EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS

Ana Fernández Bravo

UNIVERSITAT ROVIRA i VIRGILI

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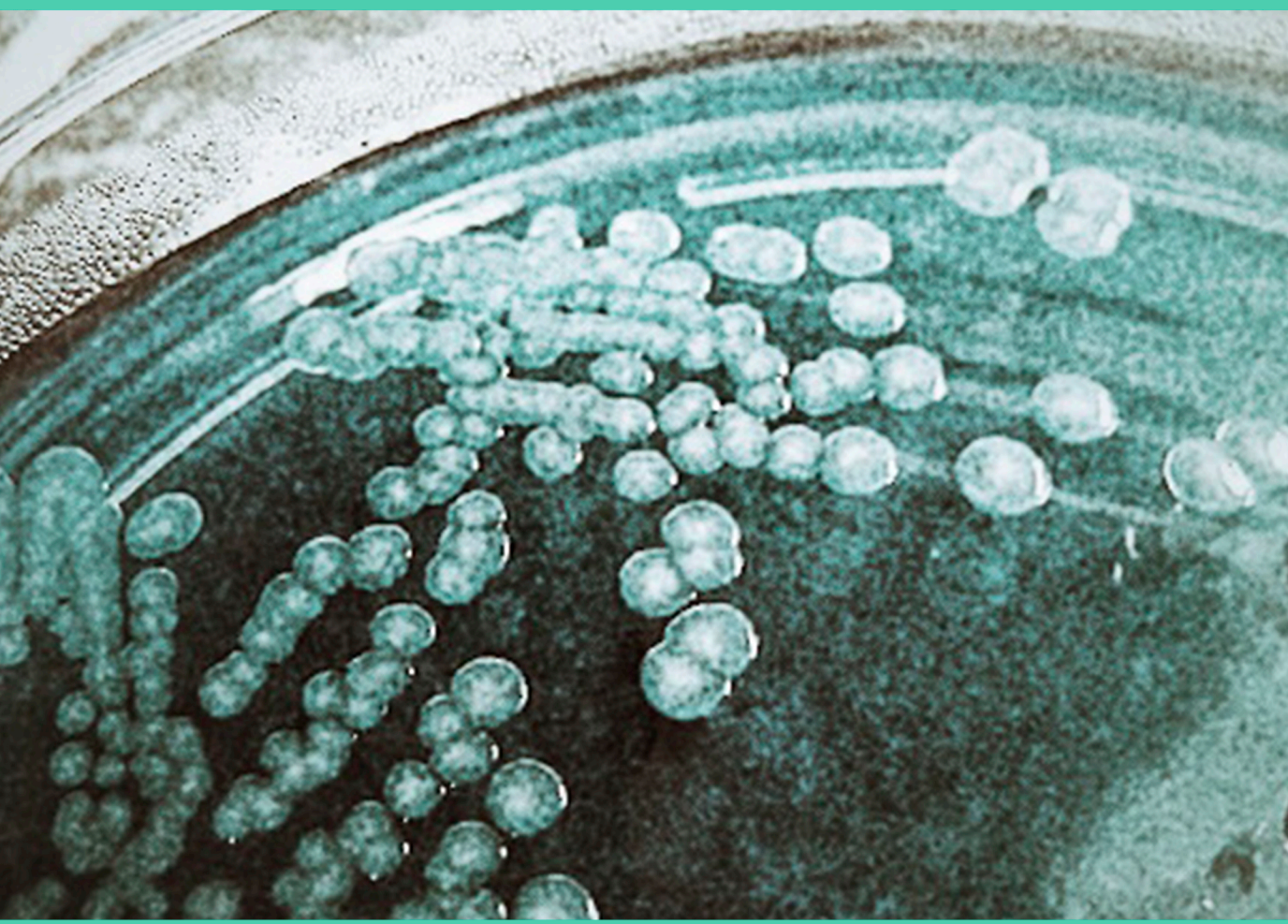
ANA FERNÁNDEZ BRAVO



DOCTORAL THESIS
2019

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Directed by Prof. Mª Jose Figueras Salvat

Department of Basic Health Sciences
Faculty of Medicine and Health Sciences

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CERTIFY THAT:

The present work entitled: "Epidemiology and pathogenic characterization of species of the genus *Aeromonas*" prepared by Ana Fernández Bravo to obtain the degree of doctor by the University Rovira i Virgili, have been carried out under my supervision at the Unit of Microbiology of the Department of Basic Health Sciences, and that it fulfils the requirements to obtain the International Doctorate mention.

Reus, April 26th, 2019.

Dr. Maria José Figueras Salvat

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To my father Sergio

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Miriam, Tere, Alba…Vosotros habéis hecho que todo esto fuera tan especial, las risas y los grandes momentos siempre han estado asegurados con vosotros.

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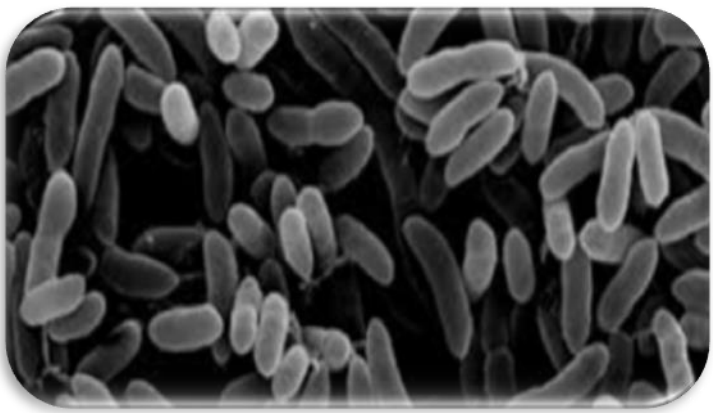
1. INTRODUCTION



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1.1. Historic perspective of the genus *Aeromonas*

The genus *Aeromona*s (aer-, from the Greek: gas; -mones, units, gas producing units), according to the most recent edition of the Bergey's Manual, belongs to the Class Gammaproteobacteria, Order Aeromonadales, Family *Aeromonadaceae*, which currently includes 3 genera: *Aeromonas*, *Oceanimonas* and *Tolumonas* (Martin-Carnahan and Jospeh, 2005). These microorganisms are characterized for being Gram-negative bacillus, oxidase and catalase positive, capable of degrading nitrates to nitrites, glucose fermenters and resistant to vibriostatic factor O/129 (2,4-diamino-6,7-di-iso-propylpteridine phosphate). Although they are microorganisms native to the aquatic environment, they are also frequently isolated in human food, fish and other infectious processes in humans, including immunocompromised and immucompetent people (Castro-Escarpulli et al., 2002; Martin-Carnahan and Joseph, 2005; Figueras 2005; Janda and Abbott et al., 2010; Figueras and Beaz-Hidalgo, 2015).

It is believed that the first isolates were reported in 1891 Sanarelli et al. and these bacteria were classified as *Bacillus hydrophilus fuscus.* The genus was created in 1936 by Kluyver and van Niel, but the International Committee of Systematic Bacteriology established the authorship to Stainer in 1943.

The genus *Aeromonas* belonged to the family *Vibrionaceae*, since 1965, together with the genera *Vibrio* and *Plesiomonas* (Martin-Carnahan and Joseph, 2005). In the mid-70s, most of the *Aeromonas* were included within two groups, mainly based on the temperature of growth and various characteristics, such as motility, pigment production in TSA or indole production. These two groups were the following:

- Mesophilic strains (optimal growth at 35-37ºC) responsible for various infections in humans and defined globally under the name of *A. hydrophila.*

- Psychrophilic strains (optimal growth at 22-28ºC) mainly fish pathogens and identified as *A. salmonicida*.

These two groups were reclassified later on the basis of studies of DNA-DNA reassociation (McInnes et al., 1979; Popoff et al., 1981; Farmer et al., 1986).

From 1992 to date, 22 new species have been described and included in the genus: *A. allosaccharophila* (Martínez-Murcia et al., 1992b), *A. encheleia* (Esteve et al., 1995b), *A. bestiarum* (Ali et al., 1996), *A. popoffii* (Huys et al., 1997a, b), *A. simiae* (Harf-Monteil et al., 2004), *A. molluscorum* (Miñana-Galbis et al., 2004), *A. bivalvium* (Miñana-Galbis et al., 2007), *A. tecta* (Demarta et al., 2008), *A. piscicola* (Beaz-Hidalgo et al., 2009), *A. fluvialis*, *A. taiwanensis* and *A. sanarellii* (Alperi et al., 2010a, b), *A. diversa* (Miñana-Galbis et al., 2010), *A. rivuli* (Figueras et al., 2011a), *A. cavernicola* (Martínez-Murcia et al 2013), *A. australiensis* (Aravena-Román et al., 2013), *A. dhakensis* (Beaz-Hidalgo et al., 2013), *A. finlandiensis*, *A. lacus* and *A. aquatica* (Beaz-Hidalgo et al., 2015b), *A. rivipollensis* (Martí-Balcázar et al ., 2016) and *A. lusitana* (Martínez-Murcia et al., 2016). In addition, four new species included in the Doctoral thesis of Fadua Latif Eugenín in 2015 are in the process of description, and all the species are represented in Table 1.

- The type species of the genus *A. hydrophila* includes 2 subspecies: *A. hydrophila* subsp. *hydrophila* and *A. hydrophila* subsp. *ranae* (LMG 19707^T), while *A. salmonicida i*ncludes 5 subspecies: *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes* (ATCC 3659^T), *A. salmonicida* subsp. *masoucida* (ATCC 27013^T), *A. salmonicida* subsp. *smithia* (ATCC 49393^T) and *A. salmonicida* subsp. *pectinolytica* (DSMZ 12609^T).

- *A. veronii* includes 2 biotypes: *A. veronii* bv. *sobria* and *A. veronii* bv. v*eronii.*

- The species *A. icthiosmia* and *A. culicicola* are considered synonyms of *A. veronii* bv. *sobria* and *A. veronii* bv. *veronii* (Huys et al., 2001; 2005).

- *A. enteropelogenes* is a synonym of *A. trota* (CECT 4255^T, Collins et al., 1993).

- *A. hydrophila* subsp. *dhakensis* (CECT 5744^T, Huys et al., 2002) and *A. aquariorum* (CECT 7289^T, Martínez-Murcia et al., 2008) were reclassified as a single species *A. dhakensis* (CECT 5744^T).

Table 1. *Aeromonas* species

Species	Type strain	Source	References
A. hydrophila	CECT 839^T	Milk	Stainer, 1943
A. salmonicida	CECT 894^T	Salmon	Griffin et al., 1953
A. sobria	CECT 4245^T	Fish	Popoff and Veron, 1981
A. media	CECT 4232^T	Fisheries water	Allen et al., 1983
A. veronii	CECT 4257^T	Sputum	Hickman-Brenner et al., 1987
A. caviae	CECT 838^T	Guinea pig	Schubert and Hegazi, 1988
A. eucrenophila	CECT 4224^T	Fresh water fish	Schubert and Hegazi, 1988
A. schubertii	CECT 4240^T	Skin abscess	Hickman-Brenner et al.,1988
A. jandaei	CECT 4228^T	Human feces	Carnahan et al., 1991
A. trota	CECT 4255^T	Human feces	Carnahan et al., 1991
A. allosaccharophila	CECT 4199^T	Eel	Martínez-Murcia et al., 1992
A. encheleia	CECT 4342^T	Eel	Esteve et al., 1995
A. bestiarum	CECT 4227 ^{T}	Sick fish	Ali et al., 1996
A. popoffi	CECT 5176^T	Drinking water	Huys et al., 1997
A. simiae	IBS S-6874^T	Monkey feces	Harf-Monteil et al., 2004
A. molluscorum	CECT 5864^T	Shellfish	Miñana-Galbis et al., 2004
A. bivalvium	CECT 7113^T	Shellfish	Miñana-Galbis et al., 2007
A. tecta	CECT 7082^T	Children feces	Demarta et al., 2008
A. piscicola	CECT 7443^T	Sick fish	Beaz-Hidalgo et al., 2009
A. fluvialis	CECT 7401 ^{T}	River water	Alperi et al., 2010
A. taiwanensis	CECT 7403^T	Wound infection	Alperi et al., 2010
A. sanarelli	CECT 7402^T	Wound infection	Alperi et al., 2010
A. diversa	CECT 4254^T	Wound infection	Miñana-Galbis et al., 2010
A. rivuli	CECT 7518^T	River water	Figueras et al., 2011
A. cavernicola	CECT 7862^T	Cavern creek water	Martínez-Murcia et al., 2012
A. australiensis	CECT 8023 ^{T}	Irrigation water	Aravena-Román et al., 2013
A. dhakensis	CECT 5744^T	Children feces	Beaz-Hidalgo et al., 2013
A. finlandensis	CECT 8028^T	Cyanobacterial bloom	Beaz-Hidalgo et al., 2015
A. aquatica	CECT 8025^T	Cyanobacterial bloom	Beaz-Hidalgo et al., 2015
A. lacus	CECT 8024^T	Cyanobacterial bloom	Beaz-Hidalgo et al., 2015
A. rivipollensis	LMG 26323^T	River water	Martí and Balcazar, 2016
A. lusitana	CECT 7828^T	Untreated water	Martínez-Murcia et al., 2016
A. intestinalis	CECT 8980^T	Human feces	Figueras et al., 2017
A. enterica	CECT 8981^T	Human feces	Figueras et al., 2017
A. crassostreae	CECT 8982^T	Shellfish	Figueras et al., 2017
A. aquatilis	CECT 8026^T	Lake water	Figueras et al., 2017

- *A. intestinalis*, *A. enterica*, *A. crassostreae* and *A. aquatilis* are the 4 new species in process of description (Figueras et al., 2017).

1.2. Taxonomy and identification

In the year 2002, Stackebrandt et al. suggested, as a member of the International Committee of Systematics of Prokaryotes (ICSP), that the definition of new prokaryotic species should include a polyphasic study involving a phenotypic, genotypic and phylogenetic differentiation. The phenotypic differentiation is based on biochemical, morphological and physiological characteristics, and on the analysis of protein and fatty acids profile; while the phylogenetic differentiation should include the sequence of at least 1300 nucleotides (nt) of the 16S rRNA gene. In addition, the sequencing of at least 7 housekeeping genes is suggested such as the so call MLSA (Multilocus Sequences Analysis), or as we prefer to denominate MLPA (Multilocus Phylogenetic Analysis). The MLPA provides greater resolution and phylogenetic differentiation.

To determine the intraspecific variability, the MLST (Multilocus Sequences Typing) has been proposed to show more resolution than other molecular typing techniques (RAPD, ERIC-PCR, REP-PCR, AFLP, etc.).

In recent years, molecular identification uses the information included in the genome for genome comparison. Several techniques have been used, such as the Average Nucleotide Identity (ANI) or the *in silico* DNA-DNA Hybridization (*isDDH*) (Richter and Rosselló-Móra, 2009; Meier-Kolthoff et al., 2013).

1.2.1. Phenotypic characterization

Phenotypic characterization embraces the morphology, physiology, and biochemical response of the bacteria. The morphology analyses the form, presence of flagella, inclusion bodies, endospores or Gram stain. The physiological characteristics include the data of the growth under different conditions (salt, pH, temperature, oxygen), while the biochemical profile includes the presence of

enzymes, metabolization of substrates, and resistance to antimicrobial agents, as well as total protein profiles (Abbott et al., 2003).

Phenotypical methods show important limitations for the characterization of *Aeromonas* spp., since many of the commercialized methods tend to confuse different species. In 2010, Beaz-Hidalgo et al. performed a re-identification of 119 strains isolated from fish, previously identified with phenotypical methods by using molecular methods (16S rRNA-RFLP and *rpoD* sequencing), and the results demonstrated that the majority of strains were mistakenly identified as *A. hydrophila.* Similar errors occur in clinical, due to the limitations of the methods, being *A. hydrophila* erroneously overestimated as one of the most important species (Figueras et al., 2009; Beaz-Hidalgo et al., 2010; Morinaga et al., 2013; Figueras and Beaz-Hidalgo, 2015).

The typical phenotypic characteristic of the genus include Gram negative stain, presence of the cytochrome oxidase, growth in nutrient broth at 0% of NaCl and negative growth at 6%, inositol acid production, the ability to oxidize-ferment glucose and resistance to the vibriostatic factor O/129, except for the species *A. cavernicola* and *A. australiensis* which are sensitive to this agent (Altwegg, 1999, Martin-Carnahan and Joseph, 2005; Martínez-Murcia et al., 2013; Aravena-Román et al., 2013).

Three species of *Aeromonas* (*A. hydrophila*, *A. sobria*, and *A. caviae*) existed as phenospecies, that is, a named species containing multiple DNA groups, the members of which could not be distinguished from another one by simple biochemical characteristics (Borrell et al., 1998; Abbott et al., 2003). An important study was performed in 2003 by Abbott, in which 62 biochemical tests and 193 strains were used. They discovered that only 9 tests showed the same response for all strains. Regarding the study performed by Beaz-Hidalgo et al. (2010), the re-identification of the 119 strains isolated from fish and identified by biochemical test showed that only 35.5% were correctly identified at species level with the biochemical characterization (Beaz-Hidalgo et al., 2010).

For many years, automatic or semiautomatic commercial identification systems have been the most commonly used in the clinical laboratories for bacterial identification. However, as indicated the phenotypic identification of *Aeromonas* species has been considered difficult, due to the limitations of the phenotypic test involved in these Systems (API 20E, Vitek, BBL Crystal, MicrosScan W/A, among others). The results obtained after comparing the biochemical identification of *Aeromonas* species using non-automated systems and automated systems showed a good correlation (Vivas et al., 2000). However, the comparison of the results obtained with those obtained with molecular methods shows a very low concordance (Lamy et al., 2010). An additional, an important problem of the identification with automated systems is the confusion of *Aeromonas* strains with species of the genus *Vibrio* (Lamy et al., 2010; Janda and Abbott et al., 2010).

1.2.2. Molecular identification

1.2.2.1. Identification techniques based on 16S ribosomal RNA

The sequences of the 16S ribosomal RNA (16S rRNA) gene have been classically considered as stable and as specific molecular markers for the identification of bacterial species. The 16S rRNA is an essential gene with universal distribution that allows the comparison with all microorganisms. Moreover, its structure presents a mosaic of variable regions, useful in the differentiation of organisms closely related, and their conserved regions are useful for comparing distant organisms, and have allowed the design of "universal" primers (Woese et al., 1987).

Sequences of the 16S rRNA gene

The first sequencing of the 16S rRNA gene in the genus *Aeromonas* was performed in 1992 by Martínez-Murcia et al. The findings demonstrated that the phylogenetic analysis agreed with the DNA-DNA hybridization (DDH) results that were used to define the species. In *Aeromonas* spp., the 16S rRNA showed to be extremely conserved, and the informative nucleotide positions are located in region V3, followed by V1, and V2 (Martínez-Murcia and Lamy, 2015).

Introduction

The presence of mutation or microheterogeneities was described in the 16S rRNA gene of some species that had led to some misidentifications (Alperi et al., 2008). Nowadays, it is considered that in the genus *Aeromonas*, this gene is useful for delimiting distant species, but species considered different showed identical 16S rRNA sequences. Therefore, this gene is not reliable for the identification of closely related *Aeromonas* spp. (Alperi et al., 2008; Martínez-Murcia and Lamy, 2015). The phylogenetic tree derived from sequences of the 16S rRNA gene of all *Aeromonas* species described until now is shown in Figure 1.

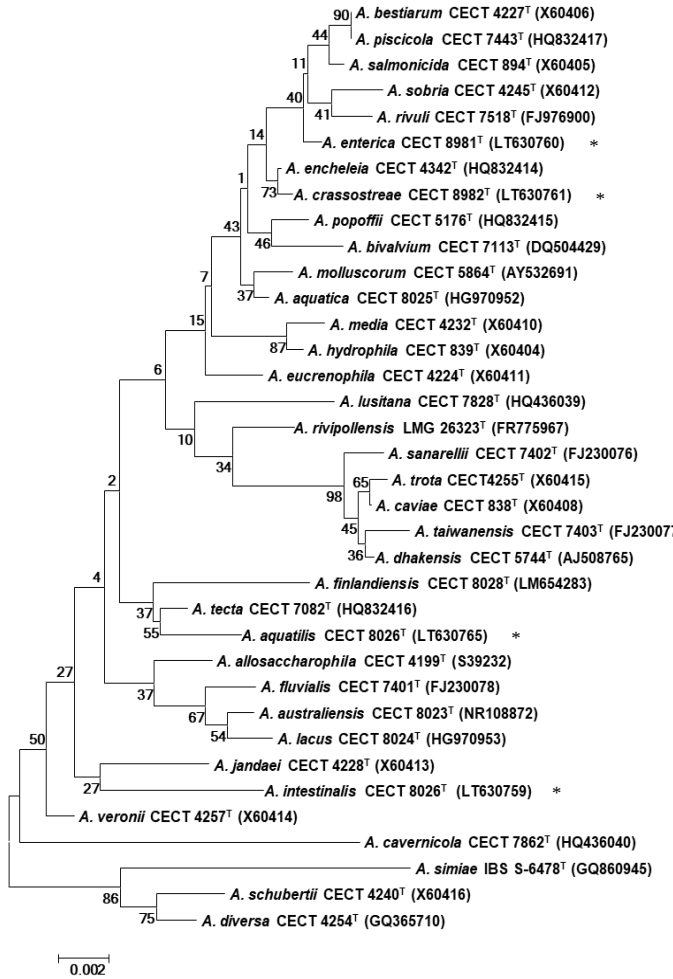


Figure 1. Phylogenetic relationship of 40 species of *Aeromonas* (*four in process of description) based on the sequences of the 16 rRNA gene. The number in the nodes indicates the bootstrap values. Bar show number of substitutions by site.

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS)

The MALDI-TOF MS is an approach used for the identification of microorganisms that has been introduced in recent years in many clinical laboratories (Vávrová et al., 2015). The method determines the molecular weights (mainly 2-20 kDa) of proteins, the majority of these associated with the 16S rRNA gene (Donohue et al., 2006; Latif-Eugenín, 2015). Its principal advantage consists in a short-time analysis without previous extensive preparation methods Vávrová et al., 2015. Currently, 2 software are available for the identification, SARAMIS™ and Biotyper. Lamy et al. (2011) analyzed 139 strains of clinical and environmental origin identified with *rpoB* sequencing. The results showed that 100% of the identification was correct at genus level, while the correct identification at species level was 91.4%. Similar results were obtained by Benagli et al. (2012) with the re-identification of 714 isolates from clinical and environmental origin, also previously identified with the *gyrB* sequences that found a correct identification at species level of 93%.

Later, Chen et al. (2014a) used 217 clinical isolates previously identified by *rpoB* sequencing, which were 100% correctly identified at genus level, and 97% at species level. In the last years, two new studies were performed (Shin et al., 2015, Latif-Eugenín, 2015). Shin et al. (2015) performed a MALDI-TOF identification of 65 clinical strains previously identified by *gyrB* sequencing and found 98.5% of concordance at genus level, and 92.3% at species level. These results are relatively similar to the ones obtained by Latif-Eugenín (2015) using 179 clinical strains from Spanish hospitals, where 98.3% showed a correct identification at genus level, and 91.1% at species level. Based on these data, it is possible to conclude that MALDI-TOF is a useful tool, since the error of identification is <10%. However, this method has a limitation because the database should be updated to include all the species of the genus, for instance *A. dhakensis* is missing in different databases (Latif-Eugenín, 2015).

1.2.2.2. Identification based on Housekeeping genes

The housekeeping genes codify proteins with essential functions for the bacterial survival (Stackebrandt et al., 2002). The phylogenetic information of these genes is higher than the 16S rRNA gene, since these genes possess a higher evolutionary rate, and the variations are distributed for the whole gene. In order for these genes to be useful, they have to comply with certain characteristics like: 1) not to be influenced by horizontal gene transfer, 2) they have to be present in all bacteria, 3) they should have a single copy in the genome, and 4) two regions with a higher conservation for the primer construction (Latif-Eugenín, 2015).

Single Housekeeping gene and Multilocus Phylogenetic Analysis (MLPA)

The first genes studied in *Aeromonas* were *gyrB* (encoding the B subunit of DNA gyrase, a type II DNA topoisomerase) and *rpoD* (encoding the s70 factor, one of the sigma factors that confers promoter-specific transcription initiation on RNA polymerase) (Yáñez et al., 2003; Soler et a., 2004). These genes have helped to recognize many new species and were used in the description of many species (Demarta et al., 2008; Beaz-Hidalgo et al., 2009; Alperi et al., 2010a, b; Miñana-Galbis et al., 2010; Figueras et al., 2011a; Martínez-Murcia et al., 2013; Aravena-Román et al., 2013; Beaz-Hidalgo et al., 2013, 2015b; Martí and Balcázar, 2016; Martínez-Murcia et al., 2016; Figueras et al., 2017). Nevertheless, the sequencing of only one housekeeping gene may not show enough resolution to show without doubt the phylogenetic positions of some closely related species, as occurs between *A. veronii* and *A. allosaccharophila* or between *A. salmonicida, A. bestiarum* and *A. piscicola.* This limitation can be resolved by using the sequencing of several housekeeping genes, performing a phylogenetic study based on the concatenated sequences of these different genes. In the genus *Aeromonas*, the other genes proposed to study the phylogenetic relationships are:, *rpoB, recA, dnaJ, cpn60, mdh, gyrA, dnaX, atpD, groL, gltA, metG, ppsA, dnaK, radA, tsf* and *zipA* (Küpfer et al., 2006; Nhung et al., 2007; Sepe et al., 2008; Miñana-Galbis et al., 2009, 2010; Martínez-Murcia et al., 2011; Martino et al., 2011; Roger et al., 2012b). Martínez-

Murcia et al. (2011) described a Multilocus Phylogenetic Analysis (MLPA) for the first time, by using concatenated sequences of 7 housekeeping genes (*rpoD, gyrB, gyrA, recA, dnaJ, dnaX* and *atpD*).

1.2.3. Whole genome sequencing

Advances in the techniques used to obtain complete genomes have evolved very quickly reducing considerably the cost of sequencing. In this sense, in 2012, only 6 *Aeromonas* genomes were available; however, in 2014, the number of genomes increased, and in fact that year, 56 genomes that represented 12 of the species of the genus *Aeromonas* were analyzed by Colston et al. (2014). The authors described that the size of the *Aeromonas* genomes oscillate between 3.90 Mbp (*A. fluvialis*) and 5.18 Mbp (*A. piscicola*) with an average size of 4.51 Mbp. Similarly, the % of G+C was 60.2%, oscillating between 58.1% (*A. australiensis*) and 62.8% (*A. taiwanensis*).

One year later, Beaz-Hidalgo et al. (2015a) re-identified the genomes of 44 strains deposited in the Genbank, and the results showed that 14 strains were identified incorrectly. The taxonomic affiliation was determined by a Multilocus Sequence Typing (MLST), and by pairwise Average Nucleotide Identity (ANI). The results demonstrated that the correct taxonomic affiliation should be verified by using ANI and MLPA before a genome is submitted to the NCBI, and that researchers should amend the existing taxonomic errors present in databases (Beaz-Hidalgo et al., 2015a).

The misidentifications of the genomes could be determined by using the new tools that have been developed for the bacterial identification based on the genome comparison, the *in silico* DNA-DNA hybridization (*is*DDH) and the Average Nucleotide Identity (ANI) (Colston et al., 2014; Creason et al., 2014; Figueras et al., 2014; Beaz-Hidalgo et al., 2015a, b).

1.2.3.1. *In silico* DNA-DNA hybridization (*is*DDH)

The tool call *Genome to genome distance calculator* allows to determine the degree of reassociation or genetic similarity between two genomic DNAs belonging

to different bacterial isolates (*in silico* DDH, *is*DDH). This informatic tool was developed by DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), and in fact simulates the classical experiment of DNA-DNA hybridization (DDH) used for the comparison of bacteria strains that showing a 16S rRNA gene similarity above 97%. If the *is*DDH was ≥ 70% it indicated that two strains belonged to the same species. The same threshold is used for the *is*DDH calculation (Auch et al., 2010a; Meier-Kolthoff et al., 2013)

1.2.3.2. Average Nucleotide Identity

The Average Nucleotide Identity is defined as the average percentage of identity that exists in the nucleotide sequence of orthologous gene shared by two genomes (Konstatinidis and Tiedje, 2005; Goris et al., 2007; Richter and Rosselló-Móra, 2009). Several software available at different public databases have been developed to perform this. The results obtained in different studies demonstrated that the percentages of ANI ≥ 95-96% were correlated with the results of *is*DDH ≥ 70% (Goris et al., 2007; Richter and Rosselló-Móra, 2009; Kim et al., 2014). For this reason, the ANI has been proposed as a new reference method. There are 4 web sites available to calculate this: JSpecies, ANI calculator, OrthoANI and OrthoANI-usearch tool. Colston et al. (2014) analyzed 56 *Aeromonas* genomes and determined that values ≥ 96% were consistent with the taxonomy of the genus. Additionally, Beaz-Hidalgo et al. (2015a) compared the ANI values with the results obtained by MLPA and the data in both were concordant. This work ratified that ANI is a good alternative to use as a gold standard for the delineation of the species as equal or different when comparing genomes. Moreover, the ANI cutoff in *Aeromonas* to the species separation should be higher than 96% (Colston et al., 2014; Beaz-Hidalgo et al., 2015a).

1.2.4. Molecular typing techniques

Molecular typing techniques are essential to determine the epidemiological relationships between the strains and allow recognizing if different isolates belong or not to the same strains or are clonally related. The techniques identify the strains

responsible for epidemiological outbreaks: PFGE, RAPD-PCR, AFLP, REP-PCR, BOX-PCR, and ERIC-PCR or a group of genes, as in the case of ribotyping and the MLST (Figueras et al., 2009). All the techniques except the MLST generated band patterns and can be compared to determine if the isolates belong or not to the same strain.

1.2.4.1. Enterobacterial Repetitive Intergenic Consensus PCR (ERIC- PCR)

Enterobacterial Repetitive Intergenic Consensus (ERIC) are imperfect palindromic sequences of 127 bp that occur in multiple copies in the genomes of enteric bacteria, vibrios, and other bacterial genera (Wilson and Sharp, 2016). This technique allows determining the epidemiological relationships between strains and to recognize the strains that are the source of infection in epidemiological outbreaks. Moreover, it allows to recognize if the isolates included in the description of new species are identical or not, or if they derive from the same clone (Figueras et al., 2006; Figueras et al., 2011d).

In 2003, Soler et al. using 26 isolates of *Aeromonas* spp. from different geographical origins showed that the ERIC-PCR was more discriminatory than the REP-PCR, RFLP or RFLP of the ITS 16S-23S. In addition, the study conducted by Figueras et al. (2006) demonstrated that the ERIC-PCR produced the same results as the PFGE and the AFLP. Studies published in 2008 showed that the ERIC-PCR could be useful for typing at the species (Nováková et al., 2009) and subspecies levels (Beaz-Hidalgo et al., 2008).

1.2.4.2. Multilocus Sequence Typing

The MLST uses the sequence of internal fragments of seven housekeeping genes (450-500 bp of seven genes) and for each gene the sequences from different strains are compared and each unique sequence is assigned a specific number for each gene or allele. The strains that show the same allelic profile (identical sequence for all genes) correspond to the same sequence type (ST) which is identified by a specific number (Maiden et al., 1998; Maiden, 2006; Figueras et al., 2011b; Jolley

and Maiden, 2014). When more ST are available for each bacterium, it will be easier to understand the structure of the various biological populations (Maiden et al., 1998; Maiden, 2006; Jolley and Maiden, 2014; Figueras and Beaz-Hidalgo, 2015).

1.3. Ecology and epidemiology

Aeromonas are microorganisms associated with the aquatic environment. However, these bacteria are worldwide distributed and have been isolated from several clinical and environmental samples (Del Val et al., 1990; Janda and Abbott, 2010; Figueras et al., 2017). Figure 2 from Janda and Abbott, 2010 shows the environmental sources of *Aeromonas* that can be the route of colonization and infection for the human population.

The literature suggests that the majority of the *Aeromonas* infections in humans have been acquired by the contact with water, as well by food i.e. meat, milk, seafood (Janda and Abbott, 2010; Ponnusamy et al., 2016; Teunis and Figueras, 2016).

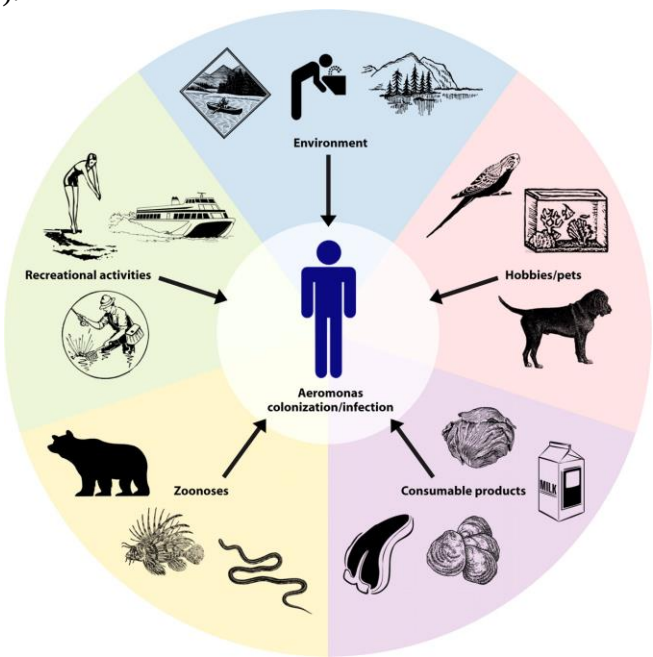


Figure 2. Environmental sources of *Aeromonas* associated with the infection in humans from Janda and Abbot, 2010.

1.3.1. *Aeromonas* in the aquatic environments

The genus *Aeromonas* has been described an aquatic pathogen and can be found in different types of water, such as surface water, groundwater, drinking water, seawater, irrigation water, among others (Ferguson et al., 1994; Araujo et al., 1990; Borrell et al., 1998; Figueras et al., 2005; Rahman et al., 2009; Aravena-Román et al., 2013; Figueras and Ashbolt, 2019). Several authors have described characteristics of *Aeromonas* that explain their broad distribution (Altwegg et al., 1989; Schubert, 1991; Szewzyk et al., 2000; Sautour et al., 2003; Janda and Abbott, 2010). These bacteria can grow in a wide interval of temperatures (4ºC to 45ºC) with an optim range between 22ºC-30ºC, and under different pH conditions (5.2-9.8).

Surface water

The concentration of *Aeromonas* in surface waters is abundant and the diversity is higher than in seawater (Kaper et al., 1981; Sharma et al., 1995; Montes et al., 1999; Chaix et al., 2017; Figueras and Ashbolt, 2019). Borrell et al. (1998) described that in Catalonia (Spain) 96% of lakes and reservoirs samples, as well as 88% of river water samples harbored *Aeromonas* strains. Finish water from many lakes and rivers harbored many strains, and this is the origin of three new species described (*A. aquatica, A. finlandensis* and *A. lacus)* in 2015 by Beaz-Hidalgo et al.

Groundwater

The prevalence of *Aeromonas* in groundwater is generally lower, and high levels of the bacterium could be associated with the intrusion of fecal contamination (WHO, 2002; Figueras and Ashbolt, 2019).

Drinking water

There are numerous cases in which has been detected the presence of *Aeromonas* in drinking water distribution systems and even in bottled water (Massa et al., 1995; Huys et al., 1995; Borrell et al., 1998; Figueras et al., 2005; Figueras and Ashbolt, 2019). The concentration of *Aeromonas* in drinking water is related to the capacity to regrow in the system (Holmes et al., 1996; van der Kooij et al., 2015)

and can be changed depending on the available nutrients, the temperature, and the concentration of residual chlorine (Sisti et al., 1998). Latif-Eugenín (2016a) showed the importance of the culture conditions. In two studies in Spain, the incidence of *Aeromonas* in drinking water ranges from 6.9% to 8.9% (Borrell et al., 1998; Figueras et al., 2005). In all the positive water samples, the concentration of chlorine was lower. Some authors associated the increase of *Aeromonas* in the distribution systems with rain events (Gavriel et al., 1998; Simon et al., 2001). Furthermore, the concentrations vary depending on the seasons, obtaining higher levels of *Aeromonas* at lower temperatures (Pablos et al., 2009).

Seawater

Aeromonas have been isolated from seawater by several authors (Küeh et al., 1995; Ferguson et al., 1994; Borrell et al., 1998). The concentration could be associated with the contributions of the terrestrial water effluents (Araujo et al., 1990). Additionally, it has been demonstrated that *Aeromonas* can grow and survive at a low salinity (Araujo et al., 1990; Monfort and Baleux, 1991).

Irrigation water

Due to factors such as population increase, alteration of rainfall patterns, and natural water sources contamination, the water is becoming a scarce resource. This has led to the reuse of residual water after undergoing different treatments in a Wastewater Treatment Plant (WWTP), generating the so call reclaimed or recycled water. A secondary biological treatment is normally performed, however, if the water has a direct contact with vegetables or humans, an additional tertiary treatment involving desinfection is required. The latter tertiary treatment is normally chemical (ozone, chlorination) and physical (ultraviolet radiation). However, natural processes such as lagooning system, which is a process of purification, produced when the wastewater is temporarily stored in shallow ponds or lagoons can be also employed depending on the type of used of the produced water (Boussaid et al., 1991; Hassani et al., 1992; Jjemba et al., 2010). This water can be used for irrigation. *Aeromonas* has been frequently isolated from irrigation water, and the

prevalence in reclaimed water used for irrigation was >60% (Aravena-Román et al., 2013; Latif-Eugenín et al., 2016, 2017). Water used for irrigation that contains *Aeromonas* can contaminate fruits and vegetables (Pianetti et al., 2004; Latif-Eugenín et al., 2016, 2017), being able to cause infection after consumption. In relation to the latter, there is epidemiological evidence showing that water acts as a vehicle for the dissemination of these pathogens, since it has been possible to identify the same *Aeromonas* strain from drinking water samples, as from the feces of individuals with diarrhea (Khajanchi et al., 2010; Pablos et al., 2011). Moreover, in another study, the same strains recovered from lettuce and tomatoes were isolated in the irrigation water (Latif-Eugenín et al., 2016).

1.3.2. *Aeromonas* in animals

Aeromonas have been described as aquatic pathogens, for fish and aquatic animals (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015). The first isolates were in association with a case of septicemia in frogs and sick fish (Farmer et al., 2006). The species *A. salmonicida* and *A. hydrophila* are considered important fish pathogens, especially for salmonids in which they generate ulcers, hemorrhages, furunculosis and septicemia, producing large losses in the aquaculture industry (Pylkkö et al., 2005, 2006; Treasurer et al., 2007; Reith et al., 2008; Beaz-Hidalgo and Figueras, 2013; Figueras et al., 2011c). However, other species can be isolated from fish, for example, *A. veronii, A. bestiarum, A. encheleia* and *A. sobria* have been all recovered from common carp (Kozinska et al., 2002; Soriano-Vargas et al., 2010), *A. allosaccharophila, A. dhakensis, A. caviae, A. veronii, A. hydrophila, A. jandeai, A. media* and *A. trota* have been isolated from eel (Martínez-Murcia et al., 1992b; Esteve et al., 1995a, b, 2012; Yi et al., 2013), *A. veronii* from catfish (Nawaz et al., 2010), *A. piscicola* from salmonids (Beaz-Hidalgo et al., 2009, 2010), *A. sobria* from tilapia (Li and Cai, 2011) and *A. schubertii* from snakehead fish (Chen et al., 2012). Recently, hypervirulent *A. hydrophila* strains have been the cause of persistent outbreaks of warm-water fishes worldwide (Rasmussen-Ivey et al., 2016).

Similarly, outbreaks caused by *A. hydrophila* in rabbit farms with a high mortality have been described (Paniagua et al., 1998). Additionally, *Aeromonas* have been isolated from dogs and cats (Ghenghesh et al., 1999), as well as from feces of horses (Forga-Martel et al., 2000; Waldridge et al., 2011).

Aeromonas have also been detected as echinoderm pathogens (Yang et al., 2008a), mollusks pathogens (Miñana-Galbis et al., 2004, 2007) and they have been associated with copepods (Gugliandolo et al., 2008). *A. hydrophila* has been described from the normal intestinal microbiota of Nile crocodiles (Lovely et al., 2008). They can also cause septicemia in poultry, however, in seagulls they have also been defined as normal microbiota (Kinzelman et al., 2008).

Finally, *A. veronii* has been recovered from sea lions, *A. hydrophila* from ovine and *A. dhakensis* from a neonate dead cetacean (Ilhan et al., 2006; Gonzalez et al., 2009; Pérez et al., 2015).

1.3.3. *Aeromonas* in food

Food may also be another possible source of infection. This colonization was firstly described by Aiken et al. (1936), as well as, by Miles and Halnan (1937). *Aeromonas* have been isolated from a wide variety of foods, mainly fish, seafood, meat products and sausages, fruits, vegetables and dairy products (Borrell et al., 1998; Neyts et al., 2000a; Awan et al., 2006; Chang et al., 2008; Yucel and Erdogan, 2010; Ottaviani et al., 2011; Nagar et al., 2011, 2013; Beaz-Hidalgo et al., 2009, 2010; Beaz-Hidalgo and Figueras, 2012, 2013; Figueras and Beaz-Hidalgo, 2014). Different studies have shown that *A. hydrophila, A. veronii* bv. *sobria, A. caviae* and *A. dhakensis* were the most prevalent species in fish for human consumption (Neyts et al., 2000a; Bashir Awan et al., 2006; Chang et al., 2008; Figueras and Beaz-Hidalgo, 2014). Nevertheless, with molecular methods (RFLP-16S and *rpoD* gene sequencing) the most prevalent species in a variety of fish were *A. salmonicida*, followed by *A. bestiarum, A. veronii, A. encheleia, A. hydrophila. A. allosaccharophila* and *A. bivalvium* (Castro-Escarpulli et al., 2003; Figueras and Beaz-Hidalgo, 2014).

In addition, *Aeromonas* have been isolated from seafood (oysters, mussels, shrimps and cockles), and the diversity is similar to the one found in fish, being the most prevalent *A. veronii, A. dhakensis, A. caviae, A. jandaei* and *A. schubertii* (Neyts et al., 2000a, b; Figueras and Beaz-Hidalgo., 2014; Yano et al., 2015). In relation to meat products (beef, lamb and poultry), *A. hydrophila* was the most prevalent species, using biochemical methods, followed by *A. caviae* (Borrell et al., 1998; Neyts et al., 2000a; Nagar et al., 2011). However, the diversity increases with *rpoD* and *gyrB* sequencing, because in addition to *A. hydrophila* and *A. caviae*, other species like *A. veronii, A. salmonicida, A. jandaei* and *A. simiae* were isolated (Fontes et al., 2010; Nagar et al., 2013).

Different studies reported the isolation of *Aeromonas* in fruits and vegetables. The bacterium has been isolated from carrot, tomato, lettuce, cucumber, potato, onion and celery. Among them, *A. hydrophila, A. caviae* were the most prevalent clinical species (Chang et al., 2008; Nagar et al., 2011, 2013).

Aeromonas are capable of growing in several conditions. This bacterium survives in low temperatures (2-10C^{o}). Additionally, salt or sodium chloride (NaCl) is a usual preservative of meat products and raw fish. In general, *Aeromonas* are able to grow in NaCl concentrations up to 4%. The pH is another parameter used to control ate the bacterial growth in food. Nevertheless, it has been demonstrated that *Aeromonas* can survive at pH=5 (Figueras and Beaz-Hidalgo et al., 2014).

The higher numbers of isolates were recovered after enrichment techniques, since the *Aeromonas* concentration is in most of the cases low (Janda and Abbott, 2010).

1.3.4. Human infections

Currently, some *Aeromonas* spp. are considered, as opportunistic emerging pathogens because they are isolated as etiological agents in various infectious processes, mainly gastroenteritis, bacteremia/septicemia and wound infections, as well as in other infections that affect the hepatobiliary system, respiratory tract, bones and joints, as shown in Table 2 (adapted from Latif-Eugenín, 2015 & Figueras

and Beaz-Hidalgo, 2015). *Aeromonas* are mainly enteric pathogens that affect as it occurs with other microbes the populations at risk such as children, the elderly, and the immunocompromised individuals (Figueras, 2005; Janda and Abbott, 2010; Igbinosa et al., 2012; Figueras and Beaz-Hidalgo, 2015). Numerous cases of bacteremia and extraintestinal infections such as meningitis, pneumonia, keratitis, and osteomyelitis caused by *Aeromonas* have been described in healthy and immunocompetent patients (Figueras, 2005; Dwivedi et al., 2008; Gunasekaran et al., 2009; Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015).

The exact worldwide incidence of *Aeromonas* infections is unknown. The first report was made in California in 1988, and it showed an annual incidence of *Aeromonas* infections of 10.5 cases per million people (King et al., 1992). In 2004, in England, the estimated incidence of bacteremia by *Aeromonas* was 1.5 cases per million people (Janda and Abbott, 2010), and in France, in 2006, 0.66 cases per million were estimated (Lamy et al., 2009). Another study carried out in Taiwan between 2008 and 2010 showed that the incidence of bacteremia caused by *Aeromonas* was 76 cases per million inhabitants, which was one of the highest incidence. In this way, it is shown that the different diseases caused by *Aeromonas* vary based on geographical location and this can be related to food and hygiene habits (Wu et al., 2012a).

Cases from human *Aeromonas* infections have been described from over the world; nevertheless, the incidence of gastroenteritis appears to be higher in undeveloped or developing regions. In relation to septicemia the incidence is higher in Asian countries, an explanation of this could be a higher incidence of cirrhosis, which is an important underlying condition related to *Aeromonas* septicemia (Figueras and Beaz-Hidalgo, 2015; Batra et al., 2016; Figueras, 2017).

Until the description of *A. dhakensis* which resulted from the reclassification of *A. aquariorum* and *A. hydrophila* subsp. *dhakensis*, only three species, *A. caviae*, *A. veronii* and *A. hydrophila*, were considered predominant in clinical samples, corresponding approximately to 92% of the isolates (Figueras, 2005; Janda and Abbott, 2010; Igbinosa et al., 2012; Figueras and Beaz-Hidalgo, 2015).

The prevalence of predominant species in clinic has changed with the description of *A. dhakensis* and with the introduction of molecular identification methods. Nowadays, based on the data from different studies performed around the world, 96.5% of the clinical isolates correspond to four species; *A. caviae* (29.9%); *A. dhakensis* (26.3%), *A. veronii* (24.8%) and *A. hydrophila* (15.5%), as seen in Table 3 (from Latif-Eugenín, 2015, adapted from Figueras and Beaz-Hidalgo, 2015), which also shows all species isolated from clinical samples that have been identified by molecular methods. In fact, *A. dhakensis* is the most prevalent species in Malaysia (Puthucheary et al., 2012), more frequent than *A. caviae* and *A. veronii*. A similar situation arises in Australia (Aravena-Román et al., 2011a) and in Taiwan (Wu et al., 2012a, 2015; Chen et al., 2013, 2014b), which demonstrates the clinical importance of *A. dhakensis*, isolated in different countries and from different types of samples (Table 3). The observation that *A. dhakensis* is more prevalent in tropical climates (Malaysia, Taiwan, and Australia) needs to be confirmed. Conversely, it also needs to verify if *A. caviae* is the most frequently isolated species in blood samples in Japan as indicated in one study (Kimura et al., 2013), or if *A. hydrophila* predominates in French Caribbean islands and in Taiwan (Hochedez et al., 2011), specially because in both studies they used phenotypic methods for the identification.

Furthermore, the confusion between *A. hydrophila* and *A. dhakensis* may have consequences, since the latter species is more virulent and shows greater cytotoxic activity against human skin fibroblast cell lines than *A. hydrophila*, as demonstrated by Chen et al. (2013). Recently, Chen et al. (2014b) showed that patients with bacteremia attributed to *A. dhakensis* had a higher mortality than bacteremia produced by the other species. Two new species i.e. *A. enterica* and *A. intestinalis* isolated from human feces (in process of description) have been included in the list (Table 2) of *Aeromonas* species associated with human infections (Figueras et al., 2017). However, it is not known the prevalence of the cryptic species *A. rivipollensis* (Martí and Balcázar, 2016) uncovered by *A. media* in clinical infections.

Table 2. Distribution of the molecularly identified *Aeromonas* spp. recovered from different samples or sites of infection (adapted from Latif-Eugenín, 2015 & Figueras and Beaz-Hidalgo, 2015[a]).

Species	Feces	Wound	Blood	Respiratory tract[b]	Urine	Peritoneal dialysis	Billis	Other[c]	Total (%)
A. caviae	130	36	112	3	6	4	2	6	299 (30.23)
A. veronii	99	40	96	9			2	3	249 (25.17)
A. dhakensis[d]	55	82	84	3	3	5	2	3	237 (23.96)
A. hydrophila	47	65	32	5	2	4	1	2	158 (15.97)
A. media	8	3	5		1				17 (1.71)
A. trota	1	2					1		4 (0.40)
A. taiwanensis	1	3							4 (0.40)
A. salmonicida[e]		2						1	3 (0.30)
A. jandaei	1		1		1				3 (0.30)
A. sanarellii	1	3							4 (0.40)
A. allosaccharophila	1		1						2 (0.20)
A. tecta	2								2 (0.20)
A. diversa		2							2 (0.20)
A. schubertii		1							1 (0.10)
A. bestiarum			1						1 (0.10)
A. popoffii					1				1 (0.10)
A. intestinalis[f]	1								1 (0.10)
A. enterica[f]	1								1 (0.10)
Total	346	239	332	20	14	13	8	15	989

[a]Data obtained from Huys et al. (2002), Hua et al. (2004), Al Benwan et al. (2007), Bossi-Küpfer et al. (2007), Demarta et al. (2008), Figueras et al. (2009), Tena et al (2009), Alperi and Figueras (2010), Miñana-Galbis et al (2010), Aravena-Román et al. (2011a), Esteve et al. (2012), Puthucheary et al. (2012), Roger et al. (2012b), Sedlácèk et al. (2012), Senderovich et al. (2012), Wu et al. (2012a, 2015), Morinaga et al. (2013), Chen et al. (2013, 2014b), Shin et al. (2013), Pérez-Valdespino et al. (2014), Figueras et al. (2017) and theresults of 76 unpublished fecal isolates; [b]10 sputum strains; [c] Others includes: *A. caviae* (n = 6) from: vagina (3), appendix (1), fluid ascitic (1) and abscess (1); *A. veronii* (n = 3): ascitic fluid (2) and ear fluid (1); *A. dhakensis* (n = 3) from: ascitic fluid (1), joint fluid (1) and bone (1); *A. hydrophila* (n = 2) from: ulcer exudate (1) and abscess (1) and *A. salmonicida* from abcess (1); [d] In many of the studies *A. aquariorum* was the name used for *A. dhakensis*; [e] An additional case associated with peritoneal fluid was described by Yang et al. (2008), but the identification method used was not specified; [f] Two new species of *Aeromonas* in process of description.

The percentage of isolation of *Aeromonas* from people with diarrheal diseases range from 0.8% to 7.4%, and the percentage of isolation from feces of asymptomatic people is 4% (King et al., 1992). Studies have shown that the isolation of *Aeromonas* from fecal samples can vary significantly based on the season of the year, showing an increase in the number of cases in the warmer months, which favors its development in water, increasing the microbial load in both freshwater and saltwater. This same type of behavior has been observed in other diseases caused by *Aeromonas*, such as bacteremia/septicemia, which increases during the summer (Esser et al., 2000; Janda and Abbott, 2010).

In relation to the association between water and infections caused by *Aeromonas*, it is important to take into account that *Aeromonas* were the most isolated microorganisms after natural disasters, such as the tsunami in Thailand in 2004. In fact, *Aeromonas* corresponded to 22.6% of all isolates recovered from infected wounds of the survivors. Additionally, the dominance in wound infections was also observed after Hurricane Katrina in the southeastern United States (2005), (Hiransuthikul et al., 2005; Presley et al., 2006). In fact, floods and droughts are considered the main impacts of climate change and there is evidence that the increase and decrease in the volume of rainfall, are related to the *Aeromonas* outbreaks associated with water consumption (Figueras and Borrego, 2010). In addition, *Aeromonas* have been isolated together with *Vibrio cholerae* in the outbreaks that occurred in Haiti (Hasan et al., 2012; Mendes-Marques et al., 2012; Figueras and Beaz-Hidalgo, 2015).

Table 3. Distribution of the molecularly identified *Aeromonas* spp. from clinical studies performed in different countries (from Latif-Eugenín, 2015, adapted from Figueras and Beaz-Hidalgo, 2015).

Study	**Figueras et al., 2009[a]**	**Aravena-Román et al., 2011[b]**	**Puthucheary et al., 2012[c]**	**Roger et al., 2012b[d]**	**Senderovich et al., 2012[e]**	**Wu et al., 2012a[f]**	**Chen et al., 2013[g]**	**Pérez-Valdespino et al., 2013[e]**	**Chen et al., 2014b[e]**	**Wu et al., 2015[f,h]**	**Total**
Species/country	Taiwan	Australia	Malaysia	France	Israel	Taiwan	Taiwan	Mexico	Taiwan	Taiwan	
A. caviae	73 (51.8)	34 (23.8)	18 (19.1)	24 (23.5)	11 (64.7)	15 (25.0)	11 (13.7)	10 (35.7)	7 (36.8)	43 (28.1)	246 (29.9)
***A. dhakensis*[i]**	25 (17.7)	34 (23.8)	47 (50.0)	6 (5.9)		16 (36.4)	37 (46.3)	3 (10.7)	1 (5.3)	48 (31.4)	217 (26.3)
A. veronii	16 (11.3)	36 (25.2)	12 (12.8)	46 (45.1)	5 (29.4)	12 (27.3)	14 (17.5)	3 (10.7)	10 (52.6)	50 (32.7)	204 (24.8)
A. hydrophila	17 (12.2)	33 (23.1)	16 (17.0)	23 (22.5)		5 (11.4)	13 (16.2)	11 (39.3)		10 (6.5)	128 (15.5)
A. media	5 (3.5)	2 (1.4)		1 (1.0)			1 (1.3)	1 (3.6)			10 (1.2)
A. trota	3 (2.1)		1 (1.1)								4 (0.5)
A. taiwanensis	1 (0.7)				1 (5.9)		2 (2.5)				4 (0.5)
A. sanarellii	1 (0.7)						2 (2.5)		1(5.3)		4 (0.5)
A. salmonicida		1 (0.7)		1 (1.0)							2 (0.2)
A. allosaccharophila		1 (0.7)		1 (1.0)							2 (0.2)
A. schubertii		1 (0.7)									1 (0.1)
A. bestiarum		1 (0.7)									1 (0.1)
A. jandaei				1 (1.0)							1 (0.1)
Total	141	143	94	103	17	48	80	28	19	151	824

[a-f] Origin of the strains: blood (41.1%, 58/141), wound (25.5, 36/141), feces (3.6%, 5/141), others (14.2%, 20/141); [b] wound (37.8%, 54/143), feces (23.1%, 33/143), blood (23.1%, 33/143), others (16.3%, 23/143); [c] stool(51.1%, 48/94), wound (23.4%, 22/94), blood (8.5%, 8/94), others (17.0%, 16/94); [d] wound (36.0%, 37/103), blood (16.5%, 17/103), others (15.5%, 16/103); [e-h] Studies include only isolates of: [e] stool, [f] blood, [g] wound; [h] In this study, a total of 153 strains were analyzed, but there were 2 that were identified as *Aeromonas* sp.; [i]In many studies *A. aquariorum* was the name used for the species *A. dhakensis.*

Mixed infections

The infections caused by *Aeromonas* often involve more than one type of bacteria within the same clinical sample (polymicrobial). Mixed infections with bacteria from other genera occur with a frequency ranging from 30% in bacteremia, 60% in wound infection and even 80% in the case of respiratory tract infections (Lamy et al., 2009). Nevertheless, other mixed infections with two different strains of *Aeromonas* in the same clinical sample have been described (Grim et al., 2014; Ponnusamy et al., 2016). These mixed infections have been estimated to occur in 5%-10% of the *Aeromonas* cases and the interaction of the virulence factors among the different co-infecting strains have been investigated in few studies (Lamy et al., 2009, Grim et al., 2014; Mosser et al., 2015; Ponnusamy et al., 2016).

A study of clinical *Aeromonas* infections performed in France during six months showed that 42.3% (33/78) of the cases were polymicrobial (Lamy et al. (2009). Mixed cultures of *Aeromonas* with other genera like *Campylobacter*, *Salmonella* etc. corresponded to 20.5% of the cases in a study performed by Latif-Eugenín (2015) in Spain (Table 5).

Mosser et al. (2015) investigated virulence of different pairs of *Aeromonas* strains (different species and clones) recovered from human mixed infections using the *C. elegans* model, and observed that the severity of the infections was lower when the strains were administered alone. Enhanced virulence was observed for several pair combinations of strains indicating the synergistic virulent effects (Mosser et al., 2015) The authors of the latter study concluded that infections do not depend only on the virulence of the single strains but are instead a more complex scenario where the interaction between the microbes involved, the host and the environment are all important players. In fact, they demonstrated that "the pathogens may display enhanced virulence in the presence of the right partner" (Mosser et al., 2015). Ponnusamy et al. (2016) described a mixed infection caused by two *A. hydrophila* strains recovered from a young immunocompetent patient with a fast progressing necrotizing fasciitis that resulted in the amputation of almost all the

extremities. They suggested that the T6SS and the ExoA toxin could be involve in this complex infection.

1.3.4.1. Gastroenteritis

Intestinal infections by *Aeromonas* are common and globally distributed (Von Graeevenitz, 2007; Figueras and Beaz-Hidalgo, 2015; Latif-Eugenín, 2015; Teunis and Figueras, 2017). However, the incidence is underestimated due to the fact that these infections are not routinely investigated (Figueras and Beaz-Hidalgo, 2015). The first isolation of *Aeromonas* in stool was in 1961, although it had already been isolated in 1954, as an agent of myositis in a Jamaican woman. From that moment there are numerous studies that have associated *Aeromonas* with gastroenteritis (Baman, 1980; Gelbart et al., 1985; Reina and Lopez, 1995; Figueras, 2005; Graevenitz, 2007; Hasan et al., 2012; Ottaviani et al., 2013; Qamar et al., 2016; Teunis and Figueras, 2017).

The most frequent clinical presentation is secretory water (enteritis), ranging from 75% to 89% of all cases in which this microorganism was the only associated pathogen (Janda and Abbott, 2010; Latif-Eugenín, 2015; Figueras and Beaz-Hidalgo, 2015). An important complication resulting from *Aeromonas* gastroenteritis is the Hemolytic-Uremic Syndrome (HUS) and several cases were described in 2007 by Figueras et al.

Diarrhea in adults

The incidence in adults varies from 2% observed in Sweden, 6.9% in Hong Kong in healthy people, and 13% in immunocompromised patients (Figueras, 2005). Among patients with traveler's diarrhea in Spain, *Aeromonas* was defined as a causative agent in 2% of the cases (Vila et al., 2003), while in Japan it was isolated in 5.5% of the cases, and 8.7% in Finland (Figueras, 2005). In 5.5% of the cases of traveler's diarrhea, the infection was monomicrobial (Chopra and Houston, 1999; Vila et al., 2003). Recently, the first case of appendicitis associated with diarrhea of the rodent caused by *A. sobria* was described (Lim, 2009).

Diarrhea in children

Diarrhea caused by *Aeromonas* occurs mainly in children under 3 years old, and may last for one or two weeks with stools showing a watery consistency (Figueras, 2005; Figueras and Beaz-Hidalgo, 2015). In addition, studies carried out in Africa and South Asia have shown that *Aeromonas* mainly affect children between 24-59 months (Kotloff et al., 2013) The incidence of *Aeromonas* as a diarrheic agent in children varies from 2.3% in Taiwan to 13% in Nigeria (Esser et al., 2000; Juan et al., 2000; Nzeako and Okafor, 2000; Figueras, 2005; Igbinosa et al., 2012; Figueras and Beaz-Hidalgo, 2015). Two studies, one performed in Cuba by Bravo et al. (2012), and the another in Egypt by Mansour et al. (2012) showed a higher incidence of isolation of *Aeromonas* in children with diarrhea (7.5% and 1.4%) than in the control group (1.76% and 0.5%).

Enterophatogenicity of *Aeromonas*

The role of *Aeromonas* as an etiological agent in bacterial diarrhea has been questioned, mainly due to the lack of outbreaks, the isolation in asymptomatic patients, the no existence of an animal to reproduce the diarrhea and the lack of fulfillment of Koch's postulates demonstration (Janda and Abbott, 2010). However, Figueras and Beaz-Hidalgo (2015) have defended the pathogenic role of this bacterium, mainly based on the same strain (genotype) that causes diarrhea isolated from water and food (Altwegg et al., 1991; Pablos et al., 2010, 2011; Kajanchi et al., 2010; Latif-Eugenín et al., 2016). Additionally, a recently study (Teunis and Figueras, 2016) demonstrated, based on the combined evidence from the few human outbreaks (Morgan et al., 1985; Carnahan et al., 1991; Granum et al., 1998; Krovacek et al., 1995; Zhang et al., 2012), that *Aeromonas* should be treated as a human enteropathogen. Exposure to low doses of the bacterium may lead to infection, but most infections are asymptomatic. For this reason, more investigations are needed to determine the specific combination of host, environment, and pathogen factors that lead to illness (Teunis and Figueras, 2016).

1.3.4.2. Wound infections

After gastroenteritis, wound infections are the most frequent presentation and are usually associated with healthy individuals. Several types of wound infections have been described, ranging from superficial infection to life-threatening infections, such as necrotizing fasciitis. Wounds occur as traumatic events or burns associated with contaminated water and soil (Lamy et al., 2009; Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015). The body sites most affected are hands, feet, arms and legs (Janda and Abbott, 2010) and the higher prevalence of *Aeromonas* wound infections are in people >10 years of age (Lamy et al., 2009; Janda and Abbott, 2010).

Azzopardi et al., 2011 analyzed the infections in burn patients and determined that 76% of the cases have been produced by *Aeromonas*, and 96% of these cases were associated with non-treated water contact. Moreover, in a previous study from Taiwan that recovered 129 cases of skin and soft tissue infections showed that 78% of patients suffered a previous trauma, and in 30% of the cases there was water exposition.

Additionally, medical procedures, including leech therapy and elective surgery, can also predispose the individuals to develop wound infections caused by *Aeromonas* (Bauters et al., 2007). In the case of leech therapies, they are generally underestimated. The incidence of infection by *Aeromonas* in this type of treatment varies between 7%-20% and has two presentations, a rapid one that occurs within the first day after treatment, and another slow after 10 days or more, of the application of leeches (Schnabl et al., 2010; Mumcuoglu et al., 2014; Figueras and Beaz-Hidalgo, 2015). Within nosocomial infections, the most common are those associated with surgical wounds, and that can present a high mortality (Tena et al., 2009). The involved etiological agents may vary depending on the surgical procedure used and the anatomical site affected.

Necrotizing fasciitis

Wound infections due to *Aeromonas* can evolve by generating necrotizing fasciitis (NF) (Figueras, 2005; Abuhammour et al., 2006; Tsai et al., 2009; Chang et al., 2012; Kao and Kao, 2012; Kumar et al., 2012; Sood and Nerurkar, 2014; Spadaro et al., 2014), especially in immunocompromised patients (Monaghan et al., 2008; Shak et al., 2011). Necrotizing fasciitis is a rapidly progressive, life-threating soft-tissue infection that causes a necrotic inflammation of the fascial planes and the surrounding tissue (Bellapianta et al., 2009; Tsai et al., 2015; Ponnussamy et al., 2016). The clinical features of necrotizing fasciitis include hypotension, fever, necrosis and gangrene (Spadaro et al., 2014; Tsai et al., 2015), *Aeromonas* spp. are mainly associated with NF, from which different cases were reported, some of them associated with water (Furusu et al., 1997; Joseph et al., 1979; Sever et al, 2013). Two previous studies (Grim et al., 2014; Ponnusamy et al., 2016) described the infection and showed the complex interactions among multiple strains of *A. hydrophila*, called flesh eating bacteria, isolated from an immunocompetent individual who developed NF resulting in amputation of various vital parts of the body (Grim et al., 2014; Ponnusamy et al., 2016). This case was considered originally monomicrobial, because there were two strains of the same species, *A. hydrophila*, and the strains were named NF1 and NF2. Also, both strains showed differences in the presence of a gene *exoA*, involved in the inhibition of protein synthesis and host cell death (Zdanovsky et al., 1993; Grim et al., 2014). In addition, the previous results showed that when NF is caused by a mixed infection with *A. hydrophila*, the progression of the NF follows a different course to that of a single strain of this species.

1.3.4.3. Bacteremia/Septicemia

The incidence of bacteremias produced by *Aeromonas* varies between 0.12% and 3.3%, depending on the study (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015). Janda and Abbott. (2010) classified the bacteremias in four groups based on the affected populations, the main one including immunocompromised individuals (>80%), followed by those who suffered a

traumatic accident, the cases that affect healthy people, and finally those that involve patients undergoing reconstructive surgery and/or leeches therapy.

The most common underlying diseases found in association with cases of bacteremia/septicemia are neoplasms, hepatobiliary disease, and diabetes (Shizema et al., 2011; Figueras and Beaz-Hidalgo, 2015; Latif-Eugenín, 2015). The most common symptoms associated with *Aeromonas* bacteremia according to Janda and Abbott. (2010) included: fever (74% to 89%), jaundice (57%), abdominal pain (16% to 45%), septic shock (40% to 45%), and dyspnea (12% to 24%). The percentage of mortality associated with *Aeromonas* bacteremia is about 30% (Figueras and Beaz-Hidalgo, 2015; Latif-Eugenín, 2015).

