Presence of virulence markers on clinical and environmental isolates of *Aeromonas jandaei*

Soler L.[1], Chacón M.R. [1], Aguilera-Arreola G. [1,2], Janda J.M.[3], Esteve C.[4], Guarro J. [1] y Figueras M.J. [1]

[1]Unitat de Microbiologia. Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Sant Llorenç 21, 43201 Reus (Spain); [2]Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, 11340 México Distrito Federal, México. [3]Microbial Diseases Laboratory, Division of Communicable Diseases Control, Berkeley, California. [4]Facultad de Ciencias Biológicas, Universidad de Valencia, Burgasot, Valencia. Spain

Running title: Virulence markers on *Aeromonas jandaei*.

*Author for correspondence:

Maria José Figueras

Departament de Ciències Mèdiques Bàsiques

Facultat de Medicina i Ciències de la Salut

Universitat Rovira i Virgili

Sant Llorenç 21

43201 Reus (Spain)

Phone: 34-977759321

Fax: 34-977759322

E-mail: mjfs@fmcs.urv.es

Presence of virulence markers on clinical and environmental isolates of *Aeromonas jandaei*

Abstract

We have investigated the presence of four virulence genes, i.e. *alt*, *ast*, aerolysin/hemolysin and *laf*A in 32 isolates of *A. jandaei* from clinical, environmental and animal origins. The *laf*A gene was the most widely distributed (61.5% of the isolates), followed by the aerolysin/hemolysin (42.3%), *alt* (11.5%) and *ast* (3.8%) genes. Only one human diarrhoeal isolate presented the *ast* gene in combination with *alt* and *laf*A. Simultaneous presence of the *laf*A gene and aerolysin/hemolysin was observed in 34.7% of the isolates regardless of their origin. A total of 72.2% of the clinical isolates presented any of the studied virulence genes, while only 40% of the environmental isolates did. Since 18 of the 32 isolates were obtained from different authors, all the isolates were typed with the PCR amplification of the enterobacterial repetitive intergenic consensus (ERIC) obtaining specific profiles for only 26 isolates. This indicated that the rest were duplicates.

1.Introduction

The genus *Aeromonas* includes 15 species (Figueras *et al*., 2000b; Pidiyar *et al*., 2002), *A. veronii* bt sobria, *A. caviae* and *A. hydrophila* being the most prevalent in clinical samples, although other species such as *A. jandaei* are also isolated (Carnahan et al., 1991; Joseph et al., 1991; Gonzalez et al., 2001; Overman et al., 1999; Figueras et al., 2000a; Carson et al., 2001; Janda & Abbott, 1998; Valera & Esteve, 2002; Sarma, 2002). The incidence of *A. jandaei* in our studies using genetic identification methods was 2.6% of intestinal and 5.6% of extraintestinal isolates (unpublished data). This species has caused septicemia and intestinal diseases in humans (Janda & Abbott, 1998) and has showed more resistance to antibiotics than other clinical species such as *A. schubertii*, *A. trota* and *A. veronii* biotype veronii (Overman et al., 1999).

Several virulence factors have been studied in *Aeromonas* including aerolysin/hemolysin, enterotoxins, proteases, lipases and deoxyribonucleases (Chopra et al., 1999; Janda, 2001; Chacón et al., 2003). A recent study by Albert et al. (2000) has revealed a significant statistical association between two cytotonic enterotoxin genes, the *ast* gene that codifies for a heat stable enterotoxin (Chopra et al., 1994) and the *alt* gene that codifies for a heat labile enterotoxin (Chopra et al., 1986), in strains isolated from patients with diarrhoea. It was postulated that both genes act synergistically to induce severe diarrhoea. Moreover, double deletion mutants for the *ast* and *alt* genes lead important fluid secretion reduction in a mouse model (Sha et al., 2002). Another virulence factor of relevant interest in this genus is the cytotoxic enterotoxin *act*, which presents hemolytic, cytotoxic and enterotoxic activity (Rose et al., 1989; Ferguson et al., 1997), and is an aerolysin-related toxin (Chopra et al., 1993). Triple deletion mutants for *alt*, *ast* and *act* genes lead to a 100% reduction in fluid secretion in a mouse model (Sha et al., 2002).

The incidence of virulence genes in *A. jandaei* have been only rarely investigated (Kingombe et al., 1999; Albert et al., 2000; Kirov et al., 2002; Chacón et al., 2003). The present study investigates the presence of the *alt* and *ast* genes in 32 *A. jandaei* isolates from clinical, environmental and animal origins in order to evaluate if any relationship exists between these two genes and the presence of diarrhoea. The presence of the aerolysin/hemolysin (*act*) and *laf*A genes is also evaluated. The amplification of the enterobacterial repetitive intergenic consensus (ERIC), which is a good typing technique for *Aeromonas* (Soler et al., 2003), has been performed to ensure the uniqueness of each strain.

2. Materials and methods

Strains. The study included 32 *A. jandaei* isolates, 14 from our collection and 18 provided by other authors (Table 1). All the isolates have been re-identified by the 16S rDNA RFLP technique (Borrell et al., 1997; Figueras et al., 2000b). Ten *A. popoffii* isolates were used as outgroups for the ERIC dendogram.

