence similarity rates (%; upper right) of the *rpoD* gene sequences of the 7 strains in study.

Strain	A.131/2	A.136/15	A.L8-3	A.L10-4	A.MW.11/6	A.MW.28/6	A.L15-1
A.131/2		92	91	92	92	92	93
A.136/15	50		98	97	98	97	93
A.L8-3	53	9		96	98	96	92
A.L10-4	50	19	20		96	98	92
A.MW.11/6	50	7	8	20		96	92
A.MW.28/6	51	18	21	11	21		92
A.L15-1	48	48	51	50	49	52	

7.3.3 Phylogenetic study based on 16S rRNA gene

The nucleotide sequences comprised between positions 76 and 1395 of 16S rRNA gene were obtained for the 7 strains (*E. coli* numbering, based on *E. coli* 16S rRNA gene sequence accession number J01695; Brosius *et al.*, 1978).

Strains A.L10-4 and A.MW.11/6 had identical 16S rRNA gene sequence as did A.136/15 and A.MW.28/6 and distinct from the remaining strains which also displayed different molecular variants from each other and all were distinct from the previously reported *Aeromonas* species.

The sequence alignment obtained with CLUSTALX software showed that strains A.136/15, A.L8-3, A.L10-4, A.MW.11/6 and A.MW.28/6 and *A. eucrenophila* CECT4224^T shared the same signature nucleotide sequence between positions 457 and

476, within the V2 region, according to *E. coli* numbering, different from the remaining type strains of the genus.

Figure 7.3 displays the phylogenetic tree based on 16S rRNA gene sequences. The tree topology is consistent with results previously reported for all known species (Alperi *et al.*, in press; Beaz-Hidalgo *et al.*, 2009).

In agreement to the other housekeeping genes obtained trees, strains A.131/2 (Group I) and A.L15-1 (Group III), grouped together, forming independent and distinct branches from each other. As with the *rpoD* and contrary to the *gyrB* phylogenetic tree, this grouping is included in the main cluster containing the majority of species of the genus. In regard to 16S rRNA gene partial sequences similarities, the formerly known species more closely related to Groups I and III are *A. trota*, *A. aquariorum*, *A. caviae*, *A. taiwanensis* and *A. sanarellii*, displaying 99% of similarity, which were also the species grouping with these strains in the phylogenetic tree. 16S rRNA gene sequence similarity between Groups I and III was 99% (two nucleotide differences).

In contrast to what has been observed for the *rpoD* and *gyrB* based trees, in the 16S rRNA gene tree, strain A.L8-3 did not cluster with the remaining strains of Group II. Instead, this strain remained in an independent line in the grouping formed by *A. bestiarum*, *A. piscicola*, *A. salmonicida*, *A. molluscorum*, *A. encheleia*, *A. eucrenophila* and *A. tecta*, with whom shared 99% of 16S rRNA gene partial sequence similarity. Strains A.136/15, A.MW.28/6 and A.L10-4, A.MW.11/6 (sharing 99% of similarity of 16S rRNA gene sequence) clustered in the same grouping harbouring *A. trota*, *A. aquariorum*, *A. caviae*, *A. taiwanensis* and *A. sanarellii*, forming a distinct group. These strains exhibited 98% of 16S rRNA gene partial sequence similarity with all the previously reported species except for *A. schubertii* (97%), *A. diversa* (97%) and *A. simiae* (96%).

Table 7.4 displays 16S rRNA gene sequence similarities and nucleotide differences between the 7 strains.

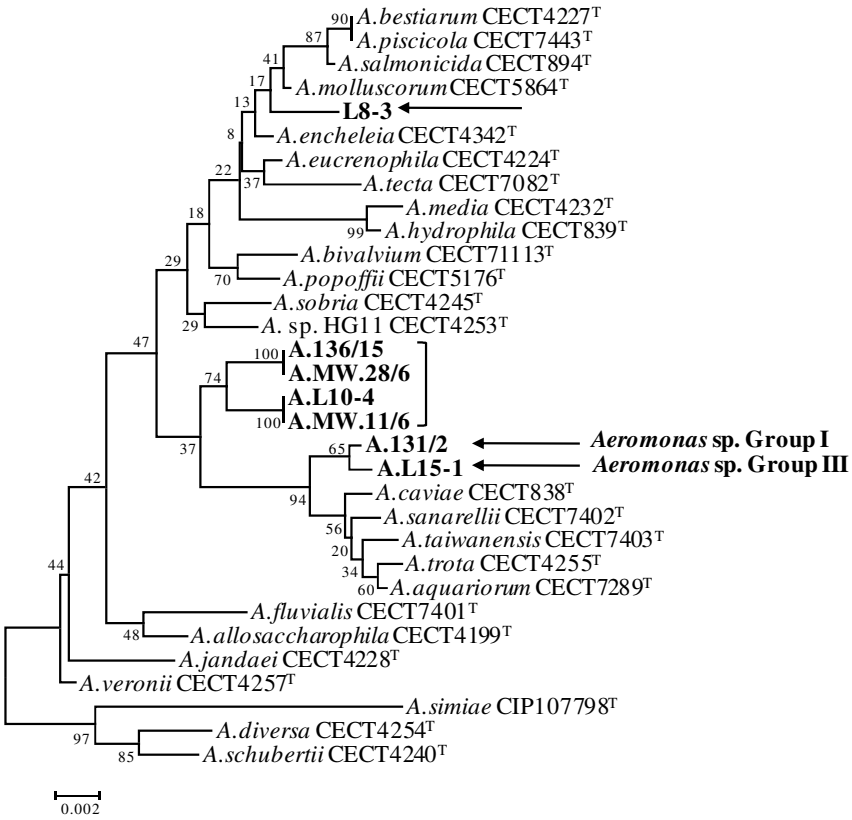

Figure 7.3 Unrooted phylogenetic tree based on 16S rRNA gene partial sequences, demonstrating the relationships of the 7 strains in study (in bold) to *Aeromonas* sp. type strains present in the MDC (Orihuela, Spain) culture collection.

Legend to figure:
All known *Aeromonas* spp. are included. *Aeromonas* strains Groups I and III are signalised as also the remaining strains in study.
The tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) with the Kimura-2 parameter model (Kimura, 1980) with the MEGA4 program (Tamura *et al.,* 2007). Bootstrap values based on 1000 replicates are shown next to the nodes. Scale bar represents 0.002 nucleotide substitutions per site.

Table 7.4 Number of nucleotide differences (lower left) and sequence similarity rates (%; upper right) of the 16S rRNA gene sequences of the 7 strains in study.

Strain	A.131/2	A.136/15	A.L8-3	A.L10-4	A.MW.11/6	A.MW.28/6	A.L15-1
A.131/2		99	98	98	98	99	99
A.136/15	13		99	99	99	100	99
A.L8-3	18	10		99	99	99	98
A.L10-4	15	7	11		100	99	98
A.MW.11/6	15	7	11	0		99	98
A.MW.28/6	13	0	10	7	7		99
A.L15-1	2	13	20	15	15	13	

7.3.4 16S rRNA gene microheterogeneities

Chromatogram analysis of the 16S rRNA gene partial sequence of strains A.131/2 (Group I), A.L8-3 and A.L15-1 (Group III) revealed double sequencing peaks in determinate positions (microheterogeneities). Isolates DW.131/7 and V.L15-5 shown to be clonally related to strains A.131/2 and A.L15-1, respectively (exhibited the same RAPD profiles – chapters 3 and 4), were also subjected to *gyrB*, *rpoD* and 16S rRNA genes partial sequencing. Experiments were performed in duplicate for each of the five specimens in order to rule out possible sequencing errors or presence of mixed DNA from different strains. All double signals were reproducible and consistent in every sequencing experiment and the *gyrB* and *rpoD* sequences analysis demonstrated the purity of the DNA of each specimen used in examination. Table 7.5 displays the microheterogeneities observed for the 16S rRNA gene sequence of each specimen as well as their positions in the sequence.

Thirteen mutations, including 11 transitions [purine ↔ purine (A/G) and pyrimidine ↔ pyrimidine (C/T)] and two transversions (purine ↔ pyrimidine) mostly localised in the V3 region (11 positions) but also in the V6 region (2 positions), were observed in strain A.131/2. They were further confirmed by the detection of the same double sequence signals in the same positions of the 16S rRNA gene sequence of isolate DW.131/7 of this strain (showing identical RAPD profile).

In strain A.L8-3 one transition (A/G) was noticed in the V3 region at position 459 (*E. coli* numbering).

Two transitions, C/T and A/G, were encountered in strain A.L15-1 and in its clone V.L15-5 gene sequences in the V6 region at positions 1011 and 1018 (*E. coli* numbering).

Table 7.5 Positions of 16S rRNA gene microheterogeneities found in strains A.131/2, A.L8-3 and A-L15-1 and in the clonally related specimens to A.131/2 (DW.131/7) and A.L15-1 (AS.L15-5).

Specimen	Localisation of microheterogeneities in 16S rRNA gene[a]												
	457	**458**	**459**	**461**	**469**	**471**	**472**	**473**	**474**	**475**	**476**	**1011**	**1018**
A.131/2	C/T	G/A or A/G	G/T	A/G	T/C	T/C	G/A	C/A	T/C	G/A	A/G	T/C	A/G
DW.131/7	C/T	G/A or A/G	G/T	A/G	T/C	T/C	G/A	C/A	T/C	G/A	A/G	T/C	A/G
A.L8-3	-	-	A/G	-	-	-	-	-	-	-	-	-	-
A.L15-1	-	-	-	-	-	-	-	-	-	-	-	C/T	A/G
AS.L15-5	-	-	-	-	-	-	-	-	-	-	-	T/C	A/G

[a] microheterogeneities spots according to *E. coli* numbering (Brosius *et al.*, 1978).

7.3.5 gyrB-rpoD concatenated sequence analysis

Figure 7.4 displays a phylogenetic tree based on the *gyrB-rpoD* concatenated sequences (approximately 1250bp).

Strains A.131/2 (Group I) and L15-1 (Group III) clustered together forming independent lines of each other and from the previously known species with a bootstrap value of 94%.

Strains A.136/15, A.L8-3, A.L10-4, A.MW.11/6 and A.MW.28.6 (Group II) formed a single grouping distinct from the remaining *Aeromonas* species with a bootstrap value of 100%. The sub-groups produced within Group II were similar to the ones formed in the *rpoD* gene alone tree (Figure 7.2), that is, strains A.136/15, A.L8-3 and A.MW.11-6 grouped together and strains A.L10-4 and A.MW.28.6 formed the other sub-group.

Comparing all phylogenetic trees, differences were observed in relation to the main groupings were Groups I, II and III clustered. As seen in the *gyrB* alone phylogenetic tree (Figure 7.1), Groups I and III formed an independent grouping from the main cluster containing the majority of species of the genus as also from the one harbouring *A. simiae*, *A schubertii* and *A. diversa*. The closest relatives of Group II were *A. tecta*, *A. eucrenophila*, *A. encheleia* and *Aeromonas* sp. HG11.