1.3.4.4. Other infections

Several other infections associated with *Aeromonas* have been described: i) pneumonia and other respiratory tract infections; ii) Spontaneous Bacterial Peritonitis (SBP); and iii) Urinary tract infections. Pneumonia caused by *Aeromonas* is a rare clinical picture, however different cases have been described (Kao et al., 2003; Nagata et al., 2011; Chao et al., 2013; Figueras and Beaz-Hidalgo, 2015). Although the carbapenems treatment is effective, there are cases where pneumonia is fulminating and fatal (Nagata et al., 2011). Spontaneous bacterial peritonitis (SBP) is associated with the ascitic fluid (Moreira and López-San Román, 2009). *Aeromonas* have been associated with SPB, which is a complication that occurs in 16% of patients with cirrhosis (Figueras, 2005; Latif-Eugenín, 2015). The percentages of mortality vary according to different studies, fluctuating between 20% and 56% (Figueras and Beaz-Hidalgo, 2015). Urinary tract infections are rare, but there are cases, in which *Aeromonas* is the causal agent (Tena et al., 2007; Mandal et al., 2007; Figueras and Beaz-Hidalgo, 2015). However, the majority of the cases are associated with patients that have different underlying diseases, such as spina bifida, bilateral renal dilatation, and diabetes mellitus.

1.3.5. *Aeromonas* in Spanish hospitals

In relation to the diversity of *Aeromonas* species recovered from Spanish hospitals, a re-identification of 422 isolates was performed by sequencing the *rpoD* gene in a previous doctoral thesis (Latif-Eugenín 2015). The results showed that only 176 (41.6%) were correctly identified at species level. However, the results varied depending on the method used in hospitals (MicroScan W/A, Vitek II and MALDI-TOF). In comparison with the molecular identification, MALDI-TOF was able to identify correctly 91.9% of the strains at species level, but the phenotypic methods MicroScan W/A and Vitek II were only able to identify correctly 6.8% and 1.5%, respectively (Table 4, adapted from Latif-Eugenín, 2015).

Table 4. Identification concordance between phenotypic and genetic identification based on *rpoD* gene sequence (data obtained from Latif-Eugenín, 2015).

Identification Method (Nº of strains)	Nº (%) of Strains Correctly Identified	
	Genus	Species
Vitek II (73)	65/66 (98.5)	1/66 (1.5)
MicroScan W/A (177)	168/177 (94.9)	12/177 (6.8)
MALDI-TOF (179)	176/179 (98.3)	163/179 (91.1)

By sequencing the *rpoD* gene, it was determined that of the 422 strains investigated, 260 (61.6%) corresponded to *A. caviae*, 91 (21.5%) to *A. veronii*, 27 (6.4%) to A. *hydrophila*, 19 (4.5%) to *A. media*, 5 (1.2%) to *A. dhakensis*, 2 (0.5%) to *A. allosaccharophila*, 2 (0.5%) to *A. salmonicida*, 1 (0.2%) to *A. bestiarum*, 2 (0.5%) to *Aeromonas* sp. and 13 (3.1%) did not belong to the genus *Aeromonas* (Figure 3, data obtained from Latif-Eugenín, 2015). These results of Latif-Eugenín in 2015 differ from the previously reported data about the *Aeromonas* distribution over the world presented in Table 3; however, the most prevalent species in all studies was *A. caviae* (61.6% Spain vs 29.9% when summarizing the results of 10

studies performed from 2009 to 2015). The prevalence of *A. dhakensis* was lower in Spain (1%) when compared with the previously reported data (26.3%), as shown in the Table 3 and Figure 3. Moreover, independently of the country 98% of *Aeromonas* species identified in clinical cases belong to five species i.e. *A. caviae*, *A. dhakensis*, *A. veronii*, *A. hydrophila*, and *A. media* (Table 3 and Figure 3). The main difference would be the higher prevalence of *A. hydrophila* and *A. dhakensis* in countries with tropical temperatures (Figueras and Beaz-Hidalgo, 2015).

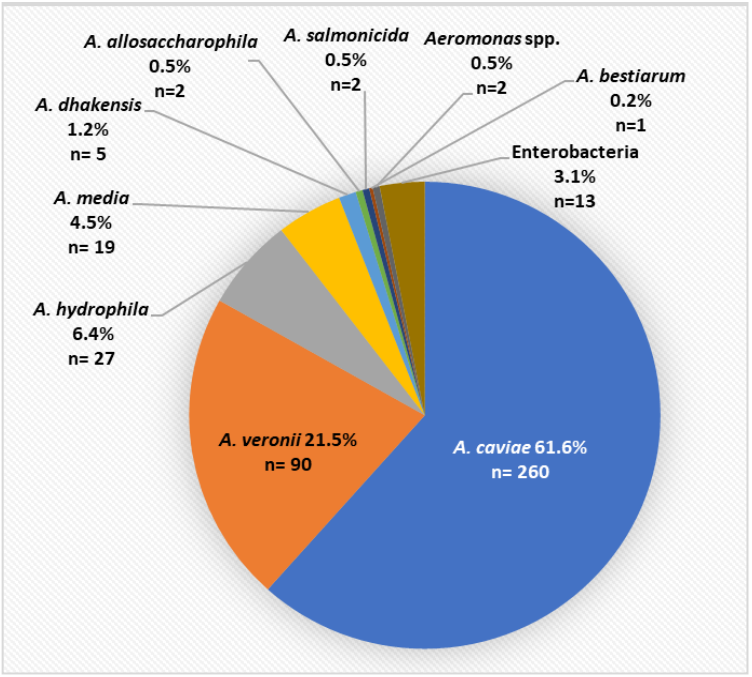


Figure 3. Distribution of 422 isolated strains based on the species. Data obtained from Latif-Eugenín, 2015.

Regarding the isolation origin, 86.8% of the 409 *Aeromonas* strains were recovered from feces (Figure 4, data obtained from Latif-Eugenín, 2015). The 409 strains of *Aeromonas* were isolated with a similar incidence in women and men. Table 5 (data obtained from Latif-Eugenín, 2015). Globally, *Aeromonas* were recovered with an incidence of 41.3% in patients with age lower than 14 years old (pediatric patients), followed by the elderly population with a prevalence of 24.4%. In addition, 63.3% of the cases correspond to monomicrobial infection, while 20.5% were a polymicrobial infection with *Campylobacter* and *Salmonella*.

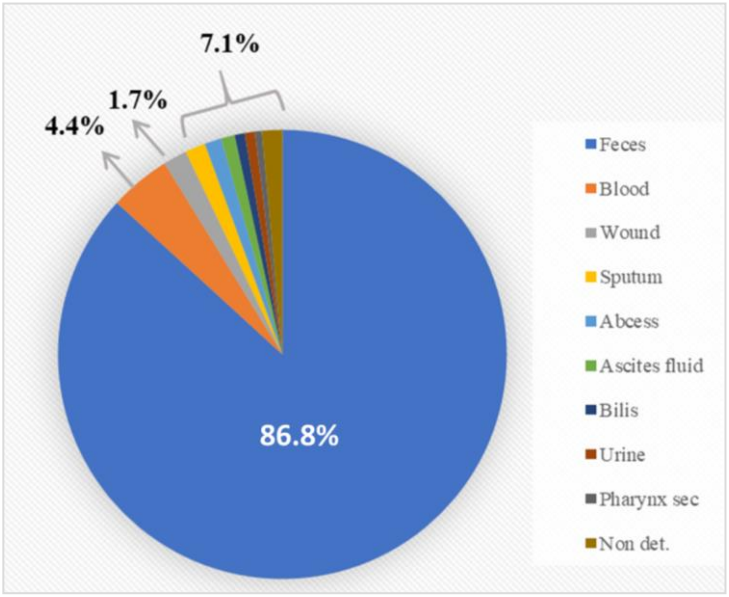


Figure 4. Distribution of 409 *Aeromonas* strains based on the origin. Data obtainted from Latif-Eugenín, 2015.

Table 5. Distribution of 409 *Aeromonas* strains based on sex, age and type of infection. Data obtained from Latif-Eugenín, 2015.

Sex %	Male	171 (41.8)
	Female	172 (42.1)
	Non-det.	66 (16.1)
Age %	0-14	169 (41.3)
	14-44	25 (6.1)
	44-65	46 (11.2)
	>65	100 (24.4)
Type of infection %	Monomicrobial	260 (63.6)
	Polymicrobial	84 (20.5)

1.4. Host-pathogen interaction

1.4.1. Innate immune response against *Aeromonas*

The immune response is the body's response activated after antigen exposure. Two types of immune response have been described, the innate, and the adaptative. The innate response is the first line of defense of our body against a foreign invader. The innate immune system relies on the recognition of a particular

type of structural molecules that are common in many pathogens the so call "molecular patterns associated with pathogens" (PAMPs), which are recognized by pattern-recognition receptors (PRRs) present in several cells like monocytes, macrophages, among others (Takeuchi et al., 2010). The bacteria lipopolysaccharide (LPS) or the endotoxins are the prototypes of PAMPs present in Gram-negative bacteria (Akira et al., 2003). Two types of PRRs have been described, the membrane-bound PRRs, and the cytoplasmic PRRs.

The toll-like receptors (TLRs) are a type of PRRs that are expressed in the membrane of a wide variety of cells (neutrophils, monocytes, macrophages, mast cells, epithelial cells). The TLRs induce the phagocytosis of the pathogen and the inflammatory response mediated by the cytokines (TNF, IFN, IL-6, IL-8, etc). Several reports have shown that the TLR could also induce apoptosis (Salaun et al., 2007; Shrestha et al., 2012).

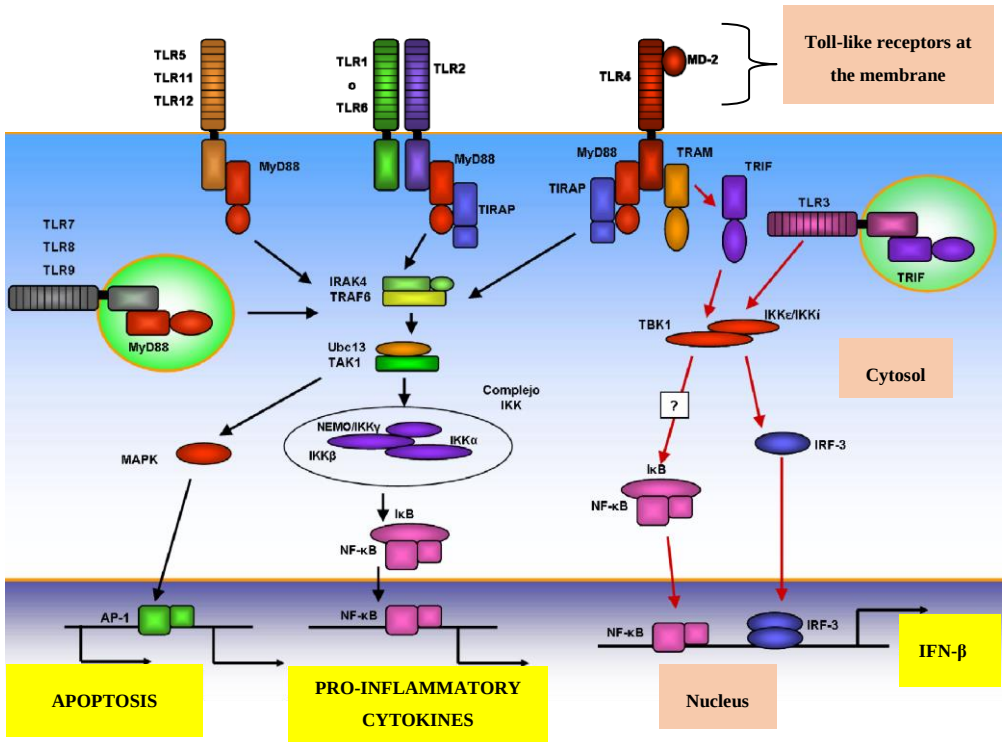


Figure 5. TLRs pathways. Adapted from Murciano, 2009; Takeda and Akira, 2004; Kawai and Akira, 2007.

The NOD-like receptors (NLRs) are cytosolic receptors that also activate cytokines and are considered another type of PRRs (Ting et al., 2008; Lamkanfi and Dixit, 2009). The NLR family of proteins are known to mediate caspase-1 activation by forming a macromolecular complex called inflammasome (Figure 6) that promotes the maturation and secretion of cytokines (IL-18, IL-lβ), inducing a different type of cell death dependent of caspase-1 called pyroptosis (Lamkanfi and Dixit, 2009; McCoy et al., 2010a) shown in Figure 6. The TLRs and NLRs proteins often act in concert to stimulate the activation of an inflammatory response (Bergsbaken et al., 2009).

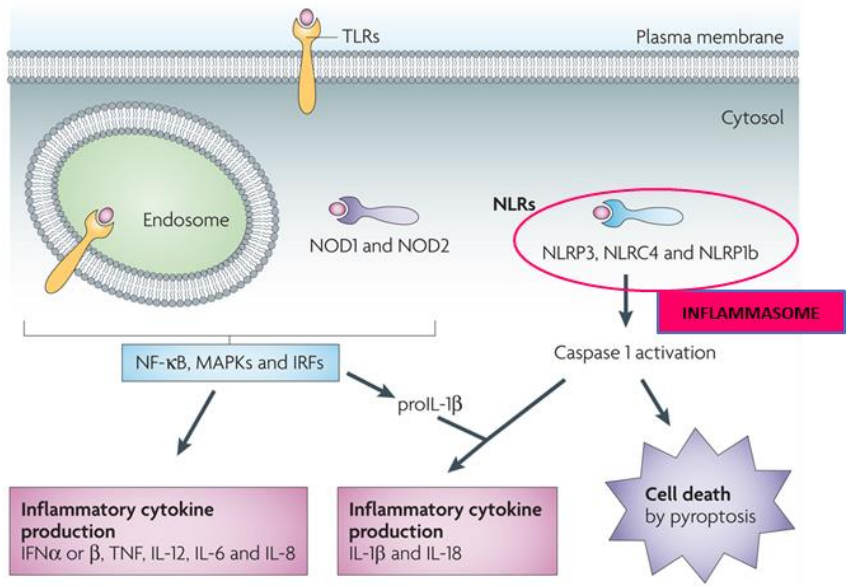


Figure 6. Pyroptosis pathway with TLRs and NLRs acting in concert, adapted from Bergsbaken et al., 2009.

A new term, “cytokine storm”, has been defined when the inflammatory response gets out of control by producing cytokines that induce the production of more cytokines by the immune cells (Tisoncik et al., 2012; Chousterman et al., 2017). Although the concept of the uncontrolled release of cytokines is well known, information about the factors that establish a cytokine storm is missing. In a study performed by infecting a mice model with *Vibrio vulnificus*, it was demonstrated

that the RtxA1$_3$ toxin was involved in the sepsis by triggering a cytokine storm that could be fatal for humans (Murciano et al., 2017).

The expression of several TLRs genes (*TLR1, TLR2, TLR3*…) after infection in a fish model with the species *A. hydrophila* and *A. salmonicida* has been described in the last years (Table 6). In recent studies, expression analyses of different TLRs in a fish model (grass carp and catfish) after *A. hydrophila* infection were performed (Zhang et al., 2017; Gong et al., 2017). The results of these studies demonstrated that the immune response showed tissue-specific patterns. However, the higher expression of TLRs indicated that they play an important role in the innate response against this species (Zhang et al., 2017; Gong et al., 2017). The toll-like receptor 4 (TLR4), is a transmembrane receptor that senses molecules such as lipopolysaccharides (LPS) present in the cell walls of Gram-negative bacteria (Takeuchi et al., 1999). Several studies have investigated the TLR4 immune response during *A. hydrophila* infections in a fish model, indicating the important role of these receptors in increasing the innate immunity in fish in response to pathogenic invasion. Previous studies demonstrated that the TLR4 signaling pathway could be activated after *A. hydrophila* infection in a minnow *Gobiocypris rarus* and the blunt snout bream (Su et al., 2009, Lai et al., 2017). Similarly, an interesting study was performed by Srivastava et al. (2017) in zebrafish. The results suggested that *A. hydrophila* manipulates the TLR4 to trigger an anti-inflammatory response facilitating the survival and the pathogenesis.

Gene expression of immune response mediators such as cytokines i.e. tumor necrosis factor alpha and beta (*TNF-α, TNF-β*), interferon gamma *IFN-γ*, interleukin 6, 8 and 10 (*IL-6, IL-8, IL-10*) among others, as well as chemokines such as C-C ligand 3 (*CCL3*) or CC produced by TLRs signaling have been studied in *A.hydrophila* and *A. salmonicida* after infecting a fish model, as summarized in Table 6 (Mulder et al., 2007; Brietzke et al., 2015; Xu et al., 2016; Kong et al., 2017). Previous studies with *A. salmonicida* in rainbow trout suggested an upregulation of *TNF-α* among other cytokine genes, involved in the systemic inflammation (Mulder et al., 2007; Britezke et al., 2015). Nevertheless, some

differences were observed in relation to the tissue studied (Mulder et al., 2007; Britezke et al., 2015). An interesting study by Kong et al. (2017) demonstrated that *A. hydrophila* induced the upregulation of pro-inflammatory cytokine gene *TNF-α* in the intestine of fish, impairing the integrity of the mucosal barrier structure. However, the oral incubation with *B. subtilis* reduced the damage and the inflammation caused by *A. hydrophila.*

Similarly, the expression of different chemokines, which are a family of small cytokines, has been studied after *A. hydrophila* infection in eel and grass carp (Arockiaraj et al., 2015; Liao et al., 2018). Two interesting chemokines, i.e., C-C motif chemokine ligand 2 (CCL2), and C-C motif ligand 20 (CCL20) have been described in *Vibrio anguillarum* infection using the fish (*Plecoglossus altivelis*) (Yu et al., 2019) and it was demonstrated that CCL2 plays a role in the immune response of a fish. Moreover, the expressions of *CCL2* and *CCL20* genes have been described after infection of THP-1 monocytes and mice with *Vibrio vulnificus* (Murciano et al., 2015, 2017).

Induction of several cytokines and chemokines after infecting mice and several cell lines, such as murine macrophage from blood (raw 264.7), human colon cancer cell line (HT-29), and a cancerous cell line (HeLa) with *A. hydrophila* have been demonstrated in several studies shown in Table 6. In the study of the genes related to the type III secretion system (T3SS), the results demonstrated that Act or AopB could be involved in the production of cytokines and chemokines in raw 264.7 and HT-29 cells (Fadl et al., 2006). Furthermore, a study of the immunomodulatory and protective role of quorum sensing in mice during *A. hydrophila* infection was carried out. The mice were treated with N-acyl homoserine lactones (AHLs) before the infection with *A. hydrophila*, and this resulted in a reduced level of cytokines and chemokines as well as the bacterial load in the organs. This work suggested that the AHL pretreatment modulated the innate immune response in mice, and increased the survival of the mice after *A. hydrophila* infection (Khajanchi et al., 2011).

Regarding apoptosis or programmed cell death, several studies have described that *Aeromonas* spp., mainly *A. hydrophila,* can induce this process *in vitro*, by using different cell lines such as raw 264.7, HeLa, HT-29 or mouse BALB/C monocyte macrophage (J744). Other studies have also demonstrated the capacity of *A. hydrophila* and *A. veronii* to cause apoptosis in kidney leukocytes or head kidney macrophages, among others obtained from fish (Table 6). In relation to the proteins associated with the apoptosis, the TP53 is a suppressor tumor protein with an important role in the programmed cell death, whose expression has been studied in zebrafish after *A. hydrophila* infection (Lü et al., 2015). However, this is the only study that investigated the *TP53* gene, therefore, other studies evaluating the expression of this gene in other cell lines and infections with other species would be necessary. Also, the induction of apoptosis has been studied by evaluating the caspase 3 protein (CASP3) activation in HeLa cells (Sierra et al., 2010). The results of this study demonstrate that the AexU gene in *A. hydrophila* induces the apoptosis via CASP3 (Sierra et al., 2010). These pro-apoptotic proteins have been deeply studied in other genera, for example in *Vibrio* (Lu et al., 2009; Murciano et al., 2015; Lee et al., 2015). These studies used these genes as a method of analysis of the immune response against this bacterium. Another interesting pro-apoptotic protein is the B-cell lymphoma 2 associated X protein (BAX) that belongs to the BCL2 protein family, and that is regulated by the TP53. The BAX has not been studied in *Aeromonas*, however, in a previous work with *V. vulnificus* this gene was upregulated in a THP-1 after infection (Murciano et al., 2015).

NLR subset inflammasomes, such as NLR pyrin domain containing 3 (NLRP3), NLR card domain containing 4 (NLRC4) or NLR pyrin domain containing 1 (NLRP1), promotes the maturation and secretion of the pro-inflammatory cytokines interleukin 1 beta (IL-1β) and IL-18, resulting in pyroptosis. The inflammasome consists of a complex of caspase-1, PYCARD domain (ASC), the AIM-2, and the NLR receptor (Figure 6). The activation of caspase-1, and the release of interleukin 1 beta (IL-1β), followed by the cell death called pyroptosis have been studied after infecting macrophages with *A. hydrophila* (McCoy et al.,

2010a). The results suggested that *A. hydrophila* induces an inflammatory response via NLRP3 inflammasome, which comprises the NLR protein (NLRP3), the adapter ASC, and caspase-1 (McCoy et al., 2010a). Another study performed by the same Japanese group demonstrated that inflammasomes-mediated caspase-1 activation (NLRP3 and NLRC4) are involved in host defenses against systemic *A. veronii* infection in mice and macrophages obtained from mice (McCoy et al., 2010b) (Table 6).

Independently if the cell damage is caused by apoptosis or by pyroptosis, the lactate dehydrogenase (LDH) assay has been used to determine the damage during *Aeromonas* infections (McCoy et al., 2010; Yarahmadi et al., 2016). The LDH is a cytoplasmatic enzyme that is released to the medium only when the cell is lysed or has a damaged membrane.

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that plays a role in regulating the immune response, controlling the activation of pro-inflammatory cytokines. The stimulation of the TLRs induces the activation of NF-κB. The RelA is a protein involved in NF-κB formation and previous studies in fish demonstrated the expression of the RELA after *A. hydrophila* infections by using a pathway that induces the production of several pro-inflammatory cytokines (Table 6). The c-Jun is an immune modulation protein that along with c-Fos, forms the AP-1 that is an activator of pro-inflammatory genes such as *TNF-α, IL-8, IL-6*, among others. However, it has been described that when the c-fos acts alone, this protein induces an increase of the anti-inflammatory genes such as *IL-10* or *IL-4*, among others (Hop et al., 2018). These genes have been previously studied by using different *Aeromonas* spp. to clarify the role of these transcription factors in virulence using human epithelial colorectal adenocarcinoma (Caco-2) cells (Hayes et al., 2009).

Table 6. Studies that investigated the gene expression and proteins involved in the immune response after *Aeromonas* spp. infections in animal models or cell lines**.**

Proteins studied	Model	*Aeromonas*	Reference
TLRs (TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, TLR18, TLR19, TLR20, TLR22…)	Fish Mice	*A. hydrophila* *A. salmonicida*	Su et al., 2009 Basu et al., 2011a,b Merino et al., 2014 Zhang et al., 2015 Brietzke et al., 2015 Lai et al., 2017 Shan et al., 2017 Zhang et al., 2017 Gong et al., 2017 Srisvastava et al., 2017
Cytokines (TNF-α, TNF- β**,** IFN-γ, IL-6, IL-8**,** IL-10...)	Fish Mice HeLa cell line Raw 264.7 cell line HT-29 cell line	*A. hydrophila*, *A. salmonicida* *A. veronii*	Fadl et al., 2006 Mulder et al., 2007 Khajanchi et al., 2011 Sierra et al., 2011 Merino et al., 2014 Brietzke et al., 2015 Xu et al., 2016 Kong et al., 2017 Chandrarathna et al., 2018
Chemokines (CC-Chem14, CsCC-Chem20, CsCC-Chem25, CCL3, CCL5…)	Fish Mice HeLa cell line Raw 264.7 cell line HT-29 cell line	*A. hydrophila*	Fadl et al., 2006 Khajanchi et al., 2011 Sierra et al., 2011 Arockiarai et al., 2015 Liao et al., 2018
Apoptosis (TP53, CASP3)	Fish Leukocytes from fish Macrophages from fish HeLa cell line	*A. hydrophila*, *A. veronii*	Sierra et al., 2010 Jones et al., 2012 Banerjee et al., 2012 Banerjee et al., 2014 Lü et al., 2015 Ni et al., 2016 Shelly et al., 2017
Pyroptosis (NLRP3, NLRC4, IL-1β)	Mice Macrophages from mice	*A. hydrophila*, *A. veronii*	McCoy et al., 2010a,b
Transcription factors (c-jun, RelA)	Fish Caco-2 cell line	*A. hydrophila* *A. salmonicida* *A, veronii*	Hayes et al., 2009 Tian et al., 2017 Zhang et al., 2017

1.4.2. Virulence factors

The presence of virulence factors has been described in *Aeromonas*, they allow the bacteria to colonize, invade, overcome the immune response of the host and cause infection (Janda and Abbott, 2010; Tomás, 2012; Figueras and Beaz-Hidalgo, 2015).

1.4.2.1. Structural components

Flagellum

Like other *Proteobacteria*, the genus *Aeromonas* has two types of flagellum (Canals et al., 2007), one polar which is constitutive and one lateral which is inducible. The lateral flagellum is used for surface movement and swarming, and the polar flagellum for movement in liquid suspensions. Merino et al. (1997) showed that mutant strains for genes involved in the flagella formation *flaAB*, *flaH*, *fliA*, *fliM*, *maf-1*, and *flrC* decreased polar flagellum production resulting in a decrease in adhesion and biofilm formation (Merino et al., 1997). It has also been shown that flagellar glycosylation is related to the ability to form biofilm and to adhere to Hep-2 cells (Galán and Collmer, 1999).

Pili

Pili are structures, that like the flagella, have a role in adherence to other bacteria or to the host cells. These structures are formed from protein subunits called pilins and can be differentiated from the flagellum because they have a smaller diameter (3-8 nm vs 15-20 nm of the flagella) and normally they do not present a coiled structure (Proft and Baker, 2009).

Capsule

The capsule is a structure composed of polysaccharides that generally covers the outer membrane of the bacterial cell (Tomás, 2012), since this is the outermost layer of the bacteria involved in interactions between the bacteria and the environment; consequently, the capsule has been described as an important virulence factor of many pathogens. In fact, it reduces opsonization, prevents phagocytosis, favors interactions with other bacteria, with host tissue, and acts as a barrier against hydrophobic toxins (Merino and Tomás, 2010).

S layer

The S layer is a surface protein layer of paracrystalline nature that is produced by a wide range of bacteria to form the outermost cell envelope (Tomás,

2012). This layer has been associated with several functions related to pathogenicity due to its exposure on the cell surface. It plays a major role in various biological functions: adhesion protection against complement and attack by phagocytes, antigenic properties, anchoring site for hydrolytic exoenzymes, bacteriophage receptors, among others (Beveridge et al., 1997).

Lipopolysaccharides (LPS)

Lipopolysaccharides are amphipathic macromolecules composed of three regions, i.e., lipid A, the central oligosaccharide (OS), and a portion of polysaccharide O-antigen (Nazarenko et al., 2011). The genus *Aeromonas* includes a total of 97 serotypes, serotyped from reference strains of *A. hydrophila, A. caviae* and *A. sobria* (Thomas et al., 1990); however, only some of them seem to be associated with virulence on fish. Furthermore, serotype O11 is associated with serious infections in humans, such as septicemia, meningitis, and peritonitis, while serotype O34, the most common in mesophilic *Aeromonas*, is associated with wound infections in humans and outbreaks of septicemia in fish (Janda et al., 1996). Additionally, the TLR4 is most well-known for recognizing LPS, which induces the activation of the immune response essential for an antibacterial defense (Su et al., 2009; Tomás, 2012).

1.4.2.2. Extracellular proteins

Exotoxins

The genus *Aeromonas* produces a wide variety of exotoxins, however, not all toxins described are produced by all strains even though these strains have genes. Two types of enterotoxins have been described in *Aeromonas* spp., cytotonic and cytotoxic (Tomás, 2012).

The cytotoxic enterotoxin (*act*) inhibits the phagocytic activity of host cells, produces hemolysis and increases the levels of tumor necrosis factor α (TNF- α) and interleukin (IL-1β) in the murine macrophages RAW 264.7 (Chopra et al., 2000). The cytotonic enterotoxins have been divided into two groups: the thermolabile

toxin (*alt*), and the thermostable antitoxin (*ast*), whose function is to increase the levels of AMPc and prostaglandins in the intestinal mucosa (Tomás et al., 2012).

Hemolysins

Hemolysins are a diverse group of enzymes that produce osmotic lysis of erythrocytes, through the production of pores in the cytoplasmic membrane (Rasmussen-Ivey et al., 2016). In *Aeromonas*, two types of hemolysins have been defined: α and β with physiological and functional differences (Singh and Sanyal, 1992). Aerolysin (*aerA*) is the prototype of hemolysin of the genus which is secreted by the Type Two Secretion System (T2SS), this active hemolysin binds to glycoproteins of the host cell membrane (Nelson et al., 1997).

Proteases

Proteases contribute to the metabolic versatility that allows *Aeromonas* to persist in different habitats and facilitate interactions with other microorganisms. In general, proteases contribute to pathogenicity (Tomás, 2012), since they promote invasion by direct damage to host tissue or by proteolytic activation of toxins (Figueras and Beaz- Hidalgo, 2014). They may also contribute to the establishment of an infection that exceeds the host's initial defenses, inactivating the complement system or providing nutrients for cell proliferation (Figueras and Beaz- Hidalgo, 2014).

Lipases

Lipases are produced by a wide range of bacteria. These can provide nutrients or constitute virulence factors when interacting with human leukocytes or by affecting various functions of the immune system through free fatty acids generated by lipolytic activity (Tomás, 2012). An important lipase in the genus *Aeromonas* is the glycerol phospholipid cholesterol acyltransferase (GCAT), which can digest the membranes of erythrocytes and produce their lysis (Igbinosa et al., 2012). This gene, in addition to being an important virulence factor, can be used for the identification of the bacteria belonging to the genus since several authors have shown that this gene is present in practically all *Aeromonas* strains, including

representatives of all species (Chacón et al., 2002; Figueras and Beaz-Hidalgo, 2014).

Shiga toxins

The function of these toxins is the inactivation of ribosomes (arrest of protein synthesis) of vascular endothelial cells, leading to cell death (Alperi et al., 2010). The Shiga toxins have only been detected in strains recovered from food (Palma-Martínez et al., 2016).

1.4.2.3. Secretion systems

Currently six secretion systems are known in Gram negative bacteria, all of these systems are involved with the transport of virulence factors to the extracellular medium or directly within the host cell (Figueras and Beaz-Hidalgo, 2013).

Type Two Secretion System (T2SS)

The T2SS is present in all known members of *A. hydrophila*, this is integral in the extracellular secretion of a wide range of virulence factors including aerolysin, amylases, DNAsa, and proteases (Galán and Collmer, 1999). The T2SS secrete proteins are found in the cytoplasm of the bacteria in the extracellular space outside of the cell.

Type Three Secretion System (T3SS)

The T3SS functions as a molecular needle which introduces effector toxins into the host cells (Vilches et al., 2009; Khajanchi et al., 2011; Tomás, 2012; Latif-Eugenín, 2015). The presence of a T3SS has been described in *A. salmonicida* and in *A. hydrophila* strains AH-1, AH-3 and SSU (Vilches et al., 2004; Sha et al., 2005; Tomás et al., 2012; Tanaka et al., 2012). Nowadays, it is known that the T3SS of *Aeromonas* is similar to the T3SS of *Yersinia* (Vilches et al., 2004). Five effector toxins of the T3SS have been identified in *Aeromonas*: AexT (ADP-ribolisante toxin), the AopU which inhibits the nuclear Kappa B factor involved in the activation of IkB (protein kinase), the AopH (tyrosine phosphatase), the AopO (serine/threonine kinase), and AexU (similar to AexT toxin). The T3SS of

Aeromonas has been shown to be co-regulated by contact with the host cells, cytotoxic enterotoxin, flagella, lipopolysaccharide, DNA methylation, temperature, calcium and magnesium levels, while the effectors are required for an appropriate secretion signal (Vilches 2008; Tanaka et al., 2012; Tomás, 2012).

Type Four Secretion System (T4SS)

The T4SS is the only system capable of transferring genetic material between bacteria, which plays a crucial role in the propagation of resistance genes and virulence. This system is a versatile transporter that secretes both nucleic acids and proteins by the sec-independent route (Rangrez et al., 2006).

Type Six Secretion System (T6SS)

The T6SS functions analogously to the phage tail, allowing the injection of virulence factors into the host cell through G repeat proteins (VgrG) and hemolysin-co-regulated protein (Hcp), which functions as an antimicrobial pore former protein when secreted, or as a structural protein. It appears to be highly conserved and can be found in one or more copies in diverse Gram-negative species, such as *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Yersinia pestis*, *Escherichia coli*, *Salmonella enterica*, among others (Tomás, 2012).

A functional T6SS has been described in *A. hydrophila* involved in virulence (Suarez et al., 2008; Tomás, 2012). Recently, the T6SS has been associated with a role in the mixed infections between two *A. hydrophila* strains (NF1-NF2) obtained from a case of necrotizing fasciitis (NF) in an immunocompetent young girl (Ponnusamy et al., 2016). The data derived from the induced NF infection with these strains separately and together, suggested the possibility that the expression of the effectors related to T6SS may be differentially regulated in both strains, causing a different progression of NF by mixed infections in comparison with a single strain of *A. hydrophila* (Ponnusamy et al., 2016).

In the last years it has been described that the T6SS have an additional role eliminating competitor bacteri, being this “antibacterial” characteristic essential in the case of polymicrobial infections (English et al., 2012). In 2013, Carrauthers et al.

suggested that T6SS in *Acinetobacter baumanii* plays a role in the competence with other bacteria. Another study demonstrated that the T6SS of *Shigella sonnei* confers an advantage to this species when it competes with *S. flexneri* and *E. coli* and this advantage was reduced in T6SS mutant strains (Anderson et al., 2017).

1.4.2.4. Quorum sensing

Quorum sensing (QS) is a communication system by which bacteria induce the expression of some virulence factors, such as the T3SS or the *act* toxin. This is regulated by "signal" molecules, such as N-acyl homoserine-lactone (AHLs) in response to external stimulus, for example, the bacteria populational density (Figueras and Beaz-Hidalgo, 2014). In addition, the AHLs are capable to modulate the host immune response (Khajanchi et al., 2011; Grim et al., 2014).

1.4.2.5. Metal ions

Metal ions are essential for the correct functioning of microbial biological processes (O'Halloran and Culotta, 2000). Many evidences indicate an important role of "metallostasis" in the host-pathogen interaction, involved in the acquisition and transfer of metals (Rosenzweig, 2002; Waldron et al., 2009; Capdevila et al., 2017). During infection the host limits the availability of essential metals, by inactivating the metal-dependent processes of the bacterial pathogen that compensates this limitation by producing different proteins (O'Halloran and Culotta, 2000; Palmer and Skaar, 2016; Capdevila et al., 2017).

In the genus *Aeromonas* it is known that the mechanisms for iron acquisition play a role in the pathogenesis and the development of the infection. Two mechanisms are known: siderophore-independent and siderophore-dependent. The siderophores are peptides which present a functional group with affinity to iron ions that need specific cell membrane bound receptors and a cell-associated apparatus to incorporate the metal into the bacterial metabolism. The siderophore-independent mechanism consists of a bacterial outer membrane protein that binds host-specific iron. Also, the gene expression related to the iron acquisition is regulated by protein Fur (Tomás, 2012).

Other metal cofactors with a role in the pathogenicity of *Aeromonas* have been described, for example, some copper and silver resistance genes coding for different proteins (Francki et al., 2000; Fones and Preston, 2013). However, less information is available on the acquisition of other metals in *Aeromonas.*

For instance, nothing is known about the nickel homeostasis that has been described in other bacterial models such as *Helicobacter. pylori* or *E. coli* with a role in the host-pathogen interaction (Johnson et al., 2015; Hu et al., 2017; Blum et al., 2017). A relevant metallochaperone HypA has been associated with the adaptation to acidic environments, considering this protein as a potential virulence factor in emerging pathogenic bacteria.

1.5. Antimicrobial susceptibility

Antimicrobial susceptibility studies carried out until now for *Aeromonas* have followed the guidelines for the *Enterobacteriaceae* included in the Clinical and Laboratory Standards Institute (CLSI) published in 2015. Generally, *Aeromonas* have been inhibited by most antimicrobial agents. However, different resistance has been described such as to penicillins (Aravena-Román et al., 2012). Nevertheless, previous studies described *A. trota* as the only species of the genus susceptible to ampicillin (Carnahan, 1991; Overman and Janda, 1999; Huys et al., 2002; Abbott et al., 2003; Lai et al., 2007; Dallagassa et al., 2018).

1.5.1. Resistance mechanism

The β-lactamases are related to the activity against several β-lactam antibiotics (Janda and Abbott, 2010). Three mainly β-lactamases have been described in *Aeromonas,* class B metallo-β-lactamase, class C cephalosporinase and class D penicillinase. Fosse et al. (2003) characterized the β-lactamases in *Aeromonas*. i) *A. hydrophila* complex strains expressing class B, C, and D β-lactamases; (ii) *A. caviae* strains expressing class C and D β-lactamases; (iii) *A. veronii* group strains containing class B and D lactamases; (iv) *A. schubertii* strains harboring class D lactamases; and (v) *A. trota* strains with class C β-lactamases. It also appears that many *A. veronii* bv. sobria isolates also produce a class C

cephalosporinase (Fosse et al., 2003; Janda and Abbott, 2010). Although *Aeromonas* are sensitive to quinolones, resistance to these drugs has been reported. According to the data, this resistance could be related with plasmid-mediated proteins (Cattoir et al., 2008; Sanchez-Cespedes et al., 2008).

1.6. Pathogenicity

One important issue in the study of the pathogenicity of *Aeromonas* is the selection of adequate *in vivo* or *in vitro* model to reproduce the infection in the different experiments (Romero et al., 2016)

1.6.1. Studies with different cell lines

Many studies of pathogenesis and virulence in *Aeromonas* have been performed by using *in vitro* cell lines in the last years (Table 7) The capacity of *Aeromonas* to adhere, invade, and to produce cytotoxicity has been described, mainly in *A. hydrophila* and *A. caviae,* and the most frequently used cell lines are the cell lines human larynx carcinoma (HEp-2) and human colon carcinoma (Caco-2). The cytotoxicity of 55 *Aeromonas* strains was analyzed in another study by using four cell lines, African green monkey kidney (Vero), Madin Darby Bovine Kidney (MDBK), Baby Hamster Kidney cells (BHK-21), and marmoset B lymphoblastoid cells (B95a), demonstrating that the Vero cell line is the best model to examine and test the cytotoxicity of *Aeromonas* spp., due to is the most sensitive cell line (Ghatak et al., 2006). In a recent study, Dos Santos et al. (2015) evaluated the capacity of *Aeromonas* spp. to adhere, invade, survive and produce cytotoxicity by using three different cell lines, HEp-2, Caco-2, and human colorectal adenocarcinoma (T-84). The results showed that *Aeromonas* isolated from different sources were able to invade intestinal (T-84, Caco-2) and epithelial (HEp-2) cell lines (Dos Santos et al., 2015). These results suggested that the cytotoxicity and the adhesion could depend on the type of cell line tested.

Table 7. Summary of interesting recent studies that infect different cell lines with *Aeromonas* spp.

Cell line	Study	*Aeromonas*	Reference
MDBK **Vero** **B95a** **BHK-21**	Adhesion, invasion and cytotoxicity	*A. hydrophila* *A. caviae* *A. veronii* *A. trota* *A. jandaei*	Ghatak et al., 2006
HEp-2 **Vero**	Adhesion and cytotoxicity	*A. hydrophila* *A. salmonicida* *A. veronii* *A. bestiarum* *A. schuberti* *A. eucrenophila* *A. encheleia* *A. jandaei* *A. sobria* *A. caviae* *A. trota* *A. media*	Ottaviani et al., 2011
HEp-2 **CHO** **Vero**	Cell -contact cytotoxicity	*A. hydrophila* *A. caviae* *A. veronii*	Krzyminska et al., 2012
HT-29	Cytotoxicity	*A. caviae* *A. veronii*	Chen et al., 2014b
C2C12	Cytotoxicity	*A. hydrophila* *A. dhakensis* *A. veronii* *A. caviae*	Chen et al., 2014c
HEp-2	Adhesion, biofilm and immune stimulation	*A. hydrophila*	Merino et al., 2014
HEp-2 **Caco-2** **T-84**	Adhesion, invasion and cytotoxicity	*A. hydrophila* *A. caviae*	Dos Santos et al., 2015
RAW 264.7	Mixed infections	*A. hydrophila*	Ponnusamy et al., 2016
Caco-2	Adhesion and cytopathic effect	*A. hydrophila* *A. dhakensis* *A. bestiarum* *A. piscicola* *A. salmonicida*	Albarral et al., 2016
HepG2 **WLR-68**	Cytotoxicity effect of metalloprotease	*A. hydrophila*	Abdallah, 2019
Caco-2	Adhesion, invasion and cytotoxicity	*A. salmonicida*	Dias et al., 2019

In 2012, Krzyminska et al. demonstrated that the presence of T3SS in the *Aeromonas* strain tested could mediate cell-contact cytotoxicity, destruction of host epithelial cells, and tissue damage in different cell lines: Chinese hamster ovary (CHO), HEp-2 and Vero. Merino et al. (2014) demonstrated by the generation of mutants that the flagella glycosylation in *A. hydrophila* plays an extremely important role in the adhesion to Hep-2 cells and in biofilm formation. Similarly, the flagella glycosylation induces the stimulation of the immune response.

As previously explained, Ponnusamy et al. (2016) studied the complex mixed infections between two strains of *A. hydrophila* (NF1-NF2) by using in the *in*

vitro experiments the murine macrophages (RAW 264.7). They suggested a different course of the disease in a mixed infection compared to the single infection.

In addition, host immune studies regarding *Aeromonas* interactions with different cell lines (Krzyminska et al., 2008; Rosenzweig and Chopra, 2013; Reyes-Becerril et al. 2016) have been described in the section 1.4.1.

1.6.2. Studies on animal models

Simulation of the *Aeromonas* infections in several animals has been performed in different previous studies. The animal models and the latest relevant studies are described in Table 8. Selection of the experimental model is one of the most important issues in the study of virulence and pathogenicity (Romero et al., 2016).

Mice are susceptible to a similar range of microbial infections as well as humans (Buer and Balling, 2003). As shown in Table 8, in many recent studies, mice are considered a good model used to study the pathogenicity in *Aeromonas*; remarkable studies were performed. In two studies performed by Grim et al. (2014) and Ponnusamy et al. (2016), the possible role of the mixed infections with two *A. hydrophila* strains in the progression of NF was described. Additionally, another interesting study with mice was performed by Chen et al. (2014c), which demonstrated the virulence of *A. hydrophila*, *A. dhakensis*, *A. caviae*, and *A. veronii* by the analysis of the survival curves by using BALB/c mice. The results suggested a variation among the most clinical prevalent species.

Romero et al. (2016) demonstrated that zebrafish larvae could be used as host model to assess the virulence of *A. hydrophila.* Additionally, zebrafish was used as a model of co-infection with *Aeromonas* species (Chandrarathna et al., 2018), demonstrating a higher mortality under co-infection in relation to the single infection. Moreover, zebrafish has been used as a model to study the immune response against *Aeromonas* (Srivastava et al., 2017).

Recently, catfish has been used as a fish model to clarify the virulence mechanism in *A. hydrophila* (Peatman et al., 2018), the blocking of aerolysin

activity after *A. hydrophila* infections (Dong et al., 2018) and the enterotoxic effects after *A. hydrophila* infection (Sellegounder et al., 2018), among others.

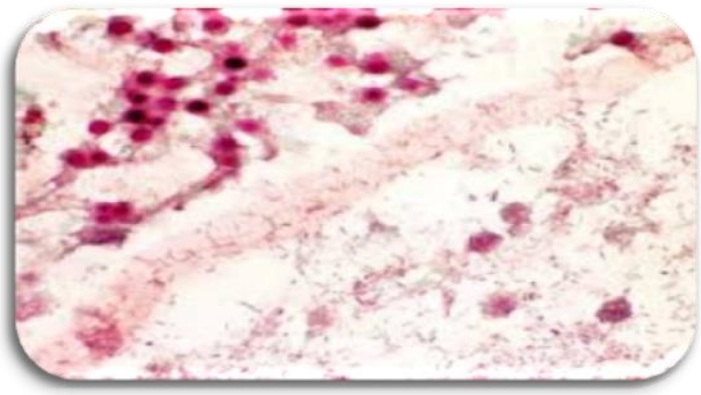
Table 8. Latest relevant studies that perform *Aeromonas* infections in different animal models

Model	Study	*Aeromonas*	Reference
Mice	Mixed infections, pathogenicity	*A. hydrophila* *A. dhakensis* *A. caviae* *A. veronii*	Chen et al., 2014c Grim et al., 2014 Romero et al., 2016 Ponnusamy et al., 2016
Catfish	Enterotoxic effects, virulence mechanism, transcriptome, aerolysin activity	*A. hydrophila* *A. veronii*	Sellegounder et al., 2018 Li et al., 2018 Peatman et al., 2018 Dong et al., 2018
Blue gourami	Septicemia, immune responses	*A. hydrophila*	Fock et al., 2001 Yu et al., 2005
Zebrafish	Immune response, mixed infections	*A. hydrophila* *A. veronii*	Romero et al., 2016 Guo et al., 2018 Chandrarathna et al., 2018 Srivastava et al., 2017
Slime mold	Pathogenicity	*A. salmonicida* *A. hydrophila*	Froquet et al., 2007
Nematode	Virulence, immune response, necrosis	*A. hydrophila* *A. dhakensis* *A. veronii* *A. caviae*	Mosser et al., 2015 Chen et al., 2017 Chen et al., 2018a Chen et al., 2018b

Chen et al. (2018a) evaluated the netamode of *Caenorhabditis elegans* as a virulence and pathogenicity model to study the *Aeromonas* infections, demonstrating that *A. dhakensis* was the most virulent species in comparison with the most prevalent clinical species. Also, in 2017, Chen et al. used *C. elegans* as a disease model of muscle necrosis after *A. dhakensis* infection; the results validated the *C. elegans* as model to study *A. dhakensis*. Mosser et al. (2015) contributed to the paradigms of mixed infections suggesting that the virulence of one *Aeromonas* strains could be higher in presence of a partner.

Other interesting models of infection have also been proposed, but less studied: i) Blue gourami was used to study the role of an endogenous serum lectin in the immune protection against *A. hydrophila* infections (Fock et al., 2001), as well as for the identification of putative virulence factors in *A. hydrophila* strains (Yu et al., 2005); ii) Slime mold was studied for a host model to evaluate the virulence of *Aeromonas* (Froquet et al., 2007).

2. INTEREST AND OBJECTIVES



UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
Ana Fernández Bravo

Interest and objectives

The genus *Aeromonas* includes more than 32 species autochthonous of aquatic systems, some of which are considered emerging human pathogens. Nowadays, water is an increasingly limited resource and for this reason different methods of water treatment have been developed, including those that treat wastewater to generate reclaimed water and usually this water is used for irrigation. It has been previously shown that strains of *Aeromonas* may be present in this irrigation water and the same strains have been recovered in the irrigated vegetables, i.e. lettuces and tomatoes, and this may represent a risk of infections for consumers. For this reason, to continue studying the presence of *Aeromonas* in water it is extremely relevant to determine the risk more precisely (**objective 1, study 4.1.**).

The species of *Aeromonas* considered as emerging pathogens can cause a wide spectrum of diseases in humans, mainly gastroenteritis, bacteremia, and wound infections, being able to infect both immunocompromised and immunocompetent patients. According to the recent literature, at least 19 of the species included in the genus *Aeromonas* can be considered potential pathogens for humans. However, 96.5% of the strains associated to clinical cases correspond only to four species i.e. *A. caviae* (29.9%), *A. dhakensis* (26.3%), *A. veronii* (24.8%), and *A. hydrophila* (15.5%). Species classically associated with fish diseases, such as *A. salmonicida* have also been sporadically isolated in association with human infections. However, a comparative analysis of the capacity of mesophilic and psychrophilic strains of *A. salmonicida,* recovered from human clinical cases, water and fish, to generate infections in a murine model, has never been performed. This kind of study will provide light on the capacity of those strains to generate human infections. Furthermore, the comparison of the genomes of such strains might contribute to a better understanding of their pathogenicity and will help to understand if the subspecies defined within this species using phenotypical methods, are recognized as such, by using a comparison of their genomes. The **objective 2**, (**study 4.2.)**, pretends to provide answers to these questions.

In clinical settings, the recognition of the *Aeromonas* spp. has been subjected to much confusion when using phenotypic identification methods. For

instance, confusion between *Aeromonas* and *Vibrio* occurs frequently, as recognized in our laboratory, when the identification is performed in parallel with molecular techniques such as the use of the sequences of housekeeping genes (*rpo*D, *gyr*B, etc). Therefore, in our laboratory we collaborate with the University hospitals of our area to re-identify with molecular methods the strains of *Aeromonas* associated with clinical cases, studying the characteristics of the patients and preparing publications of the most relevant cases. The aim is to raise awareness among clinicians on the importance of the *Aeromonas* infections (**objective 3, studies 4.3. and 4.4.**)

In the mentioned case studies, we also investigate the performance of the MALDI-TOF identification system. This system is nowadays used in many clinical laboratories, where it helped to improve considerably the identifications of *Aeromonas* and many other microbes. In fact, recent studies using MALDI-TOF show that between 98.3% and 100% of the strains of *Aeromonas* are correctly identified at the genus level and 91.1% to 97% at the species level. However, erroneous identifications due to the absence of some species, such as *A. dhakensis* in the database are still common. In fact, it is not known the full extent of these errors especially when MALDI-TOF is used to characterize environmental strains. Therefore, more studies are required to compare the results of MALDI-TOF with those obtained by using reliable methods, such as the analysis of the sequences of housekeeping genes (**objective 4, study 4.5.**).

In the last years, the number of genome sequences from the *Aeromonas* spp., has increased from the six genomes available in 2012, to 348, in 2019, when the database was consulted (04/18/2019). The introduction of the taxonomic indexes (ANI, *is*DDH, etc), as well as the development of several bioinformatic tools allows the comparison of *Aeromonas* genomes and their characterization in relation to their identity and the presence of specific genes associated with virulence, antimicrobial resistance, metabolic pathways, among others. *Aeromonas lusitana* is a recently described species isolated from water and vegetables in Portugal and Spain that has, so far, not been reported after its description from other habitats. In our laboratory we have identified a new strain of this species in a study that characterized strains of

Aeromonas recovered from rainbow trout in Mexico. Considering this, we decided to analyze and compare the genome we obtained from the new strain, along with the phenotypic characteristics and presence of virulence and resistance genes with the data derived from the type strain to increase the knowledge about this relatively new and poorly known species (**objective 5, study 4.6.**).

The virulence of *Aeromonas* has been described as multifactorial and linked to the expression of genes (*exoA, alt, act…*) that encode different toxins, structural components (*flaA*, *maf-5, flp…),* secretion systems (T3SS, T6SS…) and proteins associated with metals. Nonetheless, the specific role of some genes, as genes encoding metalloproteins, like the metallochaperone HypA that plays a role in the survival of *Helicobacter pylori* and *Escherichia coli* in the acidic environment of the stomach, has not yet been investigated in *Aeromonas.* Considering that 80% of the infections caused by *Aeromonas* are gastrointestinal, a similar possible adaptation of these bacteria to acidic environment of the stomach could be suspected suggesting that HypA could also be present in the bacteria of this genus (**objective 6, study 4.7.**).

Mixed infections with *Aeromonas* have been estimated to occur in 5%-10% of the cases and the interaction of the virulence factors among the co-infecting strains has been poorly studied. In fact, two *A. hydrophila* strains were recovered from a fast progressing necrotizing fasciitis that occurred in a young immunocompetent patient resulting in the amputation of almost all the extremities. Recently, the team of Dr. A.K. Chopra (Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, USA) demonstrated in an *in vitro* and *in vivo* model of infection that the progression of the necrotizing fasciitis varied if the infection was performed with only one or with the two strains of *A. hydrophila* recovered from the mentioned case. They observed that the infection followed a different course characterized by the antagonism/synergism of the strains causing differences in the severity of the disease. The authors suggested that the T6SS could have a role in the antagonistic effect observed between the two strains. In addition, the ExoA toxin, present only in one of the strains, could play a

role in the bacterial dissemination. However, there is a need to demonstrate this hypothesis by using mutant strains for the genes associated with the T6SS, as well as mutants for the gene encoding the ExoA toxin and performing with them combined mixed infections to determine or confirm the specific role of these virulence factors in these complex mixed infections. My contribution to that study has been performed during my six-month stay at the Dr. Chopra's laboratory in Galveston (USA), and it corresponds to the **objective 7 (study 4.8.).**

Another aspect that is poorly known in relation with the *Aeromonas* infections is the interactions of the bacteria with the human immune system. The few existing studies have been centred in *A. hydrophila*, followed by *A. veronii* and *A. salmonicida*, by using animal models such as fish or mice, as well as different cell lines, i.e., mouse BALB/C monocyte macrophages (J744), murine macrophages from blood (RAW 264.7), the cancerous cell line (HeLa) or the human colon colorectal adenocarcinoma (HT-29). Previous studies suggested that the immune response could be cell type-dependent, considering this, it could be interesting to study the human monocytic cell line (THP-1) that has so far not been studied in *Aeromonas*. The THP-1 cell line acts as the first line of defense simulating the early steps of the infection. Moreover, several studies demonstrated the expression of different immune-related genes including the ones associated with cytokines (*TNF-α*), chemokines (*CCL20*), TLRs (*TLR4*), transcription factors (*RELA*, *JUN*), apoptosis (*CASP3*, *TP53*) and pyroptosis (*IL-1β, NRLP3*). However, the chemokine gene *CCL2*, as well as the *BAX* gene related to the apoptosis route, have not been studied in infections produced by *Aeromonas*. Additionally, the response against other important *Aeromonas* species like *A. caviae* and *A. dhakensis* that have a higher frequency of isolation in clinical cases, have not yet been studied. In addition, if strains recovered from the environment have an equal capacity to develop an innate-immune response in the host cells, in comparison with the classically considered more virulent strains from clinical origin is another aspect that has never been studied. Therefore, to perform a study by using a selection of the species that represent the different frequencies of isolation in the clinical cases, together with the

use of clinical and environmental strains may help to clarify if there exists a species-specific immune response that could explain their differential prevalence in human infections (**objective 8, study 4.9.**)

In order to clarify the above commented aspects, the following **specific objectives** have been defined:

1. To quantify the presence of *Aeromonas* in different water samples by qPCR to determine the potential risk to public health.
2. To evaluate the pathogenicity of mesophilic and psychrophilic *A. salmonicida* clinical and environmental strains, comparing their genomes and using a murine model of infection.
3. To describe the characteristics of the patients and the strains associated with cases of infections produced by *Aeromonas* and *Vibrio* at our local hospitals.
4. To evaluate the performance of the MALDI-TOF identification method for the characterization of environmental *Aeromonas* strains by comparing the results with those derived from the sequences of the *rpoD* gene.
5. To contribute to the characterization of *A. lusitana* performing a genomic and phenotypic comparative analysis of a strain recovered from fish in our laboratory with the type strain.
6. To characterize the potential role of the HypA metallochaperone in the virulence of *Aeromonas.*
7. To study the role of the T6SS and the *exoA* gene in the necrotizing fasciitis (NF) mixed infection generated in a mouse model and in the murine macrophage cell line from blood (RAW 264.7) by using the two original strains of *A. hydrophila*, that generated the human NF, in combination with defective mutants of those strains for the above mentioned virulence factors.
8. To evaluate the host immune response of the human monocytic cell line (THP-1) infected independently with six *Aeromonas* species, and with strains of clinical and environmental origin, evaluating the expression profile of 11 immuno-related genes. To determine if it exists a species-specific immune response that could explain the different prevalence of the species associated with human infections.

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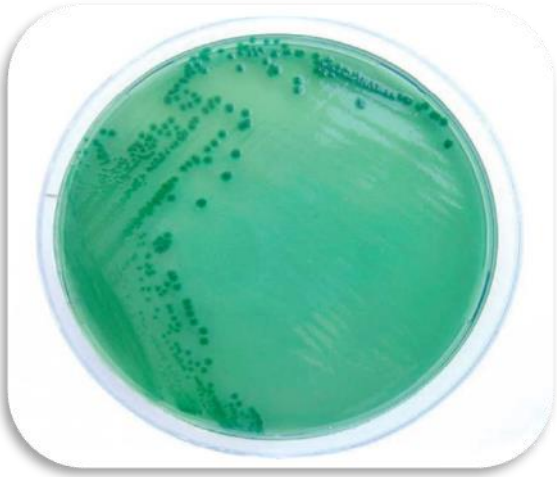
3. MATERIALS AND METHODS



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3.1. Bacterial strains, conservation and culture

The strains used in this doctoral thesis are listed in the Supplementary section. All the strains correspond to environmental and clinical isolates obtained in our laboratory, as well as isolates from culture collections, including type strains.

For the conservation, the strains used in this doctoral thesis were maintained in Tryptic Soy Broth (TSB, Difco™, France) plus glycerol (15%) at 80°C. Before experiments, bacteria were routinely grown in Tryptone Soya agar (TSA, Difco™, France) at 30°C for 24 h. Other media and buffers were used such as ADA, APA-A, LB Agar or Blood Agar. Antibiotics such as ampicillin (Ap), kanamycin (Km) and rifampicin (Rif) were added when necessary (Table 9).

Table 9. Media and buffers used in the studies.

Media	Study
TSA Tryptic Soya Agar	4.2., 4.4., 4.4., 4.5., 4.6., 4.7., 4.9.
ADA (+amp) Ampicillin Dextrin Agar Base +amp	4.1.
LB Agar Luria Bertani Agar	4.8.
LB Agar (+antibiotics) Luria Bertani Agar +amp	4.8.
APA-A Buffered Peptone Water + amp	4.1.
XLD Xilose, Lysisne, Deoxycholate Agar	4.4.
TCBS Thiosulfate Citrate Bile Salts Sucrose Agar	4.3.
BA Blood Agar	4.3.
TSB (+glycerol) Tryptic Soy Broth	Strain maintenance

3.1.1. Genetic manipulation of the bacterial strains

3.1.1.1. Construction of bioluminescent mutant

In the study 4.8., performed in the University of Texas Medical Branch, a conjugation with two strains of *Escherichia coli*, SM10 Λpir carrying the pTNS2

plasmid and SM10 Λpir harbouring the pUC18-mini-Tn7T::Km-lux was performed (Ponnusamy et al., 2016). The later minitransposon contains a lux luminescence operon with the native promoter and kanamycin resistance (Km^r) selection marker. This system allows the integration downstream of the *gmlS* gene, which encodes a conserved glucosamine-6-phosphate synthetase, with the helper plasmid pTNS2 providing the transposase complex (Ponnusamy et al., 2016).

3.1.1.2. Construction of knockout/complemented mutants

All mutants of *A. hydrophila* NF1 and NF2 in the study 4.8., were generated in the University of Texas Medical Branch. The mutants were constructed by using a crossover PCR and a homologous recombination, previously described (Ponnusamy et al., 2016; Suarez et al., 2008; Liang et al., 2015). The insertion and deletion of the genes were confirmed by PCR and genome sequencing (study 4.8.)

3.2. DNA extraction and genotyping

Genomic DNA for molecular characterization was extracted from pure cultures from TSA, using the InstaGene™ DNA Purification Matrix (Bio-Rad-Hercules CA, USA) following the manufacturer's instructions. All the strains included in the study 4.6., were genotyped using ERIC-PCR, using the primers ERIC 1R and ERIC 2 (Table 10) previously described by Versalovic et al. (1991) and in concordance with the conditions described by Houf et al. (2002).

Table 10. Primers used for the ERIC-PCR.

Method	Primer	Sequence 5'- 3'
ERIC-PCR	ERIC 1R	ATGTAAGCTCCTGGGATTCAC
	ERIC 2	AAGTAAGTGACTGGGGTGAGCG

3.3. Molecular identification

3.3.1. Phylogenetic analysis with housekeeping genes

All strains analyzed in this doctoral thesis were identified at species level by the *rpoD* gene sequencing, using the primers listed in Table 11, and the conditions used by Soler et al. (2004). Similarly, in the study 4.6., the *gyrB* housekeeping gene was analyzed. The resulting sequences were aligned with the other described species of the genus by using the ClustalW algorithm (Larkin et al., 2007) in MEGA v6.0 (Tamura et al., 2013). A phylogenetic analysis was performed with the Neighbor-joining (NJ) method with MEGA v6.0 (Tamura et al., 2013).

Table 11. Primers used for PCR amplification and sequencing of *gyrB* and *rpoD* genes.

Primer	Sequence 5'- 3'
***rpoD*- Fs**	ACG ACT GAC CCG GTA CGC ATG TAY ATG MGN GAR ATG GGN ACN GTA ATA GAA
***rpoD*- Rs**	ATA ACC AGA CGT AAG TTN GCY TCN ACC ATY TCY TTY TT AXDACDS
***rpoD*-Fs1**	GTCAATTCCGCCTGATGC
***rpoD*-Rs1**	ATCATCTCGCGCATGTTGT
***gyrB*-3F**	TCCGGCGGTCTGCACGGCGT
***gyrB*-14R**	TTGTCCGGGTTGTACTCGTC

3.3.2. MALDI-TOF

The strains used in the studies 4.3. 4.4. and 4.5. were previously identified by using MALDI-TOF Byotiper (Bruker®), detecting proteins in the range 2-20 kDa. The result obtained in the comparison with the database is translated in a *score* with different colors, indicating the reliability of the identification at genus or species level (Figure 7).

Figure 7. Score range indicating the reliability of the identification at genus or species level.

The MALDI-TOF in the studies 4.3., and 4.4. was performed in three different hospitals: University Hospital Sant Joan, Reus, University Hospital Miguel Servet, Zaragoza, and University Hospital Joan XXII, Tarragona. Additionally, the MALDI-TOF analysis of the study 4.5. was performed in the Veterinary Health Surveillance Center (VISAVET) in Madrid.

3.4. Phenotypic characterization

A study of phenotypical characteristics was performed. Conventional tests were carried out at least twice for the strains (studies 4.6. and 4.7.). Additionally, in the studies 4.3., 4.4., and 4.6., the biochemical tests were performed by using the automated system MicroScan Walkaway (Siemens®). The biochemical properties tested included catalase, oxidase and urease activity, nitrate reduction, glucose, utilization of citrate, DL-lactate, and growth in media supplemented with different concentrations of NaCl, among other tests, previously described for *Aeromonas* (Janda and Abbott, 2003, 2010). Moreover, in the studies 4.3., and 4.4. the API20 (BioMerieux ®, Marc l'Etoile, France) were used for the phenotypic characterization, following the manufacturer's instructions.

3.5. Detection of virulence genes

The 10 virulence genes from the strains used in the study 4.6. were detected by PCR using the primers and conditions previously described that targeted the different genes (Table 12).

Table 12. Genes analyzed in the study 4.6.

Gene	Reference	Gene	Reference
lafA	Merino et al., 2003	*ascF-G*	Chacón et al., 2003
aerA	Soler et al., 2002	*ascV*	Chacón et al., 2003
act	Kingombe et al., 1999	*aexT*	Braun et al., 2002
ast	Aguilera-Arreola et al., 2005	*aopP*	Fehr et al., 2006
alt	Chopra et al., 1996	*stx1*	Wang et al., 2002
lipH3	Chacón et al., 2003	*stxA2*	Muniesa et al., 2003
pla	Chacón et al., 2003	*ahe2*	Chacón et al., 2003
plc	Chacón et al., 2003	*aspA*	Chacón et al., 2003

3.6. Antimicrobial susceptibility profile

In the studies 4.3., 4.4., and 4.6., the antibiotic resistance was studied by a disk diffusion test, and the results were analyzed according to the CLSI guidelines (CLSI, 2015). The BBL Sensi-Disc Susceptibility Test Discs (BD, Madrid, Spain) were used containing the antibiotics listed in Table 13.

Table 13. Antimicrobial agents evaluated in different studies.

Antimicrobial agents		
Amikacin	Ciprofloxacin	Trimetoprim-sulfamethoxazole
Ampicillin	Cefepime	Tigecycline
Amoxicillin-clavulanic acid	Cefuroxime	Tobramycin
Aztreonam	Ertapenem	Ciprofloxacin (5µg)
Ceftazidime	Gentamicin	Cefotaxime
Ceftriaxone (30 µg)	Imipenem	Cefoxitin
Cephalothin	Nalidixic acid	Piperacillin (100µg)
Tetracycline (30µg)	Piperacillin-tazobactam	

3.7. Protein sequence analysis and 3D-structure prediction

The prediction of 3D monomeric and dimeric protein structure in the study 4.7., and the comparative analyses of this protein were carried out by using Swiss-model online tool. To determine the sequence conservation between species, the sequences were aligned by using the ClustalW algorithm in the MegAlign software. Similarly, the phylogenetic relationships among sequences of the protein were depicted in a phylogenetic tree constructed on MEGA v6.0 using the Neighbor-joining method.