Negative control strains from other related genera were used in the PCR amplification and Dot blot hybridization experiments (Table 1).

ERIC analysis. It was performed as previously described (Soler et al., 2003). Briefly, 100 ng of DNA were used per PCR reaction using primers and conditions previously described (Vila et al., 1996). Samples of each PCR end product were electrophoresed on 10% polyacrylamide gels. AmpliSize™ Molecular Ruler 50-2,000 bp Ladder (Bio-Rad, Barcelona, Spain) was electrophoresed twice in each gel. Gel images were saved as TIFF files, and further processed by BioNumerics software, version 1.5 (Applied Maths, Kortrijk, Belgium). To construct the dendograms, levels of similarity between the profiles were calculated by using the band-matching Dice coefficient (S_D) and the cluster analysis of similarity matrices was calculated with the unweighted pairgroup method with arithmetic averages (UPGMA).

Detection of virulence genes.

Primer design and PCR amplification. Sequences deposited in the GenBank and in the European Molecular Biology Laboratory (EMBL) under the gene denomination *ast* (Sha et al., 2002), *laf*A (A1 and A2) (Kirov et al., 2002; Gavin et al., 2002) and *alt* (Chopra et al., 1996) were used for primer design using Primer Designer 3 software (Scientific Educational Software, Durham, USA) (Table 2). The selected primers were then computer-aligned with the sequences in the GenBank and EMBL databases by BLAST analysis to ensure their specificity for the mentioned *Aeromonas* genes. Table 2 contains the list of primers designed for PCR detection. The primers lafA-F and lafA-R bind to the genes *laf*A, *laf*A1 and *laf*A2 and amplified similar fragments of the consensus region of those genes (Table 2). Primers and PCR conditions for aerolysin/hemolysin were described previously (Soler et al., 2002; Chacon et al., 2003). PCR amplifications for the remaining genes were performed in a final volume of 50 μl containing 3 μg of DNA, 0.3 mM deoxynucleotide triphosphate mix, 1X PCR

buffer (Gibco-BRL, Barcelona, Spain), 1.8 mM $MgCl_2$, 0.3 μM of primers and 2.5 units of *Taq* polymerase (Gibco-BRL). PCRs were carried out in a Perkin-Elmer GeneAmp 2400 PCR System by using the following programme: 5 min denaturation cycle at 95°C, 35 melting cycles at 94°C for 1 min, annealing for 1 min at 50°C followed by an elongation at 72°C for 1 min and a final extension cycle of 72°C for 10 min. 20 μl of the amplified product was electrophoresed in a 1% agarose gel. Two amplified PCR products of each amplified gene were confirmed by sequencing with an ABI-PRISM™ 310.

Dot blot. This methodology was only employed for the detection of *ast*, *laf*A and aerolysin/hemolysin genes. Dot blot experiments were not performed for the *alt* gene due to the high percentage of similarity to several lipase gene sequences. 10 μg of DNA of each strain were blotted onto a nylon membrane. The membrane was then placed on a filter paper saturated with a denaturing solution (0.5 N NaOH) for 5 min and then on a filter paper soaked in a neutralizing solution (0.5 M Tris-HCl pH 7, 1.5 M NaCl) for 5 min. The membrane was then placed for 5 min on a new filter paper saturated with 10 X SSC (Standard Saline Citrate) (1.5 M NaCl-0.15 M sodium citrate pH 7.2). Finally the membrane with the bound DNA was UV-crosslinked for 3 min on an UV-transilluminator, rinsed in sterile double-distilled H_2O and air dried until used.

Preparation of digoxigenin-labelled probe. Probes were Digoxigenin-labelled by PCR by adding 0.5 nmol of Dig-11-dUTP (Roche Diagnostics, Barcelona, Spain) to the PCR reaction. The efficiency of the labelling was determined by running 10 μl of the labelled product in parallel to 10 μl of an unlabelled control in a 1% agarose gel.

Dot blot hybridisation with the DIG-labelled probe. The bacterial DNA bound to the nylon membranes was hybridised with the denatured digoxigenin-labelled *ast* or *laf*A probe

overnight at 50°C using DIG Easy Hyb solution (Roche Diagnostics); followed by 2 X 5 min washes at room temperature in 2 X SSC-0.1% SDS (Sodium Dodecyl Sulphate) and 2 X 15 min in 0.5% SSC-0.1% SDS at 50°C. Membrane detection signal was performed using the Digoxigenin detection kit as recommended by the manufacturer (Roche Diagnostics).

3. Results and discussion

The present study includes 18 *A. jandaei* isolates received from different authors' personal collections. In order to avoid considering duplicate isolates when studying virulence gene distribution, the isolates were typed by the ERIC-PCR method, which has proved to be highly discriminative in other *Aeromonas* species (Soler et al., 2003). The blind analysis was done and recognized as identical, by pairs, six isolates of *A. jandaei* and a group of four isolates, all ten obtained from different authors under the nomenclature of