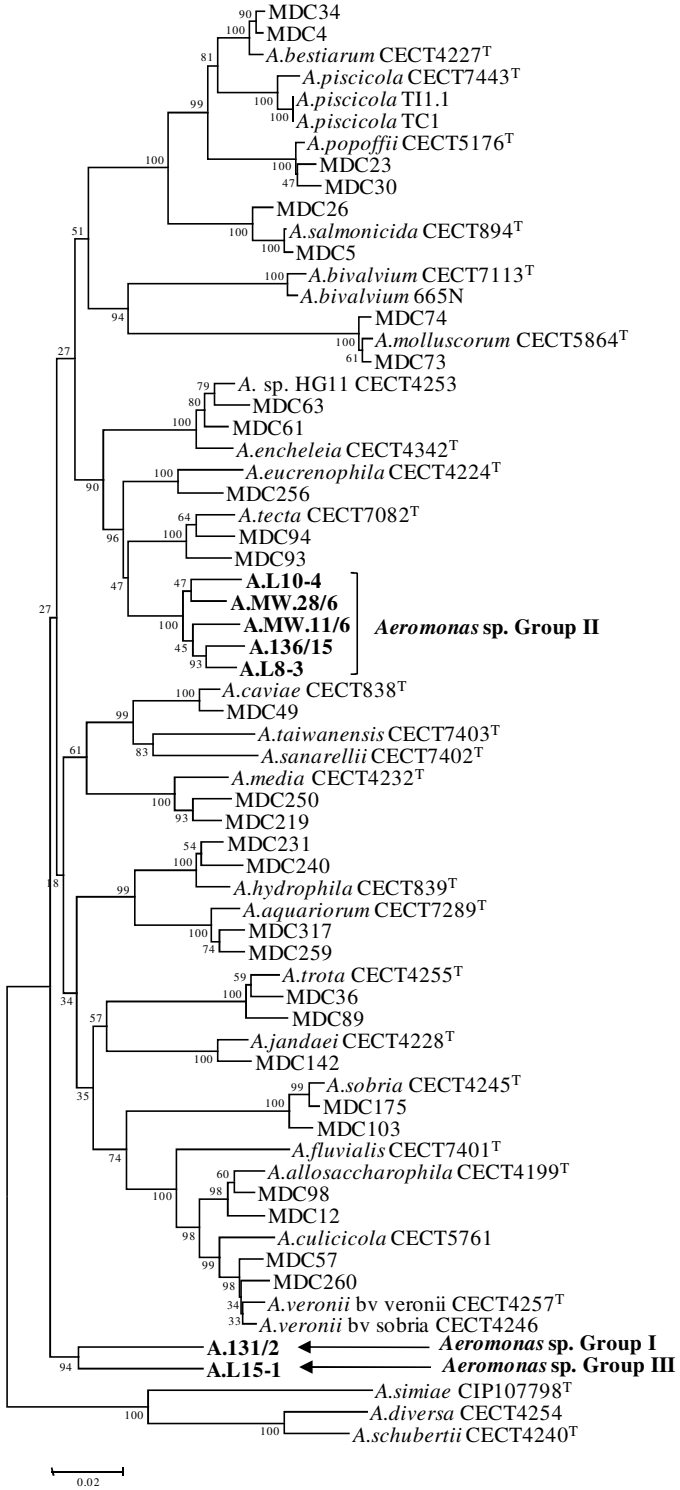

Figure 7.4 Unrooted phylogenetic tree derived from the *gyrB- rpoD* concatenated sequence showing the relationship of the 7 strains in study (in bold) to all currently described *Aeromonas* species.

Legend to figure:
Concatenated sequences included 1250bp approximately. *Aeromonas* strains Groups I, II and III are indicated. The tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) with the Kimura-2 parameter model (Kimura, 1980) with the MEGA4 program (Tamura *et al.,* 2007). Bootstrap values based on 1000 replicates are shown next to the nodes. Scale bar represents 0.02 nucleotide substitutions per site.

7.3.6 Phenotypic characterisation

A biochemical profile of the 7 strains was obtained by using the API® 20NE and API® 20E strips (Biomérieux, France). Additionally, the oxidase and catalase tests were performed, giving positive results for all strains.

The results obtained with both strips were concordant except for the citrate utilisation test (CIT in both strips) for strain A.L8-3 which was negative with the API® 20E test and positive with the API® 20NE examination.

In table 7.4 are depicted the biochemical characteristics of each strain. In some cases the tests performed would not be sufficient to distinguish some of the strains in study from each other or from some strains of the known *Aeromonas* species.

Strain A.131/2 (phylogenetic Group I), could be distinguished from the remaining 6 strains by its inability to produce lysine decarboxylase (LDC). One to two distinct tests present in the kits used would distinguish this strain from the described *Aeromonas* spp., as seen in table 7.4.

Strain A.L15-1 (phylogenetic Group III) would be distinguished from all *Aeromonas* species by the tests applied (from one to three tests) and could not only be differentiated from strain A.L10-4.

As depicted in table 7.4, strains composing Group II (A.136/15, A.L8-3, A.L10-4, A.MW.11/6 and A.MW.28/6) differed from each other in one to three of the tests used. With few exceptions, one to four tests should differentiate this group from the known *Aeromonas* spp.. The biochemical profiles obtained for strains A.L10-4, A.MW.11/6 and A.MW.28/6 were unique when compared to the ones reported for the currently described *Aeromonas* spp.. By the contrary, none of the tests applied would undoubtedly separate some *A. veronii* bv sobria and *A. jandaei* specimens from strains A.136/15 and A.L8-3, respectively.

Figure 7.5 displays results obtained with the API® strips.

Table 7.6 API® based biochemical tests which would differentiate the strains in study and these strains from the described *Aeromonas* species.

Tests[a] / *Aeromonas* spp.	ADH	LDC	CIT	VP	IND	ESC	H_2S	SAC	ARA	AMY	GNT
A. hydrophila	+	+	V	+	+	+	+	+	V	-	V
A. bestiarum	+	V	V	V	+	V	V	+	+	-	V
A. salmonicida	V	V	V	V	+	+	V	+	+	-	-
A. caviae	+	-	+	-	+	V	-	+	+	-	-
A. media	V	-	V	-	+	V	-	+	+	-	-
A. eucrenophila	V	-	-	-	+	V	V	V	V	-	-
A. sobria	-	+	+	-	+	-	+	+	-	-	-
A. veronii bv sobria	+	+	V	V	+	-	V	+	-	-	V
A. jandaei	+	+	+	V	+	-	V	-	-	-	V
A. veronii bv veronii	-	+	+	V	+	+	V	+	-	-	V
A. schubertii	+	V	V	V	-	-	-	-	-	-	-
A. trota	+	+	V	-	+	-	-	V	-	-	-
A. encheleia	V	-	-	-	+	V	V	V	-	-	-
A. allosaccharophila	V	+	V	-	+	V	V	+	V	-	-
A. popoffii	+	-	+	V	+	-	+	-	V	-	-
A. simiae	+	+	ND(-)[b]	-	-	V	ND(-)[c]	+	-	-	+
A. molluscorum	+	-	+	-	-	+	X-	+	+	ND(-)[b]	ND
A. bivalvium	-	+	+	-	+	+	X-	+	+	ND(-)[b]	ND
A. aquariorum	+	+	ND(+)[b]	-	+	+	X+	+	-	-	+
A. tecta	+	V	+	V	-	V	ND	-	-	ND(-)[b]	+
A. piscicola	+	+	ND	+	+	+	X+	+	-	ND	+
A. fluvialis	-	-	+	-	+	-	C-	+	-	-	+
A. taiwanensis	+	-	+	-	+	+	C-	+	+	+	+
A. sanarellii	+	-	-	-	+	+	C-	+	+	+	+
A. diversa	+	-	-	V	+	-	X-; C-	-	-	ND	ND
A. sp. Group I	+	-	-	+	+	+	C-	+	+	+	+
A. sp. Group II	+	+	V	V	+	V	C-	V	-	V	+
A.136/15	+	+	-	-	+	-	C-	+	-	-	+
A.L8-3	+	+	+	+	+	-	C-	-	-	-	+
A.L10-4	+	+	-	-	+	+	C-	-	-	-	+
A.MW.11/6	+	+	-	-	+	+	C-	+	-	+	+
A.MW28/6	+	+	-	+	+	-	C-	-	-	-	+
A. sp. Group III	+	+	-	-	+	+	C-	-	-	-	+

Data from species *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. veronii* (*A. veronii* bv veronii and *A. veronii* bv sobria), *A. jandaei*, *A. schubertii*, *A. trota*, *A. allosaccharophila*, *A. encheleia* and *A. popoffii* were from Martin-Carnahan and Joseph (2005). Unless otherwise indicated, data from: *A. molluscorum* were from Miñana-Galbis *et al.* (2004); *A. simiae* were from Harf-Monteil *et al.* (2004); *A. aquariorum* were from Martínez-Murcia *et al.* (2008); *A. tecta* were from Demarta *et al.* (2008); *A. piscicola* were from Beaz-Hidalgo *et al.* (2009; 2010); *A. fluvialis* were from Alperi *et al.* (2010); *A. taiwanensis* and *A. sanarellii* were from Alperi *et al.* (in press); *A. diversa* were from Miñana-Galbis *et al.* (2010a).

[a] ADH, arginine dihydrolase; LDC, lysine decarboxylase; CIT, citrate utilisation; VP, Voges-Proskauer test (acetoin production); IND, indole production; ESC, hydrolysis of esculin; H_2S, H_2S production from thiosulfate; SAC, utilisation of D-sucrose; ARA, utilisation of L-arabinose; AMY, utilisation of amygdalin; GNT, utilisation of gluconate. +, 85-100% positive strains; V, 16%-84% positive strains; -, 0-15% positive strains; ND, not determined (in brackets, data collected in the indicated study for the type strain); X-, H_2S production negative from cysteine; X+, H_2S production positive from cysteine; C-, H_2S production negative from thiosulfate.

[b] Data from Alperi *et al.* (in press).

[c] Data from Martínez-Murcia *et al.* (2008).

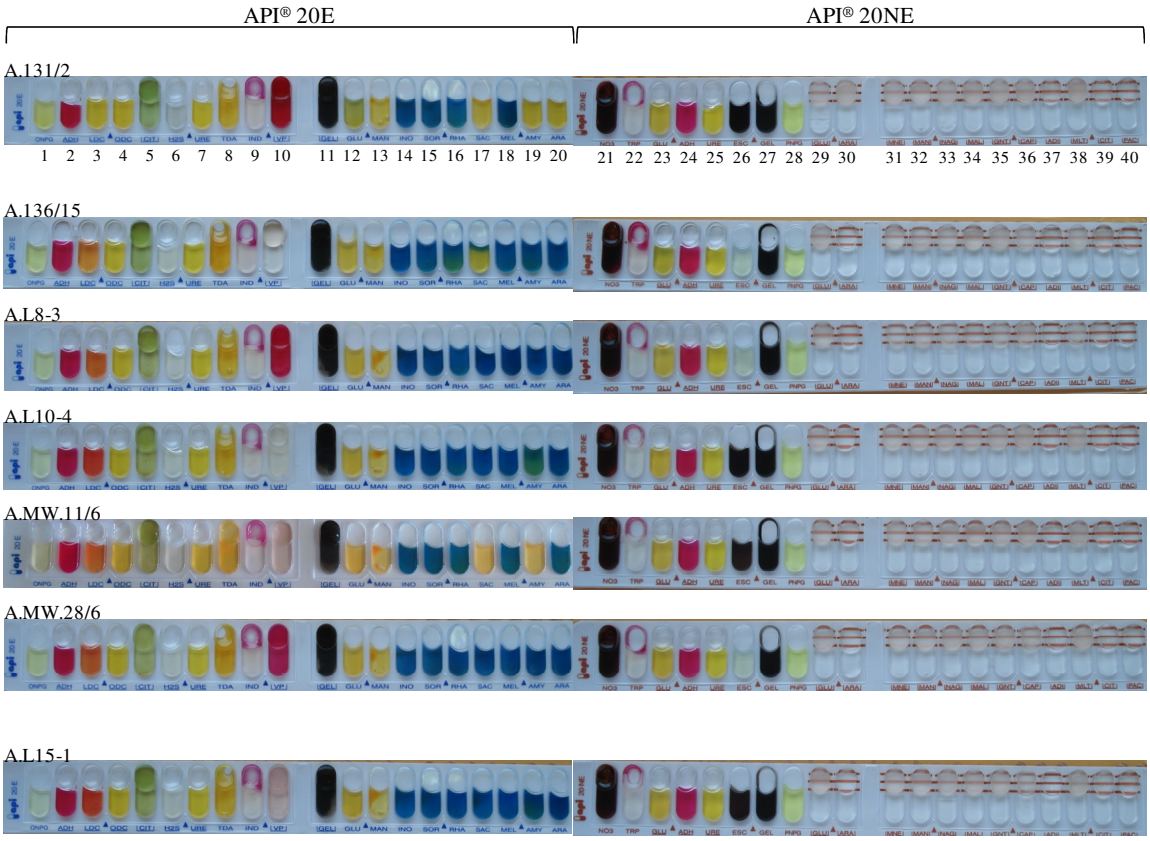

Figure 7.5 API® biochemical profiles of the 7 strains in study.