3.8. Genomics

3.8.1. DNA extraction, sequencing and annotation

A total of 4 genomes (studies 4.2. and 4.6.) were sequenced in our laboratory. Total genomic DNA was extracted from pure cultures grown in TSA by the Easy-DNA™ gDNA Purification Kit (Invitrogen, Madrid, Spain) following the manufacturer's instructions. The quality of the DNA was checked by electrophoresis of 10 µl of DNA in 1.5% agarose gel. The total amount of DNA was quantified by using Nanodrop 2000. After extraction, in the study 4.2., the DNA was sent to IBIS from Université Laval (Canada) to prepare sequencing libraries by using the KAPA Hyper Prep kit, while in the study 4.6., the sequencing libraries were performed with the Nextera DNA Library Preparation kit in our laboratory. The resulting libraries in both studies were sequenced with MiSeq platform of Illumina. The final reads in the study 4.2. were assembled by using A5-miseq version 20160825 (Coil et al., 2015), and in the study 4.6. using SPAdes (Bankevich et al., 2012) and the CGE assembler (Larsen et al., 2012).

In relation to the annotation, in the study 4.6., the assembled genome was annotated in RAST Annotation (Aziz et al., 2008) and compared with other *Aeromonas* genomes previously annotated. However, in the study 4.2., the assembled genomes were annotated by using the Prokaryotic Genome Annotation

Pipeline (PGAP) and deposited in Genbank (Tatusova et al., 2016). Additionally, the genomes were annotated by using Prokka version 1.12 (Seemann, 2014) to compare with other *Aeromonas* genomes.

3.8.2. Genomic indexes

Genomic indexes were used in the studies 4.2., and 4.6. The ANI was calculated in both studies, by using pyani (Kurtz et al. 2004) (study 4.2.) and OrthoANI (Lee et al., 2016) programs (study 4.6.). The <96 % cut-off was used for the definition of a new phylogenetic line or species (Colston et al., 2014). The *is*DDH was calculated with the GGDC software (Meier-Kolthoff et al., 2013) (Study 4.6.).

3.8.3. Genomic characterization

The functional and metabolic characterization of the genome in the study 4.6., was performed with the functional Comparison Tool of the Seed Viewer (Overbeek et al., 2014) by using the annotation obtained with RAST (Aziz et al., 2008). Additionally, the Core-Pan genome analysis was performed by using the program pancoreplot from CMG Biotools (Vesth et al., 2013).

In the study 4.2., homologous links between the translated coding sequences were defined by using GET_HOMOLOGUES version 20180103 (Contreras-Moreira and Vinuesa, 2013) with two algorithms: COG and OMCL. The sequences to the softcore were recovered by using TranslatorX version 1.1 (Abascal et al., 2010) and the alignments were filtered by using BMGE version 1.12 (Criscuolo and Gribaldo, 2010). After this, the alignments were concatenated by using AMAS (Borowiec, 2016). The maximum-likelihood phylogeny was itself performed by using IQ-TREE (Hoang et al., 2017). The antibiotic resistance was predicted by using ABRicate version 0.8.7 and the CARD database (Jia et al., 2017).

3.8.4. *In silico* search of genes in the *Aeromonas* genomes

For identification, the genes were searched in the genomes by using a sequence obtained from the Uniprot database. This nucleotide sequence was used as a query on BLAST searches in the genomes.

In the study 4.6. to ensure that the sequenced genome corresponded to the strain of interest, the seven housekeeping genes (*rpoD, gyrB, gyrA, atpD, dnaJ, dnaX* and *recA*) used in the Multilocus Phylogenetic Analysis (MLPA) were extracted from each genome and compared with the Sanger sequence originally obtained for the identification of the *Aeromonas* species. In the study 4.7. the nucleotide sequences of *hypA* and *ure* genes related to the virulence of *Aeromonas* were searched in the 36 available genomes.

3.9. *In vitro* studies

3.9.1. Cell lines and growth conditions

For the studies 4.7., 4.8., and 4.9., three cell lines were selected from the experiments. TPH-1 (human peripherial blood), J744A.1 (mouse BALB/C monocyte macrophages), RAW 264.7 (macrophage from murine blood) (Murciano et al., 2015; Rama et al., 2011; Ponnusamy et al., 2016). THP-1 cells and RAW 264.7 were maintained as suspension cells in Roswell Park Memorial Institute Medium (RPMI-1640, PAA Laboratories) supplemented with 10% FBS (fetal bovine serum, PAA Laboratories) plus 1% P/S (penicillin-streptomycin solution, PAA Laboratories), while J744A.1 cells were maintained as adherent cells in Dubelcco's Modified Eagle's Medium (DMEM, PAA Laboratories) supplemented with 10% FBS plus 1% P/S, in all cases at 37ºC and 5% CO_2.

3.9.2. Infection

Prior to infection, cells were seeded in tissue culture plates (1×10^6 cells/ml) containing serum-free DMEM without antibiotics (serum-starvation conditions) for 18 h, as previously described (Murciano et al., 2015). Cells were infected with the

strains of *Aeromonas* grown overnight at different multiplicity of infection (MOI 5,10 and 20) calculated by serial dilution and plating on TSA or LB agar (studies 4.7., 4.8., and 4.9.). In addition, to compare with macrophages infection, in the study 4.7., *Aeromonas* were seeded in culture plates with DMEM without FBS and P/S at alkaline pH (pH=8) and acidic pH (pH=4.5) adjusted with HCl solution followed by filtration to remove any precipitate.

3.9.3. Phagocytic assays

After 1 h of RAW 264.7 infection, a treatment to kill extracellular bacteria with gentamicin was performed for 1 h (0 h time point). To count the number of bacteria a serial dilution and plating were performed. The percentage of phagocytosis was calculated with the number of bacteria at 0 h time point in relation to the initial infection dose (study 4.8.).

3.9.4. Intracellular survival

After 1 h of infection of RAW 264.7 (study 4.8.) and THP-1 (study 4.9.), a treatment to kill extracellular bacteria with gentamicin was performed for 1 h (0 h time point). The percentage of intracellular survival was calculated after bacterial serial dilution and plating, followed by the calculation using the number of bacteria after 4 and 6 h in relation to the 0 h time point.

3.9.5. Cell damage assay

In the study 4.9., we performed at time curse (3,4,5, and 6 h) harvesting of supernatants after THP-1 infection. The cell damage was determined based on the quantification of lactate dehydrogenase (LDH) released, using the Cytox96® Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. Recombinant bovine LDH (Sigma Aldrich) was used to generate a standard curve and sample values were extrapolated from the curve.

3.9.6. RNA extraction and quantitative RT-qPCR

3.9.6.1. Gene expression in *Aeromonas*

In the study 4.7., the total RNA was isolated from J774.1 infection (4h) as well as from bacteria seeded in alkaline (pH= 8) and acidic (pH= 4.5) media, by using TRIzol (Invitrogen), and the quality was confirmed by using Nanodrop. The cDNA was obtained from RNA by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The RT-qPCR was performed in duplicate by using Power SYBR® green PCR Mastermix (Applied Biosystems®, Life Technologies) on StepOnePlus™ Real-Time PCR System (Applied Biosystems) to establish the expression of *hypA*, using 16s rRNA gene as a housekeeping gene. The primers are listed in Table 14

Table 14. Primers used for RT-qPCR in *Aeromonas* gene expression (study 4.7.).

Primers	Sequence 5'-3'
hypA	
Forward	ATGCACGAAATGTCTCTGGC
Reverse	TCGTAATTTGTACCCGCCAC
16S rRNA	
Forward	TGTGTCCTTGAGACGTGGC
Reverse	ACAAAGGACAGGGGTTGCG

3.9.6.2. Immune-related genes expression in monocytes

In the study 4.9., the total RNA was extracted after THP-1 infection (4h) by using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) to remove genomic DNA. The cDNA was transcribed from RNA by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. Hercules, CA, USA). As explained in 3.8.6.1., the RT-qPCR was performed in duplicate by using Power SYBR® green PCR Mastermix (Applied Biosystems®, Life Technologies) on a StepOnePlus™ Real Time PCR System (Applied Biosystems), to establish the

expression of immune related genes, using GAPDH gene as a housekeeping gene. The primers are listed in Table 15.

Table 15. Primers used for RT-qPCR in monocytes gene expression (study 4.9.).

Gene	Sequence (5'-3')
GAPDH	Forward CATGAGAAGTATGACAACAGCCT Reverse AGTCCTTCCACGATACCAAAGT
TLR4	Forward AGTTGATCTACCAAGCCTTGAGT Reverse GCTGGTTGTCCCAAAATCACTTT
JUN	Forward TGCCTCCAAGTGCCGAAAAA Reverse TGACTTTCTGTTTAAGCTGTGCC
RELA	Forward ATGTGGAGATCATTGAGCAGC Reverse CCTGGTCCTGTGTAGCCATT
TNF-α	Forward GAGGCCAAGCCCTGGTATG Reverse CGGGCCGATTGATCTCAGC
CCL2	Forward CCCCAGTCACCTGCTGTTAT Reverse TGGAATCCTGAACCCACTTC
CCL20	Forward GCAAGCAACTTTGACTGCT Reverse ATTTGCGCACACAGACAACT
CASP3	Forward GAAATTGTGGAATTGATGCGTGA Reverse CTACAACGATCCCCTCTGAAAAA
BAX	Forward CCCGAGAGGTCTTTTTCCGAG Reverse CCAGCCCATGATGGTTCTGAT
TP53	Forward CAGCACATGACGGAGGTTGT Reverse TCATCCAAATACTCCACACGC
NLRP3	Forward CAGCACATGACGGAGGTTGT Reverse TCATCCAAATACTCCACACGC
IL-1β	Forward CAGCACATGACGGAGGTTGT Reverse TCATCCAAATACTCCACACGC

3.9.7. Dynamic of mixed infections

In the study 4.8., the strains were grown overnight and mixed in LB broth at a ratio of 1:1 or 5:1, and spotted in nonselective LB plates for 4 h. The growth of the strains in the mixture was determined by serial dilution and plated on the selective plates with the appropriate antibiotic or on nonselective LB plates.

3.10. *In vivo* studies

3.10.1. Animal model

For the *in vivo* studies (study 4.2. and 4.8.), male OF-1 mice (Charles River, Criffa S.A., Barcelona) and female Swiss-Webster mice (Taconic Farms) weighting 30 grams were used. All the studies carried out were approved by the Ethical Committee of the Rovira i Virgili University and by the Institutional Animal Care and Use Committee protocol at the University of Texas Medical Branch. The animals were maintained under standard conditions with free access to food and drink, controlling daily the progress of the infection until the last day of the experiment. Euthanasia due to suffering of the animals was carried out by inhalation of CO_2. This same procedure was used at the end of the study to sacrifice the surviving animals. To monitor the welfare of the animals, different parameters were observed:

- Coat: smooth and shiny or piloerection
- Weight changes: notorious loss of body weight is a sign of suffering
- Postural changes: normal or stooped posture
- Secretions
- Seizures
- Alterations in locomotion: sudden and rapid movements or ataxia.
- Unprovoked behavior: vocalizations and self-mutilations
- Response to stimuli: aggressiveness, comatose state

3.10.2. Immunosuppression

For the study 4.2., the mice were administered with cyclophosphamide (200 mg / kg) (Genoxal® Baxter, Halle / Westfalen, Germany) intraperitoneally two days before infection (day -2) and the administration of this dose was repeated every five days until the end of the experiment (Sanchis et al., 2015).

3.10.3. Immunization

In the study 4.8., the mice were immunized with 100 ng of ExoA mixed with alum at ratio 1:1. Boosters with the same dose were given every two weeks for a total of 6 immunizations and control mice received only alum. The blood of the mice was collected after three and six immunizations to evaluate the anti-ExoA antibody.

3.10.4. Infection

To establish the inocula, the concentration of bacterial cells was determined by plating 10-folds-serial dilutions onto TSA plates, and then by counting the number of CFU after 24h (Ponnusamy et al., 2016). Different inocula were prepared depending on the experiment (studies 4.2. and 4.8.). In the study 4.8., for the mixed infections, the doses represented equal numbers of CFU for each of the mixed strains.

Female Swiss-Webster mice (Taconic Farms, Albany) were infected intramuscularly (i.m.) and intraperitoneally (i.p.) with the mixed and single strains in the study 4.8., while in the study 4.2., male OF-1 mice (Charles River, Criffa S.A., Barcelona) were infected intravenously. The inocula used for the studies 4.2., and 4.8., was 0.1 or 0.2 mL, respectively (usually between 3 and 10 animals per group).

3.10.5. Survival curves

After intravenous, intraperitoneal or intramuscular infection, depending on the experiment (single or mixed infection), the animals were observed for disease

progression over a period of 20 days, and the mortality rate was recorded daily (studies 4.2. and 4.8.).

3.10.6. Bacterial dissemination

After 24 h post-infection with mixed infections in the study 4.8., the spleen and the 200-300 g of muscle tissue (site of infection) of three or seven animals depending on the experiment were homogenized in PBS. A serial dilution was plated on LB agar with the appropriate antibiotic. After incubation for 24h and 37ºC, colonies were counted to calculate the bacterial load per organ or gram of tissue.

3.10.7. Bacterial quantification by qPCR

In the study 4.2., the liver and kidney from infected mice were collected on day 10 post-infection. The DNA was extracted by using the Easy-DNA™ Kit (Invitrogen, CA). Real-time qPCR was performed on the purified DNA by using the DNA TargetSpecies dtec-qPCR Test for *Aeromonas* spp. (Genetic PCR solutions, Orihule, Spain), and the SteponePlus™ Real-Time PCR System (Applied Biosystems) equipment. The number of copies was calculated on the basis of the standard curve and the corresponding amplification cycle threshold (Ct).

3.10.8. Histopathological analysis

In the study 4.2., the liver and kidney were collected on day 10 post-infection and fixed in 10% buffered formalin to detect lesions. After fixation, the tissues were embebed in paraffin and sectioned. For the evalutation by microscopy (CX 33, Olympus) the tissues were stained with hematoxylin, eosin and Giemsa, previously described (Sanchis et al., 2015).

3.11. Water samples

Aeromonas was analyzed for one year, in a total of 47 samples, coming from raw sewage, ground water near a pig farm, water from the Fluvià River, and ground water from a tank. In parallel, these bacteria were also studied in concentrates of

these samples by flocculation used for the detection of viruses and that corresponded to a mix of the season samples, with a total of 12 concentrated samples (Table 16) (study 4.1.)

Table 16. Samples obtained from different waters.

Samples (n)	Flocculation (n)	Origin
11	4	Raw sewage
12	-	Ground water tank
12	4	Ground water pig farm
12	4	River water

3.11.1. Quantification by plate count method

For the non-concentrated samples, a serial dilution was performed from the direct sample, as well as from 100 and 200 mL filtered. The dilutions were plated in ADA for 24 h at 37º C. After 24 h the colonies were counted (study 4.1.)

3.11.2. Quantification by "Most Probable Number"

For the non-concentrated samples, a serial dilution was performed from the direct sample, as well as from 100 and 200 mL filtered. 500 ml of these serial dilutions were inoculated in a tube with 2.5 mL of APA-A (three times for dilution). Each tube was plated in ADA for 24 h at 37º C. After 24 h a number of positives was counted, and the most probably number was obtained with the most probable number calculator as for example in the Figure 8 (study 4.1).

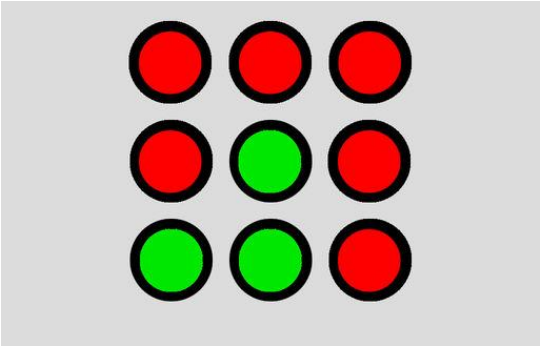
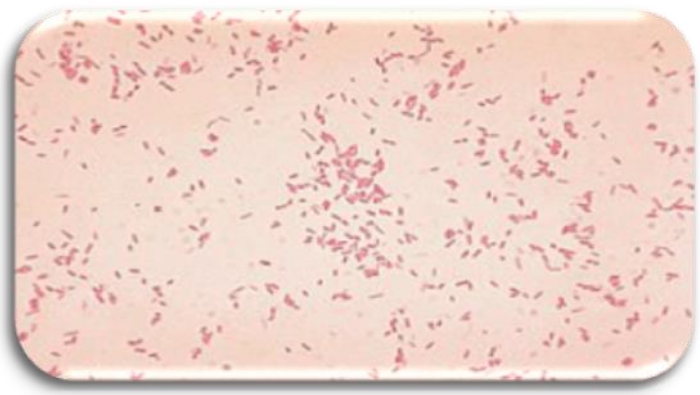


Figure 8. MPN tubes. The number used in the calculator would be 120.

3.11.3. Quantification by qPCR

For the 47 samples, 25 ml of water was filtered, and the DNA was extracted by using the FastDNATM SPIN Kit for Soil (MP Biomedicals), while the DNA of the concentrates by flocculation was extracted by FastPrep® lysing matrix A with the FastDNA Spin kit for soil (MP Biomedicals). The qPCR was performed with the StepOnePlusTM Real-Time PCR System (Applied Biosystem), using a DNA TargetSpecies dtec-qPCR Test for *Aeromonas* spp. (Genetic PCR solutions, Orihuela, Spain) (study 4.1).

4. RESULTS



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EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
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4.1. Presence of *Aeromonas* in different aquatic environments and validation of flocculation method of viruses for bacteria.

Fernández-Bravo A., Pérez-Cataluña A, Martínez-Murcia A., Rusiñol M., Girones R., Figueras MJ. (In preparation/Results will be included in a publication pending to be submitted to "Water Research").

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Results

Presence of *Aeromonas* in different aquatic environments and validation of flocculation method of viruses for bacteria.

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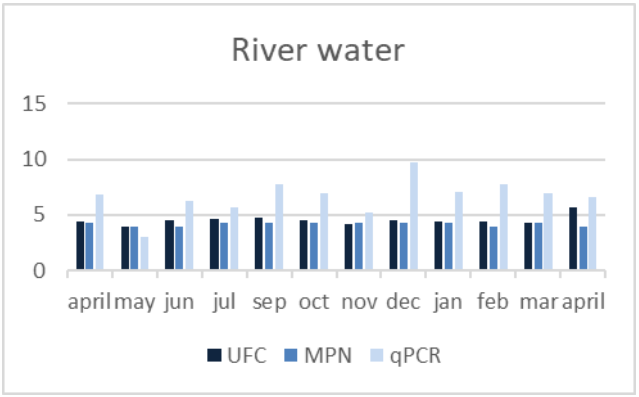
Author's contribution: MR, RG and MJF conceived the idea; AFB and APC performed research; AMM contributed new reagents; AFB and MJF wrote the results for the publication.

A total of 47 samples from four sampling points: raw sewage, ground water near a pig farm, water from the Fluvià river, and ground water from a tank for irrigation were analyzed for *Aeromonas* during one year. In parallel, 12 concentrates of these samples made by flocculation were pooled by season and were also analyzed, except the ground water from a tank for irrigation, to examine if this method of concentration used for the study of viruses was also useful for the study of *Aeromonas*. For the 47 samples, serial dilution was performed from the direct sample, as well as after concentrating 100 and 200 mL of water by filtration. After 24 h, the number of positive samples was counted. Additionally, the serial dilutions were inoculated in tubes containing enrichment media, and were incubated for 24 h. Then, the concentration was counted by using the most probable number (MPN). In parallel, 25 ml of water were filtered, and the DNA was extracted by using FastDNA^{TM} Spin Kit for Soil (MP Biomedicals), while the DNA of the concentrates by flocculation was extracted with the FastPrep® lysing matrix (MP Biomedicals) following the manufacturer's instructions. The DNA was quantified and checked for quality by using the NanoDrop instrument (NanoDrop Products, Wilmington, Delaware, USA). A real-time PCR (qPCR) was performed to quantify the *Aeromonas* spp. by using the StepOnePlusTM Real-Time PCR System (Applied Biosystem, using a DNA TargetSpecies specific dtec-qPCR Test (Genetic PCR solutions, SP) for *Aeromonas* spp. The threshold cycle (CT) was determined by the StepOne software v2.3. The results showed that all the samples were positive for *Aeromonas* with qPCR (Figure 1). Similarly, the concentration found was higher with this method in comparison with the plate count method and the MPN. The highest concentration of *Aeromonas* was found in the raw sewage (Figure 2). The year average concentration at this site was 5.72 x 10^{11} GC/100mL in filtered samples, while the year average concentration was 1.45 X 10^{11} GC/100mL in concentrated samples by flocculation (Figure 3).

Despite, in the sewage water, the concentration of these bacteria was relatively similar, higher concentration was found in flocculated concentrates in the water of the Fluvià River. In the latter the year average concentration of *Aeromonas*

spp. was 7.79 x 10^9 GC/100mL in flocculation concentrates, and 4.41 x 10^8 GC/100mL in the filtered samples (Figure 3). In some samples of raw sewage this is an exception, and this could be due to the presence of inhibition or to the extraction method. The flocculation concentration method used for viruses showed to be a good method for the detection of the bacteria that we studied.



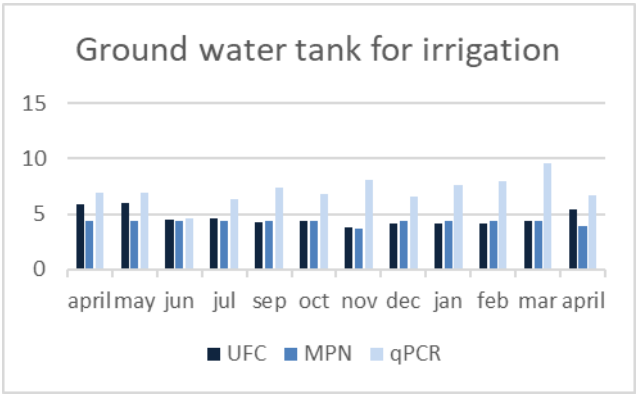
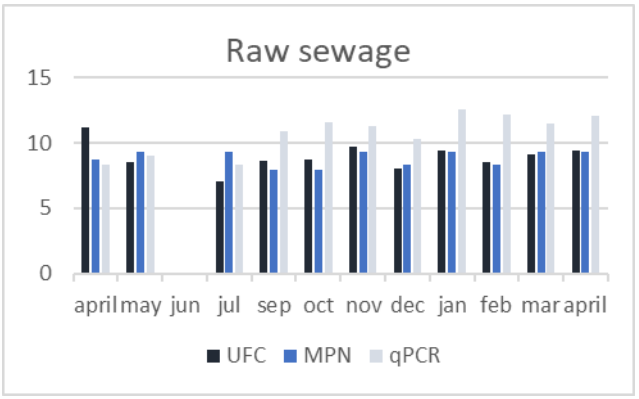


Figure 1. Detection and quantificacion (geometric mean/100ml) of *Aeromonas* by three methods



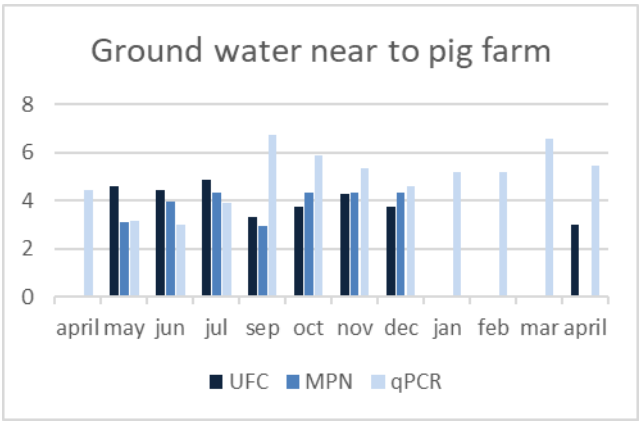


Figure 1 continued. Detection and quantificacion (geometric mean/100ml) of *Aeromonas* by three methods

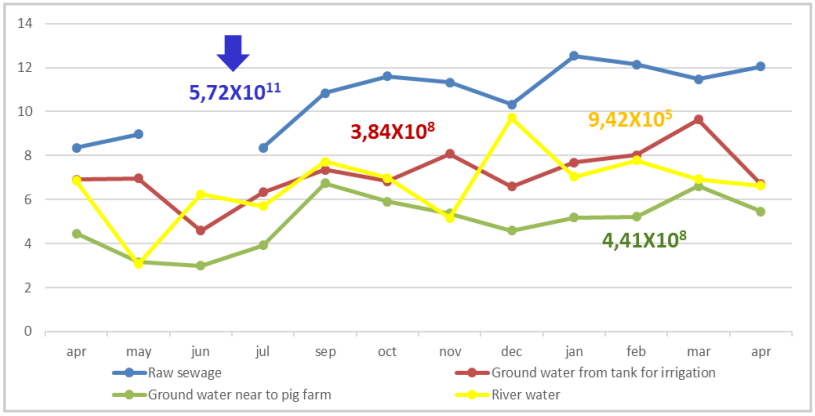


Figure 2. *Aeromonas* quantification by qPCR

Results

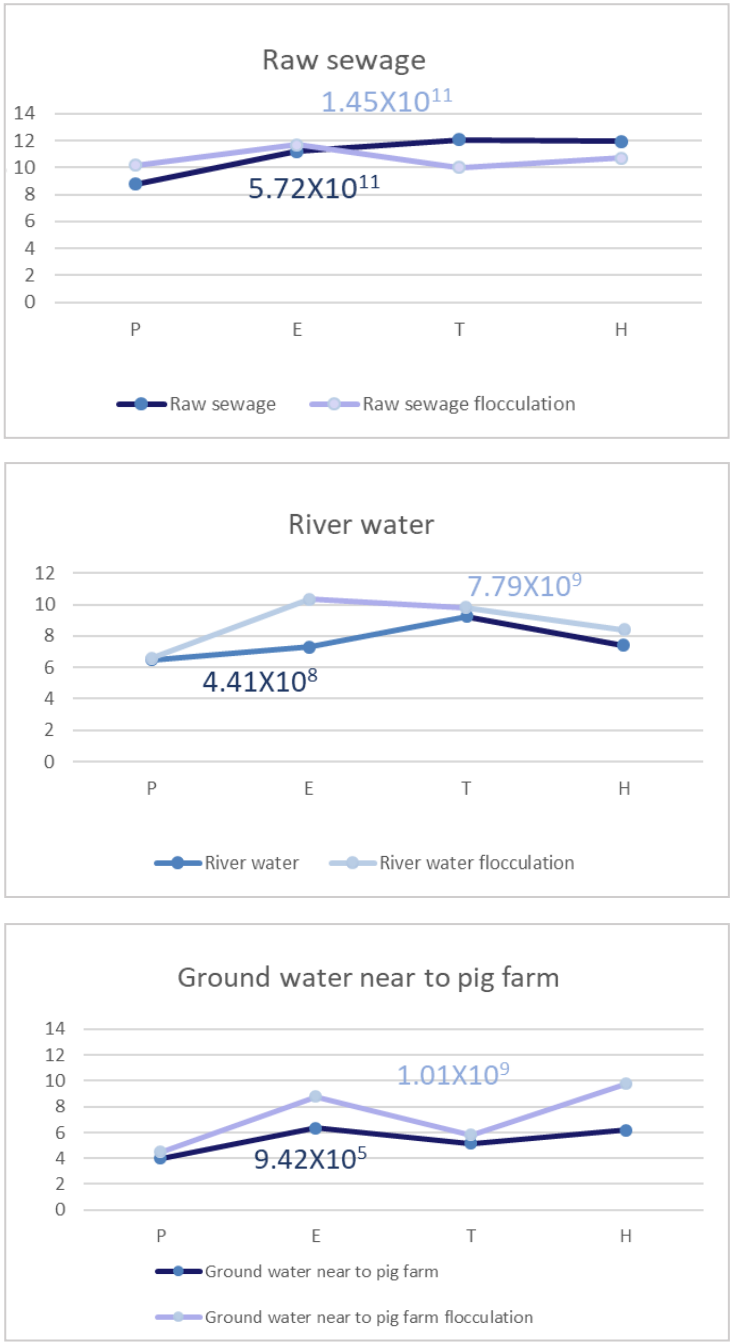


Figure 3. *Aeromonas* quantification (geometric mean/100ml) in filtered samples and concentrated by flocculation.

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4.2. Investigation of the virulence and genomics of *Aeromonas salmonicida* strains isolated from human patients.

Vincent AT*., Fernández-Bravo A*., Sanchis M., Mayayo E., Figueras MJ., Charette SJ. (2018). Infection, Genetics and Evolution. 68: 1-9.

* These authors contributed equally to this work.

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Research paper

Investigation of the virulence and genomics of *Aeromonas salmonicida* strains isolated from human patients

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ABSTRACT

The bacterium *Aeromonas salmonicida* is known since long time as a major fish pathogen unable to grow at 37 °C. However, some cases of human infection by putative mesophilic *A. salmonicida* have been reported. The goal of the present study is to examine two clinical cases of human infection by *A. salmonicida* in Spain and to investigate the pathogenicity in mammals of selected mesophilic *A. salmonicida* strains. An evaluation of the pathogenicity in a mouse model of clinical and environmental *A. salmonicida* strains was performed. The genomes of the strains were sequenced and analyzed in order to find the virulence determinants of these strains. The experimental infection in mice showed a gradient in the virulence of these strains and that some of them can cause necrotizing fasciitis and tissue damage in the liver. In addition to demonstrating significant genomic diversity among the strains studied, bioinformatics analyses permitted also to shed light on crucial elements for the virulence of the strains, like the presence of a type III secretion system in the one that caused the highest mortality in the experimental infection. Clinicians and microbiologists should consider these results for the inclusion of *A. salmonicida* in diagnosis tests since it is now clear that some mesophilic strains are also pathogens for humans.

1. Introduction

The Gram-negative bacterium *Aeromonas salmonicida* has been well known for decades to be a fish pathogen (Austin and Austin 2016). Officially, *A. salmonicida* has five subspecies (Martin-Carnahan and Joseph 2005): *salmonicida*, *smithia*, *achromogenes*, *masoucida* and *pectinolytica*. Although the taxonomy of *A. salmonicida* has always been subject to debate (Austin 2011), it was only in 2000, with the publication of the discovery of the subspecies *pectinolytica* (Pavan et al. 2000) that the diversity of this bacterium was truly revealed. While the other defined *A. salmonicida* subspecies grow only at temperatures below 25 °C, *pectinolytica* strains can grow efficiently at 37 °C and are thus considered to be mesophilic (Pavan et al. 2000). This dichotomy in the maximum growth temperatures of *A. salmonicida* was reported before the official publication of the subspecies *pectinolytica* (Altwegg et al. 1990; Guérin-Faublée et al. 1997; Janda et al. 1996; Rouf and Rigney 1971). However, at that time, the intra-species delineation of *A. salmonicida* into subspecies was not systematically used and genome sequences were not available, making conclusions difficult. Moreover, classification of *A. salmonicida* based on biochemical characteristics or 16S rRNA gene sequence has been extremely difficult and many times impossible (Beaz-Hidalgo et al. 2010).

Recently, four mesophilic *A. salmonicida* strains isolated from food in India were sequenced and characterized to shed light on genomic signatures that could explain why some evolutionarily close subspecies have such large differences in their maximum growth temperatures (Vincent et al. 2017, 2016). In accordance with previous experimental evidence based on the *salmonicida* subspecies (Tanaka et al. 2012), investigation of these genomes revealed that insertion sequences could be one of the major genomic determinants between the mesophilic and the

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1567-1348/

psychrophilic strains (Vincent et al. 2017, 2016).

Although our knowledge about *A. salmonicida* has increased significantly during recent years, the infectious potential of mesophilic strains remained unknown. While psychrophilic *A. salmonicida* subspecies are known to infect various fish species (Austin and Austin 2016), no host is certainly known for mesophilic strains. Early studies found that mesophilic *A. salmonicida* strains (known as hybridization group 3 [HG3]) could be isolated from human and animal hosts (Abbott et al. 1992; Altwegg et al. 1990; Aravena-Román et al. 2011; Janda et al. 1996; Janda and Abbott 2010). Although rigorous, these studies were made before the democratization of DNA sequencing and the recent advances in the taxonomy of *A. salmonicida* based on core genome sequence analysis. In addition, no clinical background was available for the isolates mentioned above, letting difficult to draw conclusions on the medical importance of *A. salmonicida* for humans.

In 2008, a first case of human infection by *A. salmonicida* with clear clinical background was reported (Yang et al. 2008). More precisely, a 68-year-old diabetic woman on continuous ambulatory peritoneal dialysis was diagnosed as infected by *A. salmonicida* after having been admitted for abdominal pain and cloudy peritoneal fluid. Unfortunately, there is no indication on how the strain was identified as *A. salmonicida*. Recently, in India, *A. salmonicida* was reported to have been recovered from: (i) the blood of a 34-year-old female patient (Tewari et al. 2014), (ii) a skin infection of a 67-year-old immunocompetent male (Kamble 2015) and (iii) the right eye of 55-year-old female who had recovered from a cataract surgery (Varshney et al. 2017). However, although interesting for clinical backgrounds, the taxonomic identification of these strains is putative given the inherent complexity of *A. salmonicida*.

In 2017, a study reported the isolation of a multidrug-resistant strain, ASG1, from a 15-year-old boy who had recovered from a finger surgery (Ruppé et al. 2017). This time, the strain was clearly identified as belonging to *A. salmonicida* species. Although it demonstrated once for all that mesophilic *A. salmonicida* could infect humans, the pathogenicity of these isolates and specific mechanisms that allow such infections are still unknown.

Here, we investigate two mesophilic *A. salmonicida* strains isolated from human patients in Spain, one that suffered from an acute gastroenteritis and the other that had a cellulitis in a foot after a trauma. These two clinical strains, in addition to four environmental mesophilic *A. salmonicida* strains, were tested for pathogenicity in an immunosuppressed rodent model. The complete genomes of the strains were also investigated to figure out the putative determinants implicated in the virulence of the strains.

2. Materials and methods

2.1. Isolation of the clinical strains

The strain AJ83 and 947C were isolated at a hospital in Guadalajara (Spain) (Table 1). The strain AJ83 was recovered from a cellulitis in the right foot of a 49-year-old man that also suffered of fasciitis due to trauma. The strain 947C came from the faeces of an 8-year-old girl that had an acute gastroenteritis. Both strains were first identified at the hospital as *Aeromonas hydrophila* using MicroScan W/A identification system (Dade MicroScan, Inc., Sacramento, Calif). Using the same equipment and based on the Clinical and Laboratory Standards Institute guidelines of 2015, the resistance to various antibiotics was assessed for each strain. Both strains were re-identified more thoroughly as *A. salmonicida* by sequencing the *rpoD* gene using primers and condition used in another study (Beaz-Hidalgo et al. 2010).

2.2. In vivo experiments

All *A. salmonicida* strains included in this study (Table 1) were grown on tryptic soy agar (TSA) plates and incubated at 30 °C for 24 h. The colonies were then scraped off with a sterile loop and were suspended in sterile phosphate-buffered saline (PBS) solution. For each strain, the concentration of bacterial cells was determined by plating 10-fold dilutions onto TSA plates and then by counting the number of CFU after 24 h.

Four-week-old male OF1 mice weighing approximately 30 g each (Charles River, Criffa S.A., Barcelona, Spain) were used to perform the experiments. All animals were maintained under standard conditions. The designed experiments and care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were immunosuppressed 2 days prior to infection by intraperitoneal injection of 200 mg/kg body weight of cyclophosphamide (Genoxal®; Laboratories Funk S.A., Barcelona, Spain) and thereafter the same procedure was performed once every 5 days (Sanchis et al. 2016).

Groups of 8 animals were infected intravenously at the tail with 0.2 mL of sterile PBS containing 1×10^7 or 1×10^9 CFU/mouse of the respective *A. salmonicida* strains. Parameters were selected based on previous experiments of mouse infections with *Aeromonas* (Romero et al. 2016). In all experiments, a control group of 8 mice injected with only 0.2 mL of PBS was used. At the end of the experiment, mice were euthanized by anoxia in a CO_2 chamber, followed by cervical dislocation.

The Kaplan-Meier function was used through the R package survival to verify if the survival curves were significantly different from each other. The *p*-values from the log-rank test were adjusted with the Bonferroni method ($\alpha = 0.05$).

2.3. Bacteria quantification from the different organs and histopathological analysis

The liver and kidney from the mice infected at both concentrations were directly aseptically collected when the animal died on day 10 post-infection. Each organ was divided in two parts: one part was directly frozen at −80 °C and was used for bacterial DNA quantification by real time PCR (qPCR), and the other half was directly fixed in 10% buffered formalin for histopathological studies.

The DNA was extracted using the Easy-DNA™ Kit (Invitrogen, CA),

Table 1
Mesophilic strains of *A. salmonicida* used in the present study.

Strain	Source	Country	Year	Accession number	Reference
34mel^T	River	Argentina	1988	NZ_CP022426.1	(Pavan et al. 2000)
Y47	Chicken[a]	India	2006	JZTF00000000	(Nagar et al. 2011)
A527	Giant river prawn[a]	India	2007	CP022550	(Nagar et al. 2011; Vincent et al. 2017)
A308[b]	Fresh water	France	1962	PSZJ00000000	Present study
AJ83	Human	Spain	2007	PSZI00000000	Present study
947C	Human	Spain	2008	PSZK00000000	Present study

[a] Isolated in food markets in India (Nagar et al. 2011). The real hosts are considered unknown.
[b] Strain A308 = Popoff C316 = CDC 0434–84 = CECT 5171 = LMG 13451. This strain is considered as a reference for mesophilic *A. salmonicida* (Abbott et al. 1992; Altwegg et al. 1990; Martínez-Murcia et al. 2005).

according to the manufacturer's instructions. Real-time PCR was performed on the purified DNA using the kit DNA TargetSpecies dtec-qPCR Test for *Aeromonas* sp. (Genetic PCR solutions, SP) and the StepOnePlus™ Real-Time PCR System (Applied Biosystems) equipment. The number of copies was calculated on the basis of the standard curve and the corresponding amplification cycle threshold (Ct). At the time of collection of the liver and kidney, the organs were examined to detect any macroscopic lesions. After fixation, the tissues were embedded in paraffin and sectioned before staining with hematoxylin, eosin, and Giemsa. The sections were evaluated with microscopy (CX 33, Olympus).

2.4. DNA extraction, sequencing and analysis

The strains AJ83, 947C and A308 were grown on TSA at 30 °C for 24 h and the genomic DNA extracted using Easy-DNA™ Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The DNA of strain A308 (=Popoff C316), was also sequenced since this environmental strain is considered to be a reference of the mesophilic *A. salmonicida* (known as hybridization group 3 [HG3]) by several studies (Abbott et al. 1992; Altwegg et al. 1990; Martínez-Murcia et al. 2005).

The purified DNA was used to prepare sequencing libraries using a KAPA Hyper Prep kit. The resulting libraries were sequenced using Illumina MiSeq technology (IBIS, Université Laval). The final reads were *de novo* assembled using A5-miseq version 20160825 (Coil et al. 2015). The resulting sequences were annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) of the NCBI and were deposited in GenBank (Table 1).

All the genome sequences of mesophilic *A. salmonicida* strains (including AJ83, 947C and A308 that are from the present study), the ones of selected psychrophilic *A. salmonicida* strains and finally the ones of 30 other *Aeromonas* were annotated using Prokka version 1.12 (Seemann 2014) (see Supplementary Table S1). Homologous links between the translated coding sequences were defined using GET_HOMOLOGUES version 20180103 (Contreras-Moreira and Vinuesa 2013) with two algorithms: COG and OMCL (see Supplementary Fig. S1). The 2026 gene sequences (excluding paralogs) corresponding to the soft-core, defined as the sequences present in > 95% of the genomes, were recovered and aligned by codons using TranslatorX version 1.1 (Abascal et al. 2010). The resulting alignments were filtered using BMGE version 1.12 (Criscuolo and Gribaldo 2010) and concatenated in a partitioned supermatrix using AMAS (Borowiec 2016). The best-fit model of each partition was determined using ModelFinder (Kalyaanamoorthy et al. 2017) through IQ-TREE version 1.6.1 (Nguyen et al. 2015). The maximum-likelihood phylogeny was itself done using IQ-TREE by performing 10,000 ultrafast bootstraps (Hoang et al. 2017). The Average Nucleotide Identity (ANI) values were computed for genome sequences of *A. salmonicida* using pyani (https://github.com/widdowquinn/pyani).

The antibiotic resistance genes were predicted using ABRicate version 0.8.7 (https://github.com/tseemann/abricate) and the CARD database (Jia et al., 2017). A gene sequence had to have a minimum of 80% identity on at least 70% of the length in order to annotate it as an antibiotic resistance gene. Annotation of the genes was then manually curated.

3. Results

3.1. Clinical pictures

In 2007, a 49-year-old man was hospitalized at the Guadalajara University Hospital for cellulitis and fasciitis in the right foot after trauma. The clinical background of the patient includes diabetes mellitus and Reiter syndrome, being treated with prednisone. The patient was treated by piperacillin/tazobactam and surgical debridement. The patient healed without complications. A microbial investigation at the wound exudate revealed a polymicrobial infection of *Aeromonas hydrophila*, *Staphylococcus aureus and Klebsiella oxytoca*, after MicroScan identification. No stool or blood culture was performed, since the patient did not have a fever. The *rpoD* sequence of the *Aeromonas* strain, named AJ83, revealed that this strain does not belong to the *hydrophila* species, but surprisingly to the *salmonicida* species (data not shown). This strain is resistant to three antibiotics: cefazolin, ampicillin and ticarcillin, while sensitive to piperacillin/tazobactam (see Supplementary Table S2).

One year later, at the same hospital, an 8-year-old girl without a clinical background was hospitalized for an acute gastroenteritis. The patient had bloody stools with mucus. The stool culture revealed the presence of *Campylobacter jejuni* in addition to *Aeromonas hydrophila.* A blood culture was not performed since the young girl had no fever. She had a treatment with hydration and no antibiotic was administered. Like strain AJ83, the *rpoD* sequence of the *Aeromonas* strain (named here 947C) revealed that it belongs to the species *salmonicida*. Strain 947C was shown to be resistant to cefazolin, ampicillin and cotrimoxazole (see Supplementary Table S2).

3.2. Taxonomic validation of the strains

The genome of the clinical strains AJ83 and 947C was sequenced, *de novo* assembled and used to perform a robust molecular phylogeny based on 2026 gene sequences (Fig. 1). Without any doubt, the clinical strains AJ83 and 947C are belonging to the *salmonicida* species as they clustered with the type strain of the subspecies *pectinolytica* (34mel^T) and with the other already known *A. salmonicida* mesophilic strains. Moreover, they cluster along other mesophilic strains, as strain A308. Interestingly, strain 947C cluster with strain A308, which is environmental. On its side, strain AJ83 form a group with Y567 and Y47, two strains isolated from food in Mumbai (India) and for which no host is known (Fig. 1). The ANI values revealed that available genomes of mesophilic strains are distant in terms of nucleotide sequences, although they come from strains of the same species (ANI ≥ 0.96) (Fig. 1). Only strains AJ83 and Y567 were more closely related comparatively to other strains (ANI value of 99%).

3.3. Pathogenicity of strains

The pathogenicity of six mesophilic *A. salmonicida* strains was evaluated by infecting mice (Fig. 2). Two doses were tested, 1×10^7 and 1×10^9 CFU/mouse. At 1×10^7, a clear dichotomy in the survival rate of mice can be observed between strains (Fig. 2-A). The most virulent strain is the clinical one 947C followed by strain A308, which has been isolated from fresh water. There is no statistical significant difference in the mortality caused by both strains (see Supplementary Table S3), which are in the same phylogenetic cluster (Fig. 1). The less virulent strains include A527, Y47, AJ83 and 34mel^T (subspecies *pectinolytica*). Here again, there is no significant statistical difference in the mortality caused by those strains.

Three groups of strains based on virulence can be observed at the dose of 1×10^9 CFU/mouse (Fig. 2-B). The pathogenicity of 947C is striking, with all mice being death after only three days post-infection. As seen in the test at the dose of 1×10^7 CFU/mouse, strains A527, Y47 and 34mel^T are the less virulent and without significant difference in the mortality caused by them (see Supplementary Table S4). At this dose, the environmental strain A308 showed to have an intermediate virulence, along with strain AJ83. Although they are less virulent than strain 947C, both strains A308 and AJ83 killed all mice before the end of the experiment.

Interestingly, both clinical strains 947C and AJ83 and the Indian strain Y47 produced lesions on the mouse tails, at the injection site (Fig. 2-C). This cutaneous infection that is typical of necrotizing fasciitis was only seen at the lowest dose (1×10^7 CFU/mouse) for 947C, likely because mice died too quickly at the dose of 1×10^9 CFU/mouse.

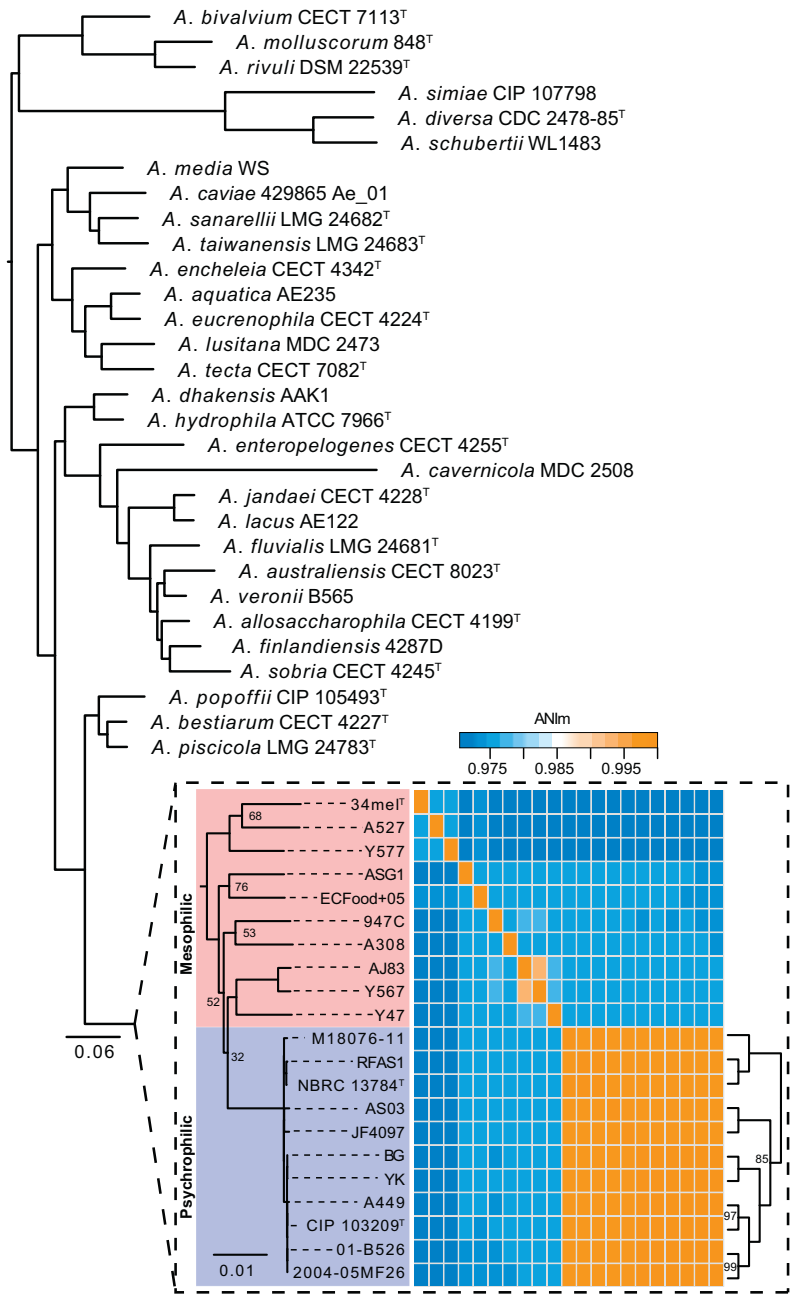

Fig. 1. Phylogenetic tree of 51 strains of *Aeromonas*. The tree is based on 2026 gene sequences using the methodology described in the Materials and Methods section. For the sake of clarity, the focus is on mesophilic (red) and psychrophilic (blue) strains of the species *salmonicida*. Bootstrap values are only shown if they are < 100. The heatmap represents the ANI values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Strains A308, A527 and 34mel^T did not produce visible cutaneous infections.

3.4. Bacteria quantification from the different organs and histopathological studies

The presence of bacterial DNA in the liver and kidney of mice was quantified by qPCR (Fig. 3). Significantly higher amounts of DNA ($p < .05$) were found in both organs for the clinical strains (947C and AJ83), than for the environmental strains. A higher amount of *Aeromonas* DNA was detected in liver than in kidney. It is interesting to note that the DNA of the environmental strain A308 was present in larger quantities than other environmental strains, in both organs. In liver and at a higher dose, more DNA of strain A308 was detected than clinical

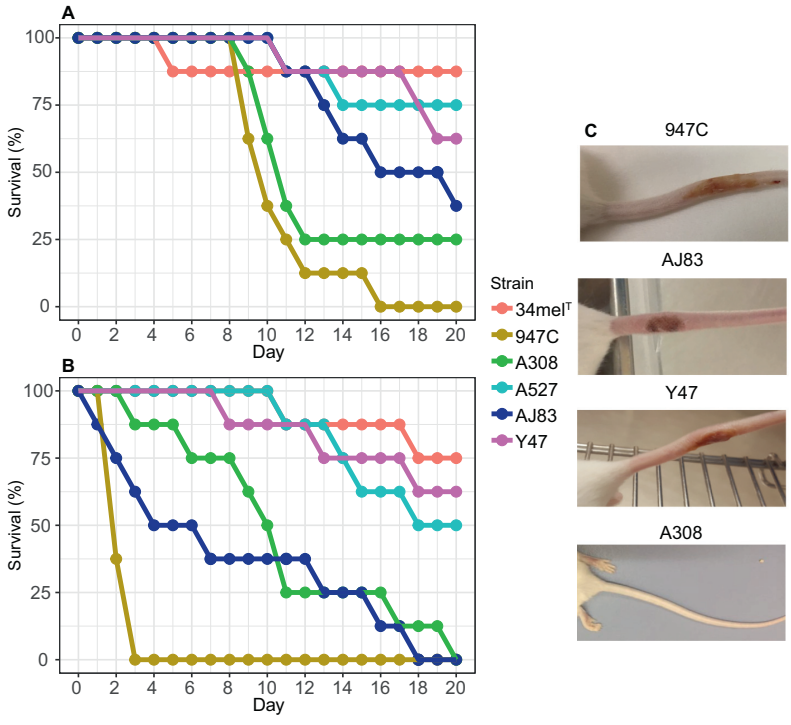

Fig. 2. Virulence tests in a mouse model. Survival rate of mice at doses of (A) 1×10^7 and (B) 1×10^9 CFU/mouse of six mesophilic *A. salmonicida* strains. (C) Pictures showing the lesions at the infection site caused by strains 947C, AJ83 and Y47. The pictures of the mouse tail infected with A308 provides a negative control for the lesions observed with the other strains.

strain AJ83 (Fig. 3A). The results obtained with the clinical strains showed a significantly greater amount of DNA of strain 947C, the most pathogenic one, at a lower dose (1×10^7) than for strain AJ83 at both doses.

Histopathological examination with hematoxylin and eosin or Giemsa staining showed no damage in the kidney (see Supplementary Fig. S2). However, the liver revealed various levels of multifocal and diffuse necrotic changes and infiltration of polymorphonuclear cells (PMNs), with inflammatory response as shown in Fig. 4. Specifically, tissues collected from animals infected with strain 947C at dose 1×10^7 showed more PMN infiltration and necrotic cells (Fig. 4A), than for strain AJ83 at dose 1×10^9 (Fig. 4B). In addition, the Giemsa staining confrimed the observation of PMN cells and the inflammatory response (Fig. 4C).

3.5. Genomic investigation

When checking the genome of strain 947C, the most virulent one, several genes involved in a type III secretion system (T3SS) were found (see Supplementary Table S5). However, it is unclear what make strains AJ83 and A308 virulent. A high number of genes that encode for hypothetical proteins were predicted to be encoded in their genomes and we cannot rule out that some of them are implied in virulence.

When looking for the presence of CDSs that encode known virulence factors (Rasmussen-Ivey et al. 2016), the gene *ast* (cytotonic enterotoxin) was found exclusively in the genomes of clinical strains 947C and AJ83 (see Supplementary Table S6). Finally, the mouse infections clearly demonstrated that strains 947C, AJ83 and Y47 can cause necrotizing fasciitis (Fig. 2). Only five orthologous genes, not yet associated with virulence in *Aeromonas salmonicida*, were found to be present in the genomes of these three strains and absent from those of strains 34mel^T, A308 and A527 (Table 2). Interestingly, four of these five genes were already listed in the literature as virulence factors in human pathogens such as *A. hydrophila*, *Helicobacter pylori*, *Leptospira* sp. and *Salmonella enterica* (Table 2).

It was also interesting to investigate the genes that could be involved in antibiotic resistance for the mesophilic strains of *A. salmonicida* (see Supplementary Table S7). All strains have genes predicted to be involved in antibiotic resistance (from 2 to 12 genes). Two genes were predicted to be encoded in the genome of all strains: *OXA-12* (resistance to cephalosporin and penam) and *cphA5* (resistance to carbapenem) genes. The two strains with the most antibiotic resistance genes are ASG1 (12 genes), isolated from a human patient, and ECFood +05 (10 genes) for which little information is available. The most virulent strain in the mouse model (Fig. 2), 947C, is predicted to have genes involved in resistance to several compounds: aminoglycoside, cephalosporin, penam and carbapenem. The second strain isolated from a human patient for the present study, AJ83, presents almost the same antibiotic resistance pattern as 947C, only differing by the absence of the gene involved in resistance to aminoglycoside compounds.

4. Discussion

Earlier studies on human cases of *A. salmonicida* infections lack clinical metadata or are taxonomically uncertain (Abbott et al. 1992; Altwegg et al. 1990; Aravena-Román et al. 2011; Janda et al. 1996; Kamble 2015; Ruppé et al. 2017; Tewari et al. 2014; Varshney et al. 2017) compared to what can be done now with core genome phylogeny (Vincent et al. 2016). Recently, the strain ASG1, clearly identified as *A. salmonicida*, was isolated from a 15-year-old boy that recovered from a finger surgery (Ruppé et al. 2017). Unfortunately, another pathogen, *Stenotrophomonas maltophilia,* was co-isolated with strain ASG1, making it impossible to draw firm conclusions on clinical aspects of the ASG1 strain. The present study clearly demonstrated for the first time by combining experimental infection essays and whole genome analyses that some mesophilic *A. salmonicida* strains are able to infect mammals.

It is not surprising that T3SS seems to be a major virulence factor, as shown by the striking mortality caused by strain 947C. T3SS is known

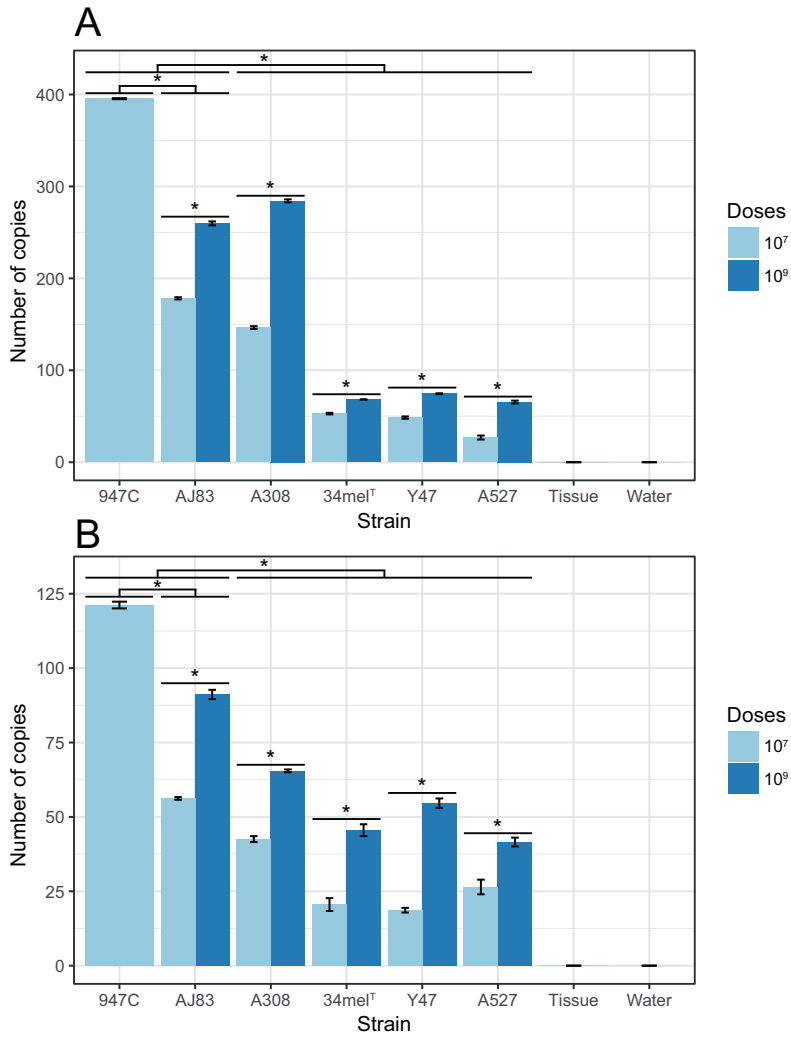

Fig. 3. Concentration of *Aeromonas* DNA determined by qPCR in mice liver (A) and kidney (B) tissues 10 days after intravenous infection at doses 1×10^7 and 1×10^9. *Statistical significance ($p < .05$). Tissue = non-infected tissue, Water = only water without tissue; both used as negative controls.

to be an important virulence factor in several Gram-negative bacteria, including the human pathogens *A. hydrophila* and *Aeromonas veronii* (Chacón et al. 2004; Vilches et al. 2004) and the fish pathogen *A. salmonicida* subsp. *salmonicida* (Frey and Origgi 2016).

Interestingly, some *A. salmonicida* strains have the ability to cause cutaneous infections that look like necrotizing fasciitis. In addition to the pathogenicity tests done in the present study, ASG1 was isolated from a finger that recovered from surgery (Ruppé et al. 2017) and AJ83 isolated from the right foot of 49-year-old man that suffered of fasciitis due to trauma. Investigation of the genomes revealed five genes that are candidates to explain why only three strains cause necrotizing fasciitis (Table 2). In addition to these genes, which may help explain the ability of some strains to cause necrotizing fasciitis, it was observed that even a low level of virulence can cause this type of infection. Strain 947C, which is the most virulent, causes a necrotizing fasciitis only at the lowest dose (Fig. 2). The other two strains that can cause this skin infection, AJ83 and Y47, cause a low or intermediate mortality level. It is possible to postulate some similarities with the subspecies *salmonicida*, which causes two forms of furunculosis in salmonids (Austin and Austin 2016). The chronic form of the disease causes a low mortality rate and is often characterized by a cutaneous appearance known as furuncles, hence the name of the disease. The acute form of the disease causes a high mortality rate (2 to 3 days) due to septicaemia and does not manifest cutaneously.

A significant amount of *Aeromonas* DNA was found in the livers of fish (more than in their kidneys) by qPCR (Fig. 3). Similar results were described with *A. hydrophila* in channel catfish, where the bacterium was detected only in the liver > 48 h post-infection and was eliminated from the other organs, including the kidney, of the fish (Zhang et al. 2016). The quantification obtained from the clinical strains correlated with the results of the histopathological examination, which showed important pathological changes in the liver while no damage was observed in the kidney (Fig. 4 and Supplementary Fig. S2). The fact that bacterial DNA was detected in the kidney at relatively low levels could be related to the process of their elimination with the urine.

Although preliminary, the degree of pathogenicity does not seem to be associated with strains of a specific phylogenetic group. However, the study of the pathogenicity of mesophilic *A. salmonicida* is still in its

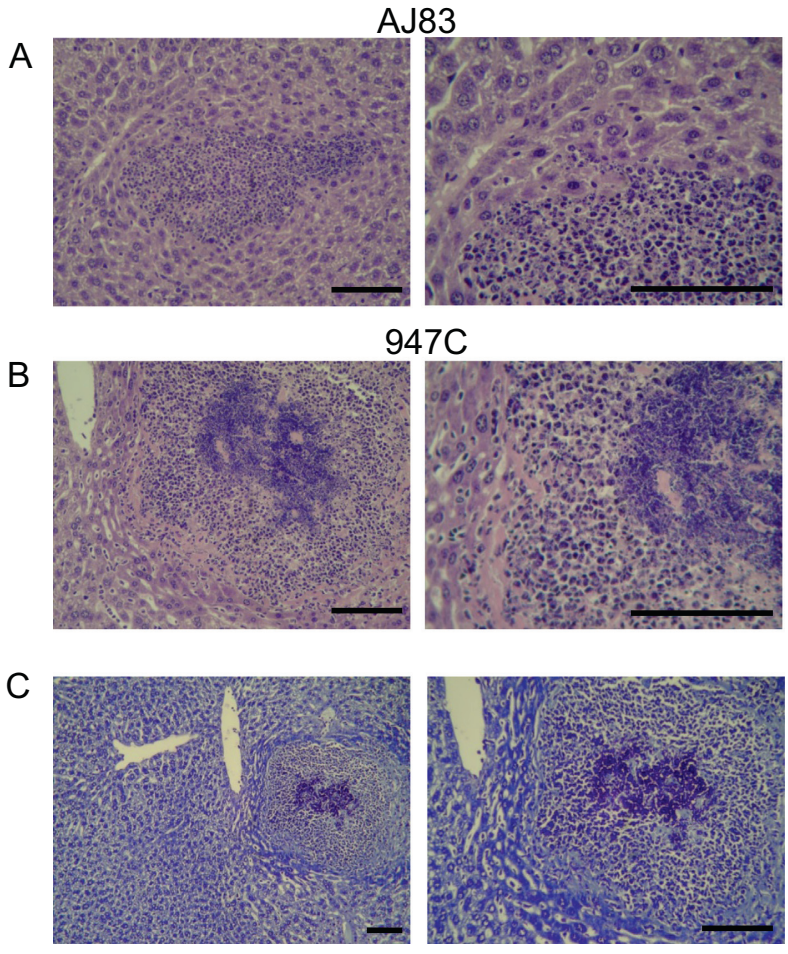

Fig. 4. Histopathological examination of mouse liver tissue 10 days after intravenous infection with two *Aeromonas* strains (AJ83 and 947C) of clinical origin. (A) Strain AJ83 at dose 1×10^9 CFU with hematoxylin/eosin staining. (B) Strain 947C at dose 1×10^7 CFU with hematoxylin/eosin staining (C) Strain 947C at dose 1×10^7 CFU with Giemsa staining. Bars represent 100 µm.

infancy and strains from various hosts will be needed to clarify the evolutionary links between these strains. The fact that the genomes of only two out of ten mesophilic *A. salmonicida* strains are similar at the nucleotide level demonstrates a great diversity in the mesophilic strains of this bacterium (Fig. 1). One of these two strains, AJ83, has a clinical origin while the second, Y567, was isolated from food.

The psychrophilic strains of *A. salmonicida* are officially divided into different subspecies: *salmonicida, smithia, achromogenes* and *masoucida,* whereas there is only one official mesophilic subspecies, *pectinolytica.* However, according to the molecular phylogeny and ANI values, the mesophilic strains of *A. salmonicida* characterized so far have greater genetic diversity than the psychrophilic strains of the same species. This fact rises, as mentioned before (Vincent et al. 2017), a certain taxonomic dilemma. It is obvious that it will be necessary to review the taxonomy of *A. salmonicida* in order to unify in a cohesive manner the mesophilic and psychrophilic strains of this species. A first scenario could be to classify mesophilic strains into different subspecies. A second scenario would be to make two subspecies, one comprising all

Table 2
CDSs present only in strains 947C, AJ83 and Y47.

Protein	Virulence trait	Ref
Two pore domain potassium channel family protein	N/A[a]	N/A
Hemerythrin	*A. hydrophila* survival in host macrophages	(Zeng et al. 2016)
Pseudaminic acid cytidylyltransferase	Colonisation of *H. pylori*	(Wahid 2017)
Catalase KatE[b]	Virulence of *Leptospira* spp. in animal models	(Eshghi et al. 2012)
UDP-*N*-acetylglucosamine-1-phosphate transferase[c]	Production of enterobacterial antigen in *S. enterica*	(Gilbreath et al. 2012)

[a] N/A, none-applicable.
[b] The catalase was annotated as KatE by PATRIC (Wattam et al. 2017).
[c] The CDS in strain Y47 appears to be divergent compared to those of strains 947C and AJ83.

the mesophilic strains and the other all the psychrophilic strains. In any case, before considering one of these scenarios, it will be necessary to continue to isolate new mesophilic and psychrophilic strains of *A. salmonicida* in order to obtain a broader view of the different genetic and phenotypic characteristics, thus making it possible to establish a robust and representative taxonomy of this bacterium. Also, it is crucial to take into account that the mesophilic *A. salmonicida* strains can be easily misidentified as *A. hydrophila* and that the use of molecular methods such as the sequence of the *rpoD* gene are required to correctly assign the taxonomy of these strains (Beaz-Hidalgo et al. 2010).

The two clinical strains investigated in the present study were shown to be resistant to some antibiotics (see Supplementary Table S2) and also to harbor genes known to be involved in resistance (see Supplementary Table S7). In fact, the resistance gene repertoires of strains 947C and AJ83 differ only in that 947C has a gene that causes resistance to aminoglycoside antibiotics. It is surprising that strain AJ83 is resistant to ticarcillin, belonging to the penem drug class, while strain 947C is sensitive to this antibiotic (see Supplementary Table S2). Similarly, strain 947C is resistant to cotrimoxazole, belonging to sulphonamide/diaminopyrimidinedrug class, while strain AJ83 is sensitive. It is still unclear why strains 947C and AJ83 differ in their resistance to these antibiotics. Other species of the genus *Aeromonas* are known to harbor genes involved in antibiotic resistance (Piotrowska and Popowska 2015). This is the case, for example, of the fish pathogen *A. salmonicida* subsp. *salmonicida*, for which several strains are multiresistant to all antibiotic approved in aquaculture in Canada (Trudel et al. 2016; Vincent et al. 2014). A similar pattern of multiple resistance seems to be apparent in mesophilic strains of the *salmonicida* species where some strains, such as ASG1 and ECFood + 05, were predicted to harbor > 10 genes involved in resistance to antibiotic compounds. This is even more interesting given the context that these two strains cluster together in the phylogenetic tree (Fig. 1), suggesting that some mesophilic *A. salmonicida* strains that arise from a particular common ancestor could be more prone to having antibiotic resistance genes. Given that both ASG1 and ECFood + 05 only share four genes (*OXA-12*, *cphA5*, *aadA* and *tet*(E)), it is reasonable to believe that the other genes could have been acquired by horizontal gene transfers. Moreover, the multiple resistance to antibiotics of ASG1 strain was confirmed experimentally (Ruppé et al. 2017). Closely monitoring mesophilic *A. salmonicida* will be essential to effectively treat cases of infection by strains of this bacterium.

4.1. Concluding remarks

In this study, it was possible to demonstrate robustly that the mesophilic strains of *A. salmonicida* can infect mammals, with varying levels of pathogenicity between strains. It will be essential in the future to isolate new mesophilic *A. salmonicida* strains and to verify their geographical distribution. The clinical strains AJ83 and 947C investigated in the present study come from Spain. However, several clinical studies have documented cases in India of infections in humans from putatively mesophilic *A. salmonicida* strains (Kamble 2015; Tewari et al. 2014; Varshney et al. 2017). Moreover, environmental strains from India have clearly been identified as mesophilic *A. salmonicida* (Vincent et al. 2017, 2016).

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.11.019.

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
Ana Fernández Bravo

Supplementary Materials

Investigation of the virulence and genomics of *Aeromonas salmonicida* strains isolated from human patients

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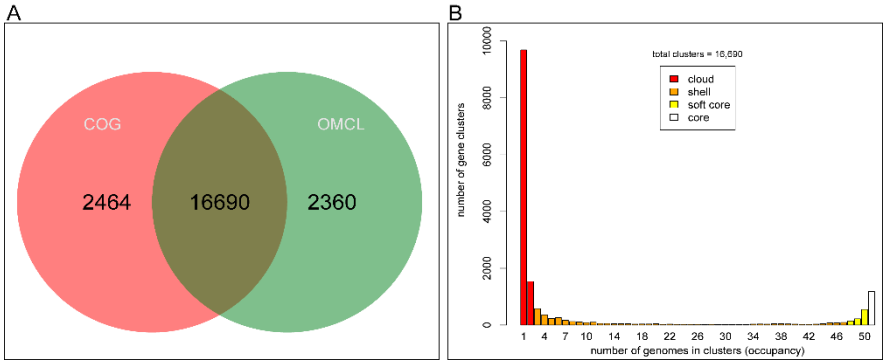

Figure S1. Pan-genome analysis for the 51 *Aeromonas* genome sequences used in the present study**.** (A) Gene clusters found by the COG (Kristensen et al., 2010) and OMCL (Li et al., 2003) algorithms that were used by the tool GET_HOMOLOGUES (Contreras-Moreira and Vinuesa, 2013) to find homologous links between the coding sequences encoded by the set of genomes (Table S1). (B) Distribution of the pan-genome in four categories.

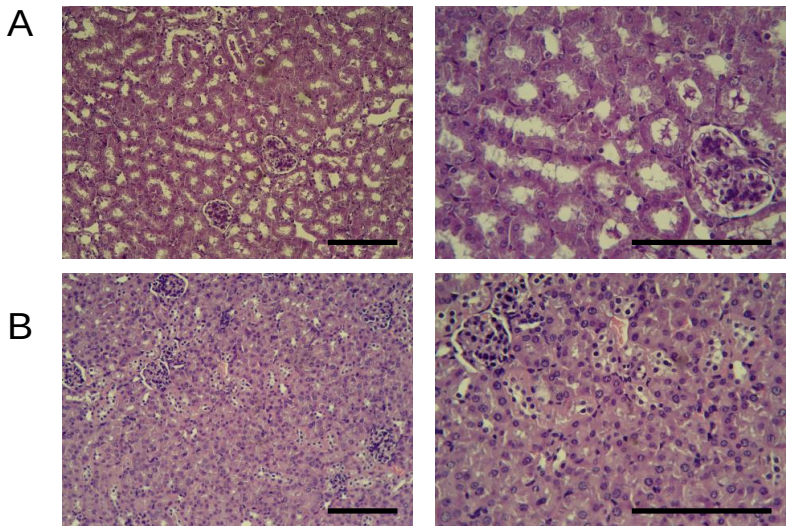

Figure S2. Histopathological examination of kidney mice tissues 10 days after i.v. infection with two *Aeromonas* strains (AJ83 and 947C) of clinical origin. Both strains showed to have caused no apparent damage in the glomeruli nor in the tubules of the kidney. (A) Strain AJ83 at dose 1 X 10^9 CFU with hematoxylin/eosin staining. (B) Strain 947C at dose 1 X 10^7 CFU. Bars represent 100 µm.

Results

Table S1. Genome sequences of *Aeromonas* used for the phylogenetic analysis.

Species	Strain	GenBank	Reference
A. allosaccharophila	CECT 4199^T	NZ_CDBR00000000	(Colston et al., 2014)
A. aquatica	AE235	NZ_JRGL00000000	(Hossain et al., 2014)
A. australiensis	CECT 8023^T	NZ_CDDH00000000	(Colston et al., 2014)
A. bestiarum	CECT 4227^T	NZ_CDDA00000000	(Colston et al., 2014)
A. bivalvium	CECT 7113^T	NZ_CDBT00000000	(Colston et al., 2014)
A. cavernicola	MDC 2508	NZ_PGGC01000000	(Martínez-Murcia et al., 2013)
A. caviae	429865 Ae_01	NZ_LIIX01000001	(Padilla et al., 2015)
A. dhakensis	AAK1	NZ_BAFL00000000	(Wu et al., 2012)
A. diversa	CDC 2478-85^T	NZ_APVG00000000	(Farfán et al., 2013)
A. encheleia	CECT 4342^T	NZ_CDDI00000000	(Colston et al., 2014)
A. enteropelogenes	CECT 4255^T	NZ_CDDE00000000	(Colston et al., 2014)
A. eucrenophila	CECT 4224^T	NZ_CDDF00000000	(Colston et al., 2014)
A. finlandiensis	4287D	NZ_JRGK00000000	(Beaz-Hidalgo et al., 2015)
A. fluvialis	LMG 24681^T	NZ_CDBO00000000	(Colston et al., 2014)
A. hydrophila	ATCC 7966^T	NC_008570.1	(Seshadri et al., 2006)
A. jandaei	CECT 4228^T	NZ_CDBV00000000	(Colston et al., 2014)
A. lacus	AE122	NZ_JRGM00000000	(Beaz-Hidalgo et al., 2015)
A. lusitana	MDC 2473	PGCP01000000	(Martínez-Murcia et al., 2016)
A. media	WS	NZ_CP007567.1, NZ_CP007568.1	(Chai et al., 2012)
A. molluscorum	848^T	NZ_AQGQ00000000	(Spataro et al., 2013)
A. piscicola	LMG 24783^T	NZ_CDBL00000000	(Colston et al., 2014)
A. popoffii	CIP 105493^T	NZ_CDBI00000000	(Colston et al., 2014)
A. rivuli	DSM 22539^T	NZ_CDBJ01000000	(Colston et al., 2014)
A. sanarellii	LMG 24682^T	NZ_CDBN00000000	(Colston et al., 2014)
A. schubertii	WL1483	NZ_CP013067.1	(Liu et al., 2016)
A. simiae	CIP 107798	NZ_CDBY00000000	(Colston et al., 2014)
A. sobria	CECT 4245^T	NZ_CDBW01000000	(Colston et al., 2014)
A. taiwanensis	LMG 24683^T	NZ_BAWK00000000	(Wang et al., 2014)
A. tecta	CECT 7082^T	NZ_CDCA00000000	(Colston et al., 2014)
A. veronii	B565	NC_015424.1	(Li et al., 2011)
A. salmonicida* subsp. *salmonicida	01-B526	AGVO00000000	(Charette et al., 2012)
A. salmonicida* subsp. *salmonicida	2004-05MF26	JRYW00000000	(Vincent et al., 2015)
A. salmonicida* subsp. *salmonicida	A449	CP000644.1	(Reith et al., 2008)
A. salmonicida* subsp. *salmonicida	CIP 103209^T	CDDW00000000	(Colston et al., 2014)

Table S1. Continued.

Species	Strain	GenBank	Reference
A. salmonicida* subsp. *salmonicida	BG	LUHO00000000	(Long et al., 2016)
A. salmonicida* subsp. *salmonicida	YK	LUHP00000000	(Long et al., 2016)
A. salmonicida* subsp. *achromogenes	AS03	AMQG00000000	(Han et al., 2013)
A. salmonicida* subsp. *smithia	JF4097	JZTI00000000	(Vincent et al., 2016)
A. salmonicida* subsp. *masoucida	NBRC 13784^{T}	BAWQ01000000	N/A
A. salmonicida* subsp. *masoucida	RFAS1	NZ_CP017143.1	(Han et al., 2011)
A. salmonicida	M18076-11	NQMJ00000000.1	(Rouleau et al., 2018)
A. salmonicida	Y47	JZTF00000000	(Vincent et al., 2016)
A. salmonicida	Y567	JZTG00000000	(Vincent et al., 2016)
A. salmonicida	Y577	JZTH00000000	(Vincent et al., 2016)
A. salmonicida	A527	CP022550	(Vincent et al., 2017)
A. salmonicida	ECFood+05	NZ_NVQH01000000	N/A
A. salmonicida	ASG1	PRJNA377399	(Ruppé et al., 2017)
A. salmonicida	A308[a]	PSZJ00000000	Present study
A. salmonicida	947C	PSZK00000000	Present study
A. salmonicida	AJ83	PSZI00000000	Present study
A. salmonicida* subsp. *pectinolytica	34mel^{T}	NZ_CP022426.1	(Gulla et al., 2016; Pavan et al., 2015)

a: Strain A308 = Popoff C316 = CDC 0434-84 = CECT 5171 = LMG 13451. This strain is considered to be a reference for mesophilic *A. salmonicida* (Abbott et al., 1992; Altwegg et al., 1990; Martínez-Murcia et al., 2005).

Table S2. Sensitivity and resistance to various antibiotics.

			Minimum inhibitory concentration mg/L	
Antibiotic	**Class**	**Accession**	**947C**	**AJ83**
Gentamicin	aminoglycoside	ARO[a]	< 4 S[b]	< 4 S
Tobramycin	aminoglycoside	ARO:0000052	< 4 S	< 4 S
Amikacin	aminoglycoside	ARO:0000013	< 8 S	< 8 S
Meropenem	carbapenem	ARO:0000073	< 4 S	< 4 S
Imipenem	carbapenem	ARO:3000170	2 S	< 1 S
Cefazolin	cephalosporin	ARO:0000058	> 16 R	> 16 R
Cefuroxime	cephalosporin	ARO:0000063	< 8 S	< 8 S

Table S2. Continued.

			Minimum inhibitory concentration mg/L	
Antibiotic	**Class**	**Accession**	**947C**	**AJ83**
Cefotaxime	cephalosporin	ARO:3000645	< 0.5 S	< 0.5 S
Ceftazidime	cephalosporin	ARO:0000060	< 1 S	< 1 S
Cefepime	cephalosporin	ARO:0000059	< 1 S	< 1 S
Cefoxitin	cephamycin	ARO:0000008	< 8 S	< 8 S
Ciprofloxacin	fluoroquinolone	ARO:0000036	< 0.12 S	< 0.12 S
Ofloxacin	fluoroquinolone	ARO:3000663	< 0.5 S	< 0.5 S
Aztreonam	monobactam	ARO:3000550	< 1 S	< 1 S
Piperacillin	penam	ARO:0000078	< 16 S	< 16 S
Piperacillin/tazobactam	penam	ARO:3004021	< 16 S	< 16 S
Amoxicillin/clavulanate	penam	ARO:3003997	< 4 S	< 4 S
Ampicillin	penam	ARO:3000637	> 16 R	> 16 R
Ticarcillin	penem	ARO:3003832	< 16 S	64 R
Cotrimoxazole	sulphonamide/diaminopyrimidine	ARO:3004024	> 2 R	<2 S

a: gentamicin A = ARO:3004015; gentamicin B = ARO:3000655; gentamicin C = ARO:0000014.

b: S = sensitive; R = resistant.

Table S3. p-values for the pairwise comparisons of mice mortality caused by the strains at 1×10^7 CFU/mouse

Strain	**34mel^T**	**947C**	**A308**	**A527**	**AJ83**
947C	0.0160	-	-	-	-
A308	0.3849	1.0000	-	-	-
A527	1.0000	0.0082	0.3906	-	-
AJ83	1.0000	0.0363	1.0000	1.0000	-
Y47	1.0000	0.0034	0.8064	1.0000	1.0000

Table S4. p-values for the pairwise comparisons of mice mortality caused by the strains at 1×10^9 CFU/mouse

Strains	**34mel^T**	**947C**	**A308**	**A527**	**AJ83**
947C	0.0011	-	-	-	-
A308	0.0100	0.0042	-	-	-
A527	1.0000	0.0011	0.1209	-	-
AJ83	0.0085	0.3835	1.0000	0.0958	-
Y47	1.0000	0.0011	0.0774	1.0000	0.0351

Table S5. Specific genes found in a group of mesophilic *A. salmonicida* strains.

Family ID	**Description**	**Proteins**	**Mean**	**Std Dev**
	Present only in the genome of strain 947C			
PGF_00557086	17 kDa surface antigen precursor	1	113	0
PGF_00426666	5-methylcytosine-specific restriction enzyme A (EC 3.1.21.-)	1	225	0
PGF_00035135	Adenine-specific methyltransferase (EC 2.1.1.72)	1	223	0
PGF_06393555	Aminoglycoside 3"-phosphotransferase (EC 2.7.1.87) => APH(3")-I	1	267	0
PGF_01082199	Aminoglycoside 6"-phosphotransferase (EC 2.7.1.72) => APH(6")-Ic/APH(6)-Id	1	247	0
PGF_00416326	Capsular polysaccharide synthesis enzyme Cap5L	1	346	0
PGF_00417450	Chaperone protein YscY (Yop proteins translocation protein Y)	1	114	0
PGF_05997426	ClpB-like protein	1	880	0
PGF_00418902	Conjugative transfer protein TrbB	1	355	0
PGF_00418905	Conjugative transfer protein TrbC	1	127	0
PGF_00418908	Conjugative transfer protein TrbD	1	90	0
PGF_00418912	Conjugative transfer protein TrbE	1	816	0
PGF_00418915	Conjugative transfer protein TrbF	1	234	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain 947C			
PGF_0041 8918	Conjugative transfer protein TrbG	1	330	0
PGF_0041 8922	Conjugative transfer protein TrbI	1	426	0
PGF_0041 8926	Conjugative transfer protein TrbJ	1	245	0
PGF_0041 8931	Conjugative transfer protein TrbK	1	108	0
PGF_0041 8937	Conjugative transfer protein TrbK	1	91	0
PGF_0307 0810	Conjugative transfer protein TrbL	1	454	0
PGF_0041 9496	CopG domain-containing protein	1	154	0
PGF_0041 9652	Coupling protein VirD4, ATPase required for T-DNA transfer	1	659	0
PGF_0042 1978	DNA-binding protein	1	381	0
PGF_0042 2437	DUF1176 domain-containing protein	1	332	0
PGF_0042 3998	EF hand domain protein	4	318.7	230.2
PGF_0042 5042	Exodeoxyribonuclease VIII (EC 3.1.11.-)	1	463	0
PGF_0000 0945	FIG00904992: hypothetical protein	1	135	0
PGF_0688 9881	Flagellar motor rotation protein MotA	1	285	0
PGF_0457 2835	Flagellar regulatory protein FleQ	1	419	0
PGF_0000 8924	Glycosidases	1	181	0
PGF_0089 6896	Homology to phage-tail assembly proteins	1	183	0
PGF_0119 6079	IcmF-related protein	1	1165	0
PGF_0055 7082	LPXTG-motif cell wall anchor domain protein	1	472	0
PGF_0081 5743	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	1	453	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain 947C			
PGF_00018067	Lysozyme (EC 3.2.1.17)	1	165	0
PGF_00022405	Modification methylase EcoRI (EC 2.1.1.72)	1	339	0
PGF_03821650	Outer membrane protein ImpK/VasF, OmpA/MotB domain	1	259	0
PGF_00032526	Phage protein	1	418	0
PGF_02742028	Phage terminase, large subunit	1	618	0
PGF_02599114	Plasmid replication initiator protein	1	284	0
PGF_00036213	Predicted purine nucleoside transporter, MFS superfamily	1	387	0
PGF_00037022	Probable dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133)	1	295	0
PGF_01929932	Protein ImpG/VasA	1	588	0
PGF_00037960	Protein StbA	1	347	0
PGF_02923769	Putative large exoprotein involved in heme utilization or adhesion of ShlA/HecA/FhaA family	1	621	0
PGF_00047100	RecT protein	1	350	0
PGF_01022609	Resolvase	1	204	0
PGF_00056927	Tir chaperone	1	128	0
PGF_04402936	Transcriptional regulator, Xre family	1	110	0
PGF_00698550	Transposase	1	775	0
PGF_04646018	Type III secretion bridge between inner and outer membrane lipoprotein (YscJ,HrcJ,EscJ, PscJ)	1	240	0
PGF_00063481	Type III secretion chaperone protein for YopD (SycD)	1	167	0
PGF_02808153	Type III secretion chaperone protein for YopE (SycE)	1	131	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain 947C			
PGF_0006 3488	Type III secretion cytoplasmic ATP synthase (EC 3.6.3.14, YscN,SpaL,MxiB,HrcN,EscN)	1	440	0
PGF_0006 3489	Type III secretion cytoplasmic LcrG inhibitor (LcrV,secretion and targeting control protein, V antigen)	1	272	0
PGF_0006 3492	Type III secretion cytoplasmic protein (YscF)	1	85	0
PGF_0006 3494	Type III secretion cytoplasmic protein (YscI)	1	112	0
PGF_0006 3498	Type III secretion cytoplasmic protein (YscL)	1	212	0
PGF_0006 3508	Type III secretion host injection and negative regulator protein (YopD)	1	298	0
PGF_0006 3511	Type III secretion host injection protein (YopB)	1	394	0
PGF_0006 3525	Type III secretion inner membrane channel protein (LcrD,HrcV,EscV,SsaV)	1	705	0
PGF_0006 3540	Type III secretion inner membrane protein (YscQ,homologous to flagellar export components)	1	308	0
PGF_0006 3543	Type III secretion inner membrane protein (YscR,SpaR,HrcR,EscR,homologous to flagellar export components)	1	217	0
PGF_0006 3548	Type III secretion inner membrane protein (YscS,homologous to flagellar export components)	1	88	0
PGF_0006 3553	Type III secretion inner membrane protein (YscT,HrcT,SpaR,EscT,EpaR1,homologous to flagellar export components)	1	262	0
PGF_0309 7682	Type III secretion inner membrane protein (YscU,SpaS,EscU,HrcU,SsaU, homologous to flagellar export components)	1	352	0
PGF_0006 3566	Type III secretion outer membrane contact sensing protein (YopN,Yop4b,LcrE)	1	293	0
PGF_0006 3575	Type III secretion outer membrane pore forming protein (YscC,MxiD,HrcC, InvG)	1	609	0
PGF_0006 3603	Type III secretion spans bacterial envelope protein (YscG)	1	117	0
PGF_0006 3613	Type III secretion thermoregulatory protein (LcrF,VirF,transcription regulation of virulence plasmid)	1	95	0
PGF_0512 5858	Type IV secretory pathway, VirD2 components (relaxase)	1	660	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain 947C			
PGF_0393 5134	Type IV secretory pathway, protease TraF	1	199	0
PGF_0006 3807	Type VI secretion lipoprotein/VasD	1	171	0
PGF_0006 3810	Type VI secretion protein VasI	1	201	0
PGF_0006 3969	UDP-N-acetyl-L-fucosamine synthase (EC 5.1.3.28)	1	377	0
PGF_0006 4344	UPF0380 proteins YafZ and homologs	1	276	0
PGF_0606 6221	Uncharacterized protein ImpA	1	481	0
PGF_0006 4962	Uncharacterized protein ImpC	1	492	0
PGF_0297 9767	Uncharacterized protein ImpH/VasB	1	332	0
PGF_0006 4987	Uncharacterized protein ImpI/VasC	1	411	0
PGF_0006 4990	Uncharacterized protein ImpJ/VasE	1	444	0
PGF_0006 5739	Uncharacterized protein similar to VCA0109	1	143	0
PGF_0006 6079	Unknown, probably involved in type III secretion	1	147	0
PGF_0007 1340	flagellin modification protein FlmH	1	174	0
PGF_0040 7860	putative lipoprotein	1	183	0
PGF_0040 8050	putative lipoprotein	1	119	0
PGF_0289 6985	putative lipoprotein	2	219.5	3.5
PGF_0041 0187	putative plasmid stabilization protein	1	688	0
PGF_0017 7460	sigma-54-dependent transcriptional regulator	1	512	0
PGF_0041 4781	y4eB gene in pNGR234a homolog	1	104	0
	122 Hypothetical proteins			

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain A308			
PGF_0041 7140	ABC transporter, ATP-binding protein	1	236	0
PGF_0102 7157	ABC transporter, permease protein	1	401	0
PGF_0102 7719	ATP-binding protein	1	457	0
PGF_0004 6216	Alpha-L-Rha alpha-1,3-L-rhamnosyltransferase (EC 2.4.1.-)	1	298	0
PGF_0005 8720	Anticodon nuclease	1	380	0
PGF_0039 9672	Baseplate assembly protein J	1	293	0
PGF_0041 5078	CMP-binding factor	1	289	0
PGF_0041 6106	CRISPR-associated protein, Csy3 family	1	356	0
PGF_0041 8464	Coenzyme F420-dependent oxidoreductase	1	440	0
PGF_0042 0893	D-arabinitol 4-dehydrogenase (EC 1.1.1.11)	1	455	0
PGF_0092 7728	D-arabinitol operon repressor	1	313	0
PGF_0042 1005	D-mannonate oxidoreductase (EC 1.1.1.57)	1	486	0
PGF_0227 2288	DNA helicase IV (EC 3.6.4.12)	1	925	0
PGF_0000 2325	FIG01223779: hypothetical protein	1	350	0
PGF_0347 9854	Flavodoxin	1	195	0
PGF_0320 7284	Lipid carrier : UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-)	1	185	0
PGF_0001 9272	Mannonate dehydratase (EC 4.2.1.8)	1	393	0
PGF_0176 7866	Metal-dependent hydrolases of the beta-lactamase superfamily II	1	292	0
PGF_0066 7779	Mobile element protein	1	267	0
PGF_0148 2614	Modulator of drug activity B	1	217	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain A308			
PGF_0271 8770	Mu-like prophage FluMu I protein	1	390	0
PGF_0277 9037	NADH-dependent butanol dehydrogenase A (EC 1.1.1.-)	1	382	0
PGF_0305 9183	Outer membrane protein romA	1	367	0
PGF_0003 1706	Phage baseplate assembly protein	1	123	0
PGF_0003 1742	Phage capsid scaffolding protein	1	273	0
PGF_0003 2032	Phage major capsid protein	1	350	0
PGF_0003 2231	Phage protein	1	524	0
PGF_0333 6619	Phage replication protein	1	795	0
PGF_0003 2787	Phage terminase, ATPase subunit	1	570	0
PGF_0003 2790	Phage terminase, endonuclease subunit	1	227	0
PGF_0312 2294	Phage-related capsid packaging protein	1	358	0
PGF_0003 6218	Predicted pyrophosphatase	1	378	0
PGF_0004 0787	Putative exported protein precursor	1	353	0
PGF_0427 4853	Putative phage-encoded peptidoglycan binding protein	1	274	0
PGF_0307 8435	RND efflux system, membrane fusion protein	1	383	0
PGF_0004 7450	RelB/StbD replicon stabilization protein (antitoxin to RelE/StbE)	1	74	0
PGF_0285 3126	Repair of Iron Centers di-iron protein	1	221	0
PGF_0004 8740	Ribose ABC transporter, periplasmic ribose-binding protein RbsB (TC 3.A.1.2.1)	1	319	0
PGF_0005 0723	Sensor protein copS (EC 2.7.3.-)	1	482	0
PGF_0005 5454	TPR domain protein	1	246	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain A308			
PGF_0411 1601	TRAP-type C4-dicarboxylate transport system, large permease component	1	433	0
PGF_0613 7558	TRAP-type C4-dicarboxylate transport system, periplasmic component	1	326	0
PGF_0005 5872	TRAP-type C4-dicarboxylate transport system, small permease component	1	168	0
PGF_0562 0245	Thiosulfate:cyanide sulfurtransferase PspE (EC 2.8.1.1)	1	107	0
PGF_0162 6418	TnpA transposase	2	500.5	3.5
PGF_0112 1714	Transcriptional regulator	1	254	0
PGF_0006 1764	Transposon Tn21 resolvase	1	208	0
PGF_0006 3471	Type III restriction-modification system methylation subunit (EC 2.1.1.72)	1	706	0
PGF_0006 4885	Uncharacterized protein AF_1681	1	214	0
PGF_0006 6263	Uronate isomerase (EC 5.3.1.12)	1	474	0
PGF_0006 6310	Usg protein	1	477	0
PGF_0006 6371	Uxu operon transcriptional regulator	1	256	0
PGF_0166 7392	Xylulose kinase (EC 2.7.1.17)	1	487	0
PGF_0329 4113	alkylhydroperoxidase like protein, AhpD family	1	113	0
PGF_0385 4940	putative flippase	1	485	0
PGF_0040 9842	putative nuclease	1	233	0
	128 Hypothetical proteins			
	Present only in the genome of strain AJ83			
PGF_0228 2781	Acetyltransferase (isoleucine patch superfamily)	1	216	0
PGF_0005 0752	Amidohydrolase family protein	1	465	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain AJ83			
PGF_00066715	Aspartate aminotransferase (EC 2.6.1.1)	1	396	0
PGF_01771319	Bacteriocin/lantibiotic efflux ABC transporter, permease/ATP-binding protein	1	685	0
PGF_04991091	Bipolar DNA helicase HerA	1	587	0
PGF_00417608	Chitinase (EC 3.2.1.14)	1	783	0
PGF_00418006	Chromosome segregation ATPase	2	425	19
PGF_00418879	Conjugative transfer ATP-dependent DNA helicase	1	667	0
PGF_00419582	Copper tolerance protein	1	177	0
PGF_06243817	Cytochrome c551/c552	1	105	0
PGF_01370898	D-glycero-D-manno-heptose 1-phosphate guanosyltransferase	1	350	0
PGF_06102630	DNA primase, phage associated	1	754	0
PGF_04276033	Fimbriae usher protein StfC	1	834	0
PGF_00552044	Helix-turn-helix domain protein	1	113	0
PGF_00014121	Inner membrane protein YbcI	1	180	0
PGF_03033306	Integral membrane protein	1	157	0
PGF_00016826	Legionaminic acid biosynthesis protein PtmG	1	368	0
PGF_08041143	MaoC family protein	1	138	0
PGF_03672087	Nitrous oxide reductase maturation protein, outer membrane lipoprotein NosL	1	181	0
PGF_00026086	Nitrous oxide reductase maturation transmembrane protein NosY	1	276	0
PGF_06331579	O-antigen export system permease protein RfbD	1	269	0
PGF_00027601	OsmC/Ohr family protein	1	162	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strain AJ83			
PGF_0003 6518	Predicted transporter component	1	148	0
PGF_0003 6619	Prevent host death protein, Phd antitoxin	2	70.5	24.5
PGF_0003 7709	Prophage Lp2 protein 6	1	363	0
PGF_0309 8502	Putative DNA processing chain A	1	444	0
PGF_0004 3825	Putative ribosomal-protein-serine acetyltransferase	1	181	0
PGF_0040 1204	Subclass B3 beta-lactamase (EC 3.5.2.6)	1	337	0
PGF_0005 6192	Tellurite resistance protein-related protein	1	222	0
PGF_0648 7367	UDP-N,N'-diacetylbacillosamine 2-epimerase (hydrolyzing) (EC 3.2.1.184)	1	380	0
PGF_0675 0368	beta-glycosyl hydrolase	1	892	0
PGF_0739 7007	gluconolactonase family protein	1	293	0
PGF_0040 4750	prophage CP4-like integrase	1	402	0
	126 Hypothetical proteins			
	Present only in the genome of strains 947C and A308			
PGF_0440 0245	GDP-4-amino-4,6-dideoxy-alpha-D-acetylglucosamine N-acetyltransferase	2	254	0
PGF_0055 7102	Lactoylglutathione lyase, YQJC B.subtilis ortholog	2	133	1
PGF_0003 2298	Phage protein	2	504.5	0.5
PGF_0251 6485	Protein containing aminopeptidase domain	2	451.5	1.5
	4 Hypothetical proteins			
	Present only in the genome of strains A308 and AJ83			
PGF_0324 4749	Bis-ABC ATPase SPy1206	2	519	0

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strains A308 and AJ83			
PGF_01949586	Chloramphenicol O-acetyltransferase (EC 2.3.1.28) => CatB family	2	211	0
PGF_03438544	DNA or RNA helicase of superfamily II	2	1047	0
PGF_02955071	Mu-like prophage FluMu protein gp29	2	532	0
PGF_00025736	Nitrate/nitrite transporter NarK	2	470	0
PGF_08117723	Phage integrase	2	481	5
PGF_00032042	Phage major capsid protein	2	302	0
PGF_00032839	Phage terminase, small subunit	2	191	0
PGF_00045452	Putative transposase	2	737	0
PGF_02967959	Putative transposase	2	626	3
PGF_00047563	Replication initiation ATPase; bacteriophage DNA transposition B protein	2	240	0
PGF_00047728	Respiratory nitrate reductase alpha chain (EC 1.7.99.4)	2	1256	0
PGF_00047730	Respiratory nitrate reductase beta chain (EC 1.7.99.4)	2	512	0
PGF_00047732	Respiratory nitrate reductase delta chain (EC 1.7.99.4)	2	238	0
PGF_00047734	Respiratory nitrate reductase gamma chain (EC 1.7.99.4)	2	225	0
PGF_00557133	Ribonuclease Z (EC 3.1.26.11) (RNase Z) (tRNase Z) (tRNA 3 endonuclease)	2	342	0
PGF_00748608	Transcriptional regulator, LysR family	2	299	0
PGF_03752321	Transposase, mutator type	3	302	97.8
PGF_00404789	prophage MuSo1, transcriptional regulator, Cro/CI family	2	248	0
PGF_00404790	prophage MuSo2, portal protein, putative	2	523	0
PGF_00557065	putative lipoprotein	2	59.5	17.5

Table S5. Continued.

Family ID	Description	Proteins	Mean	Std Dev
	Present only in the genome of strains A308 and AJ83			
PGF_01411276	putative transposition protein	2	335	0
	43 Hypothetical proteins			
	Present only in the genome of strains 947C, A308 and AJ83			
PGF_03033259	Serine/threonine protein phosphatase (EC 3.1.3.16)	3	261	0
PGF_01493148	TPR repeat	3	394	0
	4 Hypothetical proteins			

Table S6. Presence and absence of known virulence genes in six mesophilic *A. salmonicida* strains.

		Strain					
Gene	Function	947C	AJ83	A308	34mel	A527	Y47
aerA	Aerolysin	+	+	+	+	+	+
ahh1	Extracellular hemolysin	+	+	+	+	+	+
ahpB	Serine Protease	+	+	+	+	+	+
alt	Cytotonic enterotoxin (lipase)	+	+	+	+	+	+
ast	Cytotonic enterotoxin	+	+	-	-	-	-
dam	DNA adenine methyltransferase	+	+	+	+	+	+
eno	Enolase (surface-expressed)	+	+	+	+	+	+
eprA1	EprA1 (extracellular protease)	+	+	+	+	+	+
gidA	Glucose-inhibited division protein	+	+	+	+	+	+
rtxA	RTX toxin (repeat in toxin A)	-	+	*	+	*	+
ser	Serine protease	+	+	+	+	+	+
tagA	ToxR-regulated lipoprotein (TagA)	+	+	+	+	+	+
vacB	Exoribonuclease R	+	+	+	+	+	+
AHA_1741	Collagenase	+	+	+	+	+	+
AHA_3147	Invasin	+	+	+	+	+	+
AHA_3217	Thermostable hemolysin	+	+	+	+	+	+
AHA_3493	Hemolysin III	+	+	+	+	+	+

* Truncated gene

Table S7. Antibiotic resistance genes predicted from the genome sequences of mesophilic *A. salmonicida* strains.

				Strain									
Gene	**AMR Gene Family**	**Drug Class**	**Accession #**	**34mel^T**	**A527**	**Y577**	**ASG1**	**ECFood+05**	**947C**	**A308**	**AJ83**	**Y567**	**Y47**
AAC(3)-IIc	AAC(3)	aminoglycoside antibiotic	ARO:3002535	-	-	-	+	-	-	-	-	-	-
AAC(6')-Ia	AAC(6')	aminoglycoside antibiotic	ARO:3002545	-	-	-	-	+	-	-	-	-	-
ANT(2")-Ia	ANT(2")	aminoglycoside antibiotic	ARO:3000230	-	-	-	-	+	-	-	-	-	-
APH(3")-Ib	APH(3")	aminoglycoside antibiotic	ARO:3002639	-	-	-	-	-	+	-	-	-	-
APH(3')-Ia	APH(3')	aminoglycoside antibiotic	ARO:3002641	-	-	-	+	-	-	-	-	-	-
APH(6)-Id	APH(6)	aminoglycoside antibiotic	ARO:3002660	-	-	-	-	-	+	-	-	-	-
CTX-M-3	CTX-M beta-lactamase	cephalosporin	ARO:3001866	-	-	-	+	-	-	-	-	-	-
FOX[a]	FOX beta-lactamase	cephamycin, cephalosporin	ARO:3002156	-	+	-	-	-	-	+	+	+	-
OXA-12	OXA beta-lactamase	cephalosporin, penam	ARO:3001407	+	+	+	+	+	+	+	+	+	+
TEM-1	TEM beta-lactamase	penem, cephalosporin, monobactam, penam	ARO:3000873	-	-	-	+	-	-	-	-	-	-
VEB-1	VEB beta-lactamase	cephalosporin, monobactam	ARO:3002370	-	-	-	-	+	-	-	-	-	-
aadA	ANT(3")	aminoglycoside antibiotic	ARO:3002601	-	-	-	+	+	-	-	-	-	-
catII	chloramphenicol acetyltransferase (CAT)	phenicol antibiotic	ARO:3002684	-	-	-	+	-	-	-	-	-	-
cmlA5	major facilitator superfamily (MFS) antibiotic efflux pump	phenicol antibiotic	ARO:3002695	-	-	-	-	+	-	-	-	-	-
cphA5	CphA beta-lactamase	carbapenem	ARO:3003101	+	+	+	+	+	+	+	+	+	+
dfrA12	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	ARO:3002858	-	-	-	+	-	-	-	-	-	-
dfrA14	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	ARO:3002859	-	-	-	-	+	-	-	-	-	-

Table S7. Continued.

				Strain									
Gene	**AMR Gene Family**	**Drug Class**	**Accession #**	**34mel^T**	**A527**	**Y577**	**ASG1**	**ECFood+05**	**947C**	**A308**	**AJ83**	**Y567**	**Y47**
dfrA15	trimethoprim resistant dihydrofolate reductase dfr	diaminopyrimidine antibiotic	ARO:3003013	-	-	-	+	-	-	-	-	-	-
qacH	small multidrug resistance (SMR) antibiotic efflux pump	fluoroquinolone antibiotic	ARO:3003836	-	-	-	-	+	-	-	-	-	-
sul1	sulfonamide resistant sul	sulfonamide antibiotic, sulfone antibiotic	ARO:3000410	-	-	-	+	-	-	-	-	-	-
tet(E)	major facilitator superfamily (MFS) antibiotic efflux pump	tetracycline antibiotic	ARO:3000173	-	-	-	+	+	-	-	-	-	+
			Total	**2**	**3**	**2**	**12**	**10**	**4**	**3**	**3**	**3**	**3**

a. May be FOX-2, FOX-3, FOX-4 or FOX-5

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4.3. *Vibrio alginolyticus* infections: Two cases from Spain and literature review

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Case Report Open Access

Vibrio alginolyticus Infections: Report of Two Cases from Spain with Literature Review

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Abstract

Vibrio alginolyticus is rarely reported as a human pathogen. However, we report two cases from Spain. The first case involved a 69-year-old male with a rectum adenocarcinoma that developed enterocolitis and septic shock. The second case was associated with a previously healthy 37-year-old male patient with a pretibial ulcer exposed to the Mediterranean Sea. The isolates were identified as *V. alginolyticus* by MALDI-TOF MS and *rpoD* gene sequencing. The strain from the first patient was incorrectly attributed to *Aeromonas* sp. in the preliminary identification by API 20E and API 20NE. The antibiotic susceptibility against 13 antibiotics was tested and both strains were resistant to penicillin and ampicillin. This study is intended to raise awareness about the increasing incidence of *V. alginolyticus* and to that aim a review of the previous cases is also provided. Additionally, this study alerts that *V. alginolyticus* can be confused with *Aeromonas* based on the used identification method.

Keywords: *Vibrio alginolyticus*, *Aeromonas*, MALDI-TOF, rpoD gene, API 20E.

Introduction

Vibrio alginolyticus is a Gram-negative halophilic bacterium found in the aquatic environment, especially in temperate oceans but also able to grow in extremely high salty environments [1-5]. The virulence of this bacterium is directly related to its capacity to produce hemolysis, hemagglutination and proteases [2,5,6]. This microorganism is considered a rare human pathogen causing mainly diarrhea. However, it has been etiologically associated with otitis and wound infections, producing occasionally life-threatening infections in immunocompromised individuals [2,5,7,8]. The majority of isolates are resistant to penicillin, ampicillin and second-generation cephalosporins by the acquisition of resistance genes that are present in mobile elements [3,9,10]. Recent studies in USA, indicate that in 2011 the incidence of *V. alginolyticus* infections was only 0.048 cases per million [11]. However, the description of several new cases confirms that a rising incidence has occurred in the last years [1-3,8]. Therefore, a better knowledge about this infectious agent and its resistance to antibiotics is needed. This study is intended to contribute describing in detail two cases of infection produced by *V. alginolyticus* from Spain, providing a review of the literature and alerting of the potential confusion with *Aeromonas* depending on the employed identification method.

Case Presentation

Case presentation 1

A 69-year-old male patient visited his doctor complaining of watery diarrhea with a duration of 24 hours, abdominal pain, nausea and vomiting, with no fever. He had a previous history of hyperuricemia, osteoporosis, Widal syndrome, a right femur prosthesis and a proximal rectum adenocarcinoma that was treated with chemotherapy and surgical exeresis. The patient was referred to the Emergency Department of University Hospital Sant Joan de Reus (Catalonia, Spain). Upon arrival the patient exhibited hypoxemia (pO_2 31.7 mmHg, oxygen saturation 43.4%) and hypotension (blood pressure 85/65 mmHg). Laboratory data showed a white blood cell count of 16.8×10^9/L with a relative reduction in lymphocytes (5.3%) and an increase in neutrophils (91.1%). A computer axial tomography showed dilatation of the gut with internal fluid accumulation compatible with acute enterocolitis. The patient was admitted to the intensive care unit where he developed a septic shock with acute renal insufficiency, metabolic acidosis and high levels of lactate (8.75%) and then an intravenous treatment with imipenem (500 mg/6 h) was empirically initiated.

A stool sample was obtained and examined for intestinal parasites and for several bacteria i.e., *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica, Aeromonas* spp., *Plesiomonas* spp., *Vibrio* spp. and *Campylobacter* spp. Investigation of intestinal parasites was negative. However, the stool yielded a positive culture in Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) medium (Becton Dickinson Diagnostics, USA) after 24 h of incubation in aerobiosis at 37°C. The recovered isolate, labeled 1182C, was identified based on the biochemical test API 20E (BioMérieux, Marcy l'Etoile, France), and the 7-digit code obtained after three repetitions were: 0046126, identifying this strain as: 50% *Aeromonas* spp./43% *V. fluvialis*/5% *V. alginolyticus*; 1046126: 70% *Aeromonas* spp./29% *V. fluvialis* and 1045126: 99% *Aeromonas* spp. However, with the API 20NE (BioMérieux, Marcy l'Etoile, France) the result of the 7-digit code without repetition was 6030444, identifying the strain: 71% *V. vulnificus*/27% *V. alginolyticus*. Also, based on the MALDI-TOF MS Biotyper v. 3.1, the isolate was identified as *V.*

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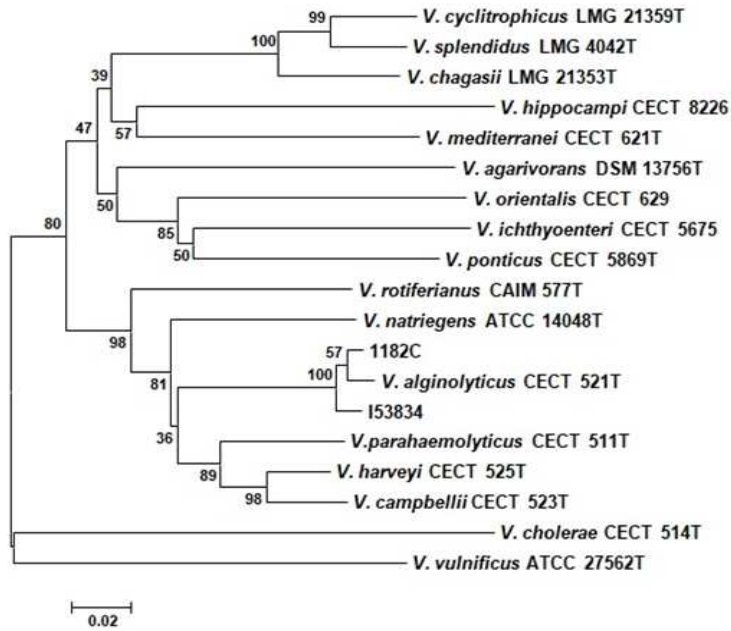
alginolyticus with a score >2.0. The antibiotic susceptibility of the strain 1182C was tested with MicroScan Walkaway and the results were analyzed according to the CLSI guidelines [12]. The results showed that the strain was resistant to ampicillin and susceptible to amoxicillin/clavulanic acid, ciprofloxacin and imipenem (Table 1). Considering these results and the good clinical status of the patient, the oral diet was restarted progressively. Also, on day 7 imipenem was changed to oral ciprofloxacin 500 mg/12 h until discharge from the hospital 9 days after admission, but treatment was continued at home for an additional period of 5 days. The strain 1182C was sent to the Unit of Microbiology at the University Rovira i Virgili for the re-identification using the sequences of the *rpoD* gene. The DNA extraction, amplification and sequencing were performed using the primers and the conditions previously described [13]. Nucleotide Blast (Blastn) analysis with the obtained *rpoD* sequence revealed a 99% similarity with *V. alginolyticus* (GenBank accession number JF930400). Considering these results, a Neighbour-joining phylogenetic tree was constructed adding also the *rpoD* sequences (479 bp) of the type strains using the MEGA 6 software. The phylogenetic tree showed that the isolate 1182C clustered with the sequence of the type strain of *V. alginolyticus* (Figure 1). In addition the antibiotic-susceptibility against 13 antibiotics was tested again using the disk diffusion method and interpretation was again done following the CLSI guidelines [12]. The strain 1182C was susceptible to all antibiotics tested except for penicillin and ampicillin (Table 2).

Case presentation 2

A 37-year-old male patient with no previous history of disease was attended at the University Hospital Miguel Servet (Zaragoza, Spain) in September 2016, because he had an apparently infected left pretibial ulcer, after swimming in the Mediterranean Sea in August. The wound occurred in January after the patient fell down the stairs. However, the wound was still not completely cured, when he noticed that the aspect of the wound had worsened. A sample of the wound exudate was collected and cultured in Blood Agar (BA). The culture was positive after 24 h of incubation in aerobiosis at 37°C and the strain recovered was named I53834 and was identified as *V. alginolyticus* / *V. parahaemolitycus* with MALDI-TOF MS Biotyper v 3.1 with a final score of 1.967 and 1.949, respectively for each microbe (Table 3). With Etest (BioMérieux, Marcy l'Etoile, France) the strain was susceptible to cefotaxime, ceftazidime, ciprofloxacin and tetracycline. An empiric antibiotic treatment was initiated with vibriamycin (doxycycline) 100 mg/12 h.

The isolate I53834 was sent to the Unit of Microbiology at the University Rovira i Virgili for re-identification using the sequences of *rpoD* gene that was performed as described above for the other case. The Blastn analysis of the obtained sequence showed a 99% similarity with a *V. alginolyticus* sequence (GenBank accession number JQ015344). In the phylogenetic tree, constructed as indicated above, the sequence of the strain I53834 clustered with the sequence of the type strain of *V. alginolyticus* and with the other sequence of this species (strain 1182C) from the other case (Figure 1). The antibiotic susceptibility against 13 antibiotics was additionally tested using the disk diffusion method and results were interpreted following the CLSI guidelines [12]. The strain I53834 showed to be susceptible to all tested antibiotics except to



*Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). Bar 0.02 estimated nucleotide substitutions per site

Figure 1: Neighbour-joining phylogenetic tree obtained with *rpoD* sequences (479 bp) showing the position of the strains 1182C and I53834.

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Antimicrobial agent	MIC (µg/ml)	Pattern
Amikacin	≤8	S
Ampicillin	≤8	R
Amox/Clavulat K	≤8/4	S
Aztreonam	≤1	S
Ceftazidime	≤1	S
Cefalotine	≤8	I
Cefotaxime	≤1	S
Cefoxitin	≤8	S
Cefazolin	≤8	S
Ciprofloxacin	≤0.5	S
Cefepime	≤1	S
Cefuroxime	8	S
Ertapenem	≤0.5	I
Gentamicin	≤2	S
Imipenem	≤1	S
Nalidixic acid	≤16	I
Piperacillin tazobactam	≤8	S
Trimet/Sulfa	≤2/38	S
Tigecycline	≤1	S
Tobramycin	≤2	I

Table 1: Response of strain 1182C to several antimicrobial agents obtained with the MicroScan Walkaway at the University Hospital Sant Joan de Reus. Susceptible (S), resistant (R) or intermediate (I).

Antimicrobial agents	Zone Diameter (mm)		Breakpoints		
	1182C	I53834	Sensitive	Intermediate	Resistance
Amikacin	S (19)	S (18)	≥ 17	15-16	≤ 14
Ampicillin	R (12.5)	R (13)	≥ 17	14-16	≤ 13
Amoxicillin-clavulanate	S (21)	S (22)	≥ 18	14-17	≤ 13
Cefotaxime	S (29)	S (29.5)	≥ 26	23-25	≤ 22
Ciprofloxacin	S (22)	S (23)	≥ 21	16-20	≤ 15
Cefepime	S (27)	S (26)	≥ 25	19-24	≤ 18
Penicillin*	R (17)	R (17)	≥ 21	19-20	≤ 18
Gentamicin	S (17)	S (17.5)	≥ 15	13-14	≤ 12
Imipenem	S (25)	S (26)	≥ 23	20-22	≤ 19
Piperacillin	S (23)	S (23.5)	≥ 21	18-20	≤ 17
Piperacillin-tazobactam	S (23.5)	S (≤ 22)	≥ 21	18-20	≤ 17
Tetracycline	S (16)	S (17)	≥ 15	12-14	≤ 14
Trimetoprim-sulfamethoxazole	S (17)	S (17)	≥ 16	11-15	≤ 10
*This antibiotic does not appear in the CLSI guidelines					

Table 2: Susceptibility profile of strains 1182C and I53834 to 13 antibiotics evaluated with the disk diffusion method at the University Rovira i Virgili (results expressed in mm).

S No	First option		Second option	
	Score	Specie	Score	Specie
1	1.998	*V. alginolyticus*	1.992	*V. parahaemolyticus*
2	1.713	*V. parahaemolyticus*	1.708	*V. alginolyticus*
3	1.985	*V. alginolyticus*	1.896	*V. parahaemolyticus*
4	1.967	*V. alginolyticus*	1.949	*V. parahaemolyticus*

Table 3: Results of the preliminary identification of the strain I53834 with MALDI-TOF MS Biotype. The method was performed four times and the two higher scores are presented.

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Patient age (years)	Country	Presentation	Condition	References
M/22	United States	Leg ulcer	Anemia and chronic leg ulcers	[14]
N/A	United States	Wound infection in the right calf	None	[14]
M/42	United States	Trauma (ulcer) in the right leg	Chronic venous insufficiency in the leg	[7]
F/55	Japan	Abdominal pain and diarrhea	Therapy of submandibular cyst	[22]
M/20	United States	Headache and fever (intracranial infection)	None	[24]
M/26	United States	Respiratory distress and dehydration (bacteremia)	Osteogenic sarcoma	[26]
M/65	United States	Cellulitis in the right leg	None	[15]
F/40	United Kingdom	Laceration in the left leg	None	[16]
M/17	Portugal	Nausea, fever and frontal headache (sphenoiditis)	None	[25]
M/31	China	Necrotizing Fasciitis in the leg	Cirrhosis and hepatitis B	[27]
M/38	United States	Watery non-bloody stool	Odynophagia and dysphagia	[23]
F/23	South Korea	Abdominal pain and diarrhea	Hepatitis B	[21]
F/52	South Korea	Abdominal pain, vomit and diarrhea	Treatment for pulmonary tuberculosis 30 years ago	[21]
F/48	Colombia	Necrotizing Fasciitis in the leg	Exacerbation of her Asthma (steroids)	[28]
M/57	Turkey	Otitis	None	[17]
M/37	Japan	Wound infection in replanted fingers	Amputation and replantation of two fingers	[18]
M/59	Korea	Shock septic with pain in the legs	Hepatitis B	[5]
M/50	Italy	Painful cutaneous ulceration	None	[19]
M/70	Italy	Wound in the right leg	None	[20]
F/70	United Kingdom	Infected wound on her lower leg	None	[1]
M/66	Spain	Painful ulcer in the left foot	Chronic radiation-induced dermatitis	[8]
F/47	Turkey	Meningitis	None	[2]
M/14	Egypt	Wound on the left foot	None	[3]

Table 4: Different cases associated to *Vibrio alginolyticus* reported at the literature. Male (M), female (F) and not available (N/A).

Characteristics	Number	Percentage
Patient		**%**
Gender	**n= 1307**	
Male	913	70
Female	392	30
Age	**n=1276**	
<1-9	213	17
10-19	250	20
20-29	135	10
30-39	149	12
40-49	156	12
50-59	130	10
>60	243	19
Clinical features		**%**
Infection	**n=1331**	
Gastrointestinal	62	5
Blood	56	4
Skin	1162	87
Unknown	51	4
Outcomes	**n=variable**	
Hospitalization	235/1202	20
Death	12/1170	1
Antibiotics	1047/1156	91
Transmission		**%**
Foodborne	**101/1331**	8
Non-foodborne	**1141/1331**	86
Water	829/998	83
Sea water	706/802	88
Other	**89/1331**	6

Table 5: Characteristics of *Vibrio alginolyticus* infections in USA between 1988-2012 (adapted from Jacobs Slikfa et al.[29]).

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penicillin and ampicillin (Table 2).

Discussion

A review of the literature of infections produced by *V. alginolyticus* showed that the most common presentations were superficial wound and ear infections [1-3,7,8,14-20]. These were followed by cases of diarrhea [21-23]. Sphenoiditis, intracranial infections, necrotizing fasciitis, bacteremia and even septic shock were the other infections [5,24-29]. The case reports found at the literature are summarized in Table 4. In addition, an epidemiological study on *V. alginolyticus* infections that occurred in USA between 1988-2012 and that included 1331 cases was performed by Jacobs Slikfa et al. [29]. The main data obtained in the Jacobs Slikfa et al. study are listed in Table 5. As shown in the Tables 4 and 5, the majority of the cases occurred in patients that showed no undelaying diseases and were associated with wound infections or ulcers that have occurred in contact with seawater [5,8,19,24,28,29]. The latter infections occurred in coastal waters of temperate and tropical regions [2,3,29,30]. In addition, in the summer months, the incidence of these infections increased significantly [2,28,31]. The impact of climate change in water temperature observed in recent years had led to an increased incidence of *V. alginolyticus* both in tropical waters and in the otherwise colder waters of northern Europe [20,29].

One of the cases reported in this study involved a patient that was immunocompromised as occurred in other cases described in the literature [5,26-29]. Therefore, it is important to consider this condition, because this agent is an emerging pathogen that in immunocompromised individuals can cause important infections. Interestingly, the patient of our second case had a wound that was exposed to seawater of the Mediterranean Sea, considered, as commented, an important risk factor in this type of infections [2,8,15,17,28].

The *V. alginolyticus* infections usually respond well to appropriate antibiotics and only occasionally causes life-threatening infections as shown in Table 5 [2,4,29]. Recent literature reported that *V. alginolyticus* is generally resistant to penicillin, ampicillin and vancomycin but susceptible to ciprofloxacin, chloramphenicol, aminoglycosides and some beta-lactams [32]. The antimicrobial profiles in this study were analyzed with an automatized system, MicroScan Walkaway and with the disk diffusion method. The results of the antimicrobial profile obtained in this study are in agreement with the resistance to ampicillin and penicillin obtained in other studies and the susceptibility to trimethoprim-sulfamethoxazole, tetracycline and gentamicin [32-35]. Regarding the identification of *Vibrio*, many clinical microbiology laboratories still routinely rely on the use of phenotypic methods, such as API 20E system that is a biochemical panel for identification and differentiation of members of the *Enterobacteriaceae* family. The results of twenty mini-test chambers provide a 7-digit code, that is introduced in the API catalog or apiweb to get an identification of the bacteria strain with a determined probability (%). In our first case the strain was incorrectly identified by API 20E as *Aeromonas* sp. This result confirms that an accurate phenotypic identification of *Vibrio* species is problematic, largely because of the great variability in biochemical characteristics [36-38]. O'Hara et al. evaluated six commercial systems for the ability to identify the 12 species of *Vibrio* found in clinical samples and the results showed that one strain of *V. cholerae* was also identified as *Aeromonas hydrophila* with API 20E [36-38]. Confusion between *Aeromonas* and *Vibrio* spp. occur approximately in 6% of the biochemical identifications, therefore a genus probe to avoid this problem have been developed at our laboratory [38-42].

MALDI-TOF MS is a mass spectrometry technique, that provides the determination of molecular weights of biomolecules, mostly of the proteins associated with the 16S rRNA gene from a bacteria isolate in a few minutes [43,44]. The profiles or protein spectra are specific to each bacteria genus and species [43,44]. The identification is obtained comparing the profiles or spectra with the ones available at the database of the system and providing a score value that describes the degree of accuracy i.e., >2.0, indicates accuracy at species level [45]. If the reference database doesn't contain sufficient spectra from all the *Vibrio* spp., the accuracy of the identification is poor (score <2.0). Furthermore, considering that *V. alginolyticus* and *V. parahaemolyticus* are very closely related species that show a 16S rRNA similarity of 99.4%, the probability that they can be correctly identified on the basis of the MALDI-TOF MS is low, because as indicated above the system relay on the proteins associated to the 16S rRNA gene [46]. These are the reasons that explains the ambiguous results obtained with MALDI-TOF MS in our second case where the strain could not be assigned either to *V. alginolyticus* or to *V. parahaemolyticus* giving for both a score <2.0.

Conclusion

Our two reports on human infections by *V. alginolyticus* is intended to raise awareness about the infections caused by this bacterium. It is essential to consider this emerging pathogen in patients with cancer or with other immunosuppressed conditions and in healthy patients with skin or soft tissue lesions that have been in contact with seawater. Furthermore, there is a need to report new cases to determine if the incidence in our country and in other regions is also increasing.

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4.4. A case of *Aeromonas trota* in an immunocompromised patient with diarrhea

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
Ana Fernández Bravo

Results

A case of *Aeromonas trota* in an immunocompromised patient with diarrhea.

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Abstract

According to the recent literature 96.5% of the *Aeromonas* strains associated to clinical cases correspond to 4 species: *Aeromonas caviae*, *Aeromonas dhakensis*, *Aeromonas veronii*, and *Aeromonas hydrophila.* However, other less prevalent species such as *Aeromonas trota,* are also described from clinical samples. On the basis of its low incidence the latter species can be regarded as rare and it is the only *Aeromonas* species susceptible to ampicillin. From the taxonomic point of view *A. trota* is considered a synonym of the species *Aeromonas enteropelogenes.* The objective of this study is to present a new clinical case associated with *A. trota* in order to increase the knowledge about this species. The strain was recovered from the feces of a 69-year-old patient with a diarrheal syndrome and peritoneal psamocarcinoma. The preliminary identification as *Aeromonas* sp. was obtained with the API 20E, but it was characterized as *Aeromonas jandei* and also as *Aeromonas enteropelogenes* with different scores with the Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF). On the basis of the sequence of the *rpoD* gene it was confirmed to be *A. trota*. The antimicrobial resistance pattern showed that the strain was susceptible to ampicillin, penicillins in combination with beta-lactamase inhibitors, quinolones, carbapenems, aminoglycosides and cephalosporins, except cephalothin. In conclusion, the recognition of an *Aeromonas* strain susceptible to ampicillin should alert the clinical microbiologist of the possible involvement of this rare species. Furthermore, the MALDI-TOF database should be updated indicating that the species *A. enteropelogenes,* is a synonym of *A. trota*.

Keywords: *Aeromonas trota, Aeromonas enteropelogenes,* ampicillin, MALDI-TOF.

Introduction

The genus *Aeromonas* includes more than 32 species, some of which are distributed in the environment and are considered autochthonous of aquatic systems (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo et al., 2015; Figueras and Ashbolt, 2019). *Aeromonas* are considered emerging pathogens that cause a wide spectrum of diseases in humans, mainly gastroenteritis, bacteremia and wound infections, being able to infect both immunocompromised and immunocompetent patients (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo et al., 2015; Teunis and Figueras, 2016). Recent literature showed that 96.5% of the strains associated to clinical cases correspond to 4 species: *Aeromonas caviae* (29.9%), *Aeromonas dhakensis* (26.3%), *Aeromonas veronii* (24.8%) and *Aeromonas hydrophila* (15.5%) (Figueras and Beaz-Hidalgo, 2015), but other less prevalent species, such as *Aeromonas trota* (0.5%), are also described from clinical samples. Therefore, on the basis of the low incidence, *A. trota* can be regarded as a rare species. This species is considered a synonym of the species *Aeromonas enteropelogenes* and both have been isolated from feces of patients with diarrhea, and are the only *Aeromonas* species susceptible to ampicillin (Schubert et al. 1990; Carnahan et al., 1991; Collins et al., 1993; Huys et al 2002).

This study describes a case of diarrhea produced by a strain of *A. trota* and provides the results of the antimicrobial pattern determined with the MicroScan WalkAway. The isolate was first identified with API 20E and re-identified with Matrix-Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF), and on the basis of the sequences of the *rpoD* gene phylogeny (Soler et al., 2004).

Case Report

69-year old female with a previously peritoneal psamocarcinoma and a colostomy performed a few years ago was hospitalized on January at the emergency department of University Hospital Sant Joan de Reus in Spain, with an episode of deterioration of her general condition and abdominal pain with bleeding soft stools and without fever. In addition, her skin was pale, but hydration was correct, and the abdomen

examination showed normal findings. The blood test performed at the moment of hospitalization revealed an acute renal failure with creatinine values of 4.5 mg/dL and 157 mg/dL, pH values were in the normal range, and she did not present anemia. With all the data the patient was diagnosed with a diarrheal syndrome and a stool sample was collected for the analysis of bacteria, viruses and parasites. An intravenously treatment with imipenem ciprofloxacin 200 mg /100 mL every twelve hours was empirically initiated, during eleven days. After this time, the patient seemed recovered from her abdominal episode.

The analysis of viruses and parasites showed to be negative but a cultured on Xilose Lysine Deoxycholate agar (XLD) (BioMerieux ®, Marc l'Etoile, France) after 24 hours at 37ºC was positive. The isolate 1183C was identified as *Aeromonas* sp. based on phenotypic tests as oxidase production and the API 20E (BioMerieux®, Marc l'Etoile, France). Considering these results, a second identification with the MALDI-TOF Biotyper (Bruker®) was performed in two independent laboratories (four replicates in each laboratory) with different versions of the Biotyper database (V4 and V5) and the results are shown in Table 1. One result of MALDI-TOF showed a lower score than 2.0 (V4), and a higher score than 2.0 (V5) for *A. jandaei*, while three results presented a higher score than 2.0 (V4 and V5) for *A. enteropelogenes* (Table 1). The antibiotic susceptibility was performed with MicroScan Walkaway (Siemens®) and the results were analyzed according to the CLSI guidelines (CLSI, 2015). This strain was susceptible to ampicillin and penicillin in combination with beta-lactamase inhibitors, quinolones, carbapenems, aminoglycosides and cephalosporins, with the exception of cephalothin (Table 2). The resistance pattern of the strain was compatible with *A. trota*, the only species of the genus along with its synonym *A. enteropelogenes* susceptible to ampicillin (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo et al., 2015). Similarly, the MicroScan WalkAway (Siemens®) could not define the species, but the isolate was identified at genus level as *Aeromonas* sp.

The isolate 1183C was sent to the Unit of Microbiology at the University Rovira i Virgili for re-identification by using the sequences of *rpoD* gene, as it was carried

out routinely for all isolates identified as *Aeromonas* sp. at the hospital. The DNA extraction, amplification and sequencing were performed by using primers and conditions previously described (Soler et al., 2004). A BlastN analysis with the obtained *rpoD* sequence revealed 99% similarity with a strain of *A. enteropelogenes.* Likewise, the phylogenetic tree constructed with the *rpoD* gene of the strain 1183C and the sequences of the type strains of all the *Aeromonas* spp. with a Neighbor-Joining (NJ) algorithm revealed that the sequence of the isolated strain clustered with the sequences of the type strain of *A. enteropelogenes* and the one of *A. trota* demonstrating that the strain belongs to these species, as indicated before they are synonyms (Figure 1).

Discussion

Species of the genus *Aeromonas* are considered opportunistic emerging pathogens that cause diarrhea, bacteremia and wound infections (Janda and Abbott, 2010; Figueras and Beaz-Hidalgo, 2015). This bacterium is mainly an enteric pathogen that affects with higher frequency children, elderly people, and immunocompromised individuals. The incidence of diarrhea caused by *Aeromonas* in children range from 2% to 13%, and in adults from 2% to 7% when the individuals are immunocompetent, however, it rises to 13% in immunocompromised individuals (Figueras and Beaz-Hidalgo, 2015). The species *A. trota,* has been isolated in association with diarrhea with a prevalence of 0.5% and it is considered a rare species (Carnahan et al., 1991; Collins et al., 1993; Reina and Lopez, 1996; Figueras and Beaz-Hidalgo, 2015). Other data that support the enteropathogenicity of *A. trota* is the capacity to develop diarrhea in a healthy laboratory worker after and accidental ingestion of a pure culture suspension or the results of an experimental infection in a murine animal model (Singh and Sanyal 1997; Teunis and Figueras, 2016) Nevertheless, the description of cases associated with *A. trota* are rare. The first case report published due to *A. trota* associated with diarrhea, after the species description in 1991, dates back to 1996, and corresponded to a 3-year-old boy that presented a mucous diarrhea not associated with other clinical manifestations (Reina and Lopez, 1996). Our case report is important since it

represents, according to our knowledge, the second case of diarrhea due to *A. trota*, and it is the first one reported in adults.

Previous studies described *A. trota* as a rare species, being, as mentioned before, the only species of the genus susceptible to ampicillin (Carnahan, 1991; Overman and Janda, 1999; Huys et al., 2002; Abbott et al., 2003; Lai et al., 2007; Dallagassa et al., 2018). Also, it has been described as resistant to cephalotin (Carnahan et al., 1991; Abbott et al., 2003). The strain isolated from our patient showed a similar resistant pattern to ampicillin and cephalotin.