Legend to figure:
API® 20E tests: 1 – β-galactosidase (Ortho-nitrophenyl-βD-galactopyranosidase); 2 – Arginine dihydrolase; 3 – Lysine decarboxylase; 4 – Ornithine decarboxylase; 5 – Citrate utilisation; 6 – H_2S production; 7 – Urease; 8 – Tryptophan deaminase; 9 – Indole production; 10 – Acetoin production (Voges-Proskauer); 11 – Gelatinase. Utilisation of: 12 – glucose; 13 – mannitol; 14 – inositol; 15 – sorbitol; 16 – rhamnose; 17 – saccharose; 18 – melibiose; 19 – amygdalin; 20 – arabinose.
API® 20NE tests: 21 – Reduction of nitrates to nitrites; 22 – Indole production; 23 – Glucose fermentation; 24 – Arginine dihydrolase; 25 – Urease; 26 – Esculin hydrolysis; 27 – Protease (Gelatinase); 28 - β-galactosidase (Para-nitrophenyl-βD-galactopyranosidase). Assimilation (utilisation on aerobiosis) of: 29 – glucose; 30 – arabinose; 31 – mannose; 32 – mannitol; 33 – N-acetyl-glucosamine; 34 – maltose; 35 – (potassium) gluconate; 36 – capric acid; 37 – adipic acid; 38 – malate; 39 – (trisodium) citrate; 40 – phenylacetic acid.

7.4 Discussion

In the present study we used a polyphasic approach including molecular and phenotypic analysis to characterise a group of *Aeromonas* strains showing *gyrB* sequences different from the previously described (chapters 3 and 4).

Misinterpretations may be done when comparing results obtained using different methodologies (Janda and Abbott, 2010), which may have occurred in this study by comparing the biochemical results obtained with others available in the literature. Also,

commercial tests as the ones used are not as accurate as standard conventional methods (Janda and Abbott, 2010). Nevertheless, these tests provided a preliminary biochemical profile of strains.

Group I harboured one strain, A.131/2, representing two isolates with the same RAPD profile, harvested from a drinking water source (Table 7.1). On the basis of single gene phylogenies (Figs. 7.1, 7.2 and 7.3) and the *gyrB-rpoD* concatenated sequence tree (Fig. 7.4) this strain was found as an independent and distinct line within the genus *Aeromonas*. The concatenated phylogeny was consistent with results obtained from *gyrB*: this group is independent from the main grouping of aeromonads (containing most species) but also from the deepest branch of the genus (*A. simiae*, *A. schubertii* and *A. diversa*). On the basis of the molecular analysis performed, this strain is equally distant from different described *Aeromonas* species (e.g. *A. eucrenophila*, *A. taiwanensis*, *A. aquariorum*, *A. tecta*, *A. media*) and strain L.15-1 (Group III) was the closest relative of this group.

Chromatogram analysis of the 16S rRNA gene sequence of this strain and isolate DW.131/7 which is the A.131/2 clone collected from the same sample (Chapter 3 of this thesis), revealed the existence of microheterogeneities in 13 sequence positions (Table 7.5), mostly located in the V3 region (11 positions) but also in the V6 region (2 positions). These mutations have been previously described in other *Aeromonas* species (Alperi *et al.*, 2008; 2010; in press), however, only 1 – 10 16S rRNA gene microheterogeneities have been reported for *Aeromonas* species (Alperi *et al.*, 2008). Nevertheless, up to 30 heterogeneous positions were found in other Gammaproteobacteria (Case *et al.*, 2007). The possibility of sequencing errors and contaminated DNA has been taken into account. However, to reinforce our results, experiments were repeated from newly extracted DNA and results confirmed the previous findings. Purity of cultures was confirmed by performing again *gyrB* and *rpoD* sequencing from the same newly extracted DNA.

On the basis of the API® examinations, strain A.131/2 would be distinguished from the described *Aeromonas* spp. by the ability to use amygdalin and to produce acetoin (positive reaction in the VP test). Additionally, it reduced nitrates to nitrites, did not produce H_2S from thiosulfate and was negative for urease and citrate utilisation. It produced indole from tryptophan, hydrolysed gelatine, was positive for arginine dihydrolase and negative for lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase. Positive for β-galactosidases (both ONPG and PNPG) and hydrolysis of

esculin, strain A.131/2 utilised D-glucose (both by fermentation and oxidation), D-mannitol, D-sucrose, L-arabinose, D-mannose, N-acetyl-glucosamine, D-maltose and gluconate but not inositol, D-sorbitol, L-rhamnose and D-melibiose. Also, it assimilated capric acid, malate, and phenylacetic acid but not adipic acid. Additionally, it was catalase and oxidase positive. The production of acid from amygdalin is an atypical feature in the genus only reported before for nine aeromonads (Abbott *et al.* 2003), the recently described *A. taiwanensis* and *A. sanarellii* (Alperi *et al.*, in press) and, in the present study, for strain A.MW.11/6 from Group II (Figure 7.5 and Table 7.6). As already mentioned interpretation of these results should be made with precaution and taken as indicative and not definitive as some of these results may not be comparable with the previously reported.

In the evolutionary trees based on single *gyrB* and *rpoD* genes and the concatenated sequences of *gyrB-rpoD* genes (Figs. 7.1, 7.2 and 7.4, correspondingly), strains A.136/15, A.L8-3, A.L10-4, A.MW.11/6 and A.MW.28.6 grouped together forming a robust and distinct branch within the genus *Aeromonas*, the so-called Group II. These strains represent a total of 12 isolates collected from untreated drinking and mineral waters and vegetables (Table 7.1). However, in the 16S rRNA gene based tree, strain A.L8-3 did not cluster with the other strains of Group II and exhibited 10 – 11 nucleotide differences (99% of sequence similarity) with the remaining strains. Nevertheless, these divergences were located in a hypervariable region (V3 region; nucleotides 456-476 according to *E. coli* numbering) which, although harbouring signature nucleotides that would distinguish most *Aeromonas* species, has been reported to be unreliable for some phylogenetic conclusions (Martínez-Murcia *et al.*, 1992a; Soler *et al.*, 2004).

Additionally, one transition (A/G) was found in this region (V3 region) of the 16S rRNA gene sequence of this strain (Table 7.5), however this heterogeneity would not produce different grouping for this strain within the 16S rRNA gene based phylogenetic tree (data not shown). This microheterogeneity at such position has been previously reported for other *Aeromonas* species and one mutation position is well within the range (1-10 spots) described for other aeromonads (Alperi *et al.*, 2008; 2010; in press).

The *gyrB* and *rpoD* clustering of these strains produced different groupings within Group II due to the differences in the similarity values when comparing the two genes sequences of all strains, reflecting the different mutational behaviours of the two housekeeping genes for closely related strains, as has been already reported for some

phylogenetic groups of the genus (Soler *et al.*, 2004). Moreover, the intraspecies similarity values for *rpoD* and *gyrB* genes in this genus were found to be approximately 97% or higher (Soler *et al.*, 2004; Alperi *et al.*, in press), which is superior to the average values recorded for this group (96.5% for *gyrB* and 96.3% for *rpoD*). The analysis of more housekeeping genes is needed in order to clarify these strains interrelationship. In fact, the use of different molecular markers should improve the phylogenetic analysis and correct identification of strains enabling a better resolution of possible doubtful issues (Stackebrandt *et al.*, 2002; Soler *et al.*, 2004; Gevers *et al.*, 2005). Nevertheless, the data here collected suggest that they constitute a single highly variable grouping within the genus *Aeromonas*.

The known *Aeromonas* species more closely related to Group II on the basis of the *gyrB* and *rpoD* genes analysis were *A. tecta* and *A. eucrenophila*. In fact, based on *rpoD* gene, the *A. tecta* strains shared 95.4% of similarity with this group of strains and the tree constructed with *rpoD* sequences (Fig. 7.2) reflects the close relation between these groups. Nevertheless, the bootstrap values obtained for Group II are high in all evolutionary trees and were improved in the tree based on the *gyrB-rpoD* concatenated sequences (Fig. 7.4), as expected from the simultaneous use of both housekeeping genes for phylogenetic inferences in this genus (Soler *et al.*, 2004).

Based on the profiles obtained with API® kits, this group of strains displayed a considerable biochemical variability, but it should be differentiated from the described *Aeromonas* species as all strains produced indole from tryptophan, were positive for arginine dihydrolase, used gluconate and not L-arabinose. Nevertheless, some strains of *A. veronii* bv sobria and *A. jandaei* would not be distinguished from some strains of Group II based on the applied tests, as already mentioned. Additionally, all strains reduced nitrates to nitrites, did not produce H_2S from thiosulfate and were negative for urease. They hydrolysed gelatine, were positive for lysine decarboxylase and negative for ornithine decarboxylase and tryptophan deaminase. Positive for β-galactosidases (both ONPG and PNPG), all strains utilised D-glucose (both by fermentation and oxidation), D-mannitol, D-mannose, N-acetyl-glucosamine, D-maltose and gluconate, but not inositol, D-sorbitol, L-rhamnose and D-melibiose. Also, strains assimilated capric acid and malate but not adipic acid. Additionally, all were catalase and oxidase positive. The VP test (production of acetoin) was positive only for strains A.MW.28/6 and A.L8-3. The latter was the unique

strain which assimilated phenylacetic acid and gave incongruent results for the utilisation of citrate test (distinct results in the two strips). Hydrolysis of esculin was positive only for A.L10-4 and A.MW.11/6. Strains A.136/15 and A.MW.11/6 were the only that used D-sucrose and strain A.MW.11/6 was the unique to use amygdalin, an atypical feature in the genus as discussed before.

Strain A.L15-1, representing 12 isolates with the same RAPD profile collected from a vegetable sample (Table 7.1) constituted Group III. Based on *gyrB*, *rpoD* and 16S rRNA gene phylogenetic trees (Figs. 7.1, 7.2 and 7.3, respectively) as well as on the basis of concatenated sequences of *gyrB-rpoD* genes (Fig 7.4), this strain formed a distinct branch from the *Aeromonas* species described to date. The closest relative of this *Aeromonas* sp. strain was strain A.131/2 (Group I). Among the previously known *Aeromonas* species, the more closely related to this strain depends on which gene sequences similarities are taken into account, but A.L15-1 is approximately equally distant from *A. eucrenophila*, *A. taiwanensis*, *A. aquariorum*, *A. tecta* and *A. media*.

Two microheterogeneity spots were found in the 16S rRNA gene sequence of strain A.L15-1 as well as in one clone of this strain (V.L15-5) isolated from the same sample (Table 7.5). Two transitions were present within the V6 region in positions already reported for other *Aeromonas* species (Alperi *et al.*, 2008; 2010; in press) and also found in other strains in this study.

The biochemical profile obtained with the API® examinations would distinguish this group from the all known *Aeromonas* species by its ability to use gluconate and inability to use D-sucrose and citrate. However, by using the tests applied, this strain could not be differentiated from Group II strain A.L10-4, as previously stated. In addition, it reduced nitrates to nitrites, did not produce H_2S from thiosulfate and was negative for urease and VP tests. Produced indole from tryptophan, hydrolysed gelatine, was positive for arginine dihydrolase and lysine decarboxylase and negative for ornithine decarboxylase and tryptophan deaminase. Strain A.L15-1 was positive for β-galactosidases (both ONPG and PNPG) and hydrolysed esculin. Also, it utilised D-glucose (both by fermentation and oxidation), D-mannitol, D-mannose, N-acetyl-glucosamine, D-maltose, but not inositol, D-sorbitol, L-rhamnose, D-melibiose, L-arabinose and amygdalin. Furthermore, it assimilated capric acid and malate, but not phenylacetic acid nor adipic acid and was catalase and oxidase positive.

Results from the present analysis clearly indicate that Groups I, II and III, represent new phylogenetic lineages within the genus *Aeromonas*, probably belonging to species not described to date. Future research attempting to formally describe new taxa at the species level should include a more extensive biochemical and physiological study, a comprehensive MLPA including at least five housekeeping genes and mandatory DNA-DNA hybridisation analysis including all other described *Aeromonas* species.