In 1993 Collins et al. demonstrated based on the 16S rRNA gene sequence analysis that *A. trota* (Carnahan et al., 1991) and *A. enteropelogenes* (Schubert et al., 1990) were identical, with a similarity of 100% between the sequences of the type strains. The first species was isolated from feces collected in south-eastern Asia and the second was isolated from human feces in India. In addition, Huys et al. (2002) confirmed with the DNA-DNA hybridization studies, as well as with phenotypic data that these strains represented the same species. The DNA-DNA hybridization values between the strains of the two species was 81%-99%, clearly above the 70% cut-off established to delimit different species, showing values of 40%-49% when comparing these two species with other non-related species as *A. caviae* and *A. sobria* (Huys et al., 2002). In addition, none of the 60 different phenotypical tests enabled to discriminate the type strain of *A. trota* and *A. enteropelogenes* which also showed the same antibiotic susceptibility pattern (Huys et al., 2002).

On the basis of the Judicial Commission, *A. enteropelogenes* has nomenclatural priority, since this species was previously described (Schubert et al., 1990; Carnahan et al., 1991). The name *A. enteropelogenes* was included in the Validation List no.38 (Schubert et al., 1990), while the name of *A. trota* was announced in the Validation List no. 40 (Carnahan et al., 1991). However, *A. trota* has been more used by the scientific community (Nair and Holmes, 1999; Nair and Holmes, 2002; Huys et al., 2002; Holmes and Farmer, 2009). A recent PubMed search (04/21/2019) using "*Aeromonas trota*" yielded 55 citations, while a similar request using "*Aeromonas*

enteropelogenes" produced only 20 records. A Request for an Opinion is necessary to change the nomenclature.

The MALDI-TOF is a fast and useful tool employed in many hospitals for the fast identification of bacteria, including those of the genus *Aeromonas* and it showed to be more precise than the phenotypic methods (Lamy et al., 2010; Pérez-Sancho et al., 2018). The validation of MALDI-TOF carried out by comparing the obtained results with those of molecular reference methods for the *Aeromonas* identification, such as the sequences of housekeeping genes (Lamy et al., 2010: Pérez-Sancho et al., 2018). The fact that the Biotyper database have few representatives of just a single representative of each species, can hinder the correct identification of the species (Lamy et al., 2010; Pérez-Sancho et al., 2018). *Aeromonas* have changed continuously with the description of new taxa and reclassifications, synonyms, such as "*Aeromonas punctata*" for *A. caviae*, "*Aeromonas trota*" for *Aeromonas enteropelogenes*, and "*Aeromonas ichthiosmia*" for *A. veronii* are examples. However, the names of species not used, such as *A. ichthiosmia, A. punctata* or *A. enteropelogenes* are included in the Biotyper database (Vávrová et al., 2014) in parallel with the correct names without advising that they are synonyms. This is a problem in the clinical field, because the clinicians do not work with taxonomy and might think that these synonyms represent different species. On the basis of these observations we believe that it is important that the Biotyper database updates the taxonomic information indicating that the species *A. enteropelogenes*, is similar to *A. trota*.

Conclusion

The study intends to alert clinicians that the recognition of an *Aeromonas* strain susceptible to ampicillin may represent a strain of *A. trota* and advise a need for updating the MALDI-TOF database indicating that the species *A. enteropelogenes*, is similar to *A. trota*.

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Conflict of interests

The author(s) declare that they have no competing interests.

Author's contribution

IFG, FBB, IP, FGB, MD, MM, EA and JMSS obtained the data of the patient, as well as worked with the strain for the preliminary identification. AFB and MJF, re-identified the strain ans wrote the manuscript.

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Results

Table 1. Results obtained with MALDI-TOF Biotyper in two independent laboratories with different versions of the Biotyper database (V5 and V4).

Species	V5 Score	V4 Score
A. enteropelogenes	2.182	2.400
A. enteropelogenes	2.118	2.360
A. enteropelogenes	2.049	2.170
A. jandaei	0.035	1.991

Table 2. Antibiotic resistance pattern determined with the MicroScan WalkAway

Antibiotimicrobial agents	Result
Penicillins[a]	Susceptible
Quinolones	Susceptible
Carbapenems	Susceptible
Amynoglycosides	Susceptible
Cephalosporins[b]	Susceptible

[a]In combination with beta-lactamase inhibitors;

[b]Except cephalotin

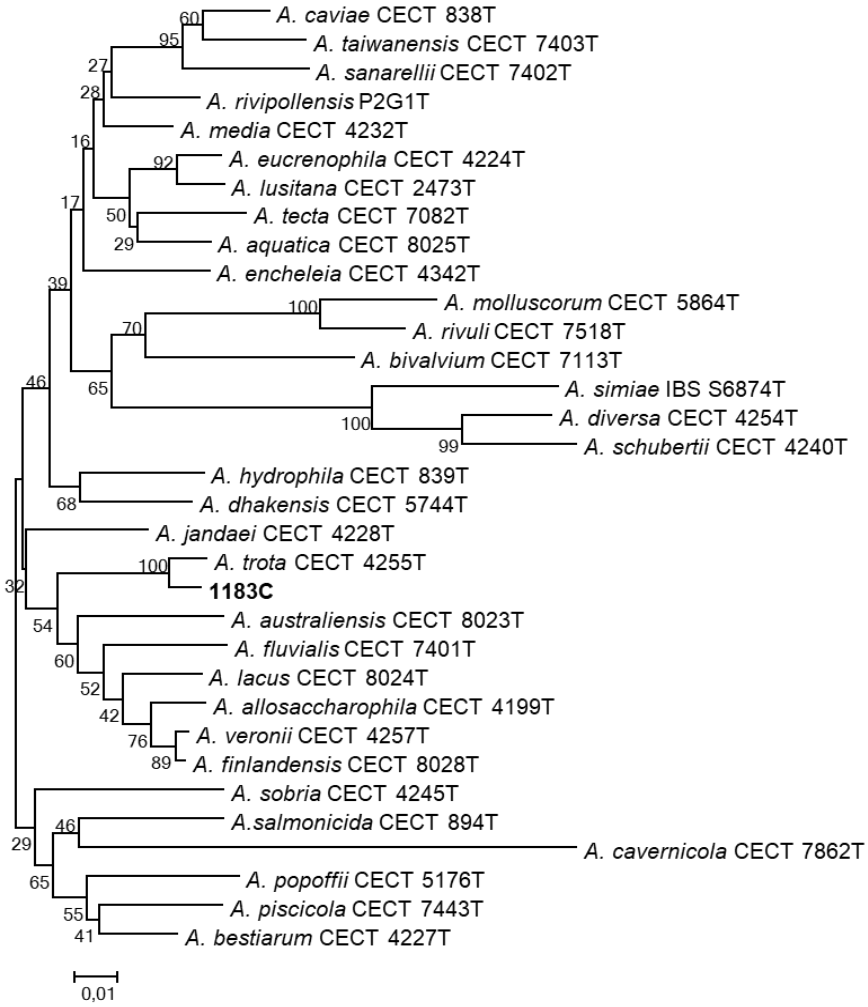

Figure 1. Phylogenetic tree based on *rpoD* gene (397 bp). Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). Bar 0.01 estimated nucleotide substitutions per site.

4.5. Limited performance of MALDI-TOF for identification of fish *Aeromonas* isolates at species level

Pérez-Sancho M., Cerdá I., Fernández-Bravo A., Domínguez L., Figueras MJ., Fernández-Garayzábal JF., Vela AI. (2018). Journal of Fish Diseases. 41:1485-1493.

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Limited performance of MALDI-TOF for identification of fish *Aeromonas* isolates at species level

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Abstract

The aim of this study was to evaluate the usefulness of the MALDI-TOF MS to identify 151 isolates of *Aeromonas* obtained mostly from diseased fish. MALDI-TOF MS correctly identified all isolates to the genus level but important differences in the percentage of isolates correctly identified depending on the species were observed. Considering exclusively the first identification option, *Aeromonas bestiarum*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Aeromonas veronii* and *Aeromonas sobria* were the best identified with results >95%. However, considering the first and second identification options, the only species that showed values >90% was *A. hydrophila*. Overall, when the database was supplemented with 14 new spectra, the number of accurate identifications increased (41% vs. 55%) and the number of inconclusive identifications decreased (45% vs. 29%), but great differences in the success of species-level identifications were found. Species-distinctive mass peaks were identified only for *A. hydrophila* and *A. bestiarum* (5003 and 7360 m/z in 95.5% and 94.1% of their isolates, respectively). This work demonstrates the utility of MALDI-TOF MS for *Aeromonas* identification to the genus level, but there is no consistency for the accurate identification of some of the most prevalent species implicated in fish disease.

KEYWORDS
Aeromonas, bacterial identification, diagnostic performance, fish, MALDI-TOF

1 | INTRODUCTION

Aeromonas are well-known fish pathogens (Beaz-Hidalgo & Figueras, 2013). The species with the highest clinical significance is *Aeromonas salmonicida*, the causative agent of furunculosis, a major disease with high mortality rates that causes great economic losses in aquaculture, particularly affecting salmonids (Beaz-Hidalgo, Alperi, Bujan, Romalde & Figueras, 2010; Khor, Puah, Tan, Puthucheary & Chua, 2015; Menanteau-Ledouble & El-Matbouli, 2016). Other species, such as *A hydrophila*, *Aeromonas bestiarum*, *A. veronii* and *Aeromonas sobria* have also been associated with diseases in fish (Beaz-Hidalgo & Figueras, 2013; Kozinska, 2007; Orozova, Barker, Austin & Austin, 2009; Rasmussen-Ivey et al., 2016; Yi et al., 2013).

The identification of *Aeromonas* isolates in clinical microbiological laboratories has been mostly based on different biochemical tests or the use of commercially identification kits or automatic or semiautomatic systems (Lamy et al., 2010; Ormen, Granum, Lassen & Figueras, 2005; Soler et al., 2003). Both approaches may give inconsistent results, confusing different species of the genus or misidentifying *Aeromonas* as members of the genus *Vibrio* (Deng et al., 2014; Janda & Abbott, 2010; Lamy et al., 2010; Rychert et al., 2015). The 16S rRNA sequencing is a not conclusive identification method (Soler et al., 2004), although correct identification can be achieved by sequencing other housekeeping genes (Beaz-Hidalgo et al., 2010; Martinez-Murcia et al., 2005; Soler et al., 2004). Nonetheless, sequencing approach can be costly and time-consuming

being not available for routine diagnosis in many clinical laboratories. Several species-specific polymerase chain reaction (PCR) assays have been developed for most of the *Aeromonas* with clinical relevance (Beaz-Hidalgo, Latif-Eugenin & Figueras, 2013) but the diversity of species of this genus associated with disease in fish and the difficulty for an etiological presumptive diagnosis based on the clinical symptoms (Bunnajirakul, Pavasutthipaisit & Steinhagen, 2015) might impair their routine use.

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) has been successfully implemented for the identification of different bacterial genera of relevance in aquaculture (Regecova et al., 2014). Several studies have also shown that MALDI-TOF MS could presumably efficiently identify *Aeromonas* species (Benagli et al., 2012; Chen et al., 2014; Deng et al., 2014; Donohue, Smallwood, Pfaller, Rodgers & Shoemaker, 2006; Donohue et al., 2007; Lamy, Kodjo, Laurent & Col, 2011; Rychert et al., 2015). Most of these studies have included environmental or human strains but no specific studies including a relatively large number of isolates from diseased fish are available. Thus, the aim of this study was to evaluate the utility of MALDI-TOF MS for the identification of a collection of clinical isolates belonging to different species of the genus *Aeromonas*, including the most prevalent and clinical relevant species in aquaculture.

2 | MATERIAL AND METHODS

2.1 | Bacterial isolates and identification

The study included 151 *Aeromonas* isolates mostly recovered from diseased animals in different fish farms from different countries (Spain, USA, Italy, Mexico, and Norway), as part of routine bacteriological analysis between 1999 and 2015. The isolates belonged to the species *A. veronii* (n = 31), *A. salmonicida* (n = 29), *A. sobria* (n = 27), *A. hydrophila* (n = 22), *A. bestiarum* (n = 17), *Aeromonas popoffii* (n = 13), *Aeromonas media* (n = 4), *A. dhakensis* (n = 3), *Aeromonas piscicola* (n = 2), *Aeromonas allosaccharophila*, *Aeromonas encheleia*, and *A. caviae* (n = 1/each). Isolates were identified to the genus level using API 20E system (bioMérieux, Marcy l'Etoile, France). Most isolates were recovered from European eel (*Anguilla Anguilla)* and rainbow trout (*Oncorhynchus mykiss)* but also from other fish species such asgilthead seabream (*Sparus aurata*), Atlantic salmon (*Salmo salar*), Koi carp (*Cyprinus carpio*) or tench (*Tinca tinca*). Identification to the species level was achieved by *rpoD* sequencing (Soler et al., 2004), which has been considered the gold standard technique in the present study. The sequences were compared with those of other Gram-negative catalase-positive species available in the GenBank database, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

2.2 | MALDI-TOF MS analysis: sample preparation

Isolates were grown on blood agar plates (bioMérieux, Marcy l'Etoile, France) and incubated at 25°C for 24 hr in aerobic conditions. Bacterial proteins were extracted using an acetonitrile/formic acid extraction protocol performed in accordance with the manufacturer's instructions (Bruker Daltonik, Bremen, Germany). Briefly, cells from representative bacterial colonies were resuspended in 300 μl of HPLC-grade water and mixed vigorously prior to 900 μl of HPLC-grade ethanol being added. Tubes with inactivated cells were centrifuged (16,000 g for 2 min), the supernatant was discarded and bacterial pellets were centrifuged again (16,000 g for 2 min). Ethanol was removed allowing pellet to dry at room temperature. Formic acid (30 μl, 70%) was added and mixed by vortexing. After this, the same volume of pure acetronitrile was added and mixed by vortexing. Centrifugation at 16,000 g for 2 min was performed. Immediately after protein extraction, one microlitre of each extract was spotted onto a 384-spot polished steel target plate, allowed dry at room temperature and overlaid with one microlitre of 10 mg/ml of HCCA (α-cyano-4-hydroxycinnamic acid) matrix solution (Standard Solvent, Sigma Aldrich).

2.3 | MALDI–TOF MS data acquisition and Biotyper classification

Samples were analysed using a Bruker Daltonics UltrafleXtreme MALDI-TOF/TOF equipment and the FlexControl software v. 3.0 (Brucker Daltonics, Bremen, Germany) for the acquisition of mass spectra, within a mass range from 2 to 20 kDa. The Biotyper Real Time Classification software v3.1 (Brucker Daltonics) was used for microbial identification by comparison of the spectra with the corresponding database provided by the manufacturer (MALDI Biotyper database, 5989 entries, Bruker Daltonics). This software generates a score, ranging from 0 to 3, showing the similarity between sample and reference spectra, and displays the top 10 matching results with the highest scores. The reliability of the identification was evaluated according to the standard manufacture interpretative criteria: ≥2.000: identification at species level with high confidence; 1.999–1.800: identification with low confidence and ≤1.799: nonreliable identification. When the score value obtained was <2.300, the extraction protocol and MALDI-TOF MS analysis were repeated twice.

In this study, we have evaluated the consistency of MALDI-TOF identification results considering the two best matches identification results provided by MALDI-TOF Biotyper establishing the following categories: A, the correct species is the unique species with score value ≥2.000, B) the correct species is first ranked but a different species in the second rank is also with score value ≥2.000; C) first and second matches have score values ≥2.000 but the correct species is second ranked; D), first and second matches have score values >2.000 but the correct species is not first neither second ranked. Those isolates within consistence categories A are considered accurately identified, those within categories B and C are considered inconclusively identified and those within the D category are considered misidentified.

2.4 | Supplementation of MALDI database

A set of randomly selected isolates (n = 14) belonging to the species *A. bestiarum*, *A. dhakensis*, *A. hydrophila*, *A. piscicola*, *A. popoffii*,

A. salmonicida, *A. sobria*, *A. veronii* were used to create Main Spectra (MSP) for supplementation of the Bruker MALDI-TOF MS database (version 3.4; 5989 entries). Two new entries per species were included except in the case of *A. dhakensis* and *A. piscicola* in which only one entry was included due to the limitation in the number of isolates available ($n = 3$ and $n = 2$, respectively). The MSP were generated using 20–24 spectra for each *Aeromonas* isolate (three subsequent measurements at 8 different spots) according to manufacturer's instructions. These MSP contain the average mass/charge and intensity of more representative peaks of each species, in addition to the prevalence of these ions. After MSP creation, the remaining isolates of these species ($n = 137$) were identified using the in-house database (entries of BDAL Biotyper Database plus 14 new *Aeromonas* MSP) as described above for the external validation of the new MSP and to assess the suitability of MALDI-TOF MS technique for the differentiation of *Aeromonas* species potentially present on clinical samples of fish.

2.5 | Identification of species-specific biomarkers and cluster analysis

Visual inspection of three spectra for each isolate was performed using the FlexAnalysis software (version 3.0, Bruker Daltonics) between 4,000 and 8,000 Da because most of the differential mass ions were detected within this spectra range. Spectra were grouped according to species they belonged in order to facilitate the comparison between species. After performing smoothing, baseline subtraction and peak picking, an in depth-evaluation of the spectra was carried out to detect the presence of differential species biomarkers. A peak shift of 500 ppm was accepted in the present study. Peaks were considered potential species-specific candidates when they showed an intensity of at least 500 arbitrary units (a.u.), and when they were present in at least 80% of the species spectra. Finally, a 2D peak distribution was performed using ClinProTools software (version 3.0, Bruker Daltonics) in order to assess the association between MALDI profiles of different test *Aeromonas* species.

3 | RESULTS AND DISCUSSION

All isolates were correctly identified to the genus level, but important differences in the percentage of isolates correctly identified were detected among the different species (Table 1). Identification of *Aeromonas* has been usually based exclusively in the first score of the identification-ranking list provided by Biotyper (Chen et al., 2014; Vavrova, Balazova, Sedlacek, Tvrzova & Sedo, 2015). Considering exclusively the first match in the identification, the best results (ranging from 89.7% to 100%) were obtained for the species *A. encheleia*, *A. bestiarum*, *A. hydrophila*, *A. salmonicida* and *A. sobria*. For instance, the single isolate of *A. encheleia* was correctly identified while the percentage of correct identification was 94.1%, 95.5%, 89.7% and 96.3% for isolates of *A. bestiarum*, *A. hydrophila*, *A. salmonicida* and *A. sobria*, respectively (score values >2.0). These rates of accurate identification are similar to those reported previously for these species (Benagli et al., 2012; Chen et al., 2014; Donohue et al., 2006; Lamy et al., 2011). However, the percentage of accurate identification for isolates of *A. media*, *A. popoffii* and *A. veronii* were 50%, 38.5% and 71.0%, respectively (Table 1). These percentages were slightly lower to those reported by other authors (Benagli et al., 2012; Chen et al., 2014; Donohue et al., 2006; Lamy et al., 2011). The three *A. dhakensis* isolates were misidentified as *A. hydrophila* and the two isolates of *A. piscicola* were identified as *A. salmonicida* and *A. bestiarum*, all of them with scores >2.0.

Previous studies indicate that identification results considering exclusively in the first identification option provided by Biotyper is not the best approach for an accurate identification, being necessary to evaluate also at least the score value of the second identification option (Lamy et al., 2011; Perez-Sancho et al., 2017). For this reason, we have evaluated the consistency of MALDI-TOF identification results taking into consideration the 2 best scores provided by MALDI-TOF Biotyper (consistence categories A-D, see M&M and Tables 1 and 3). Thus, those isolates within consistence categories A were considered *accurately identified*, those within categories B and C were considered *inconclusively identified* and those within the D category were considered *misidentified* (see Table 2). Following this criterion, the 91% and 65.5% of the *A. hydrophila* and *A. salmonicida* isolates, respectively, were accurately identified, while most isolates of *A. veronii*, *A. sobria* and *A. bestiarum* gave inconclusive identifications (Table 2). The small differences in the latter three species between the first and second identification scores (averaged differences of 0.094), that in most cases were >2.0 (Table 1; categories B–C), hindering their unambiguous identification. Most isolates of *A. popoffii* (8/13) were misidentified with different species of *Aeromonas* (Tables 1 and 2). Overall, the more stringent identification criterion used in this study significantly reduced the correct identification rates compared with the results reported by other authors (Benagli et al., 2012; Chen et al., 2014; Donohue et al., 2007; Lamy et al., 2011) but increases the identification reliability.

The detection and identification of genus- and species-specific mass peaks can be useful for bacterial identification (Holland et al., 1999, 2000). A single peak analysis was performed in the range of 4,000–8,000 Da, that included most of the potential biomarkers, and the peak list of each species (Table 3) compared with previous studies. A number of peak masses (4,256, 4,700, 5,049, 6,304 and 6,480 m/z) were found in all the *Aeromonas* species included in this study and most of them were also identified by Donohue et al. (2006). Moreover, in those species with higher number of isolates (*A. hydrophila*, *A. popofii*, *A. bestiarum*, *A. sobria*, *A. veronii* and *A. salmonicida*; Table 3), these mass peaks were consistently present in the great majority (>80%) of their isolates. Therefore, these peaks could be considered potential genus-characteristic ions. Additionally, some specific mass ions were observed only in the spectra of a particular species (Supporting Information Table S1). However, in those species with more than 10 isolates, only some of these peaks were consistently identified among the isolates of the corresponding species. The mass peaks 5,003 and 7,360 m/z were present in the

TABLE 1 Identification results using the Biotyper database for a total of 151 *Aeromonas* isolates mostly recovered from diseased fish

Species	N	1rst Identification[a]	2nd Identification[a]	Consistency[b]
A. allosaccharophila	1	*A. allosaccharophila* (2.238)	*A. veronii* (2.291)	B
A. bestiarum	5	*A. bestiarum* (2.125–2.300)	*A. bestiarum* (2.066–2.234)	A
	1	*A. bestiarum* (2.013)	*A. salmonicida* (1.990)	A
	4	*A. bestiarum* (2.110–2.261)	*A. salmonicida* (2.049–2.196)	B
	6	*A. bestiarum* (2.215–2.329)	*A. eucrenophila* (2.176–2.271)	B
	1	*A. salmonicida* (2.286)	*A. bestiarum* (2.255)	C
A. caviae	1	*A. cavie* (2.252)	*A. hydrophila* (2.159)	B
A. dhakensis	3	*A. hydrophila* (2.219–2.267)	*A. veronii* (2.078–2.213)	D
A. encheleia	1	*A. encheleia* (2.219)	*A. encheleia* (2.010)	A
A. hydrophila	20	*A. hydrophila* (2.244–2.482)	*A. hydrophila* (2.168–2.435)	A
	1	*A. hydrophila* (2.351)	*A. veronii* (2.329)	B
	1	*A. salmonicida* (2.259)	*A. salmonicida* (2.200)	D
A. media	1	*A. media* (2.227)	*A. cavie* (2.127)	B
	1	*A. media* (2.167)	*A. veronii* (2.166)	B
	1	*A. eucrenophila* (2.016)	*A. hydrophila* (1.999)	D
	1	*A. hydrophila* (2.173)	*A. veronii* (2.170)	D
A. piscícola	1	*A. salmonicida* (2.131)	*A. bestiarum* (2.072)	D
	1	*A. bestiarum* (2.263)	*A. salmonicida* (2.184)	D
A. popoffii	4	*A. popoffii* (2.174–2.344)	*A. salmonicida* (2.121–2.269)	B
	1	*A. popoffii* (2.335)	*A. bestiarum* (2.272)	B
	3	*A. bestiarum* (2.256–2,346)	*A. salmonicida* (2.090–2.250)	D
	2	*A. bestiarum* (2.212–2.332)	*A. bestiarum* (2.072–2.108)	D
	2	*A. bestiarum* (2.213–2.288)	*A. eucrenophila* (2,186–2.230)	D
	1	*A. salmonicida* (2.131)	*A. bestiarum* (2.072)	D
A. salmonicida	19	*A. salmonicida* (2.178–2.440)	*A. salmonicida* (2.097–2.350)	A
	5	*A. salmonicida* (2.141–2.403)	*A. bestiarum* (2.053–2.249)	B
	1	*A. salmonicida* (2.351)	*A. popoffii* (2.306)	B
	1	*A. salmonicida* (2.286)	*A. hydrophila* (2.188)	B
	2	*A. bestiarum* (2.226–2.318)	*A. salmonicida* (2.221–2.281)	C
	1	*A. bestiarum* (2.181)	*Aeromonas sp.* (2.145)	D
A. sobria	1	*A. sobria* (2.171)	*A. sobria* (2.021)	A
	2	*A. sobria* (2.204–2.259)	*A. hydrophila* (1.913–1.937)	A
	3	*A. sobria* (2.270–2.368)	*A. eucrenophila* (1.956–1.990)	A
	2	*A. sobria* (2.352–2.376)	*A. media* (2.030–2.049)	B
	1	*A. sobria* (2.305)	*A. caviae* (2.044)	B
	6	*A. sobria* (2.283–2.385)	*A. eucrenophila* (2.073–2.242)	B
	11	*A. sobria* (2.218–2.411)	*A. hydrophila* (2.047–2.257)	B
	1	*A. encheleia* (2.371)	*A. encheleia* (2.206)	D
A. veronii	10	*A. veronii* (2.114–2.400)	*A. veronii* (2.044–2.344)	A
	11	*A. veronii* (2.271–2.443)	*A. hydrophila* (2.244–2,350)	B
	1	*A. veronii* (2.446)	*A. ichthiosmia* (2.364)	B
	6	*A. hydrophila* (2.174–2.363)	*A. veronii* (2.156–2.350)	C
	1	*A. ichthiosmia* (2.341)	*A. veronii* (2.335)	C
	1	*A. caviae* (2.189)	*A. caviae* (2.180)	D
	1	*A. hydrophila* (2.326)	*A. ichthiosmia* (2.271)	D

Note: [a]First and second identification best matches with their respective score values provided by the Byotyper identification list.

[b]For interpretation of the identification results, two variables were considered: (i) score values following the MALDI Biotyper Compass (≥2.000: identification at species level with high confidence; 1.999–1.800: identification with low confidence and ≤1,799: non-reliable identification) and (ii) consistency ranking list of the first 2 best matches: A, the correct species is the unique species with score value ≥2.000, B) the correct species is first ranked but a different species in the second rank is also with score value ≥2.000; C) first and second matches have score values ≥2.000 but the correct species is second ranked; D), first and second matches have score values >2.000 but the correct species is not first neither second ranked. Those isolates within consistence categories A are considered accurately identified, those within categories B and C are considered inconclusively identified and those within the D category are considered misidentified.

TABLE 2 Overview of the identification results for the different species of *Aeromonas*

Species	N° isolates (n = 151)	Identification with Byotyper database N° isolates[a] (%)[b]			N° isolates (n = 137)	Identification[a] with Byotyper database plus the 14 additional MSP[c] N° isolates (%)[b]		
		Accurate	Inconclusive	Missidentified		Accurate	Inconclusive	Missidentified
A. allosaccharophila	1		1		1		1	
A. caviae	1		1		1		1	
A. encheleia	1	1			1	1		
A. piscicola	2			2	1			1
A. dhakensis	3			3	2		1	1
A. media	4		2	2	4		1	3
A. bestiarum	17	6 (35.3)	10 (58.8)	1 (5.9)	15	3 (20)	10 (66.7)	2 (13.3)
A. hydrophila	22	20 (91)	1 (4.5)	1 (4.5)	20	14 (70)	5 (25)	1 (5)
A. popoffii	13		5 (38.5)	8 (61.5)	11	2 (18.2)	3 (27.3)	6 (54.5)
A. salmonicida	29	19 (65.5)	9 (31)	1 (3.4)	27	13 (48.1)	9 (33.3)	5 (18.5)
A. sobria	27	6 (22.2)	20 (74.1)	1 (3.7)	25	22 (88)	2 (8)	1 (4)
A. veronii	31	10 (32.3)	19 (61.3)	2 (6.4)	29	21 (72.4)	7 (24.1)	1 (3.4)

Note: [a]For interpretation of the identification results, two variables were considered: (i) score values following the MALDI Biotyper Compass (≥2.000: identification at species level with high confidence; 1.999-1.800: identification with low confidence and ≤ 1,799: nonreliable identification) and (ii) consistency ranking list of the first two best matches: A, the correct species is the unique species with score value ≥ 2.000, B) the correct species is first ranked but a different species in the second rank is also with score value ≥ 2.000; C) first and second matches have score values ≥ 2.000 but the correct species is second ranked; D) first and second matches have score values > 2.000 but the correct species is not first neither second ranked. Those isolates within consistence category A are considered accurately identified, those within categories B and C are considered inconclusive identified and those within the D category are misidentified.

[b]Percentages were calculated only for those species with more than 10 isolates. [c]One isolate of *A. dhakensis* and *A. piscicola* and two isolates of *A. bestiarum*, *A. hydrophila*, *A. popoffii*, *A. salmonicida*, *A. sobria* and *A. veronii* were used to generate their respective MSP (n = 14) that were included in the Bruker database.

95.5% and 94.1% of the *A. hydrophila* and *A. bestiarum* isolates, respectively (Supporting Information Table S1). The mass peaks 4,460 and 4,490 characteristic of the species *A. sobria* and *A. veronii*, respectively, were detected in 65% of their isolates. All other potential species-specific peaks were detected in less than 50% of the isolates of the respective species (Supporting Information Table S1). For the remaining species with low number of isolates (n < 5) some specific mass ions were also identified (Supporting Information Table S1). The low consistency of most of these peaks among isolates of the same species suggest a strong variability in their MALDI-TOF MS proteome, in line with their high intra-species variability (Mandrell et al., 2005) that would limit their use as species-characteristic mass peaks. Moreover, most of the species-distinctive mass peaks identified in the present study were not observed by Donohue et al. (2006) and Benagli et al. (2012). Experimental parameters such as bacterial culture conditions, protein extraction protocol or sample preparation protocol and the equipment used may affect the reproducibility of MALDI results, hindering the comparison of the mass spectra and mass lists obtained (Jackson, Edwards-Jones, Sutton & Fox, 2005; Williams, Andrzejewski, Lay & Musser, 2003), when examining very closely related microorganisms like *Aeromonas*. Differences in the inoculation method of sample wells, type of matrix or matrix solvent used in this study compared with those used by Donohue et al. (2006) and Benagli et al. (2012) could explain the absence of concordance in the mass peaks identified in the three studies. Therefore, further studies with standardized experimental protocols will be necessary before well-defined potential species-specific peaks for *Aeromonas* can be assigned.

MALDI-TOF MS bacterial identification can be improved using a Biotyper database supplemented with locally produced MSPs (Christensen et al. 2012, Sogawa et al. 2012). Thus, in an attempt to improve the accurate identifications rates, two isolates of those species with more than 10 isolates as well as one isolate of *A. dhakensis* and *A. piscicola* (not included in the commercial Biotyper database) were used to generate additional MSP (n = 14) that were incorporated into the Bruker database. No additional MSP of *A. allosaccharophila*, *A. caviae*, *A. media* and *A. encheleia* were created because the low number of isolates of these species included in the study. The remaining 137 fish isolates were identified again against the supplemented Biotyper database and the results obtained are shown in Tables 2 and 3. Overall, the inclusion of the new 14 MSP increased the number of isolates with accurate identifications (41% vs. 55%) and decreased the number of isolates with inconclusive identifications (45% vs. 29%, Table 2). Nevertheless, there were great differences in the success of species-level identifications. Thus, there was an increase in the number of isolates of *A. veronii* (32.3% vs. 72.4%), *A. sobria* (22.2% vs. 88%) and *A. popoffii* (0% vs. 18.2%) with accurate identifications, while decrease in the number of isolates of the two former species with inconclusive identifications (Table 2). In contrast, decrease in the number of *A. hydrophila* (91% vs. 70%), *A. bestiarum* (35.3% vs. 20%) and *A. salmonicida* (65.5% vs.

TABLE 3 Identification[a] results using the Biotyper database plus 14 additional MSP[a] for a total of 137 *Aeromonas* isolates mostly recovered from diseased fish

Species	*N*	1st Identification[b]	2nd Identification[b]	Consistency[c]
A. allosaccharophila	1	*A. allosaccharophila* (2.328)	*A. veronii* (2.294)	B
A. bestiarum	3	*A. bestiarum* (2.252–2.553)	*A. bestiarum* (2.218–2.503)	A
	2	*A. bestiarum* (2.484–2.574)	*A. hydrophila* (2.484–2.571)	B
	4	*A. bestiarum* (2,485–2.606)	*A. piscicola* (2.423–2.601)	B
	2	*A. piscicola* (2.512–2.557)	*A. bestiarum* (2.453–2.519)	C
	1	*A. piscicola* (2.384)	*A. popoffii* (2.331)	D
	1	*A. popoffii* (2.458)	*A. bestiarum* (2.220)	C
	1	*A. popoffii* (2.329)	*A. piscicola* (2.276)	D
	1	*A. salmonicida* (2.516)	*A. bestiarum* (2.514)	C
A. caviae	1	*A. cavie* (2.252)	*A. hydrophila* (2.159)	B
A. dhakensis	1	*A. hydrophila* (2.267)	*A. veronii* (2.128)	D
	1	*A. dhakensis* (2.401)	*A. hydrophila* (2.219)	B
A. encheleia	1	*A. encheleia* (2.219)	*A. encheleia* (2.010)	A
A. hydrophila	14	*A. hydrophila* (2.244–2.439)	*A. hydrophila* (2.168–2.435)	A
	1	*A. hydrophila* (2.361)	*A. dhakensis* (2.318)	B
	4	*A. dhakensis* (2.325–2.557)	*A. hydrophila* (2.325–2.483)	C
	1	*A. dhakensis* (2.474)	*A. veronii* (2.420)	D
A. media	1	*A. media* (2.227)	*A. cavie* (2.127)	B
	1	*A. bestiarum* (2.332)	*A. bestiarum* (2.127)	D
	1	*A. dhakensis* (2.365)	*A. sobria* (2,350)	E
	1	*A. sobria* (2.302)	*A. dhakensis* (2.269)	D
A. piscicola	1	*A. bestiarum* (2.263)	*A. popoffii* (2.261)	D
A. popoffii	2	*A. popoffii* (2.254–2.371)	*A. popoffii* (2.174–2.235)	A
	2	*A. popoffii* (2.489–2.520)	*A. piscicola* (2.476–2.492)	B
	1	*A. popoffii* (2.441)	*A. salmonicida* (2.436)	B
	1	*A. bestiarum* (2.332)	*A. bestiarum* (2.127)	D
	1	*A. bestiarum* (2.293)	*A. salmonicida* (2.090)	D
	1	*A. bestiarum* (2.620)	*A. piscicola* (2.618)	D
	1	*A. piscicola* (2.357)	*A. bestiarum* (2.212)	D
	2	*A. piscicola* (2.418–2.642)	*A. popoffii* (2.409–2.626)	D
A. salmonicida	13	*A. salmonicida* (2.178–2.440)	*A. salmonicida* (2.097–2.407)	A
	3	*A. salmonicida* (2.519–2.610)	*A. hydrophila* (2.506–2.597)	B
	2	*A. salmonicida* (2.141–2.403)	*A. bestiarum* (2.053–2.235)	B
	2	*A. salmonicida* (2.370–2.543)	*A. piscicola* (2.333–2.532)	B
	2	*A. piscicola* (2.477–2,580)	*A. bestiarum* (2.279–2.563)	D
	1	*A. piscicola* (2.446)	*A. hydrophila* (2.419)	D
	1	*A. piscicola* (2.483)	*A. salmonicida* (2.352)	C
	1	*A. bestiarum* (2.394)	*A. salmonicida* (2.303)	C
	1	*A. bestiarum* (2.181)	*Aeromonas* sp. (2.145)	D
	1	*A. hydrophila* (2.682)	*A. bestiarum* (2.610)	D
A. sobria	22	*A. sobria* (2.244–2.606)	*A. sobria* (2.076–2.411)	A
	2	*A. sobria* (2.424–2.466)	*A. bestiarum* (2.260–2.298)	B
	1	*A. encheleia* (2.371)	*A. piscicola* (2.220)	D
A. veronii	21	*A. veronii* (2.328–2.701)	*A. veronii* (2.231–2.588)	A
	4	*A. veronii* (2.271–2.447)	*A. hydrophila* (2.244–2.350)	B

(Continues)

TABLE 3 (Continued)

Species	N	1st Identification[b]	2nd Identification[b]	Consistency[c]
	1	*A. veronii* (2.587)	*A. ichthiosmia* (2.341)	B
	2	*A. hydrophila* (2.174–2.354)	*A. veronii* (2.156–2.294)	C
	1	*A. piscicola* (2.323)	*A. bestiarum* (2.279)	D

Note: [a]One isolate of *A. dhakensis* and *A. piscicola* and two isolates of *A. bestiarum*, *A. hydrophila*, *A. popoffii*, *A. salmonicida*, *A. sobria* and *A. veronii* and were used to generate their respective MSP (n = 14) that were included in the Bruker database. [b]First and second identification best matches with their respective score values provided by the Biotyper identification list. [c]For interpretation of the identification results, two variables were considered: (i) score values following the MALDI Biotyper Compass (≥2.000: identification at species level with high confidence; 1.999–1.800: identification with low confidence and ≤1,799: nonreliable identification) and (ii) consistency ranking list of the first two best matches: A, the correct species is the unique species with score value ≥2.000, B) the correct species is first ranked but a different species in the second rank is also with score value ≥2.000; C) first and second matches have score values ≥2.000 but the correct species is second ranked; D), first and second matches have score values >2.000 but the correct species is not first neither second ranked. Those isolates within consistence categories A are considered accurately identified, those within categories B and C are considered inconclusively identified and those within the D category are considered misidentified.

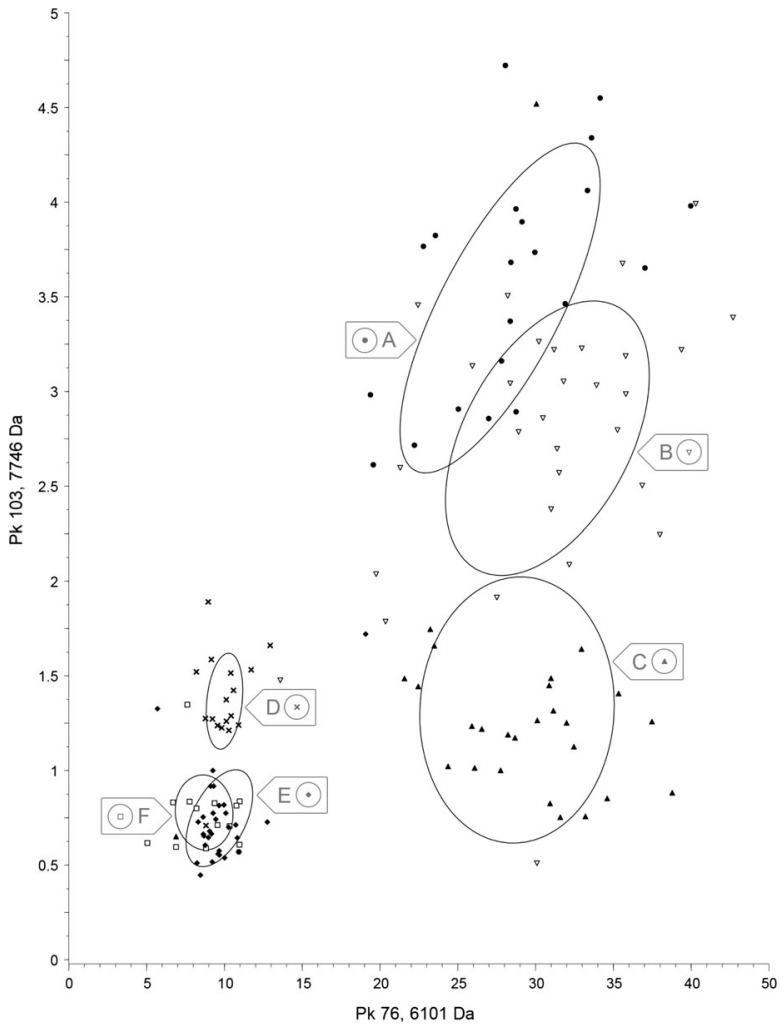

FIGURE 1 Illustrations showed the 2D peak distribution diagram of *Aeromonas* species; a, *A. hydrophila*; b, *A. veronii*; c, *A. sobria*; d, *A. bestiarum*: e, *A. popoffii*; f, *A. salmonicida*

48.1%) isolates with accurate identifications, but increasing the number of isolates of these species with inconclusive identifications (Tables 2 and 3). No differences were observed for the other species of *Aeromonas*, but the limited number of isolates of these species preclude reaching any conclusion. The close-similarity among MALDI-TOF profiles of main fish pathogenic species of *Aeromonas* was reflected by 2D

peak distribution diagram (Figure 1) showing an overlapping among isolates of different species. This overlapping is likely related with the strong phylogenetic relatedness within the genus *Aeromonas* (Martin-Carnahan & Joseph, 2005) and in line the limited discrimination power of MALDI Biotyper to separate closely related species observed in other microorganisms (Alispahic, Christensen, Bisgaard, Hess & Hess, 2014; Frey & Kuhnert, 2015).

In conclusion, this work demonstrates the utility of MALDI-TOF MS for the identification of *Aeromonas* to the genus level, but its poor performance for an accurate identification of some of the most prevalent species implicated in fish disease, such as *A. salmonicida*, *A. hydrophila* or *A. bestiarum*.

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SUPPORTING INFORMATION

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UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
Ana Fernández Bravo

4.6. First record and genome sequencing of *Aeromonas lusitana* ESV-351 isolated from rainbow trout in Mexico.

Fernández-Bravo A., Vega-Sánchez V., Pérez-Cataluña A., Latif-Eugenín F., Beaz-Hidalgo R., Martínez-Murcia A., Soriano-Vargas E., Palma-Martínez I., Castro-Escarpulli G., Figueras MJ. (In preparation, pending to be submitted to Jounal of Fish Diseases).

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Results

First record and genome sequencing of *Aeromonas lusitana* ESV-351 isolated from rainbow trout in Mexico.

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Abstract

Aeromonas lusitana, was described in 2016 with five isolates recovered from untreated water and vegetables from Portugal, and since then no further records exits of this species. Interestingly in the course of a study about the prevalence of *Aeromonas* in fish farms in Mexico, a strain (ESV-351) isolated from rainbow trout clustered phylogenetically with *A. lusitana* by using the sequences of the *rpo*D gene. In the present study, the phenotypic characteristics, antimicrobial resistance, and presence of the putative virulence genes of the new strain (ESV-351) were studied in parallel to the five isolates included in the species description. In addition, the genome of the new strain (ESV-351) was sequenced and compared with the one of the type strain of this species and with the ones of other *Aeromonas* spp. The phenotypic differential characteristics of *A. lusitana* showed to be highly conserved. However, the new strain was positive for the indole production when using conventional methods while the rest of the strains including the type strain were negative. Furthermore, an intermediate resistance to ampicillin, amoxicillin-clavulanic acid and cephalothin was found in both strains. Five different virulence gene profiles were observed among the strains, and the type strain showed the highest number of virulence genes. Genome analysis showed that *A. tecta* is the closest species to *A. lusitana* with a highly similar number of predicted proteins. The genome size, the number of genes encoding proteins, the number of tRNAs, among other characteristics suggests that the new strain ESV-351 could have the capacity to adapt to different environments. The comparison of the genome of strain ESV-351 with the one of the type strain revealed that both strains possesses a very similar sequence of the citrate synthase gene. Additionally, the chromosomal region containing the citrate synthase locus of the new strain showed to be relatively similar to that of the type strain of *A. hydrophila*, and other important human pathogens (*Vibrio cholera*, etc), suggesting the possible role of this gene in the virulence of *A. lusitana* (ESV-351). The draft genome sequence of this novel *A. lusitana* strain is a valuable addition to the genomic database and can contribute to the understanding of the role of this species in the environment.

Keywords: *Aeromonas lusitana,is*DDH, ANI, virulence genes, citrate synthase.

Introduction

The genus *Aeromonas* resides within the family *Aeromonadaceae* that belongs to the *Gamma-proteobacteria* class (Martin-Carnahan and Joseph, 2005), and includes species found in water environments and some associated with fish and human diseases (Janda and Abbott, 2010; Beaz-Hidalgo and Figueras, 2013; Figueras and Beaz-Hidalgo et al., 2015; Figueras and Ashbolt, 2019).

The virulence genes in pathogenic *Aeromonas* strains are acquired from their common ancestor or by lateral gene transfer (Hossain et al., 2013). However, the virulence of *Aeromonas* is multifactorial and related with the different structures implicated in adhesion (flagella, fimbria, outer membrane proteins (OMPs), and capsule responsible for the first contact between host and pathogen and evasion of the immune response (Beaz-Hidalgo and Figueras, 2013; Romero et al, 2016). The extracellular secreted proteins including cytotoxic and cytotonic enterotoxin, hemolysin, proteases, and lipases play a role during the bacteria invasion and in the establishment of the infection (Galindo et al., 2006; Beaz-Hidalgo and Figueras, 2013; Tomás, 2012). In addition, four of the six secretion systems (TSS) have been detected in the genus *Aeromonas* (type II, III, IV and VI). The type III (T3SS) is considered very important in the pathogenesis and virulence of the *Aeromonas* strains that generate fish and human diseases (Dallaire-Dufresne et al., 2014; Janda and Abbott, 2010).

The taxonomy of the genus *Aeromonas* has evolved very quickly in recent years with the addition of new species. Nowadays, the genus includes 32 species, and 4 are pending to be described. *Aeromonas lusitana* was described in 2016, and was described based on 5 isolates recovered from untreated water and vegetables from Portugal (Martínez-Murcia et al., 2016). In addition, to increase the knowledge about this recent species, the genome of the type strain of *A. lusitana* CECT 7828^{T} was recently sequenced (Colston et al., 2018).

In a previous study that investigated the prevalence of *Aeromonas* in fish farms in Mexico, a strain (ESV-351) isolated from the gills of rainbow trout, and that phylogenetically clustered with *A. lusitana* was identified. In the present study we analyzed the characteristics of this new strain ESV-351 in parallel with the five already known strains of this species, comparing the biochemical features, antimicrobial activity, and the presence of virulence genes. In addition, we compared the obtained genome with the one already available of the type strain. The final aim is to obtain a better knowledge of this rare species.

Materials and Methods

Bacterial strains

A cultured juvenile rainbow trout sourced from a pond located in "Huasca de Ocampo" in the state of Hidalgo in Mexico, was examined for bacterial presence in the previous study (Vega-Sánchez et al., 2014a). Samples from gills, intestine, spleen, liver, and kidney were plated on Tryptic Soy Agar (TSA) and were incubated at 30 °C for 24 h. Colonies were identified by using the 16S rDNA RFLP technique and a second identification was performed by Vega-Sánchez et al. (2014b) with a phylogenetic analysis based on *gyrB* and *rpoD* gene sequences that were obtained from all the strains. Among the strains, one named ESV-351, was clustered in the phylogenetic analysis with the type strain of *Aeromonas lusitana* (GeneBank accession number *gyrB*: KJ743554; *rpoD*: KJ743565).

For the comparative analysis in our study the strain ESV-351 was evaluated in parallel with the 5 strains used for the description of *A. lusitana* recovered from water and vegetables in Portugal (Martinez-Murcia et al., 2016). Three strains (A.11/6T, A.136/15 and A.28/6), were isolated from water (Carvalho et al., 2012), and the other two strains (L8-3 and L10-4) were later isolated from vegetables but unpublished.

Results

Phenotypic characteristic and susceptibility to antimicrobial agents

Phenotypic tests of the Mexican strain ESV-351 were evaluated by using conventional biochemical tests, as reported on previous studies (Abbott et al., 2003; Beaz-Hidalgo et al., 2010) and were compared with the five strains used for the description (Martínez-Murcia et al., 2016). Additionally, a comparison between the results obtained with the conventional biochemical tests and with the MicroScan WalkAway-40 system, was performed by using the Mexican strain ESV-351. Furthermore, the antimicrobial susceptibility test against 23 antibiotics was tested by MicroScan WalkAway-40 system, as well as, by disc diffusion. The results were interpreted according to the Clinical Laboratory Standards Institute (CLSI) Guidelines (CLSI 2005, 2012). The phenotypic test and the antimicrobial susceptibility were performed in triplicate.

Fingerprinting

An enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) was performed with the primers and conditions previously described by Versalovic et al. (1991).

Detection of putative virulence genes

The Mexican strain ESV-351 and the five strains described by Martínez-Murcia et al. (2016) were evaluated for the presence of nine putative virulence genes by PCR with three replicates. The specific gene studied was the one involved in the synthesis of the lateral flagellum *lafA* (Merino et al., 2003), aerolysin/haemolysin *aer*A (Soler et al., 2002), cytotoxic enterotoxin *act* (Kimgombe et al., 1999), heat stable enterotoxin *ast* (Aguilera-Arreola et al., 2005), heat-labile cytotonic enterotoxin *alt* (Chopra et al., 1996), lipase genes *lip*, *lip*H3, *pla*, *plc* (Chacón et al., 2003), aerolysin/haemolysin *aerA*, serine protease *asp*A, *ahe*2 (Chacón et al., 2003), T3SS *ascF-G* and *ascV* (Chacón et al., 2004),the effectors protein *aexT* (Braun et al., 2002) and *aopP* (Fehr et al., 2006) and the Shiga toxins *stx1*(Wang et al., 2002) and *stx2* (Muniesa et al., 2003).

Genome sequencing, assembling and annotation

Genomic DNA of the Mexican strain ESV-351 was extracted by using the Easy-DNA™ gDNA purification kit (Invitrogen, Madrid, Spain). The integrity of the extracted DNA was ensured by electrophoresis in 1.5% agarose gel, and it was quantified by using Qubit ™ with the Broas Range Assay kit (Invitrogen). Genomic libraries for paired-end sequencing was performed with the Nextera DNA Library Preparation kit (Illumina, Lisbon, Portugal) and sequenced by using the Miseq platform (Illumina). Clean reads were assembled *de novo* by using the CGE Assembler 1.2 (Larsen et al., 2012). Annotation of the contigs was accomplished using the Rapid Automated Annotation with the Subsystem Technology (RAST) server using the *classic RAST* scheme http://rast.nmpdr.org/ (Aziz et al., 2008; Overbeek et al., 2014).

Phylogenetic analysis based on the genome

The phylogenetic analysis was performed to verify if the ESV-351 strain was clustered with the type strain of *A. lusitana* CECT 7828^T and to analyze the phylogenetic relation with other species. A total of 7 housekeeping genes (*rpoD, gyrB, recA, gyrA, atpD, dnaJ* and *dnaX*) were extracted from the ESV-351 genome performing a search by using the Basic Local Alignment Search Tool (from the NCBI web interface) for a Multilocus Phylogenetic Analysis (MLPA). A phylogenetic tree was constructed with the concatenated sequences (MLPA) of these genes.

Genome information and comparison

The genome-derived information of *Aeromonas lusitana* ESV-351 (GenBank accession in process) was obtained with the SEED viewer from RAST http://rast.nmpdr.org/seedviewer.cgi (Aziz et al., 2008; Overbeek et al., 2014) and tRNAscan-SE http://lowelab.ucsc.edu/tRNAscan-SE/ (Lowe and Chan., 2016). In addition, other genomes were used to compare this information, i.e. the genome of *A. lusitana* CECT 7828^T type strain (Genbank Whole-Genome Shotgun project no. PRJNA417247) from untreated water (Martínez-Murcia et al., 2016), as well as

the genomes of the closest species *A. eucrenophila* CECT 4224^T (GenBank Whole-Genome Shotgun project no. CDDF00000000.1) from freshwater fish (Colston et al., 2014), *A. tecta* CECT 7082^T (Genbank Whole-Genome Shotgun project no. CDCA00000000.1) from children feces, *A. aquatica* CECT 8025^T (Genbank Whole-Genome Shotgun project no. JRGL00000000.1) from Finnish water associated with cyanobacterial blooms (Beaz-Hidalgo et al., 2015) and *A. encheleia* CECT 4342^T (GenBank Whole-Genome Shotgun project no. CDDI00000000.1) from European eel (Colston et al., 2014). These genomes were annotated in the SEED viewer from RAST to obtain the information, and the tRNAscan-SE was used to obtain the predicted tRNA. Moreover, the complete previously described genomes were also used, i.e., *A. hydrophila* ATCC 7966^T (GenBank accession no. CP000462.1), from a tin of milk with a fishy odor (Seshadri et al., 2006), *A. salmonicida* A449 (GenBank no. CP000644.1), from a brown trout (Reith et al., 2008), *A. salmonicida* 01-B526 isolated from infected brook trout (GenBank Whole-Genome Shotgun project no. AGVO00000000.1) (Charette et al., 2012), *A. aquariorum* AAK1 (GenBank Whole-Genome Shotgun project no. BAFL00000000.1), recently reclassified as *A. dhakensis* sp. nov. comb. nov., from the blood of a patient with septicemia and NF (Wu et al., 2012), *A. veronii* B565 (GenBank accession no. CP002607.1), from aquaculture pond sediment (Li et al, 2011) and *A. caviae* Ae398 (GenBank Whole-Genome Shotgun project no. CACP00000000.1) from feces of a child with diarrhoea (Beatson et al., 2011).

Genomic indexes

The genomes sequences of *A. lusitana* ESV-351 and the one of the type strain CECT 7828^T were uploaded to the Genome-to-Genome Distance Calculator GGDC 2.0 Web server (http://ggdc.dsmz.de/distcalc2.php), for the in silico DNA-DNA hybridization (**is**DDH) calculation using formula 2 (Meier-Kolthoff et al., 2014). In addition, the Average Nucleotide Identity (ANI) values between *A. lusitana* ESV-351, the *A. lusitana* CECT 7828^T and the closest species were calculated by using the Orthologous Average Nucleotide Identity Tool (Lee et al., 2016).

Core-Pan Genome analysis

Pan genome represents the entire gene set of all strains of a species. It includes genes present in all strains (core genome) and genes present only in some strains of a species (dispensable genome), and their analysis may reveal genes involved in specific functions like pathogenicity, stress, resistance, among others. (Hassan et al., 2016). To estimate the number of genes, present in the pan and core genomes, the pan- and core- genome plot method was used as previously described with other pathogens (Lukjancenko et al., 2010; Hassan et al., 2016). The core-pan genome was calculated with the genome annotations performed with Prodigal (Hyatt et al., 2010) by using the program *pancoreplot* from CMG Biotools which is dependent on BLAST (Vesth et al., 2013).

Two genes producing the best hits must be representative of the same gene family. Gene families with at least one gene in common in the genomes compared, were plotted in the core genome. The cutoff was previously established at 50/50%, indicating that two genes belong to a gene family if their amino acids are 50% identical over 50% of the length of the longest gene (50% identity/ 50% coverage). The rest of the total genes not included in the core genome was plotted in the pan genome (Ussery et al., 2009; Vesth et al., 2013).

Comparison of predicted protein

Assembled genomes in FASTA format annotated in RAST were compared by using the SEED viewer http://rast.nmpdr.org/seedviewer.cgi. (Aziz et al., 2008; Overbeek et al., 2014). The SEED viewer was used to compare the sequence identity of the annotated predicted proteins of the new *A. lusitana* strain ESV-351 and the closest species based on the sequence of *A. lusitana* type strain (CECT 7828^T), using the "Sequence based comparison" in the comparative tools.

Distribution and counts of proteins in subsystems

A search of proteins associated to different functional and structural pathways was performed with the new strain ESV-351 and the type strain of *A. lusitana* CECT 7828^T to increase the knowledge about this species. In addition, the type strain of the

closest species *A. tecta* CECT 7082^T was used as control for comparison with a different species. These proteins were evaluated after the annotation in the RAST server with the SEED viewer http://rast.nmpdr.org/seedviewer.cgi (Aziz et al., 2008; Overbeek et al., 2014).

Citrate synthase in *A. lusitana*

Citrate synthase gene in the genomes of *A. lusitana* ESV-351 and CECT 7828^T was searched, through the RAST server with the SEED viewer. Based on the nucleotide sequence of the *A. lusitana* type strain (CECT 7828^T), a search in all genomes of *Aeromonas* was performed, by using the basic local alignment search tool (BLAST) https://blast.ncbi.nlm.nih.gov/Blast.cgi. Subsequently, a phylogenetic tree was constructed with the protein sequences of citrate synthase, by using the Neighbor-Joining method. To compare the possible function as virulence factor in *A. lusitana*, the tool "Function based sequence" in the SEED viewer from RAST was used. We compared the locus of the citrate synthase gene in *A. lusitana* ESV-351 and the region surrounding this gene with the similar organisms that exist in the database of the SEED viewer http://theseed.org/wiki/Main_Page.

Results and Discussion

Fingerprinting

The ERIC-PCR analysis used to determine the genetic similarity of the six studied strains showed that all isolates presented different fingerprints, indicating that they were not clonally related (Figure 1).

Phenotypic characteristic and susceptibility to antimicrobial agents

All phenotypic characteristics of the new strain ESV-351 and those of the already know strains of *A. lusitana* obtained by conventional biochemical and the MicroScan WalkAway-40 methods are shown in Table 1 and the Supplementary Table 1. The positive indole production was the only discordant phenotypic characteristic of the new isolate ESV-351 in relation with the results obtained with the other *A. lusitana* strains (Table 1). In fact, the four isolates and the type strain of

A. lusitana (CECT 7828^T) showed to be negative for indole production as described by Martínez-Murcia et al. (2016). However, the indole production showed to be positive by using the MicroScan WalkAway-40 identification system. Therefore, this should be taken into consideration when trying to identify strains of *Aeromonas*, since the negative indole production was considered a key test for the phenotypic differentiation of *A. lusitana* from other species (Martínez-Murcia et al., 2016). Other discordant results were observed between conventional biochemical tests and MicroScan WalkAway-40 with the ADH test, LDC, ONPG and citrate utilization (Supplementary Table 1). Previous studies have indicated that the MicroScan WalkAway confuses the genus *Aeromonas* with other genera, such as *Pasteurella, Pseudomonas,* and *Vibrio* (Soler et al., 2003).

The susceptibility to 23 antimicrobial agents evaluated showed only an intermediate resistance to ampicillin, amoxicillin-clavulanic acid and cephalothin (Table 2). These results agree with the classical resistance to ampicillin defined as a typical characteristic of the majority of the species of the genus *Aeromonas* (Abbott et al., 2003; Beaz-Hidalgo et al., 2012; Dias et al., 2012). A comparative study by Aravena-Román et al. (2012) with isolates of *Aeromonas* spp. obtained from environmental and clinical samples showed that 83% (161/193) of the strains were resistant to amoxicillin-clavulanate, 94% (135/144) from clinical samples, 53% (26/49) from environmental samples, and 73% (140/ 193) showed also resistance to cephalothin (79% (114/144) from clinical samples, and 53% (26/49) from environmental samples).

Detection of putative virulence genes

The six strains of the new species *A. lusitana* showed five different virulence gene profiles (Table 3) and the type strain CECT7828^T showed the higher number of virulence genes (*laf*$^+$/*lipase*$^+$/*serine*$^+$/*ascF-G*$^+$/*ascV*$^+$/*aexT*$^+$). Lye (2011) demonstrated that strains of *Aeromonas* isolated from environmental samples were pathogenic and contained virulence abilities similar to those observed in clinical isolates in a mouse model.

Results

The lipase genes (pla/lip/lipH3/apl-1/lip) were detected in all strains included (Table 3). These genes have a hydrolytic effect on the lipids of the membrane of the host cells, and the lipase genes have been shown to be highly prevalent in clinical (93%) and environmental isolates (96%) (Chacon et al., 2003). In fact, Merino et al. (1999) reported that insertion mutants for the lipase gene in all strains reduced the lethal dose in mice and fish models. The aerolysin gene, *aerA* gene, was present in all strains. This gene has been reported in 96% (74/77) of *Aeromonas* spp. isolates from fish (Castro-Escarpulli et al., 2003), ranging from 76%-81% in clinical isolates and from 68%-78% in environmental samples (Chacon et al., 2003; Aravena-Román et al., 2014). The serine protease genes were detected in 83% (5/6) of the strains (Table 3). These genes play an important role in the pathogenicity of *Aeromonas*, because they mediate tissue damage and facilitate bacterial invasion into the host cell (Beaz-Hidalgo and Figueras, 2013). The species *A. taiwanensis* and *A. sanarelii* isolated from chironomid egg masses showed low presence or absence of this gene (25% and 0%) (Beaz-Hidalgo et al., 2012). In our study, the prevalence of lateral flagellum (*laf*) was 67% (Table 4), this is an important structure involved in the adherence and biofilm formation, expressed during bacterial growth on viscous surfaces (Beaz-Hidalgo and Figueras, 2013). The *fla* gen has been detected in 100% of the *Aeromonas* spp. isolates from drinking water in Spain (Pablos et al., 2009). Recently, Aravena-Román et al. (2014) observed that the presence of *laf* gene was more prevalent in clinical isolates than in environmental isolates. The cytotoxic enterotoxin gene (*act*) was detected in 50% of the six strains studied, this prevalence is similar to the one reported (43% to 62%) in other studies (Figueras et al., 2009; Puthucheary et al., 2012; Wu et al 2007). The genes that form part of the T3SS (ascF-G and ascV) were detected in three of the six strains (CECT 7828^{T}, MDC 2467, MDC 2468), however, only in the type strain, the effector protein *axeT* was simultaneously detected (Table 3). Nevertheless, none of the six strains bared the gene encoding the *aop-P* toxin, also delivered by the T3SS. The T3SS is considered an important virulence factor because it injects effector toxins directly into the cytoplasm of host cells (Beaz-Hidalgo and Figueras 2013). Different studies that generate mutant strains for structural genes of the T3SS showed that the T3SS is

essential for the toxicity and virulence (Burr et al., 2005; Dacanay et al., 2006). In addition, mutant strains for the effector proteins also showed to be less virulent (Dacanay et al., 2006). None of the six strains showed the genes encoding the heat-stable enterotoxin (*ast*), heat-labile cytotonic enterotoxin (*alt*) or the Shiga toxins (stx1 and stx2) (Table 4).

Phylogenetic analysis with 7 housekeeping genes extracted from the genomes

The use of phylogenetic analysis based on concatenated housekeeping genes has been recommended (Martínez-Murcia et al., 2016). The neighbor-joining phylogenetic tree constructed with the complete concatenation of 7 housekeeping genes (*gyrB, rpoD, recA, dnaJ, gyrA, dnaX* and *atpD*) obtained from the ESV-351 genome to those from other type strains of *Aeromonas,* ratified that ESV-351 belongs to the *A. lusitana* species. This result confirmed the ones obtained with the concatenation of *rpoD* and *gyrB* in the previous study (Vega-Sánchez et al., 2014b). The MLPA showed that *A. lusitana* formed a clade of the species *A. encheleia* CECT 4342^T, *A. eucrenophila* CECT 4224^T, *A. aquatica* CECT 8025^T and *A. tecta* CECT 7082^T (Figure 2). Apart from *A. aquatica* CECT 8025^T that was described after *A. lusitana*, the other three were previously reported as the closest species to *A. lusitana* (Vega-Sánchez et al., 2014b; Martínez-Murcia et al., 2016). All genomes of these closely related species were evaluated with some of the tools used to compare genomes.

Comparison of ANI and *is*DDH values obtained between *A. lusitana* and the closest species

In general, for the separation of species, an ANI below 96 % (Lee et al., 2016) and *is*DDH below 70 % (Meier-Kolthoff et al., 2014) have been recommended. The values of ANI and *is*DDH between *A. lusitana* ESV-351 and the closest species were <40 % and <90, respectively (Figure 3), being similar to previous results reported among *Aeromonas* species (Colston et al., 2014; Beaz- Hidalgo et al., 2015). As shown in Figure 3A the ANI value obtained between the genome of the ESV-351 and the type strain of *A. lusitana* was higher than >96 % (96.3 %).

However, the *is*DDH between these strains was less than 70% (68.90 %) (Figure 3B). An explanation of this borderline result could be that these strains are the same species but with some higher differences between both. In a study by Colston et al., (2014), the results showed a similar *is*DDH with strains belonging to *A. allosaccharophila*, describing the taxonomy of this species as controversial. For this reason, it is possible that *A. lusitana* species can be a controversial species like *A. allosaccharophila*, and it is important to sequence the genome of more strains of *A. lusitana* species to clarify the cutoff of the *is*DHH. However, the results of ANI are more reliable; it has been proposed as a new standard for defining microbial species, and it is gaining wide acceptance (Konstantinidis and Tiedje, 2005; Colston et al., 2014).

Genome information and comparative with other related and unrelated species

Figure 4 compares the genome of *A. lusitana* ESV-351 with the one of the type strain CECT 7828^T and with the published genomes of the closest species i.e. *A.tecta, A. eucrenophila, A. encheleia* and *A. aquatica* and with six additional genomes of other *Aeromonas* species (Seshadri et al., 2006; Reith et al., 2008; Beatson et al., 2011; Li et al., 2011; Charette et al., 2012; Wu et al., 2012). As shown in the Figure 4, the strain ESV-351 showed a slightly bigger genome size (4.74 Mb) than the type strain (4.55 Mb), and similarity with the one of the closest species *A. tecta* (4.75 Mb) and with the ones of several other species (Figure 4). Moreover, the G+C content of strain ESV-351 (60.7%) was within the range (57 to 63 mol%) that has been reported for the genus (Beaz-Hidalgo and Figueras et al., 2013). The numbers of predicted tRNAs of strain ESV-351 were 110, being the same as the ones present in the complete genome of *A. salmonicida* A449 (Reith et al., 2008), and higher than the 98 found in the type strain CECT 7828^T and the closest species, that ranges between 82-101. A study that compared the *A. hydrophila* genomes by Pang et al., (2015), showed that A. *hydrophila* ATCC 7996^T possessed a higher number of tRNAs than the other strains, and suggested that the increasing number of tRNAs may be useful to increase the protein synthesis on the basis of previous information (Lee et al., 2008). Considering this explanation, we

could suggest that, *A. lusitana* ESV-351 could synthesize a higher number of tRNAs than *A. lusitana* CECT 7828^T or the closest species. In relation to the protein-coding genes, *A. lusitana* ESV-351 showed a higher number of these (4251) than the type strain CECT 7828^T (4069) (Figure 3). The first described genomes corresponded to *A. salmonicida* A449 and *A. hydrophila* ATCC 7966^T, that are considered the first an important pathogen in fish, and the second an important pathogen in humans and fish. The latter genomes showed both a higher number of protein-coding genes, 4388 and 4128, respectively. The large number of genes, encoded by *A. salmonicida* A449, were related to the potential virulence of this species, with an abundance of insertions and pseudogenes, that suggested an evolution and adaptation of these bacteria to different hosts (Reith et al., 2008; Beaz-Hidalgo and Figueras 2013). In the case of *A. hydrophila* ATCC 7966^T, the genes encode several metabolic pathways that allow these bacteria to live in different environments (Seshadri et al., 2006; Beaz-Hidalgo and Figueras 2013). This information leads us to believe that *A. lusitana* could also adapt to different environments due to the high number of protein-coding genes. Furthermore, it is important to observe that *A. lusitana* ESV-351 possessed a higher number of these proteins than *A. lusitana* CECT 7828^T (Figure 3). An explanation of this could be that the higher number of protein-coding genes would be related to the origin of the strain. The ESV-351 (Vega-Sánchez et al., 2014b) isolated from a rainbow trout in Mexico, would have a higher number of protein-coding genes, while the CECT 7828^T (Martínez-Murcia et al., 2016) isolated from untreated water would have less protein-coding genes. In comparison with *A. salmonicida* A499 and *A. hydrophila* ATCC 7966^T, *A. lusitana* ESV-351 could be an important fish pathogen due to the high number of proteins. However, more studies of these proteins are needed to clarify if these proteins could encode virulence genes as well as different metabolic pathways that increase the pathogenicity of the strain in fish.

Core and Pan-genome analysis

The pan- and core- genome of the *A. lusitana* strains (ESV-351 and CECT 7828^T) and of both strains with the closest species (*A. aquatica* CECT 8025^T, *A.*

eucrenophila CECT 4224^T, *A. encheleia* CECT 4342^T and *A. tecta* CECT 7082^T) were analyzed to obtain more information about the *A. lusitana* ESV-351. We identified a pan-genome of 4356 genes and a core- genome of 3727 genes by using both *A. lusitana* genomes (Figure 5A). Taking into consideration the average gene numbers, 4160 for these two strains, the 3727 represent approximately 89% of the total genome. In other words, a higher part of the genomic regions is conserved. In addition, *A. lusitana* ESV-351 presented a higher number of unique genes (strain-specific) than *A. lusitana* CECT 7828^T, 392 and 237 genes, respectively. In addition, we identified a pan-genome of 6583 genes and a core-genome of 3229 by using six genomes (both *A. lusitana* strains and the closest species). The average of the gene number was 4138 for the six strains, the 3229 represent approximately 78%, an indicative of the highly conserved genomic features. As shown in Figure 5B, *A. tecta* CECT 7082^T (Demarta et al., 2008) showed a higher number of unique genes (947), an explanation of this higher number could be the contribution in the survival and the adaptation in the environment as previously reported (Fu et al., 2018). This strain was isolated from feces of a child with diarrhea (Demarta et al., 2008), and an analysis of this could be interesting to elucidate if these genes would encode virulence factors contributing to the survival.

Predicted protein sequences identity

The comparison of protein sequences of all predicted ORF's in the genomes of *A. lusitana* CECT 7828^T (used a reference sequence for the comparison) with *A. lusitana* ESV-351, *A. aquatica* CECT 8025^T, *A. eucrenophila* CECT 4224^T, *A. encheleia* CECT 4342^T, and *A. tecta* CECT 7082^T are shown in Figure 6. As expected, *A. lusitana* CECT 7828^T and *A. lusitana* ESV-351 were highly similar to each other with around 2538 proteins sharing more than 98% similarity between them. The comparison of the type strain of *A. lusitana* CECT 7828^T with the closest species *A. tecta* CECT 7082^T showed 629 proteins with a higher similarity than 98%. In the other three species the number of proteins with higher similarity than 98% in comparison with *A. lusitana* CECT 7828^T were 531 compared with *A. eucrenophila*

CECT 4224^T, 444 with *A. aquatica* CECT 8025^T and 304 with *A. encheleia* CECT 4342^T, as expected.

Distribution and counts of proteins in subsystems

A simple comparison between the genome of *A. lusitana* ESV-351 and *A. lusitana* CECT 7828^T with the closest species *A. tecta* CECT 7082^T was performed based on subsystems. The subsystems represent a group of functional roles including metabolic pathways, multisubunit complex or a specific class of proteins. A total of 4251 protein-coding genes were distributed in 523 subsystems in *A. lusitana* ESV-351, while 521 systems corresponding to the 4069 protein-coding genes of *A. lusitana* CECT 7828^T. In the case of *A. tecta* CECT 7082^T a total of 4267 protein-coding genes were classified in 525 subsystems. As shown in Figure 7 the three strains *A. lusitana* ESV-351, *A. lusitana* CECT 7828^T and *A. tecta* CECT 7082^T showed a similar distribution associated with different functional or structural systems, for example, the relation between the cell wall and capsule, membrane transport, amino acids and derivatives and carbohydrates among them. The two strains of *A. lusitana* showed almost the same amount of proteins belonging to the different subsystems, and those, as could be expected, were more similar than the number of proteins found in the closest species *A. tecta* CECT 7082^T (Figure 7). The predicted catabolic pathways for the strain *A. lusitana* ESV-351 included the use of mannose, deoxyribose, ketogluconates, fructose, D-glycerate, and D-ribose. Proteins required for using glycerol-3-phosphate, mannitol, and citrate were also detected. The latter could be responsible for the growth capacity with the presence of citrate as a carbon source found for these species, which is a differential phenotypic characteristic (Martínez-Murcia et al., 2016). Considering this observation, we decided to screen all the genomes for the presence of the citrate synthase gene.

Citrate synthase

The citrate synthase gene was found in all genomes of the *Aeromonas* genus, but this gene could not be associated with the use of the citrate as a phenotypic characteristic. Martínez-Murcia et al. (2016) demonstrated that the phenotypic test

for citrate was negative in *A. eucrenophila,* but in our study the citrate synthase was found in the genome. In the case of *A. lusitana* ESV-351, the phenotypic character was positive (Martínez-Murcia et al., 2016); however, in our study the gene of citrate synthase was found in the genome. In relation with the conservation of the gene among all the 32 *Aeromonas* spp., the phylogenetic analysis of the sequences of the 32 species showed the formation of two main clusters, that grouped each of them in 12 and 20 of the species, however, no pattern related to the phenotypic test was observed. Also, the phylogenetic analysis showed a distance of 85-97% between species (Figure 8). In addition, as shown in Figure 9 based on the SEED database, four important pathogens were compared with A. *lusitana* ESV-351, being this gene identical. Figure 9 shows these pathogens including one strain of *Aeromonas* (*A. hydrophila* ATCC 7996^{T}), which is an important pathogen in humans and fish (Figueras and Beaz-Hidalgo et al., 2015), *Vibrio cholerae* O1 biovar eltor strain 16961 that has been the dominant strain in the seventh global cholera pandemic (Barua and Cvjetanovic, 1972), *Vibrio vulnificus* CMCP16, which causes fatal septicemia and necrotizes wound infections (Duong-Nu et al., 2016) , and *Yersinina pestis* KIM, the etiologic agent of bubonic and pneumonic plague (Deng et al., 2002). However, as shown the phylogenetic analysis, the gene was found in the type strain, but was different from the ESV-351 strain. Furthermore, the citrate synthase was described as a virulence factor in other bacteria such as *Agrobacterium tumefaciens* and *Escherichia coli* (Suksomtip et al., 2005; Quandt et al., 2015). Also, citrate synthase activated a protein that increases the survival of the *Staphylococcus aureus* (Ding et al., 2014). Considering all this information, it is possible that the citrate synthase gene could also act as an important virulence factor in *A. lusitana* ESV-351, but in contraposition of what could be expected the strain was negative using the citrate utilization test.

Conclusions

This study demonstrated that the phenotypic differential characteristics were highly conserved among the *A. lusitana* strains. However, the indol production was

different between the new strain and the type strain. The genomic characterization of *Aeromonas lusitana* ESV-351 showed a slightly bigger genome size than the type strain with more protein coding genes and tRNAs and these could facilitate the survival and adaptation of this strain in the environment. Also, the chromosomal region surrounding the conserved citrate synthase locus in the new strain was relatively similar to that of four pathogenic bacteria, suggesting that this gene could play a role in the virulence of this new strain.

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Conflict of interest

The author(s) declare that they have no competing interests.

Author's contribution

AFB, VVS and MJF designed research; AFB, VVS, APC, FLE, performed research; AFB, VVS, APC, FLE, RBH, AMM, ESV, IPM, GCE, MJF analyzed the data and wrote the paper.

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Table 1. Comparative phenotypic characteristic between the Mexican strain (ESV-351) and the five strains including the type strain of the original description of *Aeromonas lusitana* (Martinez-Murcia et al., 2016)

	ESV-351	CECT 7828^T	Isolates (4)
β-haemolisis	+	+	+
Indole	+	-	-
MR	+	+	+
LDC	+	+	+
Glucose (gas)	+	+	+
Citrate	+	+	+
Hydrolysis of			
SDS	-	-	V
Aesculin	+	+	+
Starch	+	+	+
Elastase	ND	+	+
Acid from			
Glycerol	+	+	+
L-arabinose	-	-	-
Salicin	+	+	+
Utilization of			
DL-lactate	-	-	-
L-arabinose	-	-	-
Growth at 4.5% NaCl	+	+	+

MR: Methyl Red; LCD: Lysine descarboxylase; ND: not done; -: < 15% positive; V: between 15 to 85% positive; +: ≥85% of strains positive.

Results

Table 2. Susceptibility profile of strain ESV-351 and the type strain reported by Martínez-Murcia et al., 2016 to 23 antimicrobial agents.

Antimicrobial agents	Results (MIC)		Breakpoints		
	ESV-351	CECT 7828^{T}	S	I	R
Amikacin	S (≤8)	S (≤8)	≤16	32	≥64
Ampicillin	I (>16)	I (>16)	≤8	16	≥32
Amoxicillin-clavulanic acid	I (>16/8)	I (>16/8)	≤8/4	16/8	≥32/16
Aztreonam	S (≤1)	S (≤1)	≤8	16	≥32
Ceftazidime	S (≤1)	S (≤1)	≤8	16	≥32
Ceftriaxone (30 µg)	S (33)[a]	S (33)[a]	≥21	14-20	≤13
Cephalothin	I (>16)	I (>16)	≤8	16	≥32
Ciprofloxacin (5µg)	S (42)[a]	S (42)[a]	≥21	16-20	≤15
Cefotaxime	S (≤1)	S (≤1)	≤8	16-32	≥64
Cefoxitin	S (≤8)	S (≤8)	≤8	16	≥32
Ciprofloxacino	S (≤0.5)	S (≤0.5)	≤1	2	≥4
Cefepime	S (≤1)	S (≤1)	≤8	16	≥32
Cefuroxime	S (8)[a]	S (8)[a]	≤8	16	≥32
Ertapenem	S (≤0.5)	S (≤0.5)	≤2	4	≥8
Gentamicin	S (≤2)	S (≤2)	≤4	8	≥16
Imipenem	S (≤1)	S (≤1)	≤4	8	≥16
Nalidixic acid	S (≤16)	S (≤16)	≤8	16	≥16
Piperacillin (100µg)	S (29)[a]	S (29)[a]	≥21	18-20	≤17
Piperacillin-tazobactam	S (≤8)	S (≤8)	≤16/4	32/4-64/4	≥128/4
Tetracycline (30µg)	S (27)[a]	S (27)[a]	≥19	15-18	≤14
Trimetoprim-sulfamethoxazole	S (≤2/38)	S (≤2/38)	≤2/38	-	≥4/76
Tigecycline	S (≤1)	S (≤1)	≤1	2	≥2
Tobramycin	S (≤2)	S (≤2)	≤2	4	≥4

[a]Antimicrobial susceptibility test was performed by agar disk diffusion (results expressed in mm).

Table 3. Distribution of virulence genes studied by PCR in the six trains of *Aeromonas lusitana*

Strain	Origen	*laf*	*act*	*ast*	*alt*	*aerA*	Lipase genes	Serine protease genes	*ascF-G*	*ascV*	*aexT*
ESV-351	Rainbow trout	-	+	-	-	+	+	+	-	-	-
CECT 7828^T	Thermal water	+	-	-	-	+	+	+	+	+	+
MDC 2467	Drinking water	+	-	-	-	+	+	+	+	+	-
MDC 2468	Vegetable	+	-	-	-	+	+	-	+	+	-
MDC 2469	Vegetable	+	+	-	-	+	+	+	-	-	-
MDC 2472	Thermal water	-	+	-	-	+	+	+	-	-	-

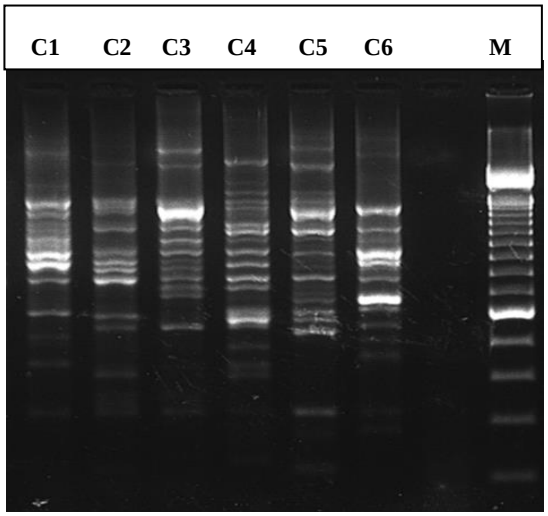


Figure 1. ERIC-PCR profile of the six strains of *Aeromonas lusitana* included in the present study. C1: 11/6^T (=MDC 2473^T =DSM 24095^T = CECT 7828^T); C2: A.136/15 (MDC 2467); C3: L8-3 (MDC 2468); C4: L10-4 (MDC 2469); C5: A.28/6 (MDC 2472); C6: ESV-351; M: Molecular weight. C1, C2 and C5 were recovered from water; C3 and C4 were isolated from vegetables. C6, was recovered from rainbow trout.

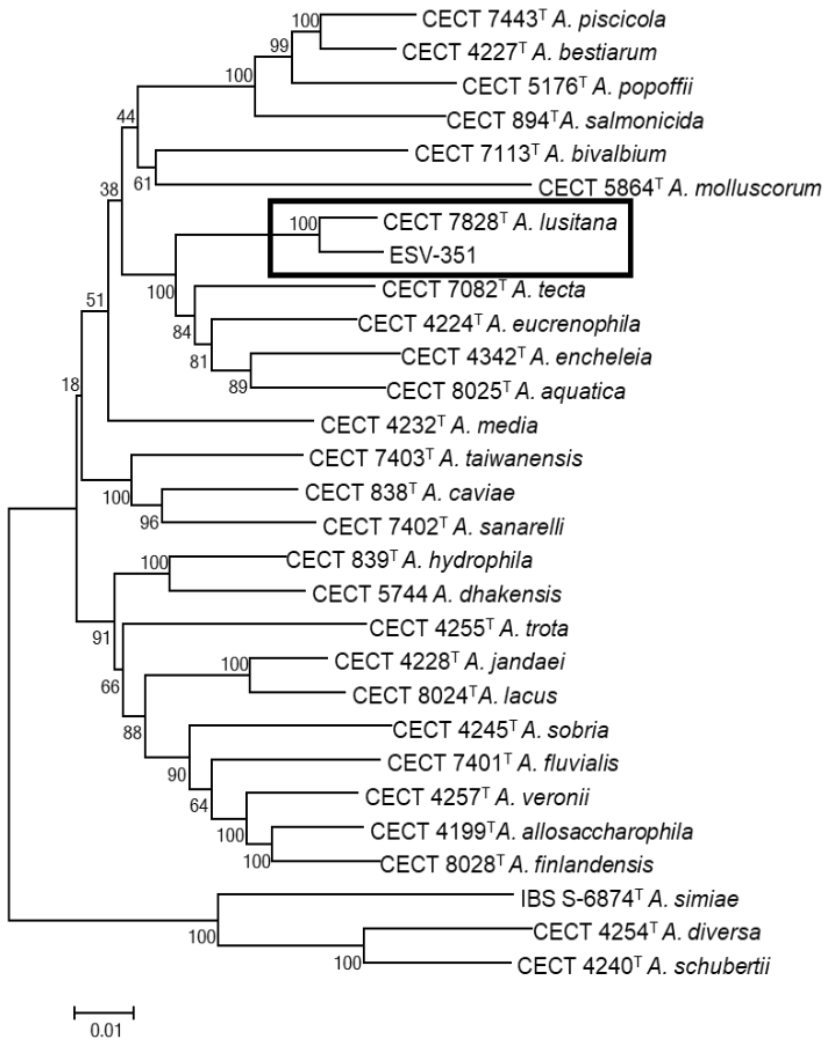
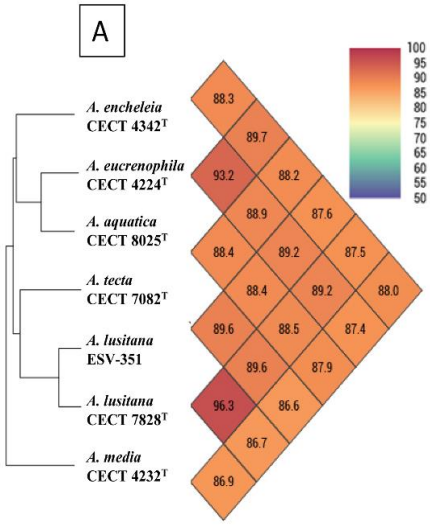


Figure 2. Phylogenetic tree derived from the MLPA of the concatenated 3849 bp sequence of 7 housekeeping genes from the genome (gyrB, rpoD, recA, dnaJ, gyrA, dnaX and atpD), showing the relationships of cluster containing the type strain of *Aeromonas lusitana* CECT 7828^T, *A. lusitana* ESV-351 and type strains of other *Aeromonas* species. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates). Bar 0.01 estimated nucleotide substitutions per site.



B

Species	(*is*DDH)
A. lusitana CECT 7828T	68.90*
A. encheleia CECT 4342T	32.80
A. tecta CECT 7082T	37.70
A. eucrenophila CECT 4224T	36.40
A. aquatica CECT 8025T	34.70

Figure 3. Comparative results of the Average Nucleotide Identity obtained between the genomes of the strains of *Aeromonas lusitana* isolated from Mexico (ESV-351) and the type strain recovered from Portugal (CECT 7827^T) and the closest *Aeromonas* species (A) and of the results of the *is*DDH (B).

Species	Size (Mb)		Protein-coding genes		tRNA		G+C (%)	
A. lusitana ESV-351	4.74	4.64	4251	4160	110	104	60.7	60.8
A. lusitana CECT 7828T	4.55		4069		98		61.0	
Closest species								
A. tecta CECT 7082T	4.75	4.56	4267	4094	96	95	60.1	61.2
A. eucrenophila CECT 4224T	4.54		4098		101		61.2	
A. aquatica CECT 8025T	4.58		4091		82		61.2	
A. encheleia CECT 4342T	4.47		4051		94		62.0	
Closed genomes availabes at the NCBI								
A. salmonicida A449	4.70	4.72	4388	4153	110	110	58.5	60.0
A. hydrophila ATCC 7966T	4.74		4128		128		61.5	
A. caviae Ae398	4.43		3960		ND		61.4	
A. veronii B565	4.55		4057		102		58.7	
A. salmonicida 01-B526	4.75		4179		111		58.5	
A. dhakensis AAK1	4.81		4214				61.8	

Figure 4. Comparison of the main characteristics of the genomes of *Aeromonas lusitana* strains ESV-351 and CECT 7828^T, the genomes of the closest species and of the six species that have closed genomes available at the NCBI database.

Results

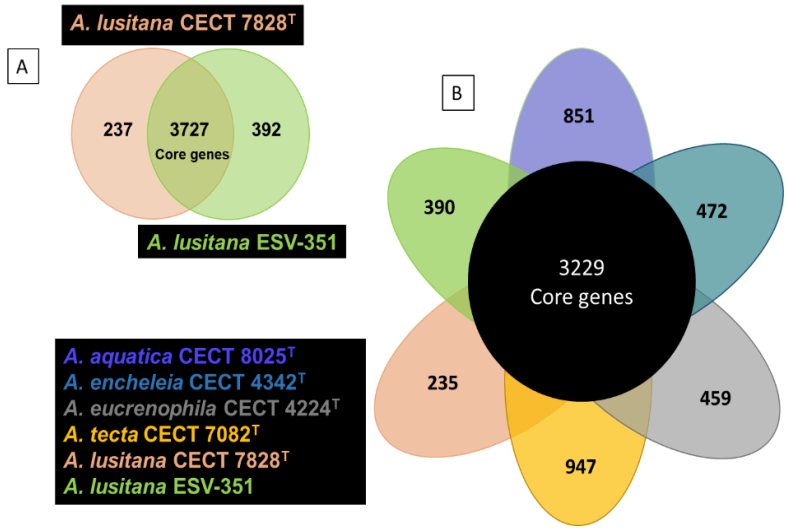


Figure 5. Core and Pan genome comparison between the strains of *Aeromonas lusitana* isolated from Mexico (ESV-351) and the type strain recovered from Portugal (CECT7827^T) **(A)** and the the closest related *Aeromonas* species **(B).**

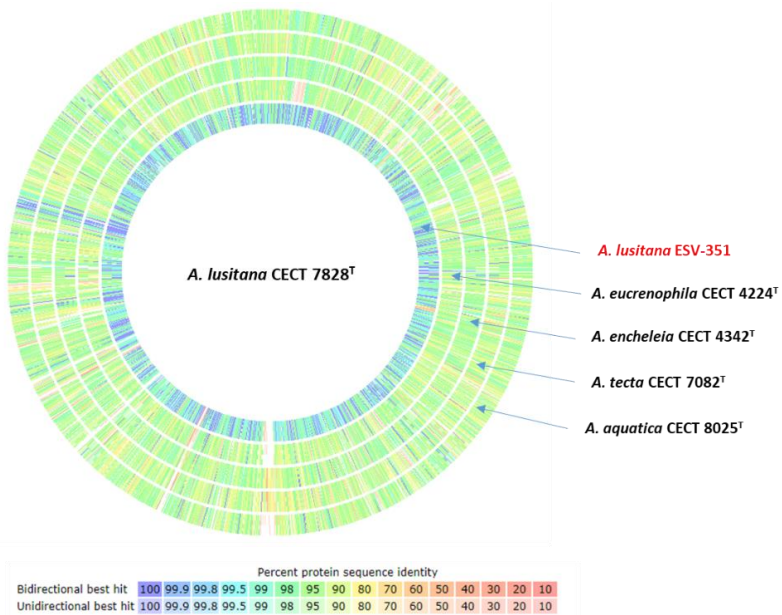


Figure 6. Comparison of Predicted Proteins. Protein sequences were compared by using the Rapid Annotation System Technology (RAST)-SEED viewer sequenced-based comparison tool with *Aeromonas lusitana* CECT 7828^T used as the reference sequence. Percent protein sequence identity is expressed by color coding provided in the legend. A threshold of 98% sequence identity was used to identify sequences highly conserved.

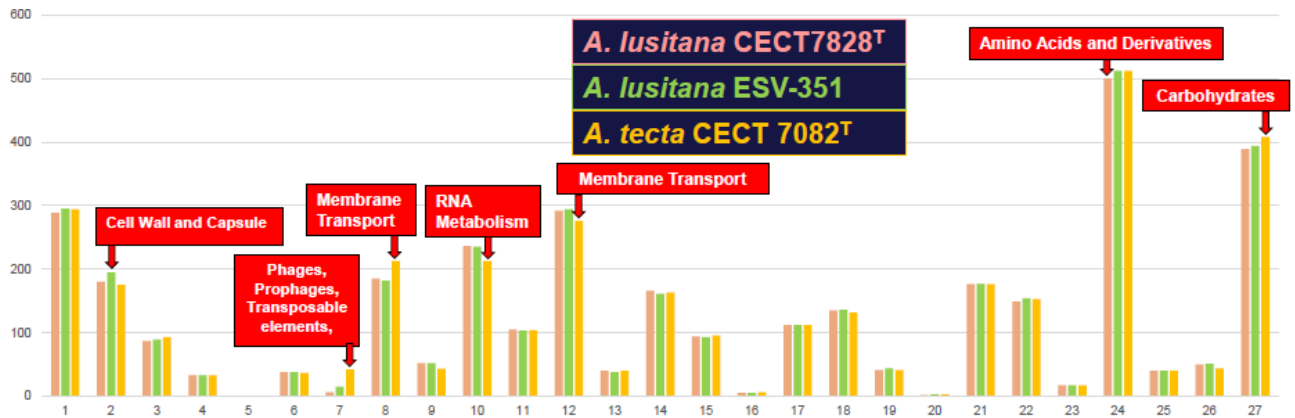


Figure 7. Subsystem category distribution of major protein coding genes of the strains of *Aeromona lusitana* isolated from Mexico (ESV-351) and the type strain recovered from Portugal (CECT 7827^T) and the closest species *Aeromonas tecta* CECT 7082^T as annotated by Rapid Annotation System Technology (RAST) server.

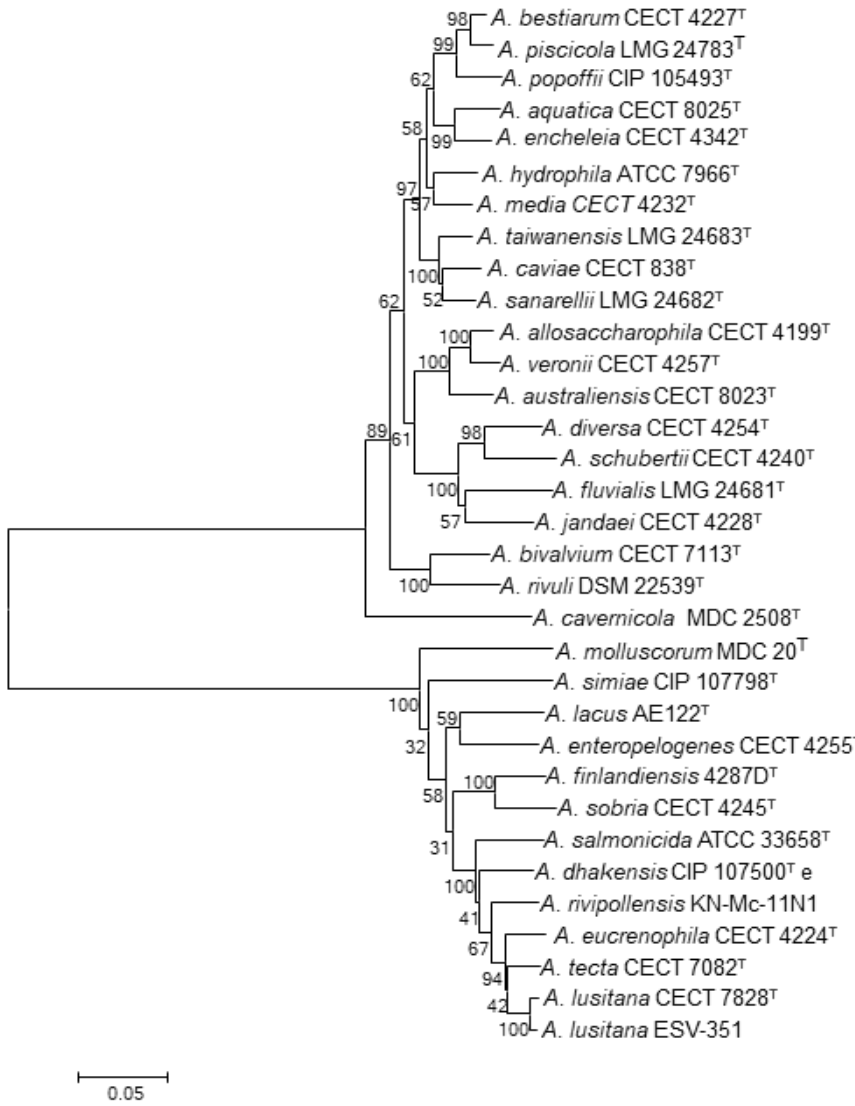


Figure 8. Phylogenetic tree derived from the citrate synthase gene, showing the relationships of cluster containing the strains of *Aeromonas lusitana* isolated from Mexico (ESV-351) and the type strain.

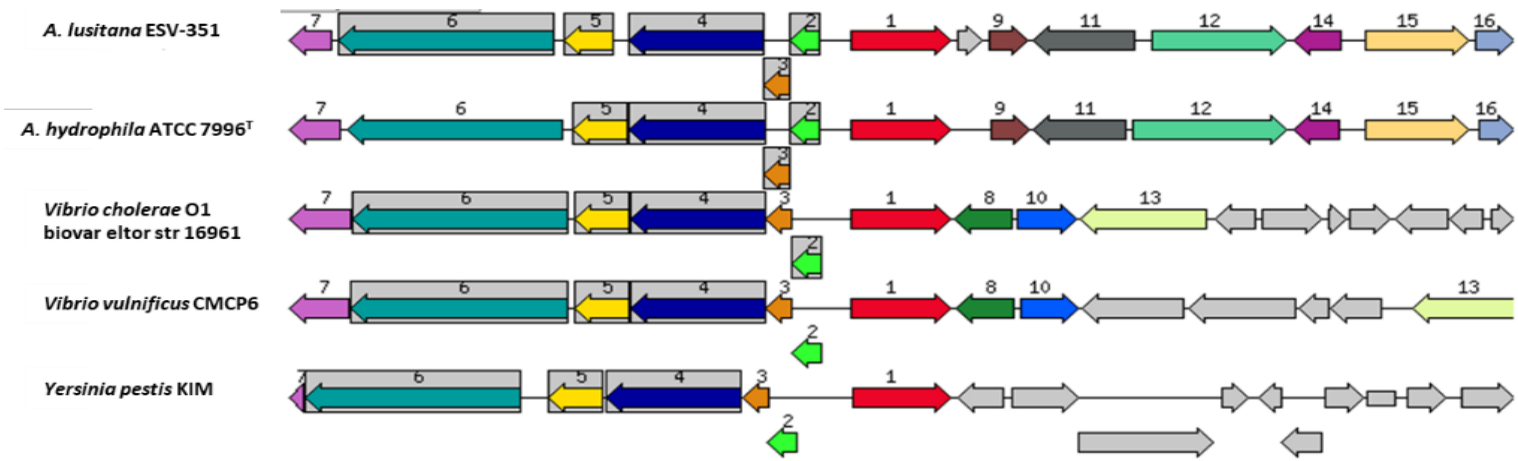


Figure 9. Comparison of the chromosomal region surrounding the conserved citrate synthase locus of *A. lusitana* Mexican strain ESV-351 with the four more similar loci of different bacteria included in the database of the SEED viewer. Orthologous genes are grouped with the same number and color. The number one in red is the citrate synthase gene. Genes whose relative position is conserved in at least four other species are functionally coupled and share gray background boxes. *Aeromonas hydrophila* is the only species of *Aeromonas* included in the database.

Results

Supplementary Table 1. Comparative phenotypic characteristic of the Mexican strain (ESV-351) isolated from rainbow trout by using a conventional biochemical test and the MicroScan (W/A) identification system.

	Biochemical results			Biochemical results	
	Conventional	MicroScan		Conventional	MicroScan
Test			**Test**		
Motility	+	ND	Acid from:		
Oxidase	+	ND	D-adonitol	ND	-
Catalase	+	ND	D-cellobiose	-	ND
ADH	+	-	D-mannitol	+	ND
LDC	+	-	D-sorbitol	-	-
ODC	-	-	Glucose	ND	+
Tryptophan deaminase	ND	-	Glycerol	+	ND
Indole	+	-[a]	L- Arabinose	-	ND
ONPG	+	-	L- Rhamnose	-	ND
Urea	-	-	D-lactose	-	ND
H_2S	-	-	D-mellobiose	ND	-
VP	-	-	*m*-Inositol	-	-
MR	+	ND	D-raffinose	ND	ND
Gelatin	+	ND	D-sacarose	+	ND
DNase	+	ND	Salicin	+	ND
Glucose (gas)	+	ND	Sucrose	-	-
Nitrate reduction	+	+			
β–Hemolysis	+		Hydrolysis of:		
			Aesculin	+	-
Utilization of:			SDS	-	ND
Citrate	+	-	Starch	+	ND
DL-Lactate	-	ND			
Malonate	ND	-	Resistance to :		
Acetamide	ND	-	Chepalothin	ND	+
Tartrate	ND	-	Colistin	ND	-
			Kanamycin	ND	+
Growth in:			Nitrofurantoin	ND	-
0% NaCl	+	ND	O /129[b] (150 µg)	+	ND
4.5% NaCl	+	ND	Penicillin	ND	+
6% NaCl	-	ND	Tobramycin	ND	-
Cetridime	ND	-			
Growth at 42°C	-	ND			

ADH: Arginine dihydrolase; LDC: Lysine decarboxylase; ODC: Ornithine decarboxylase; ONPG: *o*-nitrophenyl-β-D-galactopyranoside; VP: Voges-Proskauer; MR: Methyl Red. [a] Negative in two of three repetitions; [b] Vibriostatic agent (2,4-diamino-6,7diisopropylpteridine) (150 µg / disk); ND: Not Done.

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EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
Ana Fernández Bravo

4.7. Characteristics and potential role in infection of the metallochaperone HypA in species of the human emerging pathogen genus *Aeromonas*

Fernández-Bravo A., López-Fernández L., Figueras MJ. (Submitted/ Under review in BMC Microbiology)

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EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
Ana Fernández Bravo

Cecilia Devoto

Editor

BMC Microbiology

Reus, February 15, 2019

Dear Editor,

We respectfully request that you consider the enclosed manuscript entitled "**Characteristics and potential role in infection of the metallochaperone HypA in species of the human emerging pathogen genus *Aeromonas***" for publication in *BMC Microbiology*. The importance of this research resides in the interest of the metal ions role in virulence of the emerging pathogen *Aeromonas* spp. This is the first time that HypA metallochaperone, involved in different mechanisms, is reported as a new potential virulence factor in this genus. All the material included in the manuscript is original and unpublished, and does not infringe any personal or other copyright or property rights. All the authors have read and comply with the instructions and stated conditions provided in the instructions to authors. We are confident that *BMC Microbiology* is the most appropriate journal to share this important information to the suitable readership.

Sincerely yours,

Prof. Maria José Figueras

Dr Loida López-Fernández

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EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
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Characteristics and potential role in infection of the metallochaperone HypA in species of the human emerging pathogen genus *Aeromonas*.

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ABSTRACT

Background: Metallochaperones are essential proteins that insert metal ions or metal cofactors into specific enzymes that after maturation will become metalloenzymes. One of the most studied metallochaperones is the nickel-binding protein HypA, involved in the maturation of the nickel dependent hydrogenases and ureases. The HypA was previously described in the human pathogens *Escherichia coli* and *Helicobacter pylori*, considering it a key virulence factor in this latter. However, nothing is known about this metallochaperone in the species of the emerging pathogen genus *Aeromonas*. These bacteria are autochthonous inhabitants of the aquatic environments often associated with cases of diarrhea and wound infections.

Results: In this study, we performed an *in silico* study with 36 *Aeromonas* genome sequences to identify the presence of *hypA* gene, that showed to be present in 69.4 % (25/36) of the *Aeromonas* genomes. The similarity of the *Aeromonas* HypA proteins with the orthologous protein of *H. pylori* ranged from 21 to 23 %, while with *E. coli* it was 41-45 %. However, despite this low percentage, *Aeromonas* HypA proteins exhibited the same conserved characteristic metal-binding domains found in the other pathogens. The transcriptional analysis enabled to determine *hypA* expression levels under acidic and alkaline conditions and after macrophage phagocytosis. The transcriptional regulation of *hypA* resulted to be pH-dependent, showing upregulation at acidic pH. A higher upregulation occurred after macrophages infection.

Conclusions: This is the first study that provided evidence that HypA metallochaperone in *Aeromonas* might play a role in the acid tolerance and in the defense against macrophages.

Keywords: metallochaperone, *Aeromonas*, macrophages, acid, alkaline, ROS, hydrogenases, ureases

BACKGROUND

Metal ions are essential for the correct functioning of microbial biological processes [1]·[2]. In fact, many proteins contain metal ions bound directly to their amino acid chains by histidine or cysteine residues as cofactors. Particularly, metalloenzymes such as nitrogenases, ureases or hydrogenases are an abundant type of metalloproteins that catalyze numerous metabolic and enzymatic reactions [3-5]. The synthesis of these metalloenzymes consists of complex processes that require a set of accessory proteins. In this context metallochaperones play a key role in bacterial metal homeostasis "metallostasis", since they are involved in the acquisition and transfer of metals [5-7]. Many evidences indicate an important role of metallostasis in the host-pathogen interaction [1,7,8]. For instance, during infection the host limits the availability of essential metals, inactivating metal-dependent processes of the bacterial pathogen that compensates this limitation producing metallochaperones, among other proteins [1, 7, 8].

Metallochaperones act directly inserting metal ions into specific enzymes that will become metalloenzymes after maturation [5-7]. One of the best studied regulation mechanism by metallochaperones in bacteria is the maturation of hydrogenases, which are enzymes that catalyze the interconversion of hydrogen (H_2) into protons and electrons, playing a vital role in anaerobic metabolism and oxidative stress response[9]. There are three types of hydrogenases based on the metal attached to their active site: [FeFe] hydrogenase, [NiFe] hydrogenase and [Fe]-only hydrogenase [10, 11]. The [NiFe]-hydrogenases are heterodimeric proteins consisting of two subunits, a small (~ 26 kDa) and a large (~ 62 kDa) which possesses in its active site the metallic cofactor $NiFe(CN)_2CO$ responsible for catalyzing the reversible production of molecular H_2 [10]. Several accessory proteins encoded by genes present in the **hyp** operon are required for the $NiFe(CN)_2CO$ biosyntesis[12, 13]. In fact, the HypC, HypD, HypE and HypF proteins are considered hydrogenase maturation factors and are responsible for the synthesis and transfer of the $Fe(CN)_2$ complex to the hydrogenase precursor. After delivery of the $Fe(CN)_2CO$ complex to the hydrogenase, the metallochaperone HypA and the

GTPase HypB participate in its correct maturation by transferring a nickel ion into the large subunit of the hydrogenase [12, 14].

The nickel-binding HypA metallochaperone has been well studied in the human pathogens *Helicobacter pylori* and *Escherichia coli*, in which it has been related to virulence [12, 15-18]. Although the function of this metallochaperone is mainly associated to the maturation of hydrogenases, recent studies have demonstrated that HypA is also involved in the urease maturation [18-20]. Both metalloenzymes are relevant in the adaptation to several redox conditions in pathogenic bacteria. Previous studies demonstrated that the hydrogenases participate in the defense against oxidative stress [21], as well as in acid resistance in *E. coli* [18, 22, 23]. The data showed that in *E. coli* K-12 hydrogenase mutant exhibit an impaired acid resistance [18, 22, 23]. Moreover, previous works revealed that ureases facilitates the survival of *H. pylori* in the human gastric mucosa by protecting it from the acid environment of the stomach [18-20]. The ureases neutralize the gastric acids by catalyzing the conversion of urea into ammonia and carbon dioxide. The latter studies demonstrated that the modification of the zinc- and nickel- binding sites in HypA affect its urease activity and consequently impairs acid resistance in these bacteria [18-20].

An earlier study investigated the redox potentials of facultative aerobic and obligate anaerobic bacteria that produce H_2 along the gut of earthworms by analyzing the [NiFe]- and [FeFe]-hydrogenase gene transcripts [24]. The results showed that 21 % of the detected [NiFe]-hydrogenase-affiliated sequences corresponded to bacteria of the genus *Aeromonas* [24]. Therefore, these findings evidence the fermentative capacity of these bacteria as great hydrogen producers. Despite the presence of hydrogenases has been described in *Aeromonas*, nothing is known about the function of the metallochaperone HypA in this genus [24]. *Aeromonas* species are considered opportunistic emergent pathogens producing mainly gastroenteritis and 96.5 % of the clinical strains correspond to 4 species: *Aeromonas caviae* (29.9 %), *Aeromonas dhakensis* (26.3 %), *Aeromonas veronii* (24.8 %) and *Aeromonas hydrophila* (15.5 %) [25-27]. Hence, it is of interest to investigate whether the HypA

metallochaperone could be considered a new potential virulence factor of these bacteria. Therefore, the objective of this study was to search for the presence of the *hypA* gene in the genomes of the species of the genus *Aeromonas*, as well as to evaluate if HypA participates in their adaptive molecular response to acidic conditions and oxidative stress.

RESULTS

Identification of *hypA* gene in *Aeromonas* species

The results of the *in silico* search using the *hypA* sequence (339 bp) of *A. hydrophila* KN-Mc-1R2 (CP027804.1) as template showed that the gene was present in 69.4 % (25/36) of investigated genomes belonging to the *Aeromonas* species (Table 1). In addition, *hypA* was present in sixteen additional genomes (eight of *A. hydrophila* and eight of *A. dhakensis)* screened for determining whether the presence of the *hypA* was a strain or species-specific character (data not shown).

Sequence analyses of HypA proteins shows specific motifs associated to metal binding

The specific metal-binding motifs consisting of N-terminal MHE motif for Ni-binding and two consecutive cysteine motifs CxxCnCPxC for Zn-binding, previously reported in the HypA proteins of *E. coli* and *H. pylori*, were also observed in the *Aeromonas* spp. protein sequences Figure 1A. The three-dimensional predicted structures of HypA proteins of *A. hydrophila* CECT 7996^{T} and *E. coli* were very similar (Figure 1A). Indeed, the monomeric and dimeric predicted proteins of the latter species displayed the characteristic α-helices (α1 and α2) and a β-sheet (long β1, β2, and β6 and short β3, β4, and β5) (Figure 1B).

Conservation and phylogenetic relationships of HypA in *Aeromonas*

The similarity of the *in silico*-translated amino acid sequences of HypA between the *Aeromonas* species ranged between 86 % and 100 %. As expected, when comparing the *Aeromonas* HypA with those of *E. coli* and *H. pylori* the similarity was significantly lower, 41-45 % and 21-26 % respectively (Figure 1A). As observed in the phylogenetic tree, HypA proteins were highly conserved among the 25

Aeromonas species. Two groups of species, one formed by *A. aquatilis, A. sobria* and *A. saranellii* and the other with the species *A. encheleia* and *A. aquatica* showed to have identical hypothetical protein sequence (Figure 2). Thus, in conclusion we can state that HypA was highly conserved within the genus *Aeromonas.*

Transcriptional regulation of *hypA* under different pH-condition and macrophage

The expression patterns of *hypA* determined under stressful pH conditions in the most prevalent clinical species (*A. hydrophila*, *A. caviae, A. dhakensis* and *A. veroni*) and in two environmental species (*A. salmonicida* and *A. piscicola*) by qRT-PCR are shown in Figure 3A. All *Aeromonas* species displayed similar relative expression of *hypA* in alkaline condition (pH 8) (Figure 3A). Nevertheless, the expression of *hypA* was significantly higher ($p<0.05$) under acid condition (pH 4) in comparison to alkaline condition (pH 8), displaying a greater up-regulation in the most prevalent clinical species (Figure 3A and 3B). Furthermore, given that the phagosome of macrophages becomes acid upon phagocytosis of pathogens, we evaluated the expression of *hypA* during *Aeromonas* infection. The results showed that *Aeromonas* up-regulates *hypA* in response to phagocytosis, displaying significantly higher expression of the metallochaperone during infection than in control (alkaline media) or *in vitro* acid exposure ($p<0.05$) (Figure 3A and 3C). Although transcriptional regulation of *hypA* seems to depend on pH or infection condition, the statistical analysis revealed strain-related differences. The most clinically prevalent species showed significant higher upregulation of *hypA* under acid exposure and during infection than species considered environmental or more related with fish disease (Figure 3B and 3C). Significant differences in gene induction under acid exposure were observed among the most prevalent clinical species, except between *A. hydrophila* and *A. veronii* (Figure 3B). Additionally, there were significant differences in the group of clinically prevalent species, showing *A. veronii* notably lower induction of *hypA* when compared with *A. hydrophila, A. caviae* and *A. dhakensis* during macrophage infection ($p<0.05$) (Figure 3C).

With the purpose of understanding how *Aeromonas* adapts at low pH environments, we determined the pH variations of the medium during *in vitro* growth or during the infection of macrophages with the species under study. No significant changes in pH were observed when strains where grown in DMEM at pH 4 or pH 8 with the only exception of *A. hydrophila* (CECT 839^T). When the latter strain was incubated in DMEM at pH 4 there was an increase of pH that reached up to 7.5 and that was visually evident by the changing color of the DMEM media that functions as a pH indicator (Figure 4A and 4C). This basification of the medium or other pH changes were not observed for other species, neither for the infected or uninfected macrophages (Figure 4C).

Urease activity and urease genes in *Aeromonas* species

The HypA is also involved in the urease maturation which facilitates the survival of bacteria in the human gastric mucosa neutralizing the acid environment. Therefore, we have evaluated the ability of *Aeromonas* to hydrolase urea determining the urease activity by a biochemical method. None of the strains produced ureases because no color change of the slant in the media from light orange to magenta was observed when compared with the positive urease control (*Proteus sp.*). In addition, the battery of proteins associated with urease activity was not found in the genome of the six strains under study. This is consistent with the absence of the urease genes in *Aeromonas* sp., which is inferred from our *in silico* search in all available genomes of the *Aeromonas* genus.

DISCUSSION

The relevance of bacterial metal homeostasis is related to the essential role of metals for bacterial life and survival in different environments, including the context of host-pathogen interaction during the infection processes [28]. In the last years there has been a significant increase in the knowledge about metallochaperones, specially on how they bind metal ions, recognize the target proteins and facilitate metal transfer [5, 7]. The HypA metallochaperone has been associated with [NiFe] hydrogenases and ureases maturation and is considered a relevant protein for

adaptation to acidic environments of pathogenic bacteria like *H. pylori* and *E. coli* [18-20]. Furthermore, HypA metallochaperone participate in the defense against oxidative environments [21].

The present work is the first to address the study of the HypA metallochaperone in the genus *Aeromonas*. Our results suggest that the presence of *hypA* gene is a species-specific character, since not all the species from the genus *Aeromonas* contained the gene sequence in their genomes. However, an extended analysis that investigates more genomes from each species would be necessary to confirm the accuracy of our hypothesis. The *Aeromonas* HypA protein sequences showed to be moderately similar to those described in *E. coli* and *H. pylori.* However, they presented as the latter two human pathogenic bacteria the characteristic conserved N-terminal MHE motif for Ni-binding and two consecutive cysteine motifs CXXCnCPXC for Zn-binding [14, 15, 17, 18]. In addition, the three-dimensional structure predicted for all proteins showed to be highly similar among the *Aeromonas* species and the other two human pathogenic bacteria, confirming that it is a metallochaperone [16, 17, 29].

Although the principal role of HypA has been typically associated with the maturation of hydrogenases, in the last years it has been demonstrated that HypA could play a role in the maturation of ureases. These metalloenzymes are involved in the survival of pathogenic bacteria in acidic environment. For instance, bacterial ureases are involved in the survival of *H. pylori* in of the human stomach at acidic pH [18-20]. In *E. coli* this function is carried out by other enzymes i.e. hydrogenases [18, 22, 23]. The fact that 80 % of infections caused by *Aeromonas* are gastrointestinal diseases indicates an adaptation of these bacteria to acid environments of the gastrointestinal tract [26, 27]. Additionally, a previous study hypothesized that urease activity may contribute to acid tolerance in some *A. caviae* strains, facilitating bacterial survival during infection, as occurs in *Yersinia enterocolitica* [30]. However, *Aeromonas* has usually been described as urease negative [31]. Consistent with these previous data we observed in our study that all strains were urease negative. However, the expression study demonstrates that *hypA*

is upregulated in acidic pH. A reasonable explanation could be that hydrogenases, but not ureases, would be involved in acid resistance in *Aeromonas,* as occurs in *E. coli* [18, 22, 23]. In addition, our results showed that *A. hydrophila* CECT 839^T alkalinize the medium during acid exposure. One feasible explanation for this phenomenon would be that *A. hydrophila* has higher tolerance to acids as a consequence of an enzymatic pH shifting, which allows a better survival. Although additional analysis including more strains should be performed to determine if this is a specific characteristic of this species. Therefore, it would seem plausible to affirm that HypA could be associated with acid tolerance in *Aeromonas* species.

Numerous studies provide insights into the relevance of redox signaling and reactive oxygen species (ROS) production as defense mechanisms against pathogens [32, 33]. Moreover, bacterial infections also can induce oxidative stress contributing to increase DNA mutations rates. For instance, *H. pylori* produce superoxide anion in order to counteract the toxic effect of the ROS produced in the human stomach, which contributes even more in the development of the gastric cancer [34]. Considering that the immune system generates ROS as defense mechanism against pathogens after phagocytosis by macrophages [35, 36], resistance to acidic environments can be of great advantage for pathogens. Indeed, some studies have emphasized a strong relationship between deficiency of ROS production and susceptibility to microbial infection [37]. In these context, discovery of the chaperone Hsp33 and its role in protecting cells against the deleterious effects of reactive oxygen species [38-40], reinforces the hypothesis of the important role of redox regulation during bacterial colonization [41]. In our study, the results showed that the *Aeromonas* metallochaperone *HypA* was upregulated after phagocytosis of macrophages, which is in line with the previous works [38-40][5]. Therefore, considering that hydrogenases participate in oxidative stress defense [21] it is very possible that *HypA* also contributes to the defense against ROS produced by macrophages in the phagocytic process. In conclusion, our results suggest that *HypA* could play a role in the survival of *Aeromonas* in acid environment and in the defense against macrophages, although the exact mechanism remains unclear, but

the possible role of this metallochaperone in *Aeromonas* sp. virulence is relatively evident.

CONCLUSIONS

This study reports for the first time the distribution of orthologous sequences coding for the metallochaperone HypA in the bacterial genomes of the genus *Aeromonas* and their deduced protein structure. Interestingly, HypA was present in 69.4% of the *Aeromonas* species showing a high similarity among the species (%). Metallochaperones are relevant in the host-pathogen interaction and for this reason this preliminary study demonstrates the possible role of HypA in bacterial survival in acid environment, as well as the possible role in defense against ROS produced by macrophages. In addition, it may promote future studies to confirm and better understand the function of this metallochaperone in the survival of species from the genus *Aeromonas*.

METHODS

Bacterial strains and culture conditions

Aeromonas dhakensis CECT 5744^T, *Aeromonas caviae* CECT 838^T, *Aeromonas veronii* CECT 4257^T, *Aeromonas hydrophila* CECT 839^T *Aeromonas salmonicida* CECT 894^T and *Aeromonas piscicola* CECT 7443^T type strains were used in this study and stored at -80 °C in TSB (Tryptic Soy broth) with 20 % of glycerol. Bacteria were grown in Tryptone Soya agar (TSA) at 30 °C for 24 h. Prior to infection, bacteria were grown at 37 °C in serum and antibiotic-free Dulbecco's Modified Eagle's Medium (DMEM, PAA Laboratories) for 18 h.

For evaluation of urease activity, the bacteria were grown in Urea Agar Base media (with a yellow-orange color) at 37 °C for 24 h and the change of color of the slant indicated a positive reaction. A strain of *Salmonella* sp. was used as a negative control and one of *Proteus* sp. as positive control.

Results

In silico search of *hypA* and *ure* genes in the *Aeromonas* genomes

For identification of *hypA* sequences in *Aeromonas* species an initial *in silico* search was conducted on UniParc from Uniprot database (https://www.uniprot.org/uniparc/). As result the deduced amino acid sequence of HypA from *Aeromonas hydrophila* KN-Mc-1R2 (UPI0005A60C51) was obtained. The corresponding nucleotide sequence of this strain (CP027804.1) was used as query on BLAST searches in 36 genomes of *Aeromonas* spp. type strains deposited in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify *hypA* orthologues sequences. To investigate whether the presence of *hypA* could be a strain or a species-specific characteristic, we extended our analysis to other available genomes of non-type strains[42] from the species *A. hydrophila* (n=8) and *A. dhakensis* (n=8). For identification of urease genes in the *Aeromonas* genomes an *in silico* search was performed using as query the sequences of the genes: *ureA, ureB, ureI, ureE, ureF, ureG* and *ureH* from *H. pylori*.

Protein sequence analysis and 3D-structure prediction

To determine sequence conservation of hypA in *Aeromonas*, a comparison of HypA proteins among the 25 *Aeromonas* species and the one of *E. coli* (strain EA4377A) and *H. pylori* (strain HPGA1) was assessed by multiple alignment, using CLUSTALW algorithm on MegAlign software. Phylogenetic relationships among sequences were depicted in a phylogenetic tree constructed on MEGA6 Software using the Neighbor-joining method. In addition, the prediction of 3D monomeric and dimeric HypA protein structure and the comparative analyses of this protein between the one of *A. hydrophila* (CECT 839^{T}) and *E. coli* (EA4377A), was done using Swiss-model online tool (https://swissmodel.expasy.org/).

Cell line culture, infection and induction experiments

The cell line J744A.1 from mouse BALB/C monocyte macrophages was used for the infection experiments with the six *Aeromonas* type strains. The macrophages cells were maintained in adhesion in Dulbecco's Modified Eagle's Medium (DMEM, PAA Laboratories) (pH=8) supplemented with 10 % FBS (fetal bovine

serum, PAA Laboratories) plus 1 % P/S solution (penicillin-streptomycin stock, PAA Laboratories) at 37 °C and 5 % CO_2. Prior to infection, cells were seeded in tissue culture plates (1×10^6 cells/ml) containing serum-free DMEM without antibiotics (serum-starvation conditions) for 18 h. The macrophages J774A.1 were infected with the six *Aeromonas* type strains grown in serum-free DMEM without antibiotics at a multiplicity of infection (MOI) of 5. In addition, bacteria were seeded onto tissue culture plates in serum-free DMEM without antibiotics at alkaline pH (pH=8) or acidic pH (pH=4.5) adjusted with HCl solution followed by filtration to remove any precipitate. Co-cultures were incubated at 37 °C and 5 % CO_2 up to 4 h for gene expression analyses.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from logarithmic-phase *Aeromonas* cultures using TRIzol Reagent (Invitrogen) as previously described [43]. RNA quality and integrity was confirmed spectrophotometrically using Nanodrop 2000, calculating the 260/280 and 260/230 ratios. The cDNA was transcribed from RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. Hercules, CA, USA) according to the manufacturer's instruction. Quantitative Real time PCR was performed in duplicate using Real-Power SYBR® green PCR Mastermix (Applied Biosystems®, Life Technologies) in 10 µl total PCR reaction mixture on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The thermal cycling conditions were: 94 ºC for 5 min, followed by 45 cycles of 30 s at 94 ºC, 30 s at 60 ºC, 30 s at 72 ºC, and finally 20 s at 80 ºC. The threshold cycle (Ct) was automatically determined by the StepOne Software v2.0 (Applied Biosystems) to calculate the relative expression of the tested gene (*hypA)* using as reference the 16S rRNA housekeeping gene, as previously described [44]. Relative gene expression levels and fold change expression were estimated using $2^{-\Delta\Delta Ct}$ method [45]. The specific primers of *hypA* and 16S rRNA gene were designed with Oligo Primer Analysis Software v. 7 (Table 2). Experiments were performed in triplicate using three independently prepared bacterial growth cultures obtained on three different days.

Statistical analysis

All experiments were performed in triplicates and significant differences were determined using Student's two-tailed *t*-test calculated on Graph Pad Prism 6.0 (GraphPad Software, CA, USA) P values ≤ 0.05 were considered statistically significant (*).

LIST OF ABBREVIATIONS

ROS: Reactive oxygen species

TSB: Tryptic Soy broth

TSA: Tryptone Soya agar

DMEM: Dulbecco's Modified Eagle's Medium

FBS: Fetal bovine serum

P/S: Penicillin-Streptomycin

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data generated and/or analyzed during the current study are not publicly available due to the patient's individual privacy. All relevant data to this case is reported in the manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding information

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Authors' contributions

AFB, LLF and MJF designed the research, performed research and wrote the project.

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TABLES

Table 1. Presence or absence of *hypA* gene in the genomes of 36 *Aeromonas* species described until now.

Species	Strain	Source	Presence of *hypA* gene	Protein accession number (NCBI)
A. hydrophila	CECT 839^{T}	Milk	Yes	ABK37593.1
A. salmonicida	CECT 894 ^{T}	Salmon	Yes	WP_005315136.1
A. sobria	CECT 4245 ^{T}	Fish	Yes	WP_005301911.1$^{\#}$
A. media	CECT 4232 ^{T}	Fisheries water	Yes	AUU20677.1
A. veronii	CECT 4257 ^{T}	Sputum	Yes	WP_005351492.1
A. caviae	CECT 838 ^{T}	Guinea pig	Yes	WP_017786826.1
A. eucrenophila	CECT 4224 ^{T}	Fresh water fish	Yes	WP_042642402.1
A. schubertii	CECT 4240 ^{T}	Skin abscess	No	-
A. jandaei	CECT 4228 ^{T}	Human feces	Yes	WP_042029441.1
A. trota	CECT 4255 ^{T}	Human feces	Yes	WP_026458218.1
A. allosaccharophila	CECT 4199 ^{T}	Eel	Yes	ABK37593.1
A. encheleia	CECT 4342 ^{T}	Eel	Yes	WP_033130233.1$^{¥}$
A. bestiarum	CECT 4227 ^{T}	Sick fish	Yes	WP_043555398.1
A. popoffi	CECT 5176 ^{T}	Drinking water	Yes	WP_042034298.1
A. simiae	IBS S-6874 ^{T}	Monkey feces	No	-
A. molluscorum	CECT 5864 ^{T}	Shellfish	No	-
A. bivalvium	CECT 7113 ^{T}	Shellfish	No	-
A. tecta	CECT 7082 ^{T}	Children feces	Yes	WP_050720085.1
A. piscicola	CECT 7443 ^{T}	Sick fish	Yes	WP_021140355.1
A. fluvialis	CECT 7401 ^{T}	River water	No	-
A. taiwanensis	CECT 7403 ^{T}	Wound infection	No	-
A. sanarelli	CECT 7402 ^{T}	Wound infection	Yes	WP_005301911.1$^{\#}$
A. diversa	CECT 4254 ^{T}	Wound infection	No	-
A. rivuli	CECT 7518 ^{T}	River water	No	-
A. cavernicola	CECT 7862 ^{T}	Cavern creek water	No	-
A. australiensis	CECT 8023 ^{T}	Irrigation water	No	-
A. dhakensis	CECT 5744 ^{T}	Children feces	Yes	WP_042008198.1
A. finlandensis	CECT 8028 ^{T}	Cyanobacterial bloom	Yes	WP_033136932.1
A. aquatica	CECT 8025 ^{T}	Cyanobacterial bloom	Yes	WP_033130233.1$^{¥}$
A. lacus	CECT 8024 ^{T}	Cyanobacterial bloom	Yes	
A. rivipollensis*	KN-Mc-11N1	River water	Yes	AVP94562.1
A. lusitana	CECT 7828^{T}	Untreated water/Vegetables	Yes	WP_100861683.1
A. intestinalis$^{\pi}$	CECT 8980^{T}	Human feces	Yes	-
A. enterica$^{\pi}$	CECT 8981^{T}	Human feces	Yes	-
A. crassostreae$^{\pi}$	CECT 8982^{T}	Shellfish	No	-
A. aquatilis$^{\pi}$	CECT 8026^{T}	Lake water	Yes	-

*The genome of the type strain is not deposited. $^{\#,¥}$ Protein accession number is the same because it was deposited as multispecies. $^{\pi}$ Species pending to be described.

Table 2. Oligonucleotides used in this study for transcriptional analysis.

Primers	Sequence 5'-3'
hypA	
Forward	ATGCACGAAATGTCTCTGGC
Reverse	TCGTAATTTGTACCCGCCAC
16S rRNA	
Forward	TGTGTCCTTGAGACGTGGC
Reverse	ACAAAGGACAGGGGTTGCG

FIGURE LEGENDS

Figure 1. (A) Sequence alignment of the *in silico*-translated amino acid sequences of the HypA proteins from 36 *Aeromonas* species, *E. coli* and *H. pylori*. The alignment was constructed with MegAlign. The MHE correspond to the motif of the Nickel binding domain (green) and CxxCnCPxP to the Zinc binding domain (red). Sequences from Uniprot database of *A. hydrophila* (*). (B) Predicted monomeric and dimeric structure of HypA proteins from *A. hydrophila* type strain and *E. coli* constructed with Swiss Model online tool, the α-helices and the stranded β-sheet motifs are indicated.

Figure 2. Phylogenetic tree constructed with the *in silico*-translated amino acid sequences of the HypA proteins (113 aa) of the 36 *Aeromonas* species type strains (the only sequence not belonging to the type strain corresponds to *A. rivipollensis* KN-Mc-11N1). The phylogenetic analysis was constructed with MEGA6, using Neighbor-joining algorithm. Numbers at nodes represent bootstrap percentages (> 50 %) obtained by repeating analysis 1000 times.

Figure 3. Expression of *hypA* in *Aeromonas* species determined by RT-qPCR. (A) Relative expression of *hypA* during phagocytosis by macrophages and under acidic and alkaline culture conditions. Transcript levels of *hypA* were normalized to the

expression of 16S rRNA. B) Expression fold change of *hypA* on bacteria grown on acid media respect to the cultured in alkaline media and (C) expression fold change on bacteria phagocytized by macrophages after 4 h of infection respect to control condition on culture alkaline media calculated using the comparative ΔΔCt method. Error bars indicate standard deviations calculated from three independent experiments.

Figure 4. Culture plates with different *Aeromonas* species at 0 h and 4 h. (A) Bacteria culture in alkaline medium DMEM. (B) Bacteria culture in acid DMEM with HCl. (C) Macrophages in alkaline DMEM infected by different *Aeromonas.* Yellow indicates acid pH and pink indicates alkaline pH.