8. General discussion

The ecological distribution and phylogenetic diversity of *Aeromonas* species as well as traits related to their antibiotic resistance and pathogenicity have been studied worldwide (Chacón *et al.*, 2004; Saavedra *et al.*, 2006; Scoaris *et al.*, 2008; Rahman *et al.*, 2009; Fontes *et al.*, 2010). Researches point out to an outstandingly diversified genus present in a wide array of environments, thus adapted to survive under different conditions (Demarta *et al.*, 2000; McMahon and Wilson, 2001; Valera and Esteve, 2002; Koksal *et al.*, 2007). Also, aeromonads exhibit a characteristic antibiotic resistance profile which at least in relation to certain antibiotics may vary according to the environment scrutinised (Goñi-Urriza *et al.*, 2000a; Schmidt *et al.*, 2001b; Akinbowale *et al.*, 2007b). For the majority of times, the presence of ARG both chromosomally or extra-chromosomally encoded are responsible for such drift in the susceptibility patterns (Agersø *et al.*, 2007; Lee *et al.*, 2008; Arias *et al.*, 2009). The MGE frequently carried by *Aeromonas* spp. enables them to act as reservoirs and disseminators of different resistance markers against antibiotics and toxic compounds in distinct ecological niches (Schmidt *et al.*, 2001a; Rhodes *et al.*, 2004; Huddleston *et al.*, 2006; McIntosh *et al.*, 2008). Additionally, aeromonads present an impressive array of genetic determinants that may be potentially involved in pathogenic processes of *Aeromonas* spp. In fact, the presence of homologs to known powerful virulence factors of pathogenic bacteria has been demonstrated in *Aeromonas* spp. (Galindo *et al.*, 2006). It has been already demonstrated that the products of several of these genes do play a role in *Aeromonas* spp. virulence (Burr *et al*., 2005; Chopra, 2008; Arnadottir *et al.*, 2009); also, these microorganisms are responsible for severe clinical outbreaks in fish and for a panoply of human clinical disorders (Kozińska, 2007; Janda and Abbott, 2010). There is no doubt of the potential of aeromonads to cause disease in humans considering the numerous reports in the literature, but it is not known how and which strains are able to exert such effects (Figueras, 2005; Janda and Abbott, 2010). In this way, it is imperative to continue the scrutiny of different environments for *Aeromonas* spp., especially those in which their presence may present health hazard for human and other animal, and give further insights of their associated risk factors.

In this study, it was assessed the occurrence and phylogenetic diversity of *Aeromonas* species in untreated waters and agriculture related sources from different regions in Portugal, which in their majority are for human and other animal consumption (Chapters 3 and 4). Also, it was determined the antibiotic susceptibility profiles and

investigated the genetic basis of antibiotic resistance within the established collection (Chapter 5). Furthermore, strains were screened for virulence related extracellular activities and putative genetic determinants of virulence (Chapter 6). Additionally, for specimens that did not group with any previously known *Aeromonas* species in *gyrB* based phylogenetic analyses, it was carried out a preliminary polyphasic approach based on phenotypic and DNA sequence data in order to clarify these specimens' taxonomic positions (Chapter 7). To the best of our knowledge, no studies aiming to establish an *Aeromonas* collection from water and agricultural sources and unravel the diversity and genetic traits of antibiotic resistance and putative virulence among such collection were done before in Portugal. Annexe I includes a table containing relevant traits of the strains composing the collection established.

As discussed throughout this dissertation, molecular techniques are powerful tools to assess the genomic variation within a bacterial population and achieve an undoubted identification of the isolates scrutinised (van Belkum *et al*, 2001; Stackebrandt *et al.*, 2002). Not only is the accurate assignment of an isolate to a certain species important, but also the discrimination among strains of the same species (Schloter *et al.*, 2000; Figueras *et al.*, 2006; Singh *et al.*, 2006).

In this study, genotypic approaches were applied in order to type and classify *Aeromonas* species isolates. RAPD was primarily used to discriminate the isolates collected (Chapters 3 and 4). Some pitfalls were pointed out concerning the ability of this technique for fingerprinting purposes, namely, lack of reproducibility, difficulty of comparing patterns and the unknown nature of target regions used for generating fingerprints (Power, 1996; Martínez-Murcia *et al.*, 2000). In order to standardise the application of the technique, the applied procedure was normalised to optimise reproducibility. Additionally, interpretation of results was done by intra-gel comparison and isolates displaying different RAPD patterns (supposedly different strains) observed in the same gel were subjected to *gyrB* gene sequencing analysis. All strains displaying identical partial *gyrB* gene sequence were again subjected to RAPD analysis and observation of respective patterns in the same gel, allowing a definitive discrimination of the isolates. Thus, the possible biases of RAPD technique were potentially overcome with the efforts of standardisation and reanalysis of previously not compared patterns.

The *gyrB* gene is considered a remarkable molecular chronometer to assess the phylogenetic relationships between species of different genera, including *Aeromonas* (Yamamoto and Harayama, 1996; Yáñez *et al.*, 2003; Küpfer *et al.*, 2006). The phylogenetic analysis of our *Aeromonas* collection was based on this molecular marker partial sequence (ca. 500bp) which was compared to those from type strains and other well characterised *Aeromonas* spp. strains from the MDC culture collection (Chapters 3 and 4). Phylogenetic trees obtained by the neighbour-joining method were in accordance with previous results since the grouping of the well characterised strains, including type strains from MDC culture collection was consistent with previously described species, with the exception of the *A. sanarellii*/*A. caviae* group of strains.

A. sanarellii was described based solely in one strain and provided phylogenetic information based on *rpoD* sequence analysis and MLPA undoubtedly allocated this strain in a distinct grouping from the remaining *Aeromonas* species (Alperi *et al.*, in press). In our study, and as previously reported for some strains of distinct *Aeromonas* species (Yáñez *et al.*, 2003; Soler *et al.*, 2004), the *gyrB* nucleotide sequence has shown not to be a good discriminator for these two distinct phylogenetic lines: the *A. caviae* type and reference strains and *A. sanarellii* CECT7402^{T}, grouped together in the phylogenetic trees in addition to four *Aeromonas* strains collected in our study (A.130/1, A.130/4, A.MW1/1 and A.ALU-1). The authors did not provide any information on the *gyrB* based relationships of *A. sanarellii* CECT7402^{T} and the remaining *Aeromonas* species (Alperi *et al.*, in press). A unique strain may not represent the genotypic and phenotypic characteristics of an entire taxon and biased results in organisms' classification and taxonomic studies may be introduced by species description based on a single strain (Christensen *et al.*, 2001; Felis and Dellaglio, 2007). Our results may be due to the use of a single strain of *A. sanarellii* that precludes the establishment of a phylogenetic cluster more diverse and robust (Alperi *et al.*, in press). In this view, the affiliation of the four *Aeromonas* sp. strains recovered in this study could be dubious, but we considered them as members of the *A. caviae* phylogenetic group as the clustering of these strains in different evolutionary trees is consistent with this affiliation (data not shown). Phylogenetic studies of these strains based on other housekeeping genes would provide a more reliable classification, as the use of several molecular markers improves phylogeny delineation (Saavedra *et al.*, 2006; Alperi *et al.*, in press).

Taking into account and articulating results obtained in chapters 3 and 4 of this dissertation, preeminent information can be extracted in what concerns the occurrence, diversity and distribution of *Aeromonas* species in the environments investigated. From different regions of Portugal, a total of 100 sampling sites were scrutinised for the presence of *Aeromonas* species. Samples were collected from distinct environments, namely, untreated water sources – UnW (drinking and mineral waters), agriculture related sources - AS (untreated irrigation water and vegetables) and from a stable – SE (cow's drinking water and faeces; udder's cleaning water before milking, milk and cleaning water of the milking equipment). 133 samples were recovered from which 56 distributed by 40 sampling sites harboured aeromonads. The considerable genotypic and phylogenetic diversity of the established collection is implicit in the 169 different RAPD fingerprints (83 patterns were restricted to one isolate) observed throughout 483 isolates and the 125 distinct *gyrB* gene partial sequences present among the strains which grouped in 15 phylogenetic clusters (Annexe II). The number of collected samples was not the same for each source. Additionally, the number of isolates harvested from each sample was not the same for all samples, thus it would be erroneous to compare numbers of aeromonads positive samples or number of isolates collected per source. In fact, the aim of this study was not to compare the prevalence of aeromonads in different sources, but as mentioned in chapter 2, it was to collect multidisciplinary information about *Aeromonas* spp. communities present among the sources selected and eventually, try to compare the diversity of those communities in the distinct sources.

In this way, taking into account the number of isolates collected per species, *A. media* was the most occurring species, followed by *A. hydrophila*, *A. salmonicida* and *A. bestiarum*. In other studies, these species have been widely recovered from distinct environmental niches, but *A. media* and *A. bestiarum* are not always so occurring (Borrell *et al.*, 1998; Neyts *et al.*, 2000; Janda and Abbott, 2010). Also, *A. eucrenophila* isolates occurred more frequently in this study than in previous surveys. In fact, the isolation of these specimens was rarely reported and with low occurrence (Singh and Sanyal, 1999; Demarta *et al.*, 2000; Evangelista-Barreto *et al.*, 2006). As thoroughly mentioned before and further discussed below, the frequent detection of these specimens during this study may be due to the identification method here applied. The prevalence of *A. veronii* and *A. caviae* isolates within environmental samples according to other studies is highly variable

(Borrell *et al.*, 1998; Neyts *et al.*, 2000; Sen and Rodgers, 2004; Koksal *et al.*, 2007), but in our study, isolates included in these species were rarely detected. As previously mentioned, in some cases biased results in relation to *A. caviae* recovering might have been obtained by using the phenotypic classification schemes (Ghenghesh *et al.*, 2001; Palú *et al.*, 2006; Castilho *et al.*, 2009).

The number of species collected from the different types of samples was significantly diverse (Annexe III). UnW sources and mainly drinking water (DW) samples displayed the higher phylogenetic diversity of all sources (14 different species), which is in agreement with the high distribution of different *Aeromonas* species in the water environment (Borrell *et al.*, 1998; Koksal *et al.*, 2007; Di Bari *et al.*, 2007). Nevertheless, as thoroughly discussed, the phylogenetic diversity in our water samples is higher, which may be due to the identification method applied in this study. Also, the high occurrence of *Aeromonas* species in AS and in particular in vegetables (V) samples, are in accordance with the large dissemination of aeromonads in this environment and in produce (Callister and Agger, 1987; Pianetti *et al.*, 2004; Palú *et al.*, 2006) but the molecular diversity found (6 distinct species in V samples) is not so common (Neyts *et al.*, 2000; McMahon and Wilson, 2001) and we attribute this finding to the accurate species identification in our study. Moreover, studies on the occurrence and diversity of aeromonads in animal faeces and produce are not very common in relation to the amount of such reports in aquatic environments. Thus this may be another factor contributing to the inexistent reports of some species in these sources. As mentioned (Chapter 3), it is remarkable the number of different species (8) recovered from the three mineral water (MW) samples as well as the number of different strains detected (17 strains out of 22 isolates), demonstrating the great diversity of the aeromonads community in these sites.

Also, the species distribution patterns among the different sources were significantly heterogeneous. In some cases the isolates dissemination along the different kinds of samples appeared to be species-related: *A. veronii*, *A. popoffii* and *Aeromonas* sp. Group I were only isolated from DW samples; *A. encheleia* was found solely in a MW sample and *Aeromonas* sp. Group III in a V sample. The remaining species were distributed among different sources (Annexe III).

In relation to some phylogenetic groups the occurrence in samples scrutinised in this survey was in accordance with previous studies, namely, *A. hydrophila*, *A. bestiarum*,

A. caviae, *A. veronii*, *A. popoffii*, *A. encheleia*, *A. tecta* and *Aeromonas* sp. HG11 (Table 1.1 in Chapter 1). However, strains of some species were for the first time detected in certain samples scrutinised in this study. In fact, *Aeromonas allosaccharophila* strains were mainly collected from V samples and also from irrigation water (IW) and MW samples (Annexe III). As discussed in chapter 4, our results give a new insight into the ecological distribution of *A. allosaccharophila*, as this is the first report of the recovering of this species from produce. As extensively discussed in chapters 1, 3 and 4, and as referred by Saavedra and co-workers (2007), this species has been rarely reported probably owing to the identification methods used in the majority of studies, in contrast to the reliable identification method applied in this investigation. As well, *A. salmonicida* strains were majorly recovered from V samples but also from cows' faeces (CF), cows' drinking water (CW) and DW samples being the unique recovered species from faeces (Annexe III). This species is largely associated to aquaculture environments and diseased fish (Martin-Carnahan and Joseph, 2005) nevertheless it can also be found in other environmental sources and also in human clinical samples (Borrell *et al.*, 1998). We were not able to find any references to *A. salmonicida* collection from cow faeces and only Neyts and co-workers (2000) reported its occurrence in vegetables. Even though, this occurrence is not surprising since this species is very diverse and common in the environment (Janda *et al.*, 1996; Martínez-Murcia *et al.*, 2005). Certainly, the identification methodology applied in this study contributed for the accurate affiliation of strains of this species. Other investigations used phenotypic methods, affiliating their isolates to *Aeromonas hydrophila* complex (Palú *et al.*, 2006; Castilho *et al.*, 2009), probably precluding the establishment of the real distribution of this species. In the same way, *A. eucrenophila* integrates the phenotypic *A. caviae* complex and probably goes undetected when phenospecies identification is used to classify strains. Previously, *A. eucrenophila* strains were mainly isolated from environmental sources as water, healthy and diseased fish (Huys *et al.*, 1996; Singh and Sanyal, 1999; Valera and Esteve, 2002) and infrequently from food (Janda *et al.*, 1996; Evangelista-Barreto *et al.*, 2006) and clinical samples (Janda and Abbott, 1998; Albert *et al.*, 2000; Demarta *et al.*, 2000) and we could not find any reports referring its occurrence in vegetables. In this work, *A. eucrenophila* isolates were principally collected from V and DW samples but also from IW and MW samples (Annexe III). Additionally, *Aeromonas media* was the most widely distributed species in the distinct environments

exploited in this study. This species was collected from DW, MW, IW, CW and mainly from V samples, where it accounted for 50% of isolates collected. According to what has been previously reported, *A. media* strains have been isolated from distinct types of sources (Borrell *et al.*, 1998; Singh, 2000; Evangelista-Barreto *et al.*, 2006; Jacobs and Chenia, 2007; Picão *et al.*, 2008b) and the unique report that we were able to find mentioning recovering of this species from vegetables was that of Neyts and co-workers (2000). The authors accounted *A. media* together with *A. caviae* as the most occurring species in vegetables. In this study, *A. media* was the most recovered species from this kind of sample. Thus, our results and those of Neyts *et al.* (2000) are in disagreement with the remaining studies (Callister and Agger, 1987; McMahon and Wilson, 2001; Palú *et al.*, 2006; Castilho *et al.*, 2009), since there are no further statements on *A. media* strains isolation from vegetables. As discussed above and also mentioned by Neyts and collaborators (2000), this may be explained by the fact that *A. media* is included in the phenotypic *A. caviae* complex.

As discussed in chapter 7, *Aeromonas* sp. Groups I, II and III are constituted by strains displaying molecular variants of *gyrB*, *rpoD* and 16S rRNA genes different from the previously reported. In spite of the biochemical profiles obtained with the API® examinations should not be taken as definitive, they point to three distinct *Aeromonas* species. Even though, further biochemical tests would have to be applied to discriminate some of these previously not identified specimens from the remaining *Aeromonas* species. Distinct microheterogeneities previously reported in other *Aeromonas* strains (Alperi *et al.*, 2008; 2010; in press) were detected in the 16S rRNA gene sequences of *Aeromonas* Groups I and III specimens, indicating that a variable number of copies of this gene with intragenomic variations is distributed among these strains genomes. *Aeromonas* Groups I and III isolates were recovered from a DW and a V sample respectively, and is not possible to know if they may be disseminated in other kinds of environments. The isolates composing the previously unidentified *Aeromonas* sp. Group II were collected from DW, MW and V samples, indicating that this previously unknown group is able to occupy different ecological niches.

In chapters 3 and 4, considerations were done about the *Aeromonas* spp. population dynamics. Besides the assessment of the microdiversity within our samples enabled by the application of genetic typing, these findings demonstrate the importance of this technique

in the tracking of bacterial specimens. In fact, we were able to identify what may be the same organisms disseminated in different ecological niches and take a brief and initial look on the dynamics of the population among different areas and sources.

The 169 distinct strains representing our *Aeromonas* sp. collection were subjected to antibiotic susceptibility tests and scrutinised for the presence of sequences of β-lactamase genes as well as tetracycline resistance genes. In addition, the occurrence of class 1, 2 and 3 integrons was prospected (Chapter 5). The genes investigated are on one hand genes previously described in aeromonads, namely *cphA*/*imiS* and *tet* genes, which have been primarily implicated in the resistance phenotypes to carbapenems (Walsh *et al.*, 1997) and tetracyclines (Agersø *et al.*, 2007), correspondingly. On the other hand, the remaining *bla* genes, although not commonly found in *Aeromonas* species, are usually encoded on MGE and represent important resistance determinants occasionally found among clinical species (Pérez-Valdespino *et al.*, 2009) and present in non-clinical isolates recovered from aquatic environments (Henriques *et al.*, 2006a), animals (Barlow *et al.*, 2008) and aquacultures (Jacobs and Chenia, 2007). Additionally, *Aeromonas* species have been shown to harbour integrons, principally class 1 integrons, which for several times were responsible for the resistance patterns exhibited by aeromonads (Moura *et al.*, 2007; Chang *et al.*, 2007).

The antibiotic susceptibility profile of the aeromonads collection corresponded to the expected (Martin-Carnahan and Joseph, 2005; Janda and Abbott, 2010), that is, higher resistance rates were recorded to penicillins, to the 1st generation cephalosporin and the macrolide; resistance rates were low for imipenem, tetracycline, aminoglycosides, trimethoprim/sulfamethoxazole and for the fluoroquinolone. All strains were susceptible to 3rd and 4th generation cephalosporins, aztreonam and, except for two strains which displayed the intermediary resistance phenotype, all were sensitive to chloramphenicol. As discussed in Chapters 1 and 5, although aeromonads present a typical antibiotic resistance profile, resistance rates to the same antimicrobial agent may vary between studies with no apparent correlation to the type of species or whether the source of isolation is clinical or non-clinical (Kämpfer *et al.*, 1999; Janda and Abbott, 2010). In relation to some antibiotics, this variation seems to be more related to strains specific traits and environmental selective pressures (Janda and Abbott, 2010).

Analysing resistance patterns in relation to specific species or sources, we did not find a significant deviation from the global profile, with the exception of the greater number of tetracycline resistance strains collected from the stable environment, which may be related to extensive use of tetracyclines in veterinary medicine (Akinbowale *et al.*, 2007a). Nevertheless, *A. salmonicida*, *A. media*, *A. hydrophila*, *A. veronii, A. bestiarum* and the unique *Aeromonas* sp. Group I strain were resistant to a wider variety of antibiotics. Also, strains collected from UnW sources displayed a broader number of resistance phenotypes to distinct antibiotics. Additionally, ARG and integrons were majorly detected in strains belonging to species (especially *A. hydrophila* and *A. salmonicida*) and recovered from sources in which a wider variety of resistance phenotypes was recorded.

Multiresistance profiles were common among strains. In fact, we have reported approximately 44% of strains resistant to five or more antibiotics, including the penicillins and 1st generation cephalosporin. This statement is arguable because the majority of *Aeromonas* species strains harbour chromosomal penicillinases and cephalosporinases coordinately inducible by the presence of beta-lactams (Walsh *et al.*, 1997; Avison *et al.*, 2004). Thus, aeromonads commonly exhibit the resistance phenotype to penicillins and 1st generation cephalosporins, suggesting that these phenotypes should not be taken into account. We have incorporated ampicillin in the isolation media, possibly biasing the results by co-selecting other penicillins and cefalotin resistant isolates. But, as mentioned, in relation to penicillins and cefalotin our results were similar to others formerly reported that did not use ampicillin in the culture media (Ko *et al.*, 1996; Goñi-Urriza *et al.*, 2000a; Saavedra *et al.*, 2004; Scoaris *et al.*, 2008; Jacobs and Chenia, 2007). Additionally, if penicillins (including the combinations with beta-lactamase inhibitors) and cefalotin were not considered, only two strains were resistant to five antibiotics, lowering drastically the rate of multiresistant strains. Notwithstanding, in view of the global characterisation of the *Aeromonas* sp. population collected, we decided to include all antibiotics tested in the multiresistance pattern to express the exact antibiotic resistance profile exhibited by the collection established. In this way, the antibiotic resistance of the majority of *Aeromonas* strains scrutinised corresponded to the wild type phenotype not offering serious concern in a clinical point of view, if proper identification of strains is achieved.

The *cphA/imiS* genes and Tet determinants prospected in this study have been commonly found in *Aeromonas* spp. (Rossolini *et al.*, 1995; Agersø *et al.*, 2007). By the contrary, the $bla_{\text{TEM-1}}$ and bla_{VIM} like genes detected and the class 2 integron distinguished were rarely found in aeromonads (Barlow *et al.*, 2008; Henriques *et al.*, 2006b; Libisch *et al.*, 2008). The genetic determinants investigated and found among the collection, including integrons and *bla* genes, do not play a primary role in the antibiotic resistance of the strains recovered in this study. Nevertheless, this was not true in relation to tetracycline resistance as 14 out of 16 tetracycline resistant strains harboured one of the Tet determinants investigated. Also, three out of five trimethoprim/sulfamethoxazole resistant strains harboured a class 1 integron with a *dfrA12* gene cassette inserted. The results indicate that additional resistance mechanisms are present in these strains, especially the production of penicillinases and cephalosporinases frequently found in *Aeromonas* species. Although not always present, these enzymes are greatly prevalent in *Aeromonas* spp. isolates (Walsh *et al.*, 1997) and, as expected, the resistance profile obtained in this study indicates that these enzymes are also widely distributed in our strains.

The analysis of the nucleotide sequences of *cphA*/*imiS* like genes revealed that there is a great molecular variability within this gene family. Deduced amino acid sequences demonstrated that nucleotide divergences were silent mutations but also mutations which resulted in amino acid substitutions and, except for one, all deduced amino acid sequences were distinct from the ones previously reported. We cannot predict if these alterations conduct to structural, functional or stability differences in the enzymes but the historical evolution of beta-lactamases taught us that for several times mutations at the molecular level give rise to enzymes with a broader spectrum of activity than their parental relatives (Poole, 2004) or to changes in the enzyme kinetics in relation to certain antibiotics (Walsh *et al.*, 2005). It is tempting to speculate that *cphA*/*imiS* molecular diversity could lead to such functional changes. Additionally, these results suggest that the low occurrence of *cphA*/*imiS* like genes reported in this and other studies may be related to this genetic diversity. In fact, in three cases it would have gone undetected if not for the resistance phenotype displayed by strains which suggested the presence of a carbapenemase and justified the hybridisation based prospecting. Nevertheless, as mentioned, the majority of times the presence of *cphA*/*imiS* like genes is not accompanied by a carbapenem resistance profile *in vitro* (Rossolini *et al.*, 1995; Walsh *et al.*, 1997; Balsalobre *et al.*, 2009), which

may preclude the assessment of the presence of these genes. The deriving problem from this misdetection is that *cphA*/*imiS* like genes encode resistance to last-resort antibiotics and *in vivo* conditions or beta-lactams therapy may induce the production of these carbapenemases and compromise clinical treatment of *Aeromonas* sp. infections. This is further supported by our results as we detected the presence of *cphA*/*imiS* like genes but not the resistance phenotype to imipenem in some strains. The results enhance the possibility of aeromonads acting as reservoirs of carbapenemases with multiple molecular variants.

It is interesting the finding of part of an IS element upstream of a *cphA*/*imiS* like gene. Although we have not detected carbapenemase activity *in vitro* (imipenem susceptible phenotype), as discussed above, the production of CphA may be enhanced by other favourable conditions. IS are often capable of moving neighbouring genes and/or control their expression and IS mediated transposition of ARG is a well established HGT mechanism, playing an important role in antibiotic resistance dissemination. In this way, it is predictable that IS are able to mediate the transfer of chromosomal ARG to other genetic platforms and to distinct bacterial species of the parental strain (Benett *et al.*, 2008). Thus, our finding is particularly worrying by the fact that a carbapenemase encoding gene restricted to a bacterial genus, which additionally may go undetected *in vitro*, appears associated to what may be a functional MGE element putatively capable of promoting gene transfer and/or expression.

Also noteworthy, is the fact that $bla_{\text{TEM-1}}$ genes are typically plasmid encoded suggesting that *A. veronii* strain A.096A harbours one of such MGE.

In Chapter 6 we described the screening for the presence of genes coding for putative virulence factors and potentially pathogenic features as the production of extracellular lipases and proteases. The screening of genetic determinants and phenotypic characteristics putatively associated with virulence is a straightforward approach to recognise specimens involved in pathogenic processes and identify the sources posing health threats (Chacón *et al.*, 2003; Sen and Rodgers, 2004).

Amplified fragments corresponding to common regions of genes encoding aerolysin-like toxins were significantly detected among the 169 *Aeromonas* sp. strains. Restriction analysis of such DNA fragments and phylogenetic analysis of corresponding sequences and their homologous deposited in GenBank revealed the existence of two

major patterns corresponding to two distinct groupings in the phylogenetic tree. Whether or not this molecular segregation in two groups is related to differential activities of the aerolysin-like toxins as proposed by Kingombe and colleagues (1999) is an interesting subject for further investigations. The restriction patterns of amplicons obtained from our strains revealed an apparent species related distribution, as already noticed before (Kingombe *et al.*, 1999).

The detection of T3SS related genes among the aeromonads was low, principally of *aexT*. Nevertheless, we were able to find the *ascV* gene in 8 different phylogenetic groups, majorly in *A. bestiarum* and *A. allosaccharophila* strains, from which never or rarely have been reported (Braun *et al.*, 2002; Burr *et al.*, 2005; Silver and Graf, 2009). The *aexT* gene inspection was positive in two *A. bestiarum* and one *A. salmonicida* strain only when low stringency conditions of hybridisation were applied, indicating that these strains harbour genes homologs of *aexT*. These findings support the statement of Silver and Graf (2009) who suggested that the occurrence of T3SS related genes within *Aeromonas* sp. could be underestimated by the appliance of molecular methods based on *A. hydrophila* and *A. salmonicida* T3SS related genes to effectuate such surveys. Our survey was based in an *aexT* gene present in an *A. veronii* strain, but a similar deduction can be done.

The large majority of strains exhibited extracellular lipolytic and proteolytic activities and harboured genes coding lipolytic and/or phospholipolytic enzymes. Although not presenting an essential role in virulence (Merino *et al.*, 1999), these type of enzymes are usually involved in degradation of host cell components, contribute to cell nutrition and help evade host cell defences, thus, facilitating the invasiveness and establishment of infection (Pemberton *et al.*, 1997; Galindo *et al.*, 2006). In this way, a synergistic effect between the distinct putative virulence factors present in strains could potentiate their virulence and thus their pathogenic effect in hosts. Actually, distinct arrangements of putative virulence genes were found among strains. A higher number of gene combinations were found among *A. hydrophila*, *A. eucrenophila*, *A. bestiarum* and *A. veronii* strains. Also, UnW sources and mainly drinking water samples harboured a superior number of strains positive for the genetic virulence determinants exploited and more different combinations of genes. In this way, strains putatively virulent were majorly collected from UnW sources. Interestingly and in a different perspective, lipases and proteases may be

valuable from a technological point of view but extensive and deep studies in this direction are still needed.

Comparing results obtained in chapters 5 and 6, within the 74 multiresistant strains only one specimen did not harbour any of the genetic virulence determinants investigated. On the other hand, at least one ARG and one genetic determinant of virulence were detected in 29% of strains (including five out 9 strains carrying integrons). Among these, 51% were collected from UnW sources and 49% displayed a multiresistance profile from which 46% were retrieved from UnW sources. However, it is worthy of note that in 58% of strains collected from SE at least one ARG and one genetic determinant of virulence were identified in the same strain. Also, *A. hydrophila* strains accounted for the majority of strains in which at least one of the genetic markers mentioned were concomitantly found and in all strains of this species which carried an ARG, at least one virulence determinant gene was detected. In addition, the concurrent detection of ARG and virulence genetic determinants had more significance in *A. hydrophila* (24 out of 30), *A. salmonicida* (8 out of 14), *A. veronii* (3 out of five) and *A. allosaccharophila* (3 out of five) strains as the majority of specimens of these species harboured the genetic markers mentioned concomitantly.

In this way there is a strong relation between antibiotic multiresistance phenotypes and genetic virulence factors carriage among our aeromonads collection in all environments scrutinised. Moreover, as almost all strains displayed extracellular proteolytic and lipolytic activities, which assist in pathogenic processes, the dangerous association between virulence traits and drug resistance is generally current in our *Aeromonas* collection. Nevertheless, the concomitant detection of genetic determinants of antibiotic resistance and virulence in study was only moderate, but, as mentioned, additional drug resistance mechanisms different from the ones exploited are present in these strains. The screening for aminoglycosides, quinolones, other Tet resistance determinants and particularly for chromosomal penicillinases and cephalosporinases is still needed to clarify the extent of concomitant presence of virulence and antibiotic resistance determinants among strains as demonstrated by the antimicrobial susceptibility profile obtained.

Taken together, results demonstrate that UnW samples were the major source of *Aeromonas* species strains with: (i) broader variety of antimicrobial resistance phenotypes,

(ii) higher number and diversity of ARGs (including integrons); (iii) superior number of virulence genetic determinants and genes combinations; (iv) concurrent detection of antimicrobial resistance and virulence traits. Also, *Aeromonas* species phylogenetic diversity was superior in UnW sources. Nevertheless, it should be taken into account that few samples were collected in the stable environment, thus these considerations are made with precaution. In fact, we found that *Aeromonas* sp. strains recovered from the SE samples presented high resistance rates and a strong relation between the presence of ARG and the resistance profile exhibited by strains was noticed.

Finally, *A. hydrophila* and *A. veronii* strains, two of the *Aeromonas* species mostly implicated in human clinical disorders (Figueras, 2005), and *A. salmonicida* specimens, a major fish pathogen also related to human infections (Reith *et al.*, 2008), were always included in the group of strains exhibiting more diverse antibiotic resistance and virulence related traits, especially *A. hydrophila* specimens, which further enhances their potential health hazard. Notwithstanding, strains belonging to *A. bestiarum*, *A. eucrenophila* and *A. allosaccharophila*, species that have also been recovered from human clinical samples (Borrell *et al.*, 1998; Albert *et al.*, 2000; Saavedra *et al.*, 2007), also exhibited varied drug resistance and virulence related traits.

9. Conclusions and Future perspectives

9.1 Main conclusions of this work

The study here presented is a contribution to the knowledge on distribution and diversity of *Aeromonas* species in aquatic and agricultural environments. On the other hand, during this study new questions arisen, constituting the possible departure for future investigations. Additionally, the importance of the use of molecular methods and a polyphasic approach to reliably characterise organisms was further demonstrated.

The main conclusion that emerges from this study is that *Aeromonas* species in Portugal are widespread in water and food sources, which are considered to pose the main risk of acquiring *Aeromonas* species infections. The frequent occurrence of *Aeromonas* strains exhibiting multiresistance profiles, diverse antibiotic resistance mechanisms and virulence traits in untreated water sources and vegetables, has been demonstrated.

Additionally, some specific conclusions can be outlined.

- The different ecological niches exploited harbour a considerable genotypic and phylogenetic *Aeromonas* diversity, with different patterns of species distribution: while some species were widely distributed among different sources, others were confined to a specific environment.
- The fingerprinting and identification methods applied enabled the genotypic discrimination and the undoubted classification of most specimens of the *Aeromonas* sp. collection.
- Previously not described taxa were found, from which *Aeromonas* spp. Group II appears to be more widespread in the environment than *Aeromonas* spp. Group I and *Aeromonas* spp. Group III. *Aeromonas* sp. Group III exhibited an unusual antibiotic resistance profile and putative genetic virulence traits were identified in the remaining groups.
- Irrigation water does not seem to be a source of vegetables contamination by aeromonads in the sampling sites surveyed.
- The antibiotic susceptibility profile exhibited by the *Aeromonas* species collection mirrors the one expected for members of this genus.

- The antibiotic resistance patterns obtained were not always explained by the genetic determinants of resistance exploited, indicating that other mechanisms may be associated, including the well known aeromonads chromosomal beta-lactamases.
- Tetracycline resistance among the *Aeromonas* species collection was majorly related to the presence of *tet* (A) and *tet* (E) determinants.
- There was a considerable molecular diversity of the *cphA/imiS* genes detected and a significant molecular divergence in relation to the previously reported CphA-like beta-lactamases.
- Integrons do not play a major role in the antibiotic resistance displayed by the *Aeromonas* sp. collection.
- To the best of our knowledge, the class 1 integron with gene cassette array *dfrA12 - orfF - aadA2* was for the first time detected in the species *A. eucrenophila*. Class 2 integron with cassette array *dfrA1-sat2-aadA1* was for the first time detected in the species *A. media.*
- Genes encoding aerolysin like toxins putatively with multiple virulent activities are considerably occurring and widely distributed in the *Aeromonas* species strains in study.
- The T3SS apparatus seems to be present in distinct *Aeromonas* species other than the previously reported.
- Genetic determinants of lipolytic and/or phospholipolytic activity are a commonplace feature in the *Aeromonas* species collection.
- Multiple combinations of genetic virulence determinants and physiological activities putatively involved in pathogenic processes are highly common in the *Aeromonas* strains scrutinised.
- The sources analysed revealed to be a reservoir of antibiotic multiresistant and putatively virulent strains of *Aeromonas* species, especially untreated water sources. The concurrent detection of antibiotic resistance and virulence traits in strains enhances their potential health hazard.

- It is of paramount importance to settle surveillance on aeromonads in water distribution aquifers, since the human and animal well being may be compromised by the presence of potentially virulent antibiotic resistant specimens that are spread through different geographical regions in Portugal.

9.2 Further work

Several questions were raised by this study concerning aeromonads communities present in sources for human and animal direct or indirect use.

Three novel phylogenetic lines within *Aeromonas* were identified. Further studies including a more extensive biochemical and physiological study, a comprehensive MLPA of at least five housekeeping genes and mandatory DNA-DNA hybridisation analysis including all other described *Aeromonas* species are needed to provide formal species proposals.

Regarding antibiotic resistance, the genetic determinants encountered not always explained the phenotypes expressed. On the other hand, the genetic bases of resistance to some antibiotics were not accounted for in this study. In this way, it would be important to deepen the knowledge on resistance mechanisms to beta-lactams and tetracyclines and identify the ARG responsible for aminoglycosides and SXT resistance among strains. For that, a PCR based methodology using primers targeting previously characterised ARG should be applied. However, it is expectable that among *Aeromonas* strains some yet not described ARG can be present. In those cases an approach based on the construction and analysis of genomic libraries from the selected strains would allow the characterisation of these determinants.

Additionally, it is of paramount importance to determine the genetic context of the *cphA*/*imiS* gene found in *A. allosaccharophila* A.28/2 which could be explored by PCR mapping and cloning experiments. The use of nested PCR reactions using specific primers targeting the *cphA*/*imiS* gene combined with degenerate randomly designed primers targeting adjacent sequences of the gene as in thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995) could be applied.

The profiling of plasmid composition, dissection of their genetic content and potential for dissemination is an exciting and imperative field for investigation among our

collection. Strains displaying antibiotic resistance phenotypes and strains harbouring DNA sequences of genes related to virulence are especially interesting for the survey of the presence of plasmids and assessment of the genomic location (chromosome or plasmid) of such genetic determinants.

Distinct virulence factors were identified among our *Aeromonas* strains as well as potential features of pathogenicity. It would be of paramount importance to establish a correspondence between the presence of the virulence factors identified and the putative pathogenicity of strains. Adhesion, invasion and cytotoxicity abilities of bacteria may be determined by the use of different animal tissue cell lines. Additionally, the use of knock-out mutants is a good methodology to assess the effects of the expression of a given gene and thus to verify its role on pathogenicity to host (Chopra *et al.*, 2009).

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Annexe I

Table displaying relevant information related to each strain acquired during this study.

Strain	Species	Source[a]	N[b]	Resistance Profile[c]	ARG[d]	Integrons		Virulence Traits	
						intI	Cassette Array	Enz. Actv.[e]	Genes detected[f]
A.28/2	*A.allosaccharophila*	MW	1	AMX	*cphA/imiS*	-	-	lip, prot	*ascV*
A.1/1	*A.caviae*	MW	1	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.22/6	*A.encheleia*	MW	1	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.17/6	*A.eucrenophila*	MW	1	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.7/1	*A.media*	MW	3	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Aer-B
A.21/1	*A.media*	MW	1	TIC, TIM, AMX, CEF	-	-	-	lip, prot	Lip, Aer-B
A.23/1	*A.media*	MW	1	TIC, TIM, AMX, AMC, CEF, NET	-	-	-	lip, prot	Lip
A.30/1	*A.media*	MW	2	TIC, TIM, AMX, CEF, E	-	-	-	lip, prot	Lip
A.4/2	*A.media*	MW	2	TIC, TIM, AMX, CEF, NET, E	-	-	-	lip, prot	Lip, Aer-B
A.22/2	*A.media*	MW	1	TIC, TIM, AMX, CEF, E	-	-	-	lip, prot	Lip
A.41/2	*A.media*	MW	1	TIC, TIM, AMX, CEF, NET	-	-	-	lip, prot	Lip
A.4/6	*A.media*	MW	1	TIC, TIM, AMX, CEF, KAN, TOB	-	-	-	lip, prot	Lip
A.5/6	*A.media*	MW	1	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.11/6	*A.*sp. Group II	MW	2	AMX, CEF	-	-	-	lip, prot	Lip, Aer-A
A.28/6	*A.*sp. Group II	MW	1	AMX, AMC, CEF, E	-	-	-	lip, prot	Lip, *ascV*
A.24/6	*A.*sp. HG11	MW	1	TIC, TIM, AMX, CEF	*tet* (D)	-	-	lip, prot	Lip, Aer-B
A.37/6	*A.tecta*	MW	1	TIC, AMX, TOB, E	-	-	-	lip, prot	Lip, Aer-B
A.116A4	*A.bestiarum*	DW	1	TIC, TIM, AMX, CEF	-	-	-	lip, prot	Lip, Aer-A
A.127/4	*A.bestiarum*	DW	5	TIC, AMX, CEF	-	-	-	lip, prot	Lip, Aer-A, *ascV*
A.127/7	*A.bestiarum*	DW	7	AMX, CEF	-	-	-	lip, prot	Lip, Aer-A, *ascV*
A.128	*A.bestiarum*	DW	2	AMX, CEF	-	-	-	lip, prot	Lip, Aer-A, *ascV*
A.132/4	*A.bestiarum*	DW	2	TIC, AMX, AMC, CEF, NET, E	-	-	-	lip, prot	Lip, Aer-A, *ascV*
A.132/8	*A.bestiarum*	DW	1	TIC, AMX, CEF	-	-	-	lip, prot	Lip, Aer-A, *ascV*
A.134/1	*A.bestiarum*	DW	8	TIC, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip, Aer-A, *ascV*
A.135/1	*A.bestiarum*	DW	3	TIC, AMX, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A, *ascV*
A.135/7	*A.bestiarum*	DW	1	TIC, AMX, AMC, CEF, E	-	-	-	lip,prot	Lip, Aer-A, *ascV*
A.A1-4	*A.bestiarum*	DW	5	AMX, CEF, E	-	-	-	lip,prot	Lip
A.130/4	*A.caviae*	DW	2	TIC, AMX, CEF, STR	-	I	*aadA2*	lip	Lip
A.130/11	*A.caviae*	DW	1	TIC, TIM, AMX, CEF	-	-	-	lip	Lip
A.115A2	*A.eucrenophila*	DW	2	AMX, AMC, CEF, NET, E	-	-	-	lip, prot	Lip, Aer-B

Strain	Species	Source[a]	N[b]	Resistance Profile[c]	ARG[d]	Integrons		Virulence Traits	
						intI	Cassette Array	Enz. Actv.[e]	Genes detected[f]
A.120/8	*A.eucrenophila*	DW	1	AMX, CEF	-	-	-	lip, prot	Lip, Aer-B
A.120/12	*A.eucrenophila*	DW	1	AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.135/2	*A.eucrenophila*	DW	2	TIC, AMX, CEF	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.135/6	*A.eucrenophila*	DW	1	TIC, AMX, CEF, KAN, E	*tet* (E), *cphA/imiS*	I	*dfrA12 - orfF - aadA2*	lip,prot	Lip, Aer-B
A.137/1A	*A.eucrenophila*	DW	2	TIC, AMX, CEF, E	-	-	-	lip,prot	Lip, Aer-B, *ascV*
A.A2/2	*A.eucrenophila*	DW	1	TIC, AMX, CEF	-	-	-	lip,prot	Lip, Aer-B
A.A2/10	*A.eucrenophila*	DW	1	AMX, CEF, E	-	-	-	lip	Lip, Aer-B
A.124/1	*A.media*	DW	1	TIC, TIM, AMX, CEF	-	-	-	lip, prot	Lip
A.129/1	*A.media*	DW	4	TIC, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.130/2	*A.media*	DW	2	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip, Aer-B
A.130/13	*A.media*	DW	1	TIC, TIM, AMX, CEF, E	-	-	-	lip, prot	Lip
A.131/1	*A.media*	DW	4	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.131/6	*A.media*	DW	1	TIC, AMX, AMC, CEF	-	-	-	lip, prot	-
A.133/2	*A.media*	DW	1	TIC, TIM, AMX, CEF, STR	-	I	*aadA2*	lip, prot	Lip
A.A2/1	*A.media*	DW	4	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip,prot	Lip
A.A2/4	*A.media*	DW	3	TIM, AMX, CEF, E	-	II	*dfrA1 – sat2 – aadA1*	lip,prot	Lip
A.130/12	*A.popoffii*	DW	1	TIC, AMX, CEF	-	-	-	lip	Lip, Aer-A
A.110C1	*A.salmonicida*	DW	2	AMX, CEF	-	-	-	lip, prot	Lip, Aer-A
A.121/2	*A.salmonicida*	DW	1	AMX, CEF	-	-	-	lip, prot	Lip, Aer-A
A.136/26	*A.salmonicida*	DW	12	IPM, TIC, TIM, AMX, AMC, CEF	*cphA/imiS*	-	-	lip,prot	Lip, Aer-A
A.131/2	*A.*sp. Group I	DW	2	TIC, AMX, AMC, CEF, KAN, TET, E, SxT	-	-	-	lip, prot	Lip
A.136/15	*A.*sp. Group II	DW	1	TIC, AMX, CEF, E	-	-	-	lip,prot	Lip
A.109A5	*A.tecta*	DW	19	TIC, AMX, CEF	-	-	-	lip, prot	Lip, *ascV*
A.096A	*A.veronii*	DW	10	TIC, TIM, AMX, AMC, TET, CIP, E, SxT, STR	*tet* (A), bla_{TEM}	I	*dfrA12 - orfF - aadA2*	lip	Aer-B
A.098A	*A.veronii*	DW	1	TIC, TIM, AMX, AMC	*cphA/imiS*	-	-	lip, prot	Aer-B, *aexT, ascV*
A.135/10	*A.veronii*	DW	2	TIC, AMX, CEF	*cphA/imiS*	-	-	lip,prot	Lip, Aer-A, *ascV*
A.136/4	*A.veronii*	DW	1	TIC, TIM, AMX, AMC	-	-	-	lip,prot	Lip, Aer-B
A.136/5	*A.veronii*	DW	5	TIC, AMX	-	-	-	lip,prot	Aer-B, *aexT, ascV*
A.120/1	*A*sp. HG11	DW	1	TIC, TIM, AMX, CEF	-	-	-	lip, prot	Lip, Aer-B
A.023V	*A.hydrophila*	DW	1	TIC, TIM, AMX, CEF	*tet* (A), *cphA/imiS*	-	-	lip, prot	Lip, Aer-A, *ascV*
A.109A1	*A.hydrophila*	DW	1	TIC, AMX, CEF	-	-	-	lip, prot	Lip, *ascV*

Strain	Species	Source[a]	N[b]	Resistance Profile[c]	ARG[d]	Integrons		Virulence Traits	
						intI	Cassette Array	Enz. Actv.[e]	Genes detected[f]
A.110B1	*A.hydrophila*	DW	1	TIC, AMX, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A, *ascV*
A.110C2	*A.hydrophila*	DW	2	TIC, TIM, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.114C1	*A.hydrophila*	DW	5	AMX, CEF, TET	*tet* (E), *cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.122/3	*A.hydrophila*	DW	1	TIC, TIM, AMX, AMC, E	-	-	-	lip, prot	Lip, Aer-A
A.130/9	*A.hydrophila*	DW	1	TIC, TIM, AMX, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip
A.130/10	*A.hydrophila*	DW	1	TIC, AMX, CEF, STR	*tet* (C)	I	*catB8 - aadA1*	lip, prot	Lip, Aer-A
A.131/8	*A.hydrophila*	DW	2	TIC, AMX, CEF, KAN, TET, E, SxT, STR	*tet* (E), *cphA/imiS*	I	*dfrA12 - orfF - aadA2*	lip, prot	Lip
A.132/1	*A.hydrophila*	DW	5	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.133/3	*A.hydrophila*	DW	2	TIC, AMX, CEF, TET, E, SxT	*tet* (E), *cphA/imiS*	I	*dfrA12 - orfF - aadA2*	lip, prot	Lip, Aer-B
A.133/5	*A.hydrophila*	DW	1	TIC, AMX	*cphA/imiS*	-	-	lip, prot	Lip
A.135/5	*A.hydrophila*	DW	1	AMX, CEF, E	*cphA/imiS*	-	-	lip,prot	Lip
A.136/3	*A.hydrophila*	DW	7	TIC, TIM, AMX, AMC, CEF	-	-	-	lip,prot	Lip, Aer-A
A.136/12	*A.hydrophila*	DW	1	TIC, TIM, AMX, CEF, E	*cphA/imiS*	-	-	lip,prot	Lip
A.136/13	*A.hydrophila*	DW	1	TIC, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip,prot	Lip,*ascV*
A.136/24	*A.hydrophila*	DW	1	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip,prot	Lip, Aer-A
A.137/2	*A.hydrophila*	DW	3	AMX, CEF, E	*cphA/imiS*	-	-	lip,prot	Lip, Aer-A
A.A2/6	*A.hydrophila*	DW	1	TIC, TIM, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip,prot	Lip, Aer-A
A.126	*A.hydrophila*	DW;IW	9	AMX, E	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.A5-1	*A.hydrophila*	DW;IW	16	TIC, TIM, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip
A.A10-2	*A.allosaccharophila*	IW	4	TIC, AMX, CEF, E	-	-	-	lip, prot	*ascV*
A.A7-6	*A.bestiarum*	IW	3	IPM, TIC, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A, *ascV*
A.A10-3	*A.bestiarum*	IW	3	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-A, *ascV*
A.A4-11	*A.eucrenophila*	IW	1	TIM, AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.A10-1	*A.eucrenophila*	IW	2	AMX, CEF, E	-	-	-	lip, prot	Lip
A.A10-8	*A.eucrenophila*	IW	1	AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.A7-4	*A.hydrophila*	IW	1	TIC, TIM, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip
A.A9-1	*A.hydrophila*	IW	10	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.A9-11	*A.hydrophila*	IW	1	TIC, TIM, AMX, AMC, CEF, E	*cphA/imiS*, bla_{VIM}	-	-	lip, prot	Lip
A.A9-12	*A.hydrophila*	IW	1	TIC, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.A10-9	*A.hydrophila*	IW	1	TIC, AMX, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip

Strain	Species	Source[a]	N[b]	Resistance Profile[c]	ARG[d]	Integrons		Virulence Traits	
						intI	Cassette Array	Enz. Actv.[e]	Genes detected[f]
A.A4-1	*A.media*	IW	8	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip,prot	Lip
A.A4-8	*A.media*	IW	1	TIC, AMX, CEF, E	-	-	-	lip,prot	Lip
A.A4-9	*A.media*	IW	2	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip	Lip
A.A10-5	*A.media*	IW	1	TIC, TIM, AMX, AMC, CEF, GEN, E	-	-	-	lip, prot	Lip
A.L11-5	*A.allosaccharophila*	V	3	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip, prot	*ascV*
A.L20-1	*A.allosaccharophila*	V	2	AMX, CEF, TET	*tet* (E)	-	-	lip, prot	*ascV*
A.L20-7	*A.allosaccharophila*	V	1	AMX, CEF	*cphA/imiS*	-	-	lip, prot	*ascV*
A.L11-1	*A.bestiarum*	V	5	TIC, AMX, CEF, TET	*tet* (A), *cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.L10-7	*A.eucrenophila*	V	2	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.L12-2	*A.eucrenophila*	V	1	CEF, E	-	-	-	lip, prot	Aer-B
A.L12-6	*A.eucrenophila*	V	3	AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.L18-1	*A.eucrenophila*	V	2	E	-	-	-	lip, prot	Lip
A.L18-7	*A.eucrenophila*	V	1	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.L18-9	*A.eucrenophila*	V	1	AMX, CEF, E	-	-	-	lip, prot	Lip
A.L11-8	*A.hydrophila*	V	5	AMX, E	*cphA/imiS*	-	-	lip, prot	Lip
A.L12-1	*A.hydrophila*	V	5	TIC, AMX, AMC, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip
A.L12-10	*A.hydrophila*	V	1	TIC, AMX, CEF, E	*tet* (A), *cphA/imiS*	-	-	lip, prot	Lip
A.L18-6	*A.hydrophila*	V	2	TIC, AMX, CEF, E	*cphA/imiS*	-	-	lip, prot	Lip
A.L1-1	*A.media*	V	4	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L1-2	*A.media*	V	5	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L1-3	*A.media*	V	3	TIC, TIM, AMX, CEF, E	-	-	-	lip, prot	Lip
A.L1-6	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L1-8	*A.media*	V	5	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip
A.L1-11	*A.media*	V	1	TIC, TIM, AMX, CEF	-	-	-	lip, prot	-
A.L2-3	*A.media*	V	4	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L2-4	*A.media*	V	1	TIC, TIM, AMX, CEF, E	-	-	-	lip, prot	Lip
A.L2-7	*A.media*	V	1	TIC, TIM, AMX, CEF	-	-	-	lip, prot	Lip
A.L2-9	*A.media*	V	4	TIC, AMX, CEF	-	-	-	lip, prot	Lip
A.L2-11	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L2-12	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip

Strain	Species	Source[a]	N[b]	Resistance Profile[c]	ARG[d]	Integrons		Virulence Traits	
						intI	Cassette Array	Enz. Actv.[e]	Genes detected[f]
A.L3-1	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L3-4	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L7-1	*A.media*	V	4	TIM, AMX, CEF	-	-	-	lip, prot	-
A.L7-2	*A.media*	V	3	TIC, AMX, CEF	-	-	-	lip, prot	Lip
A.L7-3	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L7-4	*A.media*	V	4	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L8-1	*A.media*	V	4	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.L8-2	*A.media*	V	2	TIC, AMX, CEF	-	-	-	lip, prot	Lip
A.L8-4	*A.media*	V	2	TIC, TIM, AMX, CEF, E	-	-	-	lip, prot	Lip
A.L8-7	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip
A.L8-10	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	-
A.L9-1	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L9-2	*A.media*	V	6	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L9-3	*A.media*	V	2	TIC, TIM, AMX, AMC, CEF	-	-	-	lip, prot	Lip
A.L9-10	*A.media*	V	1	TIC, TIM, AMX, CEF	-	-	-	lip, prot	Lip
A.L9-11	*A.media*	V	2	TIC, TIM, AMX, CEF	-	-	-	lip	Lip
A.L10-1	*A.eucrenophila*	V	2	AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.L10-2	*A.eucrenophila*	V	2	TIC, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip, Aer-B
A.L18-2	*A.media*	V	1	AMX	-	-	-	lip, prot	Lip
A.L18-3	*A.media*	V	1	TIC, AMX	-	-	-	lip, prot	Lip
A.L18-4	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip
A.L18-5	*A.media*	V	3	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.L18-12	*A.media*	V	1	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip
A.L20-3	*A.media*	V	1	TIC, AMX, E	-	-	-	lip, prot	Lip
A.L20-9	*A.media*	V	1	TIC, AMX, E	-	-	-	lip, prot	Lip
A.L21-1	*A.media*	V	2	TIC, AMX, CEF	-	-	-	lip, prot	Lip, Aer-A
A.L21-2	*A.media*	V	1	TIC, AMX, E	-	-	-	lip, prot	Lip
A.L21-4	*A.media*	V	9	TIC, TIM, AMX, TET, E	*tet* (E)	-	-	lip, prot	Lip
A.L14-1	*A.salmonicida*	V	9	IPM, TIC, AMX, CEF	*tet* (A), *cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.L14-4	*A.salmonicida*	V	3	TIC, AMX, CEF	*tet* (A)	-	-	lip, prot	Lip, Aer-A
A.L19-1	*A.salmonicida*	V	11	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip, Aer-A

Strain	Species	Source[a]	N[b]	Resistance Profile[c]	ARG[d]	Integrons		Virulence Traits	
						intI	Cassette Array	Enz. Actv.[e]	Genes detected[f]
A.L20-2	*A.salmonicida*	V	5	CEF	*cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.L20-5	*A.salmonicida*	V	2	TIC, AMX	-	-	-	lip, prot	Lip, Aer-A
A.L8-3	*A.*sp. Group II	V	2	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip, *ascV*
A.L10-4	*A.*sp. Group II	V	6	TIC, TIM, AMX, CEF, E	-	-	-	lip, prot	Lip
A.L15-1	*A.*sp. Group III	V	12	CEF, E	*tet* (A)	-	-	lip, prot	Lip, Aer-B
A.V2-2	*A.salmonicida*	CF	3	TIC, AMX, CEF, TET, E, STR	*tet* (A)	I	*aadA2*	lip, prot	Lip, Aer-A
A.V2-4	*A.salmonicida*	CF	2	IPM, AMX, CEF, TET, E	*tet* (E), *cphA/imiS*	-	-	lip, prot	Lip, Aer-A
A.V2-5	*A.salmonicida*	CF	1	TIC, AMX, CEF, GEN, KAN, TET, E, SxT	*tet* (E)	-	-	lip, prot	Lip, Aer-A
A.V2-10	*A.salmonicida*	CF	1	TIC, AMX, CEF, E		-	-	lip, prot	Lip, Aer-A
A.V2-12	*A.salmonicida*	CF	1	TIC, AMX, CEF, TET, E	*tet* (E)	-	-	lip, prot	Lip, Aer-A
A.AV-11	*A.salmonicida*	CW; CF	5	AMX, CEF, E	-	-	-	lip, prot	Aer-A, *ascV*
A.AV-1	*A.media*	CW	1	TIC, AMX, CEF, E	-	-	-	lip, prot	Lip
A.AV-2	*A.media*	CW	6	TIC, TIM, AMX, AMC, CEF, E	-	-	-	lip, prot	Lip
A.AV-5	*A.media*	CW	2	TIC, TIM, AMX, AMC, CEF, TET, E	*tet* (E)	-	-	lip, prot	Lip
A.AV-9	*A.media*	CW	1	TIC, TIM, AMX, AMC, CEF, TET, E	*tet* (E)	-	-	lip, prot	Lip
A.AV-10	*A.media*	CW	1	TIC, TIM, AMX, AMC, CEF, TET	*tet* (E)	-	-	lip, prot	Lip
A.ALU-1	*A.caviae*	UW; M	30	TIC, TIM, AMX, CEF, TET	-	-	-	lip, prot	Lip

[a] DW, drinking water; MW, mineral water; IW, irrigation water; V, vegetables; CW, cows' drinking water; CF, cow's faeces; UW, udder's cleaning water before milking; M, milk.

[b] Number of isolates collected from each strain.

[c] Amoxicillin, AMX (10µg); amoxicillin/clavulanic acid, AMC (30µg); ticarcillin, TIC (75µg); ticarcillin/clavulanic acid, TIM (85µg); cefalotin, CEF (30µg); imipenem, IPM (10µg); gentamicin, GEN (10µg); kanamycin, KAN (30µg); tobramycin, TOB (10µg); netilmicin, NET (30µg); tetracycline, TET (30µg); erythromycin, ERY (15µg); trimethoprim/sulfamethoxazole – SxT (25µg), ciprofloxacin – CIP (5µg) (Oxoid, UK), streptomycin – STR (10µg).

[d] ARG, antibiotic resistance genes.

[e] Extracellular enzymatic activity: lip, lipolytic activity; prot, proteolytic activity.

[f] Lip, Genes coding for lipolytic activity, Aer, aerolysin related genes: Aer-A, *Hpa*II restriction pattern A; Aer-B, *Hpa*II restriction pattern B.

Annexe II

Number of samples, isolates, RAPD patterns, *gyrB* sequences and species, collected from each kind of source, including the number of samples harbouring or that failed to display aeromonads.

Kind of source/sample[a]		Nr. Samples	Nr. + Samples[b]	Nr. – Samples[c]	Nr. isolates	Nr. RAPD patterns	Nr. *gyrB* sequences	Nr. Species
UnW	DW	93	30	63	184	63	52	12
	MW	3	3	-	22	17	16	8
AS	IW	10	4	6	47	17	16	5
	V	21	15	6	176	62	45	8
SE	CW	1	1	-	12	6	5	2
	CF	2	1	1	12	6	5	1
	UW	1	1	-	12	1	1	1
	M	1	1	-	18	1	1	1
	MEW	1	0	-	-	-	-	-
	Total	133	56	76	483	169[d]	125[d]	15

[a] UnW (Untreated water sources): DW, drinking water; MW, mineral water. AS (agricultural sources): IW, irrigation water; V, vegetables. SE (Stable environment): CW, cows' drinking water; CF, cow's faeces; UW, udder's cleaning water before milking; M, milk; MEW, last cleaning water of the milking equipment.
[b] Number of samples that contained *Aeromonas* sp.
[c] Number of samples that failed to display *Aeromonas* species.
[d] These values do not correspond to the sum of values of each column as in some occasions the same RAPD patterns and *gyrB* gene partial sequences occurred in different types of sources.

Annexe III

Number and distribution of different strains and isolates among different sources.

Species	Nr. Strains (%)[a]	Nr. Isolates (%)[b]	Kind of source/sample[c]								
			UnW		AS		SE				
			DW (NS; NI)	MW (NS; NI)	IW (NS; NI)	V (NS; NI)	CW (NS; NI)	CF (NS; NI)	UW (NS; NI)	M (NS; NI)	MEW (NS; NI)
A. media	65 (39)	145 (30)	9; 21	9; 13	4; 12	38; 88	5; 11	-	-	-	-
A. hydrophila	30 (18)	90 (19)	21; 56	-	7; 21	4; 13	-	-	-	-	-
A. salmonicida	14 (8)	58 (12)	3; 15	-	-	5; 30	1; 1	6; 12	-	-	-
A. bestiarum	13 (8)	46 (10)	10; 35	-	2; 6	1; 5	-	-	-	-	-
A. caviae	4 (2)	34 (7)	2; 3	1; 1	-	-	-	-	1; 12	1; 18	-
A. eucrenophila	20 (12)	30 (6)	8; 11	1; 1	3; 4	8; 14	-	-	-	-	-
A. tecta	2 (1)	20 (4)	1; 19	1; 1	-	-	-	-	-	-	-
A. veronii	5 (3)	19 (4)	5; 19	-	-	-	-	-	-	-	-
Aeromonas sp. Group II	5 (3)	12 (3)	1; 1	2; 3	-	2; 8	-	-	-	-	-
Aeromonas sp. Group III	1 (0.6)	12 (3)	-	-	-	1; 12	-	-	-	-	-
A. allosaccharophila	5 (3)	11 (2)	-	1; 1	1; 4	3; 6	-	-	-	-	-
Aeromonas sp. Group I	1 (0.6)	2 (0.4)	1; 2	-	-	-	-	-	-	-	-
A. HG11	2 (1)	2 (0.4)	1; 1	1; 1	-	-	-	-	-	-	-
A. popoffii	1 (0.6)	1 (0.2)	1; 1	-	-	-	-	-	-	-	-
A. encheleia	1 (0.6)	1 (0.2)	-	1; 1	-	-	-	-	-	-	-
Total	169	483	63; 184	17; 22	17; 47	62; 176	6; 12	6; 12	1; 12	1; 18	0

[a] In brackets, the percentage of strains collected from each species in relation to total number of strains.
[b] In brackets, the percentage of isolates collected from each species in relation to total number of isolates.
[c] UnW (Untreated water sources): DW, drinking water; MW, mineral water. AS (agricultural sources): IW, irrigation water; V, vegetables. SE (Stable environment): CW, cows' drinking water; CF, cow's faeces; UW, udder's cleaning water before milking; M, milk; MEW, last cleaning water of the milking equipment. NS, number of strains; NI, number of isolates.