Figure 1

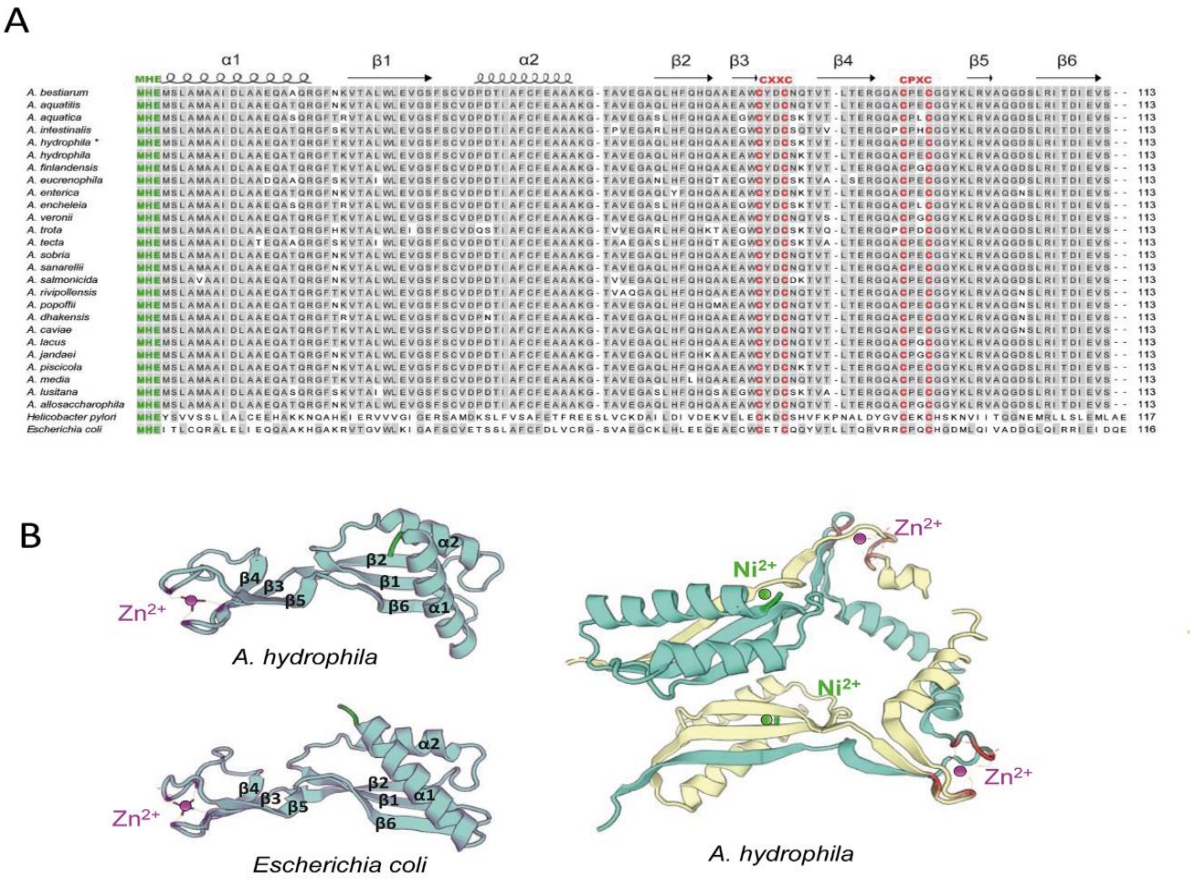
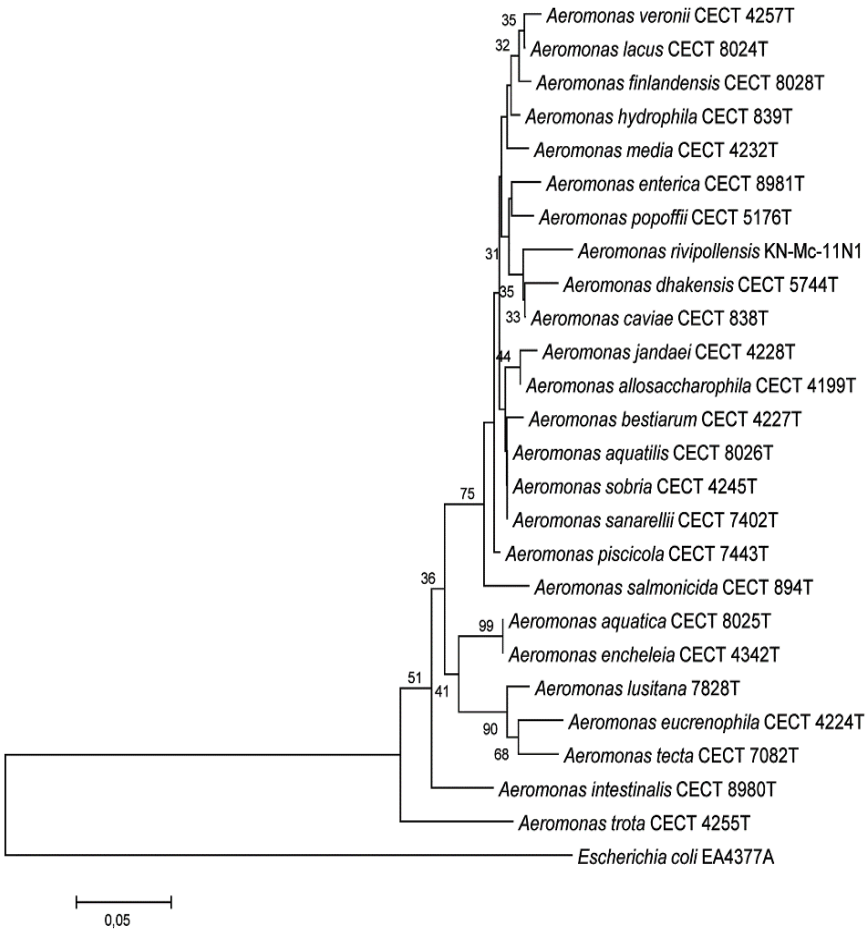

A
α1
β1
α2
β2
β3
CXXC
β4
CPXC
β5
β6
A. bestiarum
A. aquatilis
A. aquatica
A. intestinalis
A. hydrophila *
A. hydrophila
A. finlandensis
A. eucrenophila
A. enterica
A. encheleia
A. veronii
A. trota
A. tecta
A. sobria
A. sanarellii
A. salmonicida
A. rivipollensis
A. popoffii
A. dhakensis
A. caviae
A. lacus
A. jandaei
A. piscicola
A. media
A. lusitana
A. allosaccharophila
Helicobacter pylori
Escherichia coli
B
Zn2+
A. hydrophila
Escherichia coli
Ni2+
A. hydrophila

Figure 2


35
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90
68
Aeromonas veronii CECT 4257T
Aeromonas lacus CECT 8024T
Aeromonas finlandensis CECT 8028T
Aeromonas hydrophila CECT 839T
Aeromonas media CECT 4232T
Aeromonas enterica CECT 8981T
Aeromonas popoffii CECT 5176T
Aeromonas rivipollensis KN-Mc-11N1
Aeromonas dhakensis CECT 5744T
Aeromonas caviae CECT 838T
Aeromonas jandaei CECT 4228T
Aeromonas allosaccharophila CECT 4199T
Aeromonas bestiarum CECT 4227T
Aeromonas aquatilis CECT 8026T
Aeromonas sobria CECT 4245T
Aeromonas sanarellii CECT 7402T
Aeromonas piscicola CECT 7443T
Aeromonas salmonicida CECT 894T
Aeromonas aquatica CECT 8025T
Aeromonas encheleia CECT 4342T
Aeromonas lusitana 7828T
Aeromonas eucrenophila CECT 4224T
Aeromonas tecta CECT 7082T
Aeromonas intestinalis CECT 8980T
Aeromonas trota CECT 4255T
Escherichia coli EA4377A
0,05

Figure 3

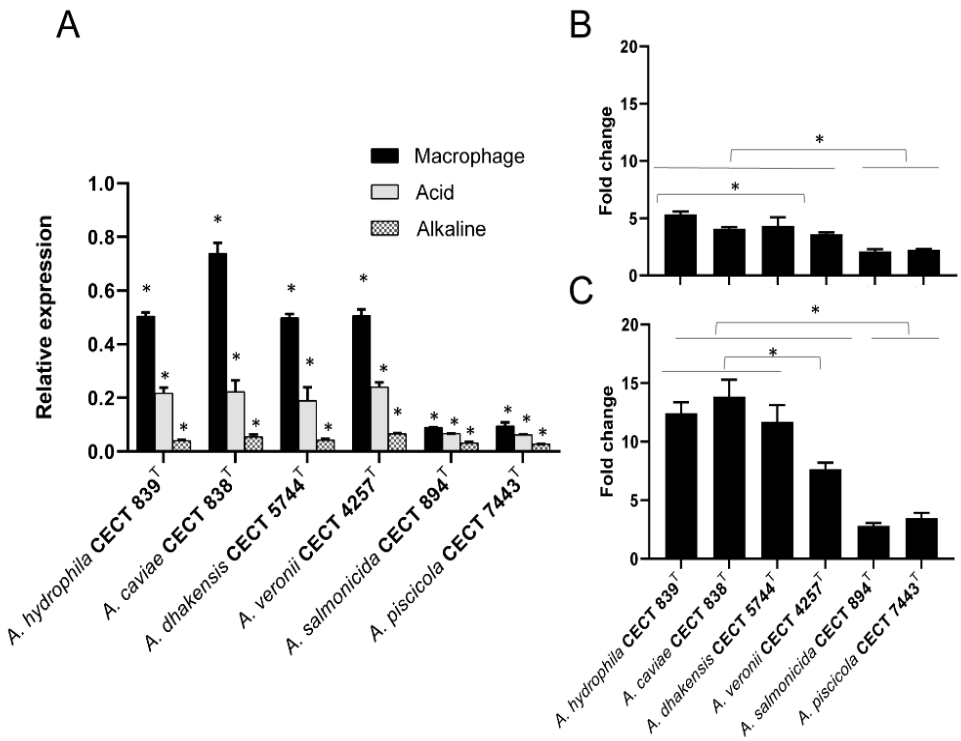
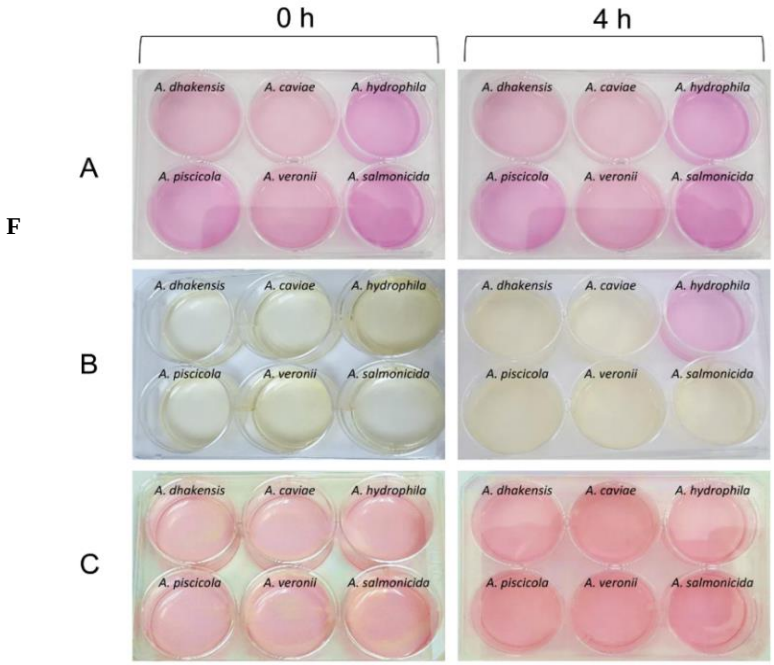


Figure 4



UNIVERSITAT ROVIRA I VIRGILI
EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
Ana Fernández Bravo

4.8. Role of T6SS and *exoA* gene in the mixed infection caused by *Aeromonas hydrophila* that develop necrotizing fasciitis

Fernández-Bravo A., Sha., J., Figueras MJ., Chopra AK. (In preparation, pending to be submitted to Proceedings of the National Academy of Sciences of the United States of America).

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Role of T6SS and *exoA* gene in the mixed infection caused by *Aeromonas hydrophila* thet develop necrotizing fasciitis .

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Abstract

A recent case of necrotizing fasciitis (NF) infection with two *Aeromonas hydrophila* strains (NF1 and NF2) in a young immunocompetent patient was described. Different experiments suggested that NF1 possesses a specific Type Six Secretion System (T6SS) that block NF2 in a murine model and that the ExoA toxin, present only in NF2, could play a role in the dissemination of the infection. To increase the knowledge about the complex interactions of these two strains during NF infection we will try to further determine the role of the T6SS and the ExoA toxin performing different experiments using different WT and mutant strains. The mutant strains were the following: two mutants of the T6SS genes *vasK* (structural gene) and *tseC* (gene encoding the effector toxin TseC) of the NF1 strain; one for the gene encoding the immunoprotein *tsiC* generated by strain NF2 against the TseC effector toxin as well as mutants of NF1 and NF2 strains positive and negative for ExoA toxin. Taking into account the results, we concluded that the T6SS and the ExoA toxin of *A. hydrophila* strains (NF1 and NF2) contribute to the development of NF in mice during mono-microbial infections. However, during the mixed infections, the T6SS plays a dominant role in developing of NF.

Keywords: *A. hydrophila*, necrotizing fasciitis, mixed infection, T6SS, ExoA.

Introduction

Necrotizing fasciitis (NF) is a rapidly progressive, life-threating soft-tissue infection that causes a necrotic inflammation of the fascia and the surrounding tissue (Bellapianta et al., 2009; Tsai et al., 2015; Ponnusamy et al., 2016). The clinical features associated with NF include hypotension, fever, necrosis and gangrene (Tsai et al., 2015). The Streptococcal infection was the most commonly identified in cases (Arif et al 2016) among other bacteria involved in NF as *Staphylococcus*, *Clostridium*, *Klebsiella*, *Vibrio vulnificus* and *Aeromonas* (Chen et al., 2017). Two types of NF have been described, the polymicrobial (type I), usually seen in the elderly or in those with underlying illnesses and monomicrobial (type II), that occur in people without underlying illness. (Sarani et al., 2009; Stevens et al, 2017). *Aeromonas hydrophila* is the *Aeromonas* species more frequently associated in the reported NF cases and some of them water was considered the source of infection (Furusu et al., 1997; Joseph et al., 1979; Sever et al, 2013; Spadaro et al., 2014; Tsai et al., 2015). The complex interactions among multiple strains of *A. hydrophila*, called flesh eating bacteria, isolated from an immunocompetent individual who developed NF resulting in amputation almost all the extremities were previously described in two studies (Grim et al., 2014; Ponnusamy et al., 2016). The NF case was originally considered monomicrobial because the different strains belonged to the same species *A. hydrophila*, but genomic analysis showed that four strains representing two paraphyletic linages of *A. hydrophila*, one including NF2, NF3, and NF4 that presented a minimal difference in the sequence, and they were all represented by NF2 and another one including NF1 that was distinct. Also, both linages showed differences in the presence of the *exoA* gene that was absent in the NF1 strain and present in the other strains. This gene encodes the ExoA toxin that has been involved in the inhibition of protein synthesis and host cell death (Zdanovsky et al., 1993; Grim et al., 2014). Previous results showed that when NF is caused by mixed infection with this *A. hydrophila* strains, the progression of NF follows a different course from when the infection is produced with a single strain. In a single infection, the absence of *exoA* gene in the strain NF1 resulted in the

bacterium remaining at the local site of the infection from were eventually was eliminated by host defense mechanism. However, the secretion of ExoA toxin by strain NF2 allowed the bacterium to disseminate to peripheral organs, destroying the local tissue barriers. Nevertheless, in the mixed infection with both strains, ExoA secreted by NF2 allowed the dissemination of NF1 to the peripheral organs destroying the local tissue barriers. Also, strain NF1 facilitates the direct killing and/or host-mediated elimination of NF2 from the site of infection (Ponnusamy et al., 2016). This antagonistic effect suggested the possible involvement of the Type-Six Secretion System (T6SS) capable of introducing effector toxins like TseC from NF1 to NF2 (Ponnusamy et al., 2016). The T6SS classically considered to be used by bacteria to target eukaryotic cells, have been recently involved in the elimination or inhibition of competitor bacteria, being this 'antibacterial' characteristic essential for the competitive fitness in the case of polymicrobial infections (English et al., 2012). Such a system could provide the pathogen with a large competitive advantage against other bacteria in the host or the environment, enabling it to proliferate and mount a successful infection. The present study pretends to provide more information about the effector molecules produced by NF1 that could block the growth of the strain NF2. For this, we performed the experiments with mutants of the genes *vasK* (encodes a structural T6SS protein) (Suarez et al., 2008) and *tseC* (encodes the T6SS effector toxin TseC) as well as the gene *tsiC* encoding the immunity protein that inhibits the toxin TseC (Liang et al., 2015). Similarly, the present study clarifies the role of the *exoA* gene in NF infections using WT strains and NF1 and NF2 mutants for this gene.

Material and Methods

Bacterial strains and growth conditions

The bacterial strains and their characteristics are listed in Table 1. Bacteria were routinely grown on Luria-Bertani (LB) broth at 37°C for 24 h with 180 rpm shaking. The cultures were washed twice in sterile PBS and were resuspended in the same buffer before the *in vitro* and *in vivo* assays were performed. All the strains were

stored in LB plus glycerol (90%) at -80°C. Antibiotics were added into the LB as needed, ampicillin (Ap) 250 µg/mL, kanamycin (Km) 100 µg/mL, and rifampicin (Rif) 200 µg/mL. All strains and plasmids used in the study are listed in the Table 1.

Genetic manipulation of the bacterial strains

Construction of bioluminescent mutant *A. hydrophila* NF2Δ*exoA-lux*

A conjugation with two strains of *Escherichia coli*, SM10 Λpir carrying the pTNS2 plasmid and SM10 Λpir harbouring the pUC18-mini-Tn7T:Km-lux was performed (Ponnusamy et al., 2016). The latter minitransposon contains a lux luminescence operon with the native promoter and kanamycin resistance (Km^r) selection marker. This system allows the integration downstream of the *gmlS* gene, which encodes a conserved glucosamine-6-phosphate synthetase, with the pTNS2 helper plasmid providing the transposase complex (Ponnusamy et al., 2016) (Table 1).

Construction of NF1and NF2 mutants related to the T6SS

The mutants NF1Δ*tseC*, NF1Δ*vasK* and NF2-*lux*-pBR322-*tsiC* (Table 1) were constructed using crossover PCR and homologous recombination as previously described with other strains. (Suarez et al., 2008; Liang et al., 2015). The insertion and deletion of the genes were confirmed by PCR.

In vitro experiments

Cell line and growth conditions

The cell line RAW 264.7 of murine macrophages was obtained from the Cell Culture facility of the University of Texas Medical Branch. The RAW 264.7 cells were maintained as adherent cells in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (Fetal Bovine Serum, Biowest Europe) plus 1mM of Napyr (Sodium pyruvate, Biowest Europe), at 37°C and 5% CO_2. Prior to infection, cells were seeded in tissue culture plates.

Phagocytic assays in macrophages RAW 264.7

The wild type and mutant strains (NF1, NF1-exoA, NF2, NF2Δ*exoA*, NF1Δ*tseC*, NF1Δ*vasK*) were grown overnight before the RAW 264.7 macrophages were infected at a multiplicity of infection (MOI) of 5. They were incubated at 37°C with 5% of CO_2 for 1h. After this time a treatment to kill extracellular bacteria with gentamycin (50µg/mL) was performed for 45 min. The number of the bacteria inside the macrophages was counted after gentamycin treatment (time point 0), by a serial dilution and plating. The percent of phagocytosis was calculated with the number of bacteria at time point 0 in relation to the initial infection dose.

Intracellular survival in macrophages RAW 264.7

The murine RAW 264.7 macrophages were infected with overnight cultures of the wild type and mutant strains (NF1, NF1-exoA, NF2, NF2Δ*exoA*, NF1Δ*tseC*, NF1Δ*vasK*) at a multiplicity of infection (MOI) of 5 and were incubated at 37°C with 5% of CO_2 for 1h. Thereafter, a treatment to kill extracellular bacteria with gentamycin was performed for 1 h. The number of the bacteria inside macrophages was counted at 4 and 6 hours after gentamycin treatment. The percentage of phagocytosis was calculated with the number of bacteria at 4 and 6 h in relation to the initial infection dose.

Dynamics of *A. hydrophila* strains in mixed infections

The wild type and mutant strains were grown overnight (NF1/NF2-lux, NF2-*lux*/NF1Δ*tseC*, NF2-*lux*/NF1Δ*vasK*, NF1/NF2-*lux*-pBR322-*tsiC* and). These strains were mixed in LB broth at a ratio of 5:1 and spotted in nonselective LB plates for 4h. The growth of the strains in the mixture was determined by serial dilution and plating on the selective plates with the appropriate antibiotic or on nonselective LB plates.

In vivo experiments

Mice infection

For-week-old and healthy Swiss-Webster mice (Taconic Farms, NY, USA) were used to perform the experiments. All animals were maintained in standard conditions and the studies were performed and approved by Institutional Animal Care and Use Committee of the University of Texas Medical Branch. For the infection studies, the overnight cultures were centrifuged, and the pellets were washed three times in sterile phosphate-buffered saline (PBS) before being suspended in 1/10 of the original culture volume of PBS. Subsequently, the number of cells in each culture was estimated and inocula were prepared in such a way that a 100µl volume contained the intended infectious dose of the organism.

Mouse model of intraperitoneal injection with single infections

Mice, 10 per group, were intraperitoneally infected with single ExoA positive (NF2 and NF1-exoA) and negative strains (NF1 and NF2Δ*exoA*) at different doses ($5x10^6$, $8x10^6$, $1x10^7$, $5x10^7$ and $8x10^7$). The survival of the mice was observed until 14 days and the percent were plotted.

Mouse model of intramuscular injection with mixed infections

Mice, 7 per group, for ExoA mutants and 10 per group for T6SS mutants were anesthetized with isoflurane, 100µl containing a mixture of WT and mutant strains was injected i.m. into one of the legs at a dose of $5x10^7$ ($2.5x10^7$cfu per strain). The mixtures were the following: NF1/NF2-lux, NF1-*lux*/NF2Δ*exoA*, NF2-*lux*/NF1Δ*tseC* and NF2-*lux*/NF1Δ*vasK*. The animals were observed for the disease progression, and the mortality was recovered daily.

ExoA immunization of mice and bacterial challenge

Mice, 8 per group, were immunized i.m. with 100 ng of ExoA mixed with alum at ratio 1:1. Boosters with the same dose were given every two weeks for a total of 6 immunizations and mice received alum only as a control. The blood of the mice was collected before immunization (pre-immune) and after the third and the sixth

immunization to evaluate the anti-ExoA antibody. After this the mice were injected i.m. with NF2 ($1x10^8$) and with the mix NF1/NF2 at a dose of $2x10^8$ ($1x10^8$ cfu per strain). The survival pattern was recovered for 14 days.

Bacterial dissemination using a murine intramuscular model

The NF2-*lux* strain was mixed at ratio1:1 with NF1 and various T6SS mutants of those strains (NF1/NF2-lux, NF2-*lux*/NF1Δ*tseC* and NF2-*lux*/NF1Δ*vasK*). Mice (7 per group) were injected i.m at a dose of $5X10^7$ ($2.5X10^7$ cfu per strain). After 24-48 h of the infection, the spleen and around 200-300 mg of muscle tissue at the site of the injection as described above were placed in PBS and homogenized. Mice (7 per group) were i.m. infected with NF1 mixed with NF2, as well as NF1 mixed with the NF2Δ*exoA* mutant strain (NF1-*lux*/NF2 and NF1-*lux*/NF2Δ*exoA*) at a dose of $5x10^7$ ($2.5x10^7$cfu per strain). The tissues were collected from terminal animals during the experiment. Also, mice (3 per group) were i.m. infected with NF1 and NF2 mixed with their positive and negative ExoA mutant (NF1-*lux*/NF1-*exoA* and NF2/NF2Δ*exoA*-*lux*) at a dose of $2x10^8$ ($1x10^8$cfu per strain). After 24 h post infection, mouse tissues were collected. Subsequently, serial dilutions were plated on LB agar plates with the appropriate antibiotic. After incubation for 24h and 37°C, colonies were counted to calculate bacterial load per organ (spleen) or gram of muscle tissue.

Statistical analysis

Student t-test, one-way ANOVA with Tukey *post hoc* test, two-way ANOVA with Sidak *post hoc* test or Log-rank (Mantel-Cox) test were performed to analyze the data. Wherever applicable, p value is reported, and p values of <0.05; <0.01 and <0.001 were considered significant.

Results

In vitro

Role of T6SS and ExoA in phagocytic elimination and intracellular survival in macrophages of strains NF1 and NF2

Murine macrophages (cell line RAW 264.7) infected with mutants of T6SS of strain NF1, i.e., NF1Δ*vasK* and NF1Δ*tseC* resulted in a significantly higher ($p < 0.05$) rate of phagocytosis of the mutant strains when compared with the intact strain NF1 (Fig. 1A). Different rates of phagocytosis were observed in the two mutant strains NF1Δ*tseC* and NF1Δ*vasK*, showing the strain NF1Δ*vasK* the higher phagocytosis (Fig.1 A). In the case of the NF2 and NF2Δ*exoA*, the rate was significantly higher ($p < 0.05$) compared with NF1 and NF1-*exoA* (Fig. 1B). However, between NF1 and NF1-*exoA* as well as NF2 and NF2Δ*exoA* no differences were found. In relation to the percentage of intracellular survival at 4h and 6h, NF1 showed a significantly higher rate of survival followed by NF1Δ*vasK* and NF1Δ*tseC* (Fig. 2A). However, the intracellular survival was similar between NF1, NF2, NF1-*exoA* and NF2Δ*exoA* and the percentage was approximately of 25% at 4h and 5% at 6h post infection (Fig. 2B).

Role of T6SS in the *in vitro* dynamics: NF2 strain suppression growth by strain NF1

When NF1 and NF2-*lux* were mixed 5:1 and plated on LB agar without antibiotic and with kanamycin, and incubated for 4 h, colony counts for NF2-*lux* were reduced (Fig. 3). This was not observed when both NF1 mutants of the T6SS (NF1Δ*vasK* and NF1Δ*tseC*) were mixed with NF2-*lux*, the colony numbers of NF2-*lux* were not reduced so these mutant strains increased the survival of NF2 compared with the first mix (NF1/NF2-*lux*). Also, the survival of NF2-*lux* in the mix with NF1Δ*vasK* was significantly higher than NF2-*lux* in the mix with NF1Δ*tseC* (Fig. 3). The colony counts of the mutant for the protective immunoprotein of T6SS (NF2-*lux*-*pBR322-tsiC*) in the mix with NF1 were significantly higher than NF2-*lux* in the mix with NF1. However, the number of NF2-*lux*-pBR322-*tsiC* was higher than NF2-*lux*

in the mix with the mutant NF1Δ*tseC*, but not significantly. Finally, NF2-*lux*-pBR322-*tsiC* mutant showed a lower not significant survival than the wild type strain NF2-*lux* in the mix with the mutant NF1Δ*vasK* (Fig.3).

Role of ExoA on NF1 strain T6SS-mediated NF2 killing in mixed cultures

When strain NF1 and bioluminescent NF2 *exoA* mutant (NF2Δ*exoA*-*lux*) were mixed in a ratio of 5:1 and plated on LB agar without antibiotic and with kanamycin, and incubated for 4h, colony counts for NF1 were significantly higher ($p < 0.005$) in relation to 0h (Fig. 4). In contrast, the number of NF2Δ*exoA*-*lux* was inhibited by NF1 at 4h (Fig. 4). Also, a dynamic infection in a ratio of 1:1 was performed and the results were similar (data not shown).

In vivo

Role of ExoA in an intraperitoneal infected mouse model caused by NF1 and NF2 single strains

When NF1 was injected i.p., at higher dose ($1x10^7$) all animals succumbed to the infection within 24h p.i., while at an infection doses of $5x10^6$ and $8x10^6$ about the 20-25% of animals survived. The mice infected with the strain NF1 that have incorporated the *exoA* gene (NF1-*exoA*) showed the same pattern of mortality than the one caused by the WT NF1 strain Fig. 5A). In contrast, all mice injected with NF2 at dose of $5x10^7$ survived. However, at higher dose ($8x10^7$) the survival percentage of mice was about 90% after NF2 infection, while after NF2Δ*exoA* the survival was significantly lower ($p < 0.05$), being about of 10% (Fig. 5B)

Role of T6SS in the pathogenesis caused by mixed infections with NF1 and NF2 strains in an intramuscular mouse model

As shown in the Fig. 6, the mixed infection NF1/NF2-*lux* were 100% lethal to mice at dose 5×10^7 cfu (2.5×10^7cfu per each strain) within 24 h p.i. However, the mix NF1Δ*vasK*//NF2-*lux* and NF1Δ*tseC*/NF2-*lux* showed a significantly higher percentage of survival ($p < 0.05$) when compared to the infection with the WT strains (NF1/NF2-*lux*), being about of 60% and 80%, respectively.

Dissemination pattern of infection in an intramuscular mouse model with mixed cultures of *A. hydrophila* T6SS mutants

Mixed infection with NF1 and NF2-***lux*** at infective dose 5×10^7 cfu (2.5×10^7 cfu per each strain) resulted in a significant decrease ($p < 0.005$) of NF2-*lux* in the muscle at 24-48 h p.i. (Fig.7A). Also, the dissemination pattern showed that the strain NF2-*lux* was not detected in the spleen (Fig. 7B). However, the mixed infections between NF2-***lux*** with NF1 T6SS mutants (NF1Δ*tseC* and NF1Δ*vasK*) showed that all strains were detected in the site of the injection (Fig. 7A), while in the spleen the mixed infection between these strains showed a reduced number of bacteria (Fig. 7B).

ExoA immunization of mice before intramuscular infected mouse model with single and mixed infection with NF1 and NF2 strains

The evaluation of the anti-ExoA antibody in mice showed a significantly higher rate of antibodies ($p<0.05$) after the immunization with the ExoA mixed with alum (1:1) (Doses-ExoA) in comparison with the pre-immune mice, as well as the positive control only with alum (Doses-Alum) (Fig. 8A). Also, the results showed that at 6 doses the rate of antibody was higher than the rate with 3 doses. In relation to the survival pattern after the immunization, the infection with NF2 in mice that received only alum resulted in all mice dead at 72h p.i., while after the infection with NF2 in the mice immunized with ExoA mixed with alum (1:1) resulted in all mice survived ($p < 0.05$). However, after the infection with NF1/NF2-Alum all mice dead at 24 h p.i. and the result with NF1/NF2-ExoA Immun. was similar, being 20% the percentage of the survival (Fig. 8B).

Role of ExoA during mixed infection between NF1 and NF2 strains in an intramuscular infected mouse model

When NF1-*lux* mixed with NF2 were injected i.m., the survival percentage of mice was about 10% and the same result was obtained with the infection NF1-*lux*/ NF2Δ*exoA* (Fig. 9A). The bacterial load from terminal mice showed that mixed infection with NF1-*lux* and NF2 resulted in a significant decrease of NF2 in the site

of the injection and in the spleen. The same occurred with the mixed infection with NF1-*lux* and NF2Δ*exoA,* in this case the latter strain was not detected in the site of injection and in the spleen (Fig. 9B).

Role of ExoA in NF1 and NF2 strain dissemination in an intramuscular infected mouse model

Mixed infection with NF1 and NF1-*exoA* was injected in mice by i.m at dose 2 x 10^8 cfu (1 x 10^8 cfu per each strain) and resulted in a similar number of bacteria recovered in the site of injection and both strains were localized in the spleen at 24 h p.i. (Fig. 10A). However, when the mice were injected with the mixed of NF2 and NF2Δ*exoA,* both strains were detected in muscle, but only NF2 disseminated to spleen at 24 h p.i. (Fig. 10B).

Discussion

Necrotizing fasciitis is a rare, life-threatening bacterial infection (Ahn, 2007). Recently, several strains of *Aeromonas hydrophila* (NF1, NF2, NF3, and NF4) were recovered from the wound and bloodstream of a young immunocompetent girl that developed a NF (Grim et al., 2014). Whole genome sequencing of the isolates showed that isolates NF2 to NF4 were clonal and NF1 was genetically distinct (Grim et al., 2014). The strain NF2 possesses an ExoA toxin, while strain NF1 did not contain the *exoA* gene. The ExoA toxin has been associated in the inhibition of protein synthesis and host cell death (Zdanovsky et al., 1993; Yates et al., 2005; Grim et al., 2014; Ponnusamy et al., 2016). The ExoA is considered the most toxic virulence factor of the pathogenic bacterium *Pseudomonas aeruginosa* (Michalska and Wolf, 2015). Grim et al. (2014) suggested that the presence of the *exoA* gene in strain NF2 could increase the virulence of this strain in the mouse model. The genomes of strains NF1 and NF2 showed the presence of two clusters of T6SS genes and effectors, (three copies of Hemolysin-coregulated protein (Hcp) and five copies of valine-glycine repeat G proteins (VgrGs). The T6SS has been reported to regulate gene expression involved in bacterial motility, biofilm formation and biofilm-antibiotic resistance (Aschtgen et al., 2008; Weber et al., 2009; Zhang et al., 2011).

Suarez et al. (2008) provided evidence that the virulence-associated secretion (*vas*) genes are essential for expression of the genes encoding the T6SS and/or they constituted important components of the T6SS. Also, Liang et al. (2015) identified and characterized the effector toxin TseC and its antagonistic immunity protein TsiC in *A. hydrophila* SSU. In the last years different studies have been suggested that the T6SS plays a role in the competition between strains in the polymicrobial infection (English et al., 2012; Anderson et al., 2017).

Increasing evidence suggests that complex relationships exist between bacteria from distinct species or genera in the setting of a mixed infection (Ramsey et al., 2011; Peters et al., 2012; Korgaonkar et al., 2013). Synergistic or antagonistic effects on virulence cause a complex interaction that influences the progression of the disease. In relation to this complex interaction in *Aeromonas*, it has been reported the virulence for a paired *Aeromonas* strains used to infect the *Caenorhabditis elegans* animal model (Mosser et al., 2015). Mosser et al. (2015), observed a synergy between two *Aeromonas* isolates, producing a higher level of virulence than only one strain. Interestingly, the results obtained by Ponnusamy et al. (2016) showed that when NF is caused by mixed infection with two strains (NF1 and NF2) of *A. hydrophila*, the progression of NF follows a different course from that NF generated by using only a single strain. This previous study suggested that the ExoA toxin of strain NF2 could play a significant role in both local necrotic inflammation and bacterial dissemination to peripheral organs. It was also suggested that the T6SS effector molecule (s) produced by NF1 could block the growth of NF2.

Data from macrophage phagocytosis and intracellular survival of the NF1 and NF2 strains performed by Ponnusamy et al. (2016) suggested that there is a host intervening process in the elimination of NF2 when NF1 is also present at the site of infection. In mixed cultures of NF2 and NF1 strains, the latter strain may favor recruitment and/or activation of macrophages, which would result in rapid elimination of NF2 before this bacterium could enter the systemic circulation. Our data of macrophage phagocytosis showed a higher rate of phagocytosis when the cell line was infected with mutants of T6SS of strain NF1 (NF1Δ*vasK* and

NF1Δ*tseC*). This result was similar to the one obtained with the *A. dhakensis* SSU strain, because the mutant strain for the constituent protein of the T6SS (SSUΔ*vasK*) was more easily phagocytosed by murine macrophages (RAW 264.7) compared with the phagocytosis observed for the wild type strain (Suarez et al., 2008). This result suggests that the T6SS protein VasK and the effector TseC, plays an important role in bacterial phagocytosis and intracellular survival within macrophages. However, our results with NF2 mutants for the ExoA toxin (not related with the T6SS) showed that independently of the presence/absence of this toxin the phagocytosis and the intracellular survival were similar, concluding that ExoA has not a role in these processes.

The results of *in vitro* dynamic infections showed that when NF1 and NF2 were in close contact on a solid medium the survival of NF2 decreased, however, this survival increased when the mix was made with mutant strains of the T6SS of strain NF1 (NF1Δ*vasK* and NF1Δ*tseC*). The same occurred when the mix included strain NF1 and the mutant strain NF2 for the immunoprotein (NF2-*lux*-pBR322-*tsiC*). In the case of the mixed infection of NF1 with NF2 and the ExoA mutant (NF2Δ*exoA*), our results demonstrated that NF1 was able to effectively kill both NF2 and NF2ΔexoA strains. Taking into account these results, T6SS and its effector TseC toxin of *A. hydrophila* NF1 strain could contribute to directly kill or inhibit NF2 strain in an *in vitro* model, and the immunity protein (TsiC) produced from the NF2 strain reverses this effect. However, these data may suggest minimal effect of ExoA on the activity of the NF1 T6SS.

Interestingly, in the present study we have demonstrated that in the mixed infections using intramuscular mouse model the NF1 strain was lethal to NF2 by a T6SS in the site of the injection as Ponnusamy et al., (2016) suggested. Reduction in mortality was observed when NF2-*lux* and mutants of the T6SS (NF1Δ*vasK* and NF1Δ*tseC)* were mixed and injected intramuscularly, in comparison with the mixed infection between NF1 and NF2-*lux.* The T6SS and its effector TseC of *A. hydrophila* NF1 strain could contribute significantly to the overall bacterial virulence in a mouse NF model (intramuscular route infection) when NF1 and NF2 were mixed. Also, our

results suggested that the role of TseC is more prominent in a mixed infection *in vivo* compared to its contribution in the *in vitro* phagocytosis and intracellular survival assays when NF1Δ*tseC* culture was used singly. In addition, the results to the dissemination pattern showed that the NF2 strain of *A. hydrophila* competed better with the T6SS mutants of NF1 (Δ*vasK* and Δ*tseC*) at the injection site and both NF1 T6SS mutants and the NF2 strain poorly disseminated to the peripheral organs. For this reason, it is likely that there are other NF1 T6SS genes that could contribute to the dissemination to the peripheral organs.

In an intraperitoneal infected mouse model, our results demonstrated that *cis* expression of the *exoA* gene in NF1 (NF1-*exoA*) did not further increased its virulence. However, deletion of the *exoA* gene from NF2 strain reduced its virulence significantly. These results suggest that ExoA could play an important role in the pathogenesis of infection caused by *A. hydrophila* NF1 strain in the intramuscular mouse model when the *exoA* gene was inserted in *cis* (NF1-*exoA*) as explained Ponnusamy et al. (2016), but not in the peritoneal infection model in a mono-microbial scenario**.** In contrast, taking into account the results obtained by Ponnusamy et al. (2016) and our results, ExoA is crucial for *A. hydrophila* NF2 strain in both infection models (intraperitoneal and intramuscularly) under the single strain infections.

The results obtained after the mice immunized intramuscularly with ExoA showed a protection from the single *A. hydrophila* NF2 strain after challenge, but not from the NF1 and NF2 mixed culture infection, concluding that ExoA has a function in the virulence of NF2, but not in the mixed infection between NF1 and NF2. Also, after the study of the immunization, we clarified the role of ExoA during mixed infections in a mouse model. Our results demonstrated that ExoA of NF2 may not further promote pathogenesis and dissemination of NF1 strain during mixed infection in the intramuscular mouse model due to the rapid killing of the NF2 strain. This result was different in comparison with the previous hypothesis that suggested that ExoA secreted by NF2 assists the dissemination of NF1 to peripheral organs in the mixed infections (Ponnusamy et al., 2016). Moreover, during NF1 and NF1-*exoA* mixed

infection using an intramuscular mouse model, both ExoA negative and positive NF1 strains were equally competent in replicating at the injection site as well as in disseminating to the peripheral organs. The lower number of NF2Δ*exoA* in mouse muscle and spleen as compared to its parent (NF2) strains was due to attenuation as the result of *exoA* deletion.

In summary, the T6SS and the ExoA toxin of *A. hydrophila* strains (NF1 and NF2) contribute to the development of NF in mice during mono-microbial infections. However, during the mixed infections, the T6SS plays a dominant role in developing of NF.

Conflict of interest

None declared.

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Author's contribution

AFB and JS performed research; AKC conceived the idea; AFB, JS, MJF, AKC wrote the manuscript.

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Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids		Relevant characteristics	Source
A. hydrophila			
	NF1	Isolated from necrotizing fasciitis patient wound site and spontaneous rifampicin resistance clone (Rif^R)	(Grim et al., 2014)
	NF1-*lux*	NF1 strain chromosomally integrated with Tn*7*-luciferase operon (Rif^R, Km^R)	(Grim et al., 2014)
	NF1-*exoA*	NF1 strain chromosomally integrated with Tn*7*-*exoA* operon (Rif^R, Ap^R)	(Ponnusamy et al., 2016)
	NF1Δ*vasK*	NF1 strain isogenic deletion mutant for *vasK* gene (Rif^R)	This study
	NF1Δ*tseC*	NF1 strain isogenic deletion mutant for *tseC* gene (Rif^R)	This study
	NF2	Isolated from NF patient wound site following the amputation and surgical debridement and spontaneous rifampicin resistance clone (Rif^R)	(Grim et al., 2014)
	NF2-*lux*	NF2 strain chromosomally integrated with Tn*7*-luciferase operon (Rif^R, Km^R)	(Ponnusamy et al., 2016)
	NF2Δ*exoA*	Isogenic deletion mutation for gene *exoA* in strain NF2 (Rif^R)	(Ponnusamy et al., 2016)
	NF2Δ*exoA*-*lux*	NF2Δ*exoA* chromosomally integrated with Tn*7*-luciferase operon (Rif^R, Km^R)	This study
	NF2-*lux*-pBR322-*tsiC*	NF2-*lux* strain transformed with plasmid pBR322-*tsiC* (Rif^R, Km^R, Tc^R)	This study
E.coli			
	SM10 λpir	Strain with λpir for replication of plasmid with R6K *ori* (Km^R)	Laboratory stock
	DH5α λpir	Strain with λpir for replication of plasmid with R6K *ori*	Laboratory stock
Plasmids			
	pTNS2	A mobilizable helper plasmid encoding the TnsABC+D transposase for Tn7 specific transposition pathway (Ap^R)	Choi et al 2005 (Nature methods)
	pUC18R6K-mini-Tn*7*::Km-*lux*	Tn*7* based transposon system carries luciferase operon and kanamycin resistance selection marker (Km^R)	(Ponnusamy et al., 2016)
	pBR322	Cloning vector (Ap^R, Tc^R)	Laboratory stock
	pBR322-*tsiC*	*tsiC* gene of *A. hydrophila* NF1 with its putative promoter region, cloned in pBR322 at the HindIII/ PstI sites, Tc^R.	This study
	pDMS197	A suicide vector, *oriT*, *oriV*, *sacB*, Tc^R.	Laboratory Stock
	pDMS*tseC*	Suicide vector pDMS197 containing upstream and downstream flanking DNA fragments to the *tseC* gene of *A. hydrophila* NF1, Tc^R.	This study
	pDMS*vasK*	Suicide vector pDMS197 containing upstream and downstream flanking DNA fragments to the *vasK* gene of *A. hydrophila* NF1, Tc^R.	This study

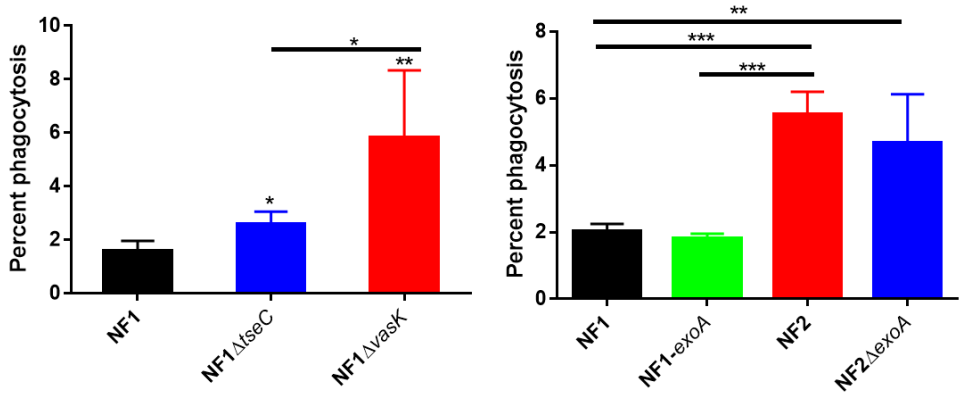

Fig. 1. Role of T6SS and ExoA in bacterial phagocytosis by macrophages. Murine macrophages (RAW 264.7) were infected at a multiplicity of infection (MOI) of 5 with WT or various T6SS mutants of *A. hydrophila* NF1 strain (A) or with various ExoA positive/negative NF1 and NF2 stains of *A. hydrophila* (B). The percentages of engulfed bacteria were determined by plate counting. Results were expressed as the arithmetic mean ± SD. Data were analyzed by using either Student t-test (NF1 vs NF1Δ*tseC*) or by one-way ANOVA with Tukey *post hoc* test. Asterisks indicate statistical significant differences as compared to WT NF1 stain in (A) or indicated by a line comparing designated groups (A and B). *p< 0.05; **p<0.01 and ***<0.001.

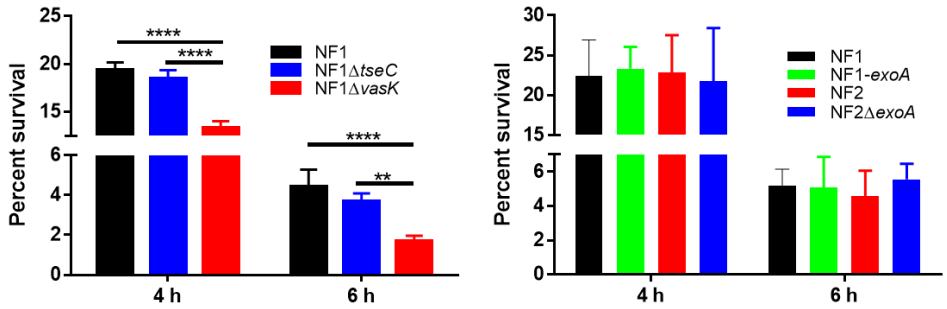

Fig. 2. Role of T6SS and ExoA in bacterial intracellular survival in macrophages. Murine macrophages (RAW 264.7) were infected at an MOI of 5 with WT or various T6SS mutants of *A. hydrophila* NF1 strain (**A**) or with various ExoA positive/negative NF1 and NF2 stains of *A. hydrophila* (**B**). The intracellular survival of bacteria was evaluated via the gentamycin protection assay after 4h and 6 h of phagocytosis. The percent survival of intracellular bacteria was calculated and expressed as the arithmetic mean ± SD. Data were analyzed by using two-way ANOVA with Sidak *post hoc* test. Asterisks indicate statistical significant differences as compared to designated groups indicated by a line. **p<0.01 and ****p< 0.0001.

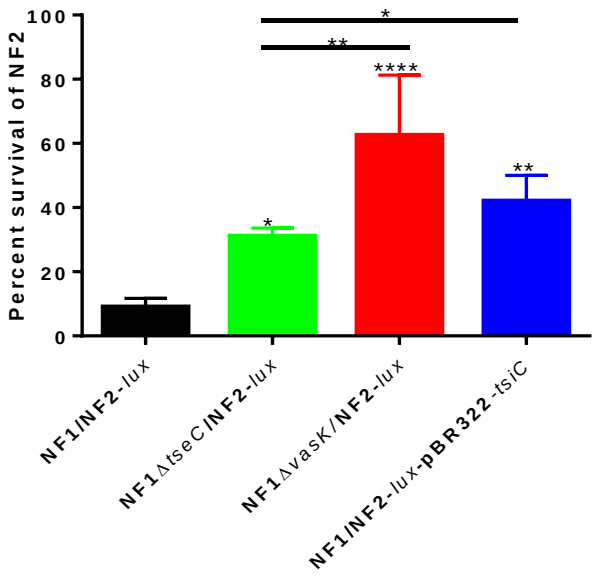

Fig. 3. Role of NF1 T6SS in killing NF2 by direct contact. WT NF1 or its T6SS mutants were mixed with the bioluminescent WT NF2 strain (NF2-*lux*) or the NF2 strain that expresses the gene encoding immunity protein (TsiC) of NF1 T6SS effector TseC via pBR322 vector (NF2-pBR322-*tsiC*) in a ratio of 5:1. The mixed cultures were spotted on LB agar plates and incubated at 37 °C for 4 h. The NF2 strains were enumerated in the mixed culture before and after 4h of incubation period. The percent survival of NF2 strains after the incubation period was then calculated and the results plotted as arithmetic means ± SD. Data were analyzed by using either Student t-test (NF1Δ*tseC*/NF2-*lux* vs NF2-pBR322-*tsiC*) or by one-way ANOVA with Tukey *post hoc* test. Asterisks indicate statistical significant differences as compared to the NF1/NF2-l*ux* group or between groups as indicated by a line. *p< 0.05; **p<0.01 and ****<0.0001. A similar result was observed when the cultures were mixed in a ratio of 1:1 (data not show).

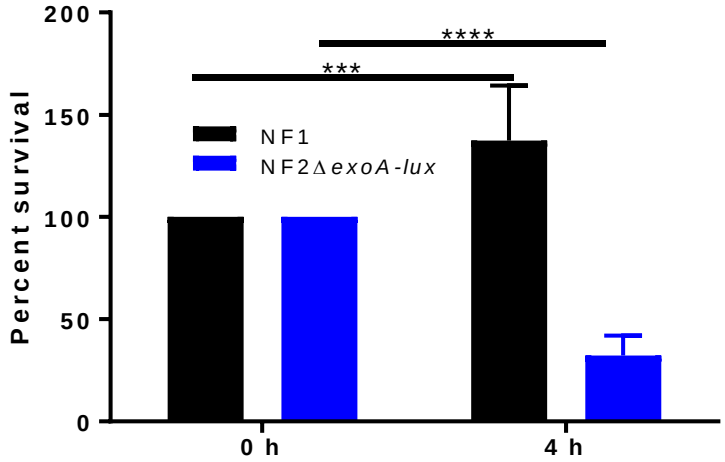

Fig. 4. Effect of ExoA on NF1 T6SS-medicated NF2 killing. WT NF1 and bioluminescent NF2 *exoA* mutant (NF2Δ*exoA-lux*) were mixed in a ratio of 5:1. The mixed cultures were spotted on LB agar plates and incubated at 37 °C for 4 h. Both NF1 and NF2Δ*exoA-lux* strains were enumerated in the mixed culture at the time of mixing (0 h) and after 4 h incubation on the agar plates. The percent survival of NF strains after the 4 h incubation period was then calculated and the results plotted as arithmetic means ± SD. Data were analyzed by using two-way ANOVA with Sidak *post hoc* test. Asterisks indicate statistical significant differences as compared to designated groups indicated by a line. ***p<0.001 and ****p< 0.0001. A similar pattern was observed when the cultures were mixed in a ratio of 1:1 (data not show).

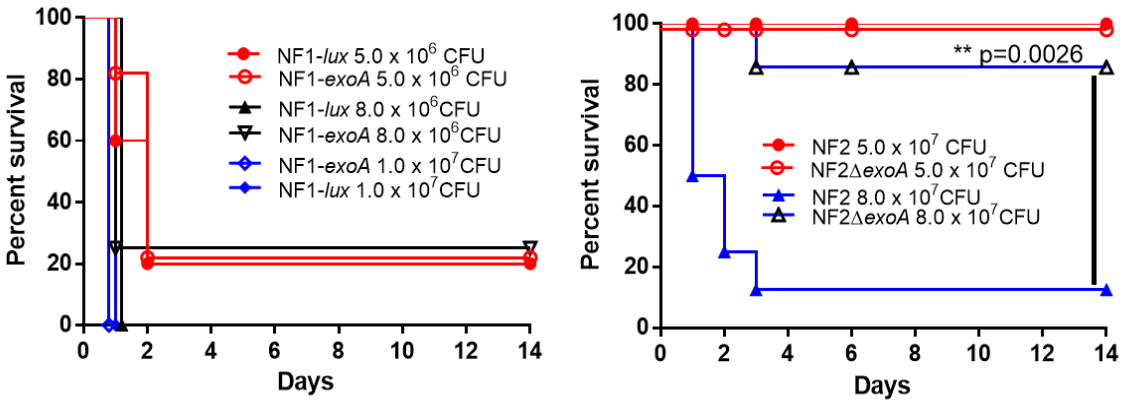

Fig 5. Role of ExoA in the pathogenesis of infection caused by *A. hydrophila* NF strains in a mouse model of peritonitis. Mice (10 per group) were intraperitoneally infected with various NF ExoA positive- and -negative strains at different doses. The percent survival of mice was then plotted. Data were analyzed by using Log-rank (Mantel-Cox) test. Asterisk indicates statistical significance with a p value for compared groups as indicated by the vertical line.

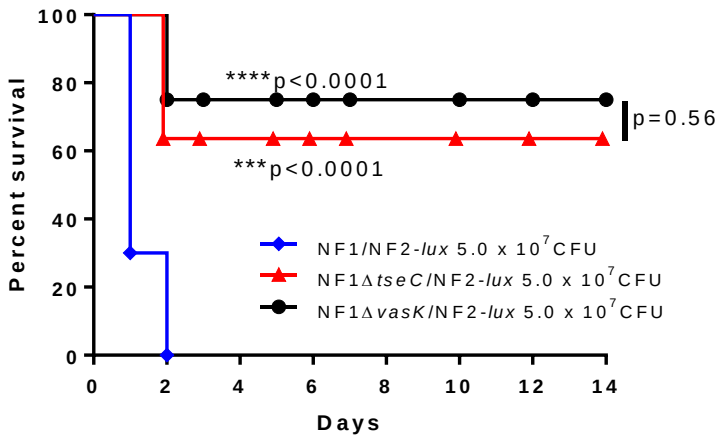

Fig 6. Role of T6SS in the pathogenesis of infection caused by *A. hydrophila* NF strains in a mouse model of NF. WT bioluminescent NF2-*lux* strain was 1:1 mixed with WT or various T6SS mutants of NF1. Mice (10 per group) were intramuscularly infected with various above mixed cultures at the dose of 5 x 10^7 CFU (2.5 x 10^7 CFU per strain). The percent survival of mice was then plotted. Data were analyzed by Log-rank (Mantel-Cox) test. Asterisks indicate statistical significance with p values when indicated groups were compared to the control group (NF1/NF2-*lux*).

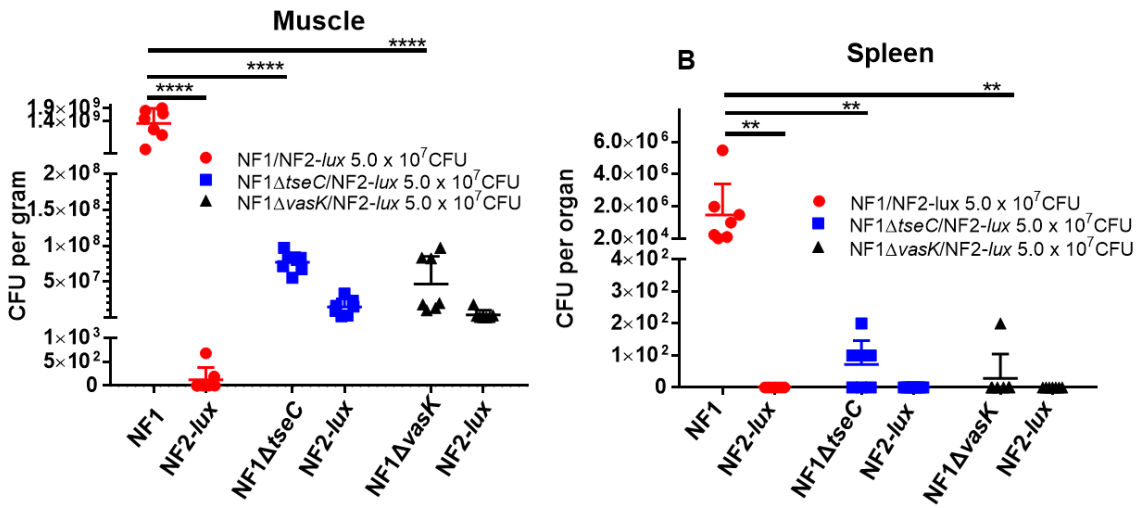

Fig 7. Dissemination of bacteria in mice intramuscularly infected with various mixed cultures of *A. hydrophila* NF1 and NF2 strains. WT bioluminescent NF2-*lux* strain was 1:1 mixed with WT or various T6SS mutants of NF1. Mice (7 per group) were intramuscularly infected with various above mixed cultures at a dose of 5 x 10^7 CFU (2.5 x 10^7 CFU per strain). After 24-48 h post infection, mice spleens and muscles at the injection site were collected, and bacterial loads in the muscles (A) and spleen (B) were enumerated. Results were expressed as arithmetic mean ± SD. Data were analyzed by using two-way ANOVA with Sidak *post hoc* test. Asterisks indicate statistical significant differences among indicated groups as indicated by lines. **p<0.01 and ****p< 0.0001.

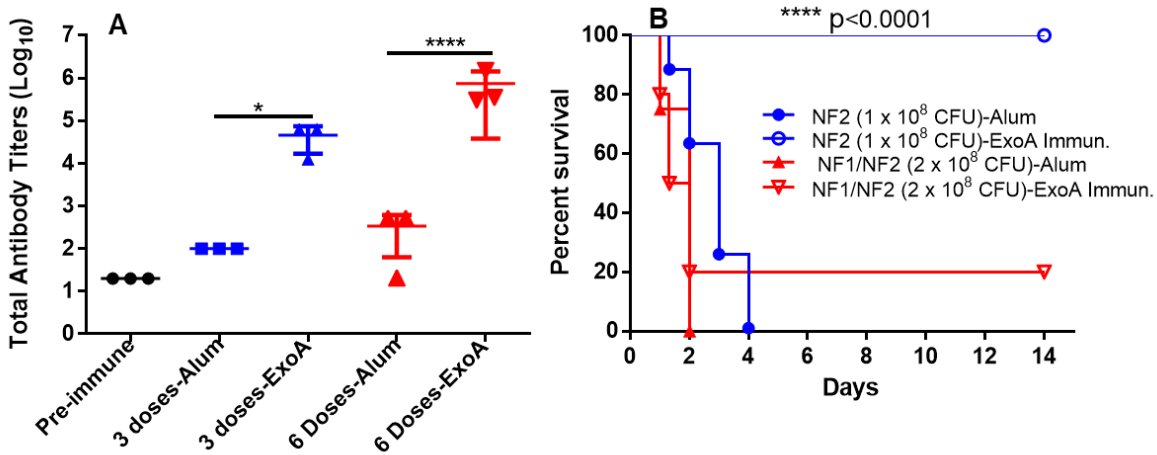

Fig. 8. ExoA immunization of mice and bacterial challenge. Mice were immunized intramuscularly with 100 ng of ExoA mixed with alum (1:1). Boosters with the same dose of ExoA were given every two weeks for a total of 6 immunizations. Mice receiving alum only served as a control. Blood was collected before immunization (pre-immune) and after the third and final immunization to evaluate anti-ExoA antibody titers arithmetic mean ± SD (A). Mice (8 per group) were then intramuscularly challenged with either *A. hydrophila* NF2 strain alone at the dose of 1 x 10^8 CFU or with a mixed culture of NF1 and NF2 strains at a dose of 2.0 x 10^8 CFU (1 x 10^8 CFU per strain). The percent survival of mice was recorded and plotted (B). Data were either analyzed by using Student t-test for antibody titers or Log-rank (Mantel-Cox) test for mice survival. Asterisks indicate statistical significance for groups in comparison to their respective controls. *p<0.05 and ****p< 0.0001.

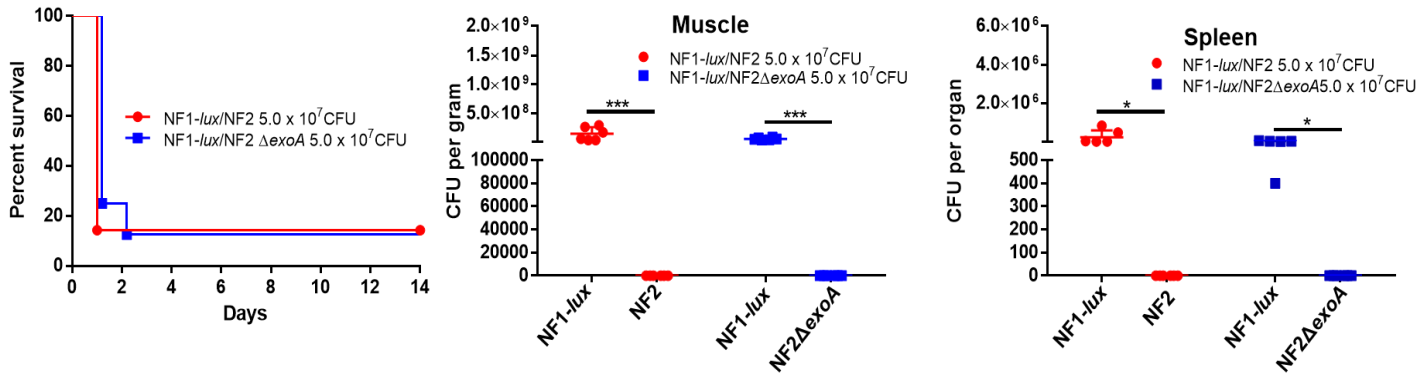

Fig 9. Role of ExoA during mixed infection with *A. hydrophila* NF1 and NF2 strains. WT bioluminescent NF1-*lux* strain was 1:1 mixed with WT NF2 or its Δ*exoA* mutant. Mice (7 per group) were intramuscularly infected with the above mixed cultures at a dose of 5 x 10^7 CFU (2.5 x 10^7 CFU per strain). The survival of mice was recorded and plotted (A). Tissues (spleens and muscles from the injection site) were collected from terminal animals during the course of infection, and bacterial loads in the muscles (B) and spleens (C) were enumerated. Results were expressed as arithmetic mean ± SD. Data were analyzed by Log-rank (Mantel-Cox) test for mouse survival, while two-way ANOVA with Sidak *post hoc* test was used for analyzing bacterial loads. Asterisks indicate statistical significance for compared groups as indicated by the lines. *p<0.05 and ***p< 0.001

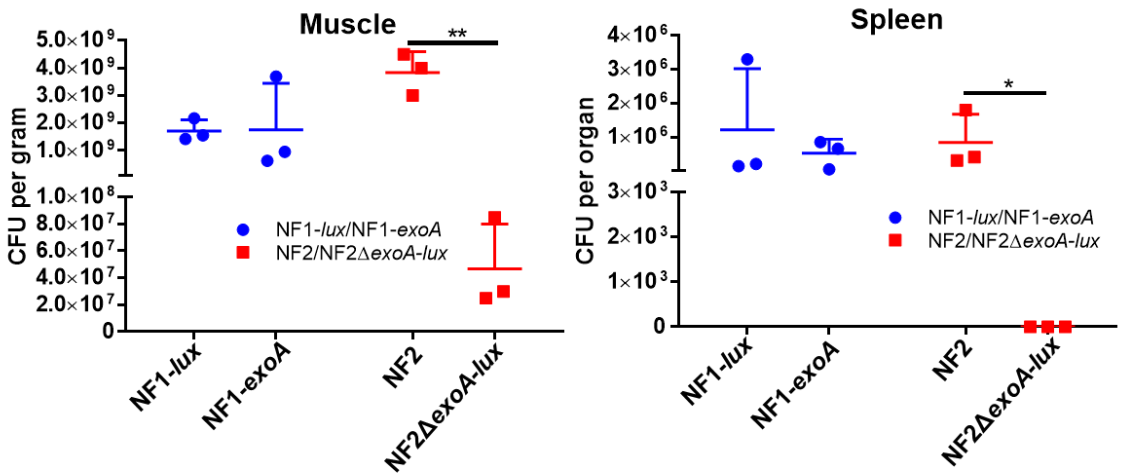

Fig 10. Role of ExoA in bacterial dissemination in mice. Mice (3 per group) were intramuscularly infected with ExoA positive/negative (1:1) pairs of indicated *A. hydrophila* NF1 or NF2 strains at a dose of 2 x10^8 (1x10^8 per strain). After 24 h post infection, mouse tissues were collected and bacterial loads for each stain at the injection site muscles (**A**) and spleens (**B**) were evaluated. Results were expressed as arithmetic mean ± SD. Data were analyzed by two-way ANOVA with Sidak *post hoc* test. Asterisks indicate statistical significance for compared groups as indicated by the lines. *p<0.05 and **p< 0.01.

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EPIDEMIOLOGY AND PATHOGENIC CHARACTERIZATION OF SPECIES OF THE GENUS AEROMONAS
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4.9. Evaluation of the immune response in the monocytic cell line THP-1 against *Aeromonas* spp.

Fernández-Bravo A., Figueras MJ. (In preparation, pending to be submitted to Frontiers in Inmunology)

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Immune response of the monocytic cell line THP-1 against six *Aeromonas* spp.

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Abstract

Aeromonas are autochthonous inhabitants of aquatic environments, that are considered to be emerging pathogen to humans, producing, diarrhea, bacteremia and wound infections. Recent publications show that 96.5% of the strains associated to clinical cases correspond to the species: *Aeromonas caviae* (29.9%), *Aeromonas dhakensis* (26.3%), *Aeromonas veronii* (24.8%) and *Aeromonas hydrophila* (15.5%). However, few studies have investigated the immune response against *Aeromonas* and the majority have involved only *Aeromonas hydrophila.* The aim of the present study was to increase the knowledge about the immune response against six *Aeromonas* species, using an *in vitro* infection model with a monocytic human cell line (THP-1) and evaluating the expression of 11 immune-related genes. The species tested were *Aeromonas dhakensis, Aeromonas media, Aeromonas veronii, Aeromonas jandaei, Aeromonas piscicola* and *Aeromonas caviae*. Transcriptional analysis showed an up-regulated expression of a variety of immune-related genes in monocytes, with a variable response depending upon the *Aeromonas* species. The species that showed the highest cell damage were coincidentally the ones that induced a higher expression of immune-related genes and were the more prevalent clinical species *A. dhakensis, A. veronii* and *A. caviae.*

Keywords: *Aeromonas* spp., immune-related genes, LDH.

Introduction

The genus *Aeromonas* includes species considered autochthonous of aquatic systems and emerging pathogen that cause a wide spectrum of diseases in humans, mainly gastroenteritis, septicemia and wound infections (Janda and Abbot, 2010; Figueras and Beaz-Hidalgo, 2015; Graf, 2015). The infection occurs more frequently in children, elderly and immunocompromised individuals as do also cases of bacteremia and extraintestinal infections such as meningitis, pneumonia, keratitis and osteomyelitis (Figueras, 2005; Janda and Abbott, 2010; Igbinosa et al., 2012; Figueras and Beaz-Hidalgo, 2015; Teunis and Figueras, 2016). *Aeromonas* was the microorganisms more isolated after natural disasters as the tsunami in Thailand (2004), representing about 22.6 % of all isolates from wound infections in the survival patients (Hiransuthikul et al., 2005)

According to the recent literature, 96.5% of the strains associated to clinical cases correspond to four species: *Aeromonas caviae* (29.9%), *Aeromonas dhakensis* (26.3%), *Aeromonas veronii* (24.8%) and *Aeromonas hydrophila* (15.5%) (Figueras and Beaz-Hidalgo., 2015). Multiples virulence factors, including aerolysin, hemolysin, lipases, enterotoxins, different types of secretion systems as well as quorum sensing are involved in the colonization, invasion and proliferation of the bacteria during the infectious process (Martin-Carnahan and Joseph, 2005; Beaz-Hidalgo et al., 2012, 2013; dos Santos et al., 2015).

The immune response is activated after antigen exposure. Two types have been described, innate and adaptative (Janeway et al., 2001). The innate immune response is activated after recognition of structures associated with the microbes named Pathogen-Associated Molecular Patterns (PAMPs) by receptors named Pattern Recognition Receptors (PRRs) present and expressed in a variety of cells, like neutrophils, monocytes or macrophages (Bergsbaken et al., 2009; Kumar et al., 2011; Mahla et al., 2013). Toll-like receptors (TLRs) are membrane PRRs that induce the phagocytosis of the pathogen and activate the expression of cytokines in the host, initiating the inflammatory response (Kawai and Akira., 2007a). It has also been demonstrated that the TLRs recognition result in the induction of apoptosis or

cell-death (Salaun et al., 2007). The apoptosis is a caspase dependent process that induces nuclear condensation, and the release of cytoplasmic content from the host cell into the extracellular environment, which prevents inflammation (Strasser et al., 2000). Moreover, the NOD-like receptors (NLR) are another type of PRRs, expressed in the cytosol that induces a different type of cell-death called pyroptosis, mediated by the activation of caspase-1 and by the formation of a macromolecular complex called the inflammasome (Bergsbaken et al., 2009, Lopez-Castejon and Brough, 2011). In the last years, several studies have investigated the expression of several immune-related genes against *A. hydrophila, A. veronii* and *A. salmonicida* (Altmann et al., 2016; Gong et al., 2017; Liao et al., 2017). The upregulation of the Toll-like receptor 4 (TLR4), which is a transmembrane receptor that senses molecules such as lipopolysaccharides (LPS) present in the cell walls of Gram-negative bacteria, have been detected after infecting a fish model with *A. hydrophila* (Altmann et al., 2016; Gong et al., 2017; Liao et al., 2017). The TLRs also induce the activation of the nuclear factor NF-Kappa-B p65 subunit (RelA) protein, which is a protein involved in the formation of the nuclear factor kappa-light-chain-enhancer (NF-κB). After an *A. hydrophila* infection in fish this factor is activated by inducing the activation of pro-inflammatory cytokines (Zhang et al., 2017). The JUN proto-oncogene (c-Jun) is another transcription factor that binds with the FOS proto-oncogen (c-Fos) and previous study suggested that changes in the expression of both genes are involved in the virulence of *Aeromonas* spp. in mice. *Aeromonas* strains which were pathogenic showed an upregulation of these genes (Hayes et al., 2009). Moreover, the expression of the pro-inflammatory cytokine genes such as *TNF-α* or interleukin 8 (*IL-8*), as well as chemokine genes such as the C-C motif ligand 20 (*CCL20*) involved in the recruitment of lymphocytes and dendritic cells has been studied in this fish model, demonstrating the activation of these genes after *A. hydrophila* and *A. salmonicida* infection (Arockiaraj et al., 2015; Rebl et al., 2017; Liao et al., 2018). However, the chemokine CCL2 has not been studied in *Aeromonas*, however it is an interesting chemokine well studied in other genera such as *Vibrio* with a role in the recruitment of monocytes, T cells, and dendritic cells (Murciano et al., 2015). Therefore, the chemokine CCL2 will be investigated for the

first time in this study. In relation to the apoptosis several studies demonstrated the capacity of *A. hydrophila*, *A. salmonicida* and *A. veronii* to induce this process by using different cell lines as well as a fish model (Suarez et al., 2010; Shelly et al., 2017). The tumor protein P53 (TP53) is a protein related to the apoptosis that induces the activation of the BCL2 associated X protein (BAX), being an important pro-apoptotic protein. Lü et al. (2015) demonstrated the induction of TP53 after *A. hydrophila* infection in a fish model. However, the expression of the gene encoding the BAX protein has not been studied in *Aeromonas* and will be also investigated in the current study. Another gene associated with apoptosis is the caspase 3 (*CASP3*), a previous study suggested that *A. hydrophila* can cause apoptosis in head kidney-derived macrophages from fish (Banerjee et al., 2012). Otherwise, a study of two proteins associated with the inflammasome such as pyrin domain containing 3 (NLRP3) and IL-1β after *A. hydrophila* and *A. veronii* were studied in mice and murine macrophages (McCoy et al., 2010a,b).

A study of the host-interaction with *V. vulnificus*, demonstrated that the immune response could vary depending on the investigated cell line (Murciano et al., 2015). In *Aeromonas* the immune response studies have been not done with the human monocytic cell line (THP-1), therefore it is interesting to perform the studies in this cell type, because the monocytes act as the first line of defense at the beginning of the infection process. Moreover, no information exists so far about the immune response generated against other important *Aeromonas* species, that are frequently isolated in clinical cases i.e. *A. dhakensis* or *A. caviae*. This information could help to understand their clinical importance. Additionally, if the capacity to develop an innate-immune response in the host cells is equal for the environmental and the clinical strains is another aspect that has never been studied. This study pretends to investigate *Aeromonas* species that show different frequencies of occurrence in clinical cases, using strains of clinical and environmental origin in order to clarify if there exists a species-specific immune response that could explain their differential prevalence in human infections.

Material and Methods

Bacterial strains

The study was performed with 24 strains (10 clinical and 14 environmental) of six different species (n=6) five of which have not been studied until now as *Aeromonas dhakensis, Aeromonas media*, *Aeromonas jandaei*, *Aeromonas piscicola*, *Aeromonas veronii* and *Aeromonas caviae,* by using the strain V*ibrio vulnificus* CECT 4999, used in previous studies (Murciano et al., 2015; Murciano et al., 2017), as positive control (Table 1). All strains came from a collection that maintained in TSB plus glycerol (20%) at -80°C and from there they were grown in Tryptone Soya agar (TSA) at 30°C for 24 h. Their identity was previously determined on the basis of the sequences of the *rpoD* gene using primers and conditions described previously (Soler et al., 2004). Prior to infection, bacteria were regrown at 37°C in serum-free Dulbecco's Modified Eagle's Medium (DMEM, PAA Laboratories GmbH, Pasching, Austria) with shaking (100 rpm) for 18 h.

Cell lines and conditions

The human peripheral blood monocytes cell line THP-1 (Tsuchiya et al., 1980) was selected for the experiments. This cell line was maintained as a cell suspension in Roswell Park Memorial Institute Medium (RPMI-1640, PAA Laboratories) supplemented with 10% FBS (fetal bovine serum, PAA Laboratories) plus 1% P/S solution (penicillin-streptomycin, PAA Laboratories) at 37°C and 5% CO2. Before the infection experiments, cells were seeded in tissue culture plates containing DMEM without FBS and P/S at a concentration of 0.5×10^6 cells/mL to obtain 1×10^6 cells/mL after 18 h.

Infection

Cell line THP-1 was infected by using the 24 *Aeromonas* strains from overnight cultures in DMEM without FBS and P/S, at multiplicity of infection (MOI) of 10 and 20. The control, strain of *V. vulnificus* CECT 4999^T was used at MOI 5. The cultures were incubated at 37°C and 5% CO_2 and were sampled at specific times depending on the experiment.

Intracellular survival

Infected monocytes at MOI 10 and 20 (initial dose) were incubated at 37ºC with 5% CO_2 for 1 h., followed by gentamicin treatment (50 µg/mL) for 1 h to kill extracellular bacteria (time 0). The percentage of intracellular survival was calculated as a number of bacteria inside the monocyte (time 0) in relation to the initial dose.

Cell damage assay (LDH assay)

After the infection at MOI 10 and 20, supernatants were obtained at different times (t= 3, 4, 5 and 6 h). Cell damage was determined by quantifying the enzyme lactate dehydrogenase (LDH) released into the culture media (supernatants), by using the kit, "Cytox 96 Non-Radioactive Cytotoxicity Assay "(Promega), as described in the manufacturer's instructions. To perform a standard curve a bovine recombinant LDH (Sigma-Aldrich) was used and the LDH levels of the samples were extrapolated from the curve.

Analysis of the expression of the genes related to the immune system

Different genes related to the immune response against pathogens were selected to quantify its transcription in response to the bacteria. The selected genes were genes related to cytokines and chemokines (*TNF- α*, *CCL2* and *CCL20*) genes related to apoptosis and pyroptosis (*TP53*, *BAX*, *CASP3*, *NLRP3* and *IL-1 β*) as well as genes of the transcription factors (*JUN* and *RELA*). After 4 h of infection at MOI 20, cells were washed twice with PBS, and RNA was isolated from the samples by using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The cDNA was transcribed from total RNA by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc. Hercules, CA, USA). A Real-time PCR was performed with cDNA for quantification by using the Power SYBR® green PCR Mastermix (Applied Biosystems®, Life Technologies) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The thermal cycling conditions were: 94 ºC for 5 min, followed by 45 cycles of 30s at 94ºC, 30s at 60ºC, 30s at 72ºC, and finally 20s at 80ºC. The threshold cycle (CT) values were obtained to establish the relative

RNA levels of the tested genes, using glyceraldehyde-3-phosphate (GAPDH) gene as a housekeeping gene. The relative gene expression was determined by using the $2^{-\Delta\Delta ct}$ method. The primers to evaluate the expression of selected genes were taken from Murciano et al., 2015 and the Primerbank are listed in Table 2.

Statistical analysis

All the experiments were performed in triplicates and the statistical significance was determined by using the Student's two-tailed *t*-test at $p < 0.05$ on GraphPad Prism 6.0 (GraphPad Software, CA, USA).

Results

Intracellular survival

The intracellular survival of *Aeromonas* strains at MOI 20 resulted to be significantly higher ($p < 0.05$) than MOI 10, except for the species *A. jandaei* and *A. veronii* (Figure 1). As shown in Figure 1, the infection at MOI 10 showed a significant difference ($p < 0.05$) between the most prevalent clinical species in relation to the less prevalent, except between *A piscicola* and *A. veronii*. Additionally, at MOI 20 the most prevalent clinical species showed a higher percentage of intracellular than the others ($p < 0.05$) (Figure 1).

Cell damage caused by *Aeromonas* species

The ability of *Aeromonas* to induce cell damage in THP-1 cells was measured as the release of LDH to the cell culture supernatant in THP-1 cells as shown in a Figure 2. All *Aeromonas* strains (24/24), independently of the species were able to induce at MOI 10 and 20 significant dose- and time-dependent cell damage when compared with non-infected cells ($p < 0.05$). The clinical strains of all the species were able to induce a higher degree ($p < 0.05$) of THP-1 cell damage than the environmental strains (Figure 2). All six *Aeromonas* species caused a significant cell damage in respect to the resting cells ($p < 0.05$) at MOI 20, and lower damage in relation to the positive control *V. vulnificus*. Also, the more prevalent clinical

species, *A. dhakensis*, *A. caviae* and *A. veronii* caused a higher cell damage than the rest independently of the origin ($p<0.05$) as shown in Figure 3.

Gene-expression by monocytic cell line THP-1 against *Aeromonas* species

Genes for cytokines and chemokines

The infection of the monocytic cell line THP-1 with all strains, independently of the origin and the species studied, induced the expression of cytokine (*TNF-α*) and chemokines (*CCL2, CCL20*) genes, with a significant difference ($p<0.05$) in the transcription pattern in relation to the non-infected cells (Figure 4).

As shown in Figure 4, the monocytic cell line showed a significant expression ($p <0.05$) of the pro-inflammatory cytokine gene, *TNF-α*, in response to *A. dhakensis*, followed by *A. caviae* and *A. veronii.* The less transcriptional levels of *TNF-α* were induced by *A. piscicola*, *A. jandaei* and *A. media*. Nevertheless, no significant differences in the expression levels of *TNF-α* were detected after stimulation with the first three species and the same result was obtained between the last three species. Additionally, the pro-inflammatory cytokine gene, *IL-8*, was tested, however, the levels were below detection (data not shown).

In the case of chemokines, the transcriptional levels of *CCL2* and *CCL20* were upregulated after infection with all strains in comparison with the non-infected cells, being higher in the case of *A. dhakensis* and *A. caviae* ($p <0.05$) than in the other species (Figure 4). A difference with the *TNF-α* expression, the transcriptional levels of *CCL2* and *CCL20* after *A. veronii* infection showed no significant differences with *A. media* or *A. piscicola*. However, all strains showed a significantly higher expression ($p <0.05$) than *A. jandaei* (Figure 4).

Genes involved in apoptosis

The transcriptional level of *TP53* and *CASP3* genes, related to apoptosis, increased after infection with all *Aeromonas* species ($p<0.05$) in relation to the non-infected cells. Nonetheless, not significant differences in relation to the non-infected cells were observed after *A. piscicola, A. dhakensis* and *A. jandaei* infections for the *BAX* gene (Figure 4). In the case of *A. veronii* the expression of the *BAX*, *TP53* and

CASP3 were almost undetectable (Figure 4). Additionally, significant differences ($p < 0.05$) in the transcriptional level of the *BAX, TP53* and *CASP3* were observed between the most prevalent clinical species and the least prevalent ones (Figure 4).

Genes for transcription factors

The THP-1 cells responded by upregulating the transcriptional levels of the transcriptional factor *RELA* after *Aeromonas* infection ($p < 0.05$). However, the upregulation of the *JUN* was not significant in the case of *A. media* and *A. veronii.* Significant differences ($p < 0.05$) have been detected between the species in the upregulation of *JUN* and in the transcriptional levels of *RELA* (Figure 4).

Gene involved in pathogen recognition (TLR- like receptor)

The transcriptional level of the *TLR4* after *Aeromonas* in the THP-1 showed significant differences ($p<0.05$) in comparison with the non-infected cells being only significant in *A. caviae*, *A. dhakensis* and *A. veronii*. As shown in Figure 4 at MOI 20, *A. caviae* showed a higher level of *TLR4* expression in relation to the non-infected cells, followed by *A. veronii* and *A. dhakensis*.

Genes related to the inflammasome

The *NLRP3* and *IL1-β* genes which are related to the inflammasome were upregulated in THP-1 cells in response to *A. dhakensis, A. media, A. caviae* and *A. veronii,* independently of the origin of the strains ($p<0.05$), while the *NLRP3* and *IL-1β* expression was almost undetectable after infection with *A. piscicola* and *A. jandaei* (Figure 4). The upregulation of *NLRP3* and *IL-1β* showed an eightfold increase ($p<0.05$) when THP-1 cells was infected with *A. veronii* strains. No differences in the level of the *IL-1β* expression were observed after infection with *A. dhakensis, A. media* and *A. caviae* in relation to the non-infected cells. However, significant differences in the over expression of the *NLRP3* were detected ($p < 0.05$) for the three species (Figure 4).

Discussion

Our results showed that the expression levels of the proinflammatory cytokine *TNF-α* were upregulated in THP-1 cells after all *Aeromonas* infection. Similar results were found after *A. hydrophila* infections in macrophages, yellowtail leucocytes and in grass carp intestine (Suarez et al, 2010, Reyes-Becerril et al., 2016, Song et al., 2017), as well as in the monocytic cell line THP-1 after *V. vulnificus* infection (Murciano et al., 2015). The TNF-α is involved in the inflammation and in the absence of this cytokine the host-defense would be impaired (Peffer et al., 2003). This suggests that independently of the species TNF-α has a role in the immune response against these six *Aeromonas* spp. However, the *IL-8* was below upregulated, unlike the previous studies that demonstrated the upregulated expression of this cytokine in macrophages or in fish model (Zhang et al., 2015; Kong et al., 2017). An explanation of this could be associated to the immune response depending on the infected host-cell (Galindo et al., 2004) as it occurs with *Vibrio vulnificus* (Murciano et al., 2015). In the case of the chemokine genes *CCL2* and *CCL20*, our data showed an upregulation of the expression after *Aeromonas* infection, involving both in the chemotaxis of the immune cells (Carr et al., 1996; Xu et al., 1996; Baba et al., 1997). In a previous study with *V. vulnificus*, the results demonstrated a higher transcriptional level of these genes in the monocytic cell line (THP-1), suggesting that this cell type could be an important role in recruitment of monocytes, T cells, dendritic cells or lymphocytes among other immune cells (Murciano et al., 2015). Thus, our data suggested that the six *Aeromonas* species would induce the expression of *CCL2* and *CCL20* playing a role in the development of the strong immune response recruiting other cells. In relation to the pathogen recognition receptors, we tested *TLR4* gene, and the results showed that it was upregulated, but the expression was not significantly higher than the non-infected cells. The TLR4 is a transmembrane receptor that recognizes a particular type of molecules from many pathogens and induces the inflammatory response, being in *Aeromonas* the Lipopolysaccharide (Kawai and Akira, 2007; Rosenzweig and Chopra, 2013; Zhang et al., 2017). Previous studies demonstrated the upregulation

of the *TLR4* gene after *A. hydrophila* infection in catfish (Zhang et al., 2017). However, more TLRs had been described in this previous study (Zhang et al., 2017). For this reason, it is possible that other TLRs could be more related to the activation of the immune response in the *Aeromonas* species tested in our work.

Furthermore, the expression analysis of the transcriptional factors showed an expression of RELA and JUN after *Aeromonas* infection. It has been described that RelA and c-jun are crucial proteins for NF-κB activation. The NF-κB allows the expression of genes as cytokines, which contribute to an effective immune response (Lorentz et al., 2003; Tak et al., 2011; Hoesel et al., 2013). Previous studies in fish demonstrated the expression of the RELA after *A. hydrophila* infections by using a fish model that induces the NF-KB pathway inducing the production of several pro-inflammatory cytokines (Zhang et al., 2015; 2017). Also, the data obtained by Hayes et al. (2009), suggested that JUN could be involved in the different virulence caused by *Aeromonas* spp. in epithelial colorectal adenocarcinoma (Caco-2) cells. Our results suggested that the elevated activity of the RelA and c-jun transcriptional factors increased the immune response against these six *Aeromonas* spp. by the induction of the cytokines and chemokines.

In relation to the inflammasome, the *NLRP3* and *IL-1β* were upregulated after *A. veronii* infection. Previous studies showed the activation of the caspase-1 and the genes related to the pyroptosis in macrophages after *A. hydrophila* infection (McCoy et al., 2010a). The results demonstrated that *A. hydrophila* induces an inflammatory response via NLRP3 inflammasome, producing the pyroptosis. Additionally, another previous work suggested that NLRP3 and NLRC4 inflammasomes are involved in host defense against *A. veronii* in mice (McCoy et al., 2010b). However, the expression of *NLRP3* and *IL-1β* were almost undetectable in THP-1 after *A. jandaei* and *A. piscicola* infection. Additionally, our results showed only an upregulation of IL-1β after *A. dhakensis* and *A. caviae* infection. An explanation could be the pathway of cell death; in the case of *A. piscicola* and *A. jandaei*, the results of the gene expression analysis showed an upregulation of the genes related to the apoptosis (*BAX*, *TP53*, and *CASP3*), while the expression of these proteins after *A.*

veronii infection were undetectable. The apoptosis route seemed to be the only way of producing cell damage and death in the case of *A. piscicola* and *A. jandaei*, while *A. veronii* apparently only used the pyroptosis route. In the case of *A. dhakensis* and *A. caviae, BAX, TP53* and *CASP3* that are related to the apoptosis were upregulated in the THP-1. However, the significant expression of the proinflammatory cytokine gene *IL-1β* could be associated with other processes non-related to the pyroptosis, as has been previously described (Mulder et al., 2006). Further studies with a higher number of strains using additional inflammasome genes are necessary to confirm this hypothesis.

The expression of immune-related genes in this work showed that the selected species caused a different immune response, characterized by a species-specific activation pattern. Independently of the immune-related gene studied in this work, generally the expression was higher in the most prevalent clinical species *A. dhakensis, A. caviae* and *A. veronii* (Figueras and Beaz-Hidalgo, 2015). An explanation of this could be associated with the cytokines storm, described as a systemic inflammatory response syndrome that increases the severity of the infections (Vidal et al., 2010; Tisoncik et al., 2012; Chousterman et al., 2017) and in this case these species would induce this mechanism and the consequence could be the higher prevalence in clinical cases. This is the first time that a hypothesis is provided to explain the different frequency of occurrence of the *Aeromonas* spp.

Furthermore, *Aeromonas* induce the cell damage in all strains, by measuring the LDH in the supernatant (Epple et al., 2004). The LDH release can be induced by lysing the cells by apoptosis or pyroptosis. Our results demonstrated that this cell damage could be related to these pathways depending on the species. Also, the most prevalent species in clinical showed a higher cell damage than the less prevalent, and this result agrees with the intracellular survival. Also, the cell damage was higher in clinical strains than in environmental strains. Taking into account the expression results, it could be indicated that a cytokine storm that induces a strong immune response would cause more cell damage, as well as an increase in the intracellular survival in the most prevalent clinical species

Conclusions

Independently of the species, the cell damage increased with the bacteria exposure time and the one produced by the clinical strains was higher than the one observed for the environmental strains. Moreover, the more prevalent clinical species: *A. dhakensis, A. caviae* and *A. veronii* seemed to cause more damage than the other less prevalent clinical species. The transcriptional analysis showed an upregulated expression of a variety of THP-1 immune-related genes, with a variable response depending on the *Aeromonas* species, and the species that showed the highest cell damage were coincidentally the ones that induced a higher expression of immune-related genes. The apoptosis route seemed to be the only way of producing cell damage and death in the case of *A. piscicola* and *A. jandaei*. However, *A. veronii* apparently only used the pyroptosis route. Further studies with a higher number of strains using additional inflammasome genes would be necessary to confirm this observation.

Conflict of interest

The author(s) declare that they have no competing interests.

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Table 1. Strains used in the study.

Strain	Origin	Reference
A. dhakensis		
CECT 5744^T	Child feces with diarrea	Beaz-Hidalgo et al., 2013
GMV 704	Cetacean	This study
128177	Human feces	This study
28B	Human wound infection	This study
A. caviae		
CECT 838^T	Guinea Pig	Schubert and Hegazi, 1988
ESV-378	Fish gills	Vega-Sánchez et al., 2014
E01980	Feces	This study
D50233	Feces	This study
A. veronii		
CECT 4257^T	Human sputum	Hickman-Brenner et al., 1987
123384	Human feces	This study
AE6	FW lake wàter	This study
01.1	Sick fish	This study
A. media		
CECT 4232^T	Fisheries water	Allen et al., 1983
ESV-382	Fish gills	Vega-Sánchez et al., 2014
32679	Feces	This study
ESV-360	Fish kidney	Vega-Sánchez et al., 2014
A. jandaei		
CECT 4228^T	Human feces	Carnahan et al., 1991
4300E	FW lake water	This study
CECT 4813	Human feces	This study
AE214	FW lake water	This study
A. piscícola		
CECT 7443^T	Sick fish	Beaz-Hidalgo et al., 2009
AE169	BW Baltic Sea	This study
CECT 7444	Rainbow trout	Beaz-Hidalgo et al., 2010
AE71	FW lake wàter	This study
V. vulnificus		
CECT 4999^T	Eel	Lee et al., 2013

Table 2. Primers used for gene expression.

Gene	Sequence (5'-3')
GAPDH	Forward CATGAGAAGTATGACAACAGCCT Reverse AGTCCTTCCACGATACCAAAGT
TLR4	Forward AGTTGATCTACCAAGCCTTGAGT Reverse GCTGGTTGTCCCAAAATCACTTT
JUN	Forward TGCCTCCAAGTGCCGAAAAA Reverse TGACTTTCTGTTTAAGCTGTGCC
RELA	Forward ATGTGGAGATCATTGAGCAGC Reverse CCTGGTCCTGTGTAGCCATT
TNF-α	Forward GAGGCCAAGCCCTGGTATG Reverse CGGGCCGATTGATCTCAGC
CCL2	Forward CCCCAGTCACCTGCTGTTAT Reverse TGGAATCCTGAACCCACTTC
CCL20	Forward GCAAGCAACTTTGACTGCT Reverse ATTTGCGCACACAGACAACT
CASP3	Forward GAAATTGTGGAATTGATGCGTGA Reverse CTACAACGATCCCCTCTGAAAAA
BAX	Forward CCCGAGAGGTCTTTTTCCGAG Reverse CCAGCCCATGATGGTTCTGAT
TP53	Forward CAGCACATGACGGAGGTTGT Reverse TCATCCAAATACTCCACACGC
NLRP3	Forward CGTGAGTCCCATTAAGATGGAGT Reverse CCCGACAGTGGATATAGAACAGA
IL-1B	Forward TTCGACACATGGGATAACGAGG Reverse TTTTTGCTGTGAGTCCCGGAG

Results

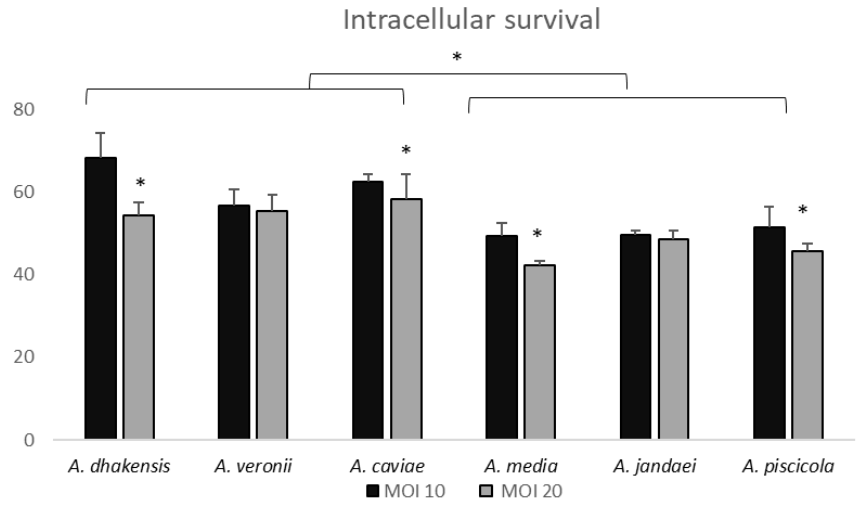


Figure 1. Average with resting cells of intracellular survival in each *Aeromonas* species. Significant differences compared with resting cells $p < 0.05$*

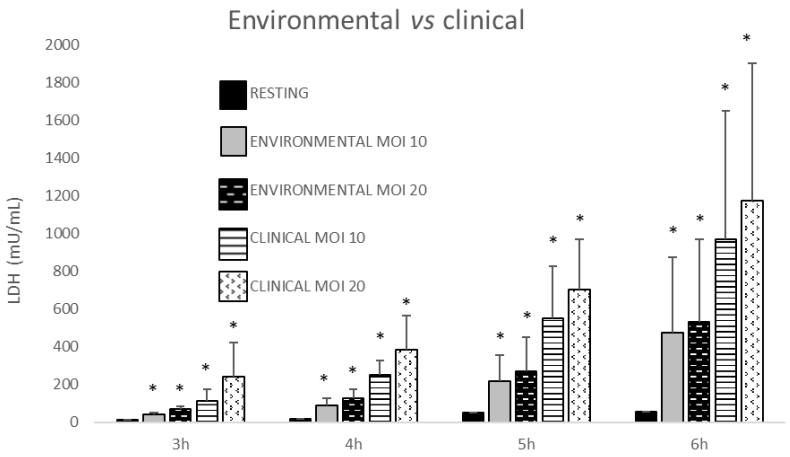


Figure 2. Observed THP-1 cell damage induced by clinical and environmental strains of *Aeromonas* spp. at different exposure times and MOIs. Significant differences compared with resting cells $p < 0.05$*.

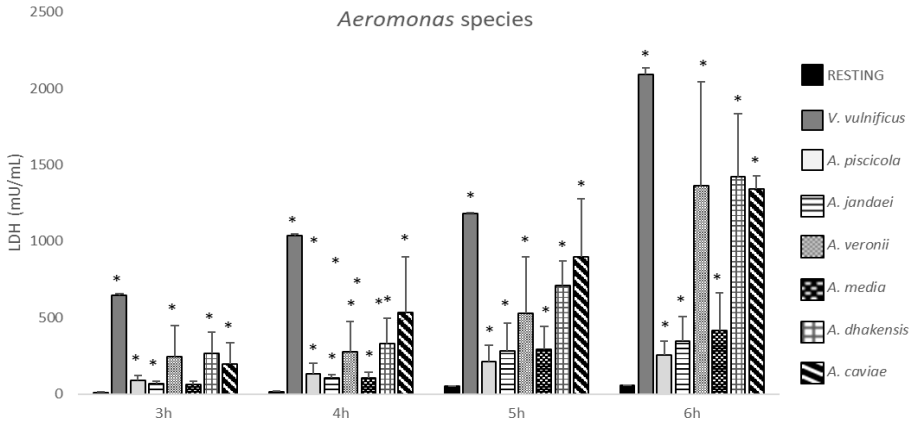


Figure 3. Observed THP-1 cell damage induced by the six different *Aeromonas* spp. at MOI 20 and at different exposure times in relation to the resting cells. Significant differences compared with resting cells $p < 0.05$*.

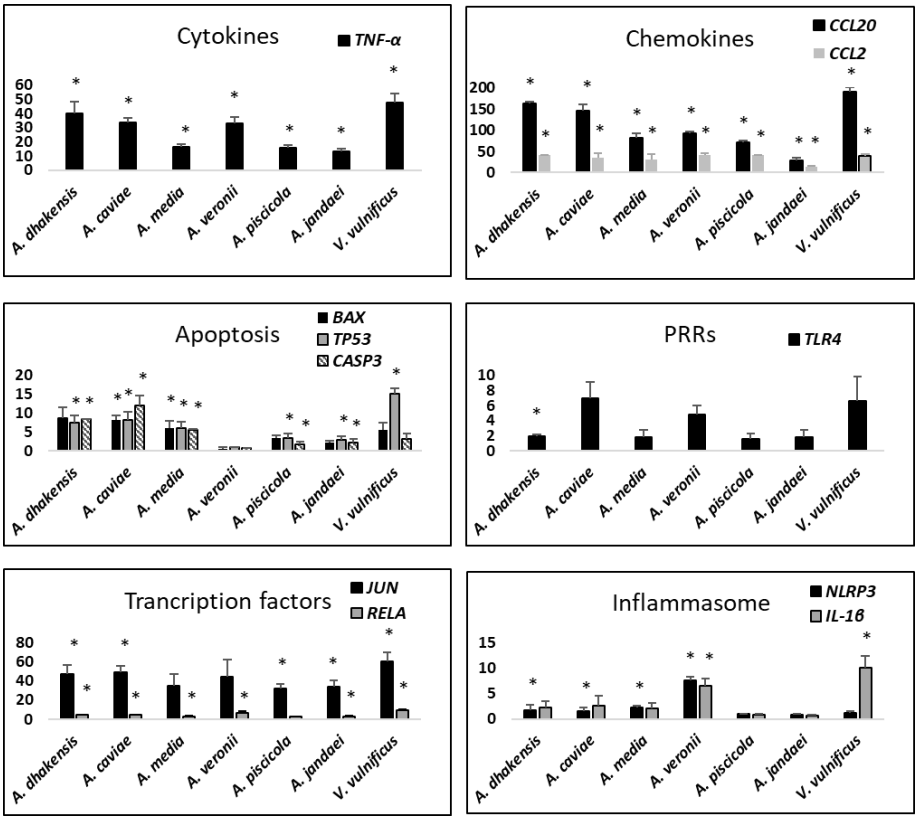
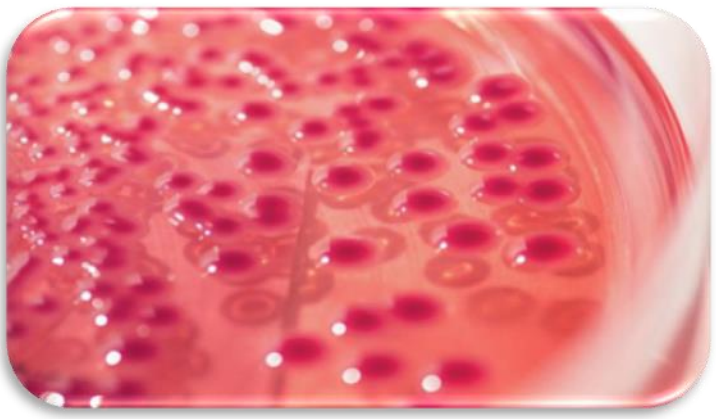


Figure 4. Gene expression profile of THP-1 cells (mean qPCR values) in relation to the resting cells induced by the different studied *Aeromonas*spp. at MOI 20. Significant differences compared with resting cells $P < 0.05$*.

5. GENERAL DISCUSSION



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General discussion

The presence of *Aeromonas* in different sources of water evaluated during one year with different methods of quantification, and with samples concentrated by skimmed milk flocculation, which is a method used in for the concentration of virus showed to be useful for the analysis of *Aeromonas*. Our results demonstrated that *Aeromonas* were detected in 100% (47/47) of the samples by qPCR, and the quantification obtained with this method was higher in comparison with MPN method and counts in plate. As expected the water with a higher concentration of *Aeromonas* corresponded to sewage water (Holmes et al., 1996; Borrell et al., 1998; Latif-Eugenín, 2017; McLellan et al., 2010; Figueras and Ashbolt, 2019). The finding that the skimmed milk flocculation concentration method used for viruses can be used for bacteria like *Aeromonas*, is very important since bacteria and viruses normally need different concentration methods. In addition, higher volumes of water can be studied, and this is one of the conclusions of this doctoral thesis.

The evaluation of the pathogenicity of mesophilic *A. salmonicida* isolated from water and human infections in the experimental assays using a mice model performed in the study 4.2 demonstrated that *A. salmonicida* can infect mammals with different levels of pathogenicity. This is suggested to depend upon the strain, probably due to the presence of different virulence factors. Our results showed the presence of several genes involved in a type III secretion system (T3SS), that generated a striking mortality, in one clinical strain at a dose of $1x10^9$. A group of 8 mice were died after only three days post-infection. The T3SS has been described as responsible for the pathogenic phenotype of strains of the genus *Aeromonas* that include human and fish pathogens (Vilches et al., 2004; Frey and Origgi et al., 2016). Another interesting finding was that an environmental strain of *A. salmonicida* showed a similar mice mortality in comparison with the two clinical strains. In the environmental strain, only one gene associated with the T3SS was found. However, a higher number of genes that encode hypothetical proteins were found in this strain and some of them could be implied in the virulence of this strain. Interestingly, some strains used showed necrotizing fasciitis in the tail after infection, and five candidate genes to cause this necrotizing fasciitis were found

only in their genome. Four of these genes were already listed in the literature as virulence factors in human pathogens (Gilbreath et al., 2012; Eshghi et al., 2012; Zeng et al., 2016; Wahid et al., 2017). The role in the human infections of the mesophilic *A. salmonicida* strains was previously suspected based on different documented clinical studies performed in India (Tewari et al., 2014; Varshney et al., 2017). Based on the genomic analysis the species *A. salmonicida* contain only two subspecies and not five as classically considered. One of the subspecies is *A. salmonicida* subsp. *pectinolytica* that embraces all the mesophilic strains recovered from the environment and from the clinical cases. The other is *A. salmonicida* subsp. *salmonicida* that grouped all the psychrophilic strains and subspecies i.e. the subspecies *salmonicida, smithia, mausoucida* and *achromogenes*. Moreover, clinicians should consider the inclusion of *A. salmonicida* in the diagnosis tests, since this study shows that mesophilic strains of this species are able to infect mammalians and show that the species can also be pathogenic for humans.

As a result of the collaboration of our laboratory with the University hospitals from our area we have identified with the *rpo*B gene a *V. alginolyticus* strain identified at the hospital with the API 20E method as *Aeromonas* sp. Therefore, it is important to alert clinicians that *V. alginolyticus* can be confused with *Aeromonas* based on the identification method used (study 4.3). In the case of the study 4.4., the strain that belonged to *A. trota*, based on the *rpoD* sequences, was identified only at genus level (*Aeromonas* spp.) at the hospital. The use of phenotypic identification methods has led to confusions in the *Aeromonas* spp. identification. Previous literature demonstrated that these methods do not always identify correctly the strains to the genus, being frequently confused with *Vibrio* (Abbott et al., 1998; Soler et al., 2003b; Lamy et al., 2010). In fact, Lamy et al. (2010) demonstrated the limitation of the API 20E system for the identification of *Aeromonas* spp. (Lamy et al., 2010).

The MALDI-TOF identification system was evaluated, in the mentioned cases (studies 4.3. and 4.4). The results obtained in the study 4.3. showed that the strain isolated in the first clinical case that belonged to *V. alginolyticus*, was

preliminary identified as *Vibrio parahaemolyticus*. An explanation of this could be that both species are very closely related because they show a 16S rRNA similarity of 99.4%, which indicate a low probability of a correct identification with MALDI-TOF at species level, considering that the majority of the MALDI derived information comes from the ribosomal protein (Erler et al., 2015). Erroneous identification of *Vibrio* by using MALDI- TOF has been previously described (Dieckman et al., 2010). Moreover, as shown by our results in the study 4.4., the strain of the clinical case was identified as *A. enteropelogenes* by MALDI-TOF, and *A. trota* by *rpoD* sequencing, being the latter the accepted name for a synonymous species that is the most used name by the scientific community (Holme and Farmer, 2009). In a previous study, using isolates from Spanish hospitals it was demonstrated that 98.3% of the *Aeromonas* strains were correctly identified at the genus level, and 91.1% at the species level, being MALDI-TOF a useful tool for the *Aeromonas* identification (Latif-Eugenín, 2015). However, this method has a limitation related to the database. In the genus *Aeromonas,* the taxonomy has changed continuously with the description of new taxa and with new reclassifications. For example, the lack of *A. dhakensis* in the MALDI database has been described (Benagli et al., 2012; Latif-Eugenín, 2015). Additionally, the names of species not used as *A. ichtioismia, A. punctata* or *A. enteropelogenes* are included in the database (Vávrová et al., 2015). This is a problem in the clinical field, because the clinicians are not experts on the taxonomy of *Aeromonas* and might think that these synonyms represent different species

Based on these results, it is important to take into account the capacity of the *rpoD* gene for the identification of *Aeromonas*. The results in the studies 4.3. and 4.4. demonstrated that the use of the *rpoD* housekeeping gene is an excellent tool for the identification at species level when compared with phenotypic methods as well as with MALDI-TOF. However, the MALDI-TOF is a rapid system that provides a relatively good identification method for *Aeromonas* and many other microbes, but the database should be updated.

The antibiotic profile of the *A. trota* and *V. alginolyticus* isolated from the clinical cases (studies 4.3. and 4.4.) were determined by the disk diffusion method or automatically determined by using the MicroScan Walkaway using the Clinical and Laboratory Standards Institute (CLSI) guidelines in the studies 4.3. and 4.4. The results obtained in the study 4.3. showed that both strains of *V. alginolyticus* were susceptible to all tested antibiotics, except to penicillin and ampicillin. This result is in agreement with the resistance to ampicillin and penicillin obtained in other studies and the susceptibility to trimethoprim-sulfamethoxazole, tetracycline and gentamicin. (Lajnef et al., 2012; Liu et al., 2012). The strain isolated from the patient in the study 4.4. showed a resistance to ampicillin and cephalotin. Previous studies described *A. trota* as a rare species, being the only species of the genus susceptible to ampicillin (Carnahan, 1991b; Overman and Janda 1999; Huys et al., 2002; Abbott et al., 2003; Lai et al., 2007; Dallagasa et al., 2018). A resistance to cephalotin has also been described (Carnahan et al., 1991b; Abbott et al., 2003). These studies pretend to guide the clinicians in the correct treatment for these pathogens.

Another important aspect studied in this doctoral thesis is the evaluation of the usefulness of the MALDI-TOF for *Aeromonas* identification isolated from fish in comparison with the *rpoD* sequencing performed in the study 4.5. Our results showed poor accurate identification at species level in our case for the most prevalent species implicated in fish diseases, such as *A. salmonicida, A. hydrophila* and *A. bestiarum*. Overall, when the database was supplemented with 14 new spectra, the number of accurate identifications increased. This study agreed with the previous study showing that MALDI-TOF is a useful method for the identification of *Aeromonas* at genus level. However, at species level important differences in the percentage of strains correctly identified depend on the species (Benagli et al., 2012). Additionally, the database should be checked, taking into account the new changes in the taxonomy of *Aeromonas*.

In relation with the characterization of the recent species *A. lusitana,* a genomic analysis was performed by using a new strain from rainbow trout in

Mexico found in our laboratory, as well as the type strain recently described by Martínez-Murcia et al. (2016) recovered from water and vegetables in Portugal. Our genomes were annotated with RAST Server (Aziz et al., 2008) and the size genome as well as the G+C content were 4.74Mb and 60.7%, respectively in the new strain, while in the type strain were 4.55 Mb and 61mol%, respectively. These results demonstrated to be in the range previously established based on the completed studied genomes, being the size (4.43- 4.81 Mb) and the content of the G+C (57-63 mol%) (Beaz-Hidalgo and Figueras, 2013). Moreover, the ANI between both genomes of *A. lusitana* was higher than 96% (96.3%) However, the *is*DDH between both genomes of *A. lusitana* in our study was less than 70% (68.9%). Previously, several authors suggested that 96% and 70% of ANI, and *is*DDH, respectively are the cut-off values used to separate species (Colston et al., 2014; Beaz-Hidalgo et al., 2015a). An explanation of these results could be that both strains are the same species with higher differences. Colston et al., 2014 showed a similar result in *A. allosaccharophila,* describing the taxonomy of this species as controversial, as it could be *A. lusitana*. For this reason, new genomic studies with more strains of this species should be carried out to elucidate this. Additionally, The Functional Comparison Tool from the SEED viewer (Overbeek et al., 2014) was used for the functional analysis, demonstrating the presence of the citrate synthase gene in *A. lusitana* previously associated with the virulence in species of *Agrobacterium tumefaciens* and *Escherichia coli* (Suksomtip et al., 2005; Quandt et al., 2015).

Besides, in the study 4.6. to reinforce the information obtained by genome sequencing, the presence/absence of virulence genes in 6 strains of *A. lusitana* was performed by PCR. The results showed that the distribution of virulence factors is strain-dependent, because the pattern of each strain was different. This pattern was similar to other environmental strains previously described (Chacón et al., 2003; Pablos et al., 2009; Beaz-Hidalgo et al., 2012, 2013a; Aravena-Román et al., 2014). Furthermore, the phenotypic characteristic and the susceptibility to antimicrobial agents were evaluated and the results showed discordant results between the new isolate and the type strain, as well as between conventional methods and MicroScan

Walkaway. The susceptibility to 23 antimicrobial agents showed an intermediate resistance to ampicillin in contrast with the classical resistance to ampicillin defined as a typical characteristic of the majority of the *Aeromonas* spp. (Abbott et al., 2003; Beaz-Hidalgo et al., 2012).

The draft genome sequence of this novel *A. lusitana* strain is a valuable addition to the genomic database and will contribute to the understanding of the role of this species in the environment as well as the relationship with other *Aeromonas* spp. Also, this study confirms that the biochemical characteristic in the genus *Aeromonas* is complex, it demonstrates the diversity of virulence profiles in strains of the new species *A. lusitana*. Cytotoxicity studies with these strains could be interesting to clarify the role of this species in human infections.

As explained in the introduction, the pathogenesis of *Aeromonas* infections is multifactorial, i.e. caused by a wide variety of virulence factors. In the study 4.7. we have characterized the potential role of the metallochaperone HypA in the virulence of *Aeromonas* by using an *in vitro* experiment. Our results showed that *hypA* was upregulated in acidic pH. The metallochaperone HypA was previously described in other pathogenic species such as *H. pylori* and *E. coli* with a role in the survival of the human stomach at acidic pH (Watanabe et al., 2015; Lacasse et al., 2016). Considering that 80% of the infections caused by *Aeromonas* are gastrointestinal, a similar possible adaptation of these bacteria to acid environment of the stomach could be suspected suggesting that HypA could play a role in this adaptation. Additionally, the results of the urease test to elucidate the enzymes related to this mechanism showed that all strains were urease negative. The principal role of the HypA has been associated with hydrogenases maturation, however, in the last years it has been suggested that it could play a role in the maturation of ureases. In *H. pylori*, the ureases are involved in the survival in the human stomach at acidic pH, while in *E.* coli the hydrogenases are involved in this mechanism (Blum et al., 2017; Hu et al., 2017). Taking into account these studies, a reasonable explanation of our results could be that hydrogenases, but not ureases, would be involved in acid resistance in *Aeromonas*. Finally, in the study 4.7., the macrophages infection

demonstrated an upregulation of *hypA* at 4 h post-infection. Previous studies suggested that the immune system generates reactive oxygen species (ROS) as a defense mechanism against pathogens after phagocytosis by macrophages, as well as the hydrogenases participates in oxidative stress defense (Vignais and Billoud, 2007). For this reason, HypA would also contribute to the defense against ROS produced by macrophages in the phagocytic process.

Recently, mixed infections with different *Aeromonas* strains or species have been estimated to occur in 5%-10% of the cases (Mosser et al., 2015). In fact, Grim et al. (2014) and Ponussamy et al. (2016) described the complex interactions in the mixed infections with two strains of *A. hydrophila* (NF1 and NF2) isolated from an immunocompetent individual that developed necrotizing fasciitis (NF) resulting in amputation of several extremities. In the study 4.8. the role of *exoA* and T6SS genes of the strains NF1 and NF2, and in the generated mutants used to generate mixed infection in an *in vitro* and *in vivo* model were investigated. The strain NF2 presents a functional ExoA toxin, while the strain NF1 did not contain this gene. The genome of both NF1 and NF2 strains showed the presence of two clusters of type-VI secretion system (T6SS) synthesis genes and effectors (three copies of Hemolysin-coregulated protein (Hcp) and five copies of valine-glycine repeat G proteins (VgrGs). Our results demonstrated VasK and TseC plays a role in the phagocytosis, the intracellular survival in macrophages and the elimination of NF2. Additionally, the immunoprotein TsiC in the NF2 plays a role in the defense against NF1 in the *in vitro* model. However, the ExoA toxin, not related with the T6SS, does not play a role in the *in vitro* model. Also, the results showed that in the intramuscular mixed infection generated in a murine model the T6SS effector TseC toxin in the NF1 strain produced the elimination of the NF2 strain from the site of the injection. Results confirmed that the progression of NF in mixed infection follows a different course in comparison with the infections produced with a single strain (Ponnusamy et al., 2016). The immunization experiments showed that ExoA plays a role in the single infection, but not in the mixed infection. Also, ExoA does not promote the pathogenesis and dissemination of NF1 contrary to the previous hypothesis

(Ponnusamy et al., 2016). These results suggested that the T6SS and ExoA contribute significantly to the development of NF in mice during mono-microbial infections. However, during mixed infection, the T6SS plays a dominant role in developing NF.

Despite the worldwide distribution of *Aeromonas* infections, its interaction with the human immune system remains poorly understood. For this reason, in this doctoral thesis we have tried to clarify if there exists a species-specific immune response that could explain the different prevalence of the different species in human infections (study 4.6). To carry out this objective, we have designed an *in vitro* model by using the most prevalent clinical species *A. dhakensis, A.caviae* and *A. veronii* and the less prevalent *A. piscicola, A. media* and *A. jandaei* (Figueras and Beaz-Hidalgo et al., 2015), excluding *A. hydrophila* that has been previously studied (Su et al., 2009; Rosenzweig et al., 2013; Zhang et al., 2017; Kong et al., 2017). Our results demonstrated the upregulation of the immune related genes as cytokines (TNF-α), chemokines (CCL2, CCL20), TLRs (TLR4), transcription factors (RELA, JUN), apoptotic genes (CASP3, TP53, BAX) and pyroptotic genes (IL-β, NRLP3), previously described after *A. hydrophila* infection using a fish, mice and cell lines as model (McCoy et al., 2010a,b; Jones et al., 2012; Zhang et al., 2017; Kong et al., 2017; Liao et al., 2018) with the exception of the CCL2 and BAX gene. However, the results in this thesis doctoral showed that the response of monocytes against *Aeromonas* infection is species-specific, demonstrating that the most prevalent clinical species activate a higher immune response genes than the less prevalent species. An explanation of this could be associated with the cytokine storm that is a systemic inflammatory response syndrome that increases the severity of the infections, and in this case the more prevalent species would induce this mechanism and the consequence could be the higher prevalence in clinical cases. Moreover, the cell damage was higher in the most prevalent clinical species, as well as in the clinical strains in comparison with the environmental strains. A previous study described that, when *V. vulnificus* arrives to the blood it liberates a toxin that triggers a cytokine storm that could be fatal (Murciano et al., 2017). In view of the results, it

could indicate that the clinical strains activate a cytokines storm responsible for the higher cell damages, as well as the increase of the intracellular survival of these bacteria in the human monocytes. In addition, in relation to the mechanism of the cell-death, our results demonstrated that *A. veronii* activated the pyroptosis in human monocytes. However, the species *A. piscicola* and *A. jandaei* showed a lower expression of the genes related to pyroptosis and higher expression in the genes related to apoptosis. Earlier literature demonstrated the activation of pyroptosis and apoptosis after *A. veronii* and *A. hydrophila* infections in mice and fish models (McCoy et al. 2010a, b; Jones et al., 2012; Lü et al., 2015). A previous study in *V. vulnificus* demonstrated that the immune response against the bacteria was type cell-dependent (Murciano et al., 2015), and this could be an explanation of the down-regulation of pyroptosis genes in the monocytic cell line after *A. veronii* infection.

Based on the results, we concluded that the expression of immune-related genes showed that the selected species caused a different immune response, which is characterized by a species-specific activation pattern. Also, the apoptosis route seemed to be the only way of producing cell damage and death in the case of *A. piscicola* and *A. jandaei*. However, *A. veronii* apparently only used the pyroptosis route. Further studies with a higher number of strains using additional inflammasome genes would be necessary to confirm this observation.

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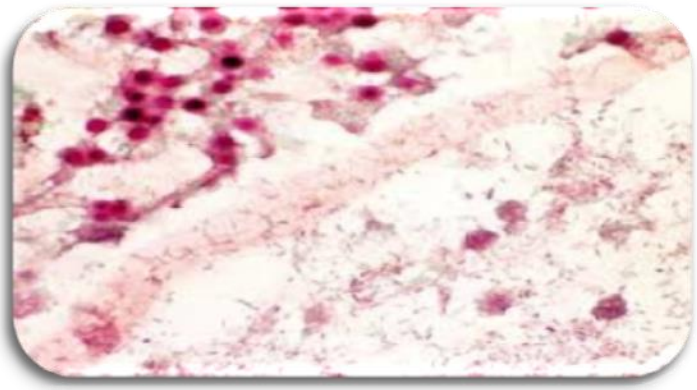
6. CONCLUSIONS



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Conclusions

1. The best method for *Aeromonas* quantification in different water sources was the qPCR because it is faster than the MPN and the plate count methods.

2. The "skimmed milk" flocculation method used for the concentration of viruses from big volumes of water showed to be a good method for the detection and quantification of *Aeromonas*.

3. *Aeromonas salmonicida,* classically considered a fish pathogen species, caninfect mammals and the infective strains showed different levels of pathogenicity.

4. The five accepted subspecies of *A. salmonicida* formed, using a genomic analysis, only two groups that corresponded to two subspecies. The subsp. *pectinolytica* that grouped all mesophilic strains and the subsp. *salmonicida* that embraced all the psychrophilic strains and subspecies i.e. *smithia, achromogenes* and *masoucida*. Therefore, the latter three subspecies should be synonymized with the subspecies *A. salmonicida* subsp. *salmonicida.*

5. The description of new clinical cases of *Aeromonas* human infections intends to raise awareness among the clinicians of the possible confusion of species of this genus with *Vibrio alginolyticus* depending on the identification method.

6. *Vibrio alginolyticus* infections, despite rare, occur in patients with cancer or other immunosuppressed conditions and in healthy individuals with skin or soft tissue lesions that have been in contact with seawater.

7. Human infections with *Aeromonas trota* are rare and occur mainly in immunocompromised patients and should be suspected when the isolated strain shows to be sensible to ampicillin.

8. The limited performance of MALDI-TOF for the identification of fish *Aeromonas* isolates at the species level has been demonstrated. This can be improved when increasing the number of strains of the different species at the database.

9. The genome of the new strain of *Aeromonas lusitana* ESV-351 showed more protein coding genes and tRNAs than the genome of the type strain and these could suggest a better survival and adaptation of this strain in the environment.

Also, the chromosomal region surrounding the conserved citrate synthase locus in the new strain was similar to that of four pathogenic bacteria, suggesting that this gene could play a role in the virulence of this new strain.

10. It has been demonstrated for the first time that the HypA metallochaperone could play a role in the acid tolerance and in the defense of *Aeromonas* against macrophages.

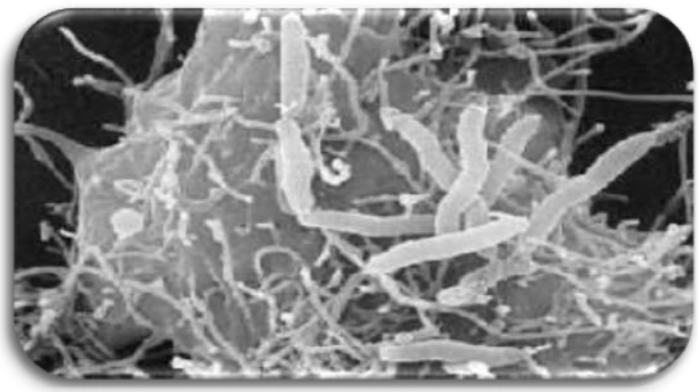
11. The T6SS and ExoA toxin plays a role in the development of the necrotizing fasciitis in mice during mono-microbial infections. However, during mixed infection, the T6SS plays a dominant role in development of this disease.

12. Independently of the species, more cell damage was observed in the human monocytic cell line (THP-1) after six hours of infection and the damage produced by the clinical strains was higher than the one observed for the environmental strains.

13. The transcriptional levels of the immune-related genes and the cell damage in the human monocytic cell line (THP-1) were higher in the infections produced with the most prevalent clinical species *A. dhakensis, A. veronii* and *A. caviae*. This could be associated with a cytokine storm and could explain their higher prevalence in clinical cases.

14. Death of human monocytic cells (THP-1) may occur only by apoptosis if infected with *A. piscicola* and *A. jandaei*. However, if infected with *A. veronii,* this would only happen by the pyroptosis route. Infections with *A. caviae* and *A. media* may induce cell death by both routes.

7. REFERENCES



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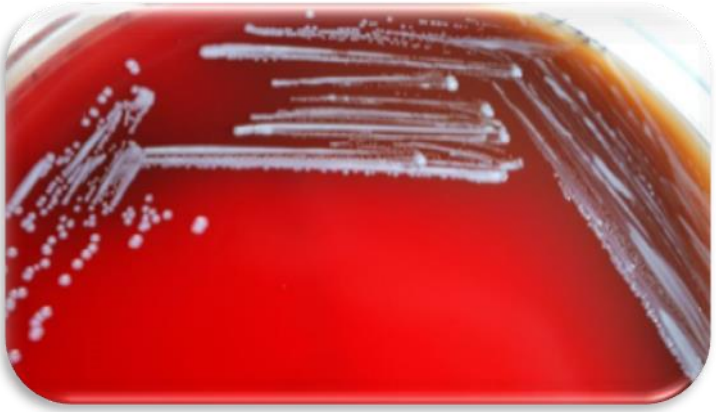
8. SUPPLEMENTARY



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Supplementary

Supplementary table S1. Strains used for the experiments

Species	Strain	Source	Study
A. salmonicida	AJ83	Wound infection	4.2.
A. salmonicida	947C	Wound infection	4.2.
A. salmonicida	A308	Water	4.2.
A. salmonicida	34 mel^T	Fish	4.2.
A. salmonicida	Y47	Chicken	4.2.
A. salmonicida	A527	Giant river prawn	4.2.
V. alginlolyitus	1182C	Feces	4.3.
V. alginlolyitus	I53834	Wound infection	4.3.
A. trota	1183C	Feces	4.4.
A. hydrophila	PS	Fish	4.5.
A. hydrophila	17164	Fish	4.5.
A. hydrophila	87	Fish	4.5.
A. hydrophila	ESV-356	Gill from fish	4.5.
A. hydrophila	ESV-357	Spleen from fish	4.5.
A. hydrophila	ESV-370	Gill from fish	4.5.
A. hydrophila	ESV-371	Intestine from fish	4.5.
A. hydrophila	ESV-380	Intestine from fish	4.5.
A. hydrophila	ESV-381	Gill from fish	4.5.
A. hydrophila	ESV-399	Gill from fish	4.5.
A. salmonicida	ESV-396	Boil from fish	4.5.
A. salmonicida	CECT 4239	Fish	4.5.
A. salmonicida	CECT 4253	Brook trout	4.5.
A. salmonicida	CECT 4238	Fish	4.5.
A. salmonicida	CECT 896	Blood from fish	4.5.
A. salmonicida	18894	Salmon	4.5.
A. salmonicida	18895	Fish	4.5.
A. salmonicida	18900	Canadian perch	4.5.
A. salmonicida	18902	Salmon	4.5.
A. salmonicida	33753	Salmon	4.5.
A. sobria	ESV-355	Intestine from fish	4.5.
A. sobria	ESV-361	Gill from fish	4.5.
A. sobria	ESV-369	Kidney from fish	4.5.
A. sobria	ESV-372	Spleen from fish	4.5.
A. sobria	ESV-390	Gill from fish	4.5.
A. sobria	CECT 4245	Gill from fish	4.5.
A. sobria	CECT 4251	Gill from fish	4.5.

Supplementary table S1. Continued

Species	Strain	Source	Study
A. sobria	CECT 4249	Gill from fish	4.5.
A. veronii	01.1	Fish	4.5.
A. veronii	01.2	Fish	4.5.
A. veronii	04.1	Fish	4.5.
A. veronii	04.2	Fish	4.5.
A. veronii	06.1	Fish	4.5.
A. veronii	06.2	Fish	4.5.
A. veronii	ESV-386	Fish	4.5.
A. veronii	ESV-400	Spleen from fish	4.5.
A. veronii	09.2	Fish	4.5.
A. veronii	09.1	Fish	4.5.
A. bestiarum	ESV-352	Kidney from fish	4.5.
A. bestiarum	ESV-353	Spleen for fish	4.5.
A. bestiarum	ESV-363	Liver from fish	4.5.
A. bestiarum	ESV-364	Spleen for fish	4.5.
A. bestiarum	ESV-365	Fish	4.5.
A. bestiarum	ESV-366	Spleen for fish	4.5.
A. bestiarum	ESV-367	Gills from fish	4.5.
A. bestiarum	ESV-375	Gills from fish	4.5.
A. bestiarum	ESV-376	Gills from fish	4.5.
A. bestiarum	ESV-384	Gills from fish	4.5.
A. popoffi	ESV-354	Gills from fish	4.5.
A. popoffi	ESV-358	Gills from fish	4.5.
A. popoffi	AE3	Lake water	4.5.
A. popoffi	AE9	Lake water	4.5.
A. popoffi	AE14	Lake water	4.5.
A. popoffi	AE17	Lake water	4.5.
A. popoffi	AE20	Lake water	4.5.
A. popoffi	AE25	Lake water	4.5.
A. popoffi	AE121	Lake water	4.5.
A. popoffi	AE123	Lake water	4.5.
A. lusitana	ESV-351	Rainbow trout	4.6.

Supplementary

Supplementary table S1. Continued

Strain	Source	Source	Study
A. lusitana	CECT 7828^T	Untreated water	4.6.
A. lusitana	MDC 2467	Untreated water	4.6.
A. lusitana	MDC 2468	Vegetables	4.6.
A. lusitana	MDC 2469	Vegetables	4.6.
A. lusitana	MDC 2472	Untreated water	4.6.
A. hydrophila	CECT 839^T	Milk	4.7.
A. salmonicida	CECT 894^T	Salmon	4.7.
A. jandaei	CECT 4228^T	Human feces	4.7. and 4.9.
A. caviae	CECT 838^T	Guinea pig	4.7. and 4.9.
A. dhakensis	CECT 5744^T	Children feces	4.7. and 4.9.
A. veronii	CECT 4257^T	Sputum	4.7. and 4.9.
A. hydrophila	NF1	Necrotizing fasciitis	4.8.
A. hydrophila	NF2	Necrotizing fasciitis	4.8.
A. hydrophila	NF1-*exoA*	Necrotizing fasciitis	4.8.
A. hydrophila	NF2Δ*exoA*-*lux*	Necrotizing fasciitis	4.8.
A. hydrophila	NF2-*lux*	Necrotizing fasciitis	4.8.
A. hydrophila	NF1-*lux*	Necrotizing fasciitis	4.8.
A. hydrophila	NF1Δ*vasK*	Necrotizing fasciitis	4.8.
A. hydrophila	NF1Δ*tseC*	Necrotizing fasciitis	4.8.
A. hydrophila	NF2-*lux*-pBR322-*tsiC*	Necrotizing fasciitis	4.8.
A. media	CECT 4232^T	Fisheries water	4.9.
A. piscicola	CECT 7443^T	Sick fish	4.9.

Supplementary table S2. Strains used for the genome analysis.

Species	Strain	Source	Study
A. hydrophila	CECT 839^T	Milk	4.2.,4.6. and 4.7.
A. salmonicida	CECT 894^T/CIP 103209^T	Salmon	4.2.,4.6. and 4.7.
A. sobria	CECT 4245^T	Fish	4.2.,4.6. and 4.7.
A. media	CECT 4232^T	Fisheries water	4.2.,4.6. and 4.7.
A. veronii	CECT 4257^T	Sputum	4.2.,4.6. and 4.7.

Supplementary table S2. Continued

Species	Strain	Source	Study
A. caviae	CECT 838 ^{T}	Guinea pig	4.2.,4.6. and 4.7.
A. eucrenophila	CECT 4224 ^{T}	Fresh water fish	4.2.,4.6. and 4.7.
A. schubertii	CECT 4240^{T}	Skin abscess	4.2.,4.6. and 4.7.
A. jandaei	CECT 4228^{T}	Human feces	4.2.,4.6. and 4.7.
A. trota	CECT 4255^{T}	Human feces	4.2.,4.6. and 4.7.
A. allosaccharophila	CECT 4199^{T}	Eel	4.2.,4.6. and 4.7.
A. encheleia	CECT 4342^{T}	Eel	4.2.,4.6. and 4.7.
A. bestiarum	CECT 4227^{T}	Sick fish	4.2.,4.6. and 4.7.
A. popoffi	CECT 5176^{T}	Drinking water	4.2.,4.6. and 4.7.
A. simiae	IBS S-6874^{T}	Monkey feces	4.2.,4.6. and 4.7.
A. molluscorum	CECT 5864^{T}	Shellfish	4.2.,4.6. and 4.7.
A. bivalvium	CECT 7113 ^{T}	Shellfish	4.2.,4.6. and 4.7.
A. tecta	CECT 7082 ^{T}	Children feces	4.2.,4.6. and 4.7.
A. piscicola	CECT 7443^{T}	Sick fish	4.2.,4.6. and 4.7.
A. fluvialis	CECT 7401^{T}	River water	4.2.,4.6. and 4.7.
A. taiwanensis	CECT 7403^{T}	Wound infection	4.2.,4.6. and 4.7.
A. sanarelli	CECT 7402^{T}	Wound infection	4.2.,4.6. and 4.7.
A. diversa	CECT 4254 ^{T}	Wound infection	4.2.,4.6. and 4.7.
A. rivuli	CECT 7518^{T}	River water	4.2.,4.6. and 4.7.
A. cavernicola	CECT 7862^{T}/MDC 2508	Cavern creek water	4.2.,4.6. and 4.7.
A. australiensis	CECT 8023^{T}	Irrigation water	4.2.,4.6. and 4.7.
A. dhakensis	CECT 5744^{T}	Children feces	4.2.,4.6. and 4.7.
A. finlandiensis	CECT 8028^{T}/4287D	Cyanobacterial bloom	4.2.,4.6. and 4.7.
A. aquatica	CECT 8025^{T}/AE235	Cyanobacterial bloom	4.2.,4.6. and 4.7.
A. lacus	CECT 8024^{T}/ AE122	Cyanobacterial bloom	4.2.,4.6. and 4.7.
A. rivipollensis	LMG 26323^{T}	River water	4.2.,4.6. and 4.7.
A. aquatilis	CECT 8026^{T}	Lake water	4.2.,4.6. and 4.7.

Supplementary table S2. Continued

Species	Strain	Source	Study
A. lusitana	CECT 7828^{T}/MDC 2473	Untreatedwater	4.2.,4.6. and 4.7.
A. intestinalis	CECT 8980^{T}	Human feces	4.2.,4.6. and 4.7.
A. enterica	CECT 8981^{T}	Human feces	4.2.,4.6. and 4.7.
A. crassostreae	CECT 8982^{T}	Shellfish	4.2.,4.6. and 4.7.
A. caviae	429865 Ae_01	Child feces	4.2.
A. dhakensis	AKK1	Septicemia (NF)	4.2.
A. media	WS	Water	4.2.
A. schubertii	WL1483	Snakehead fish	4.2.
A. simiae	CIP 107798	Pig	4.2.
A. veronii	B565	Sediment	4.2. and 4.6.
A. salmonicida subsp. *salmonicida*	01-B526	Brook trout	4.2.
A. salmonicida subsp. *salmonicida*	2004-05MF26	Fish	4.2.
A. salmonicida subsp. *salmonicida*	A449	Fish	4.2. and 4.6.
A. salmonicida subsp. *salmonicida*	BG	Freshwater fish	4.2.
A. salmonicida subsp. *salmonicida*	YK	Freshwater fish	4.2.
A. salmonicida subsp. *achromogenes*	AS03	Crucian carp	4.2.
A. salmonicida subsp. *smithia*	JF4097	Arctic char	4.2.
A. salmonicida subsp. *smithia*	NBRC 13784^{T}	Ulcer from fish	4.2.
A. salmonicida subsp. *masoucida*	RFAS1	Rockfish	4.2.
A. salmonicida subsp. *masoucida*	M18076-11	Lumpfish	4.2.
A. salmonicida	Y47	Chicken	4.2.
A. salmonicida	Y567	Food	4.2.
A. salmonicida	Y577	Food	4.2.
A. salmonicida	A527	Giant river prawn	4.2.
A. salmonicida	ECFood+05	Food	4.2.
A. salmonicida	ASG1	Wound infection	4.2.
A. salmonicida	A308	Water	4.2.
A. salmonicida	947C	Wound infection	4.2.
A. salmonicida	AJ83	Wound infection	4.2.
A. salmonicida subsp. *pectinolytica*	34mel^{T}	Fish	4.2.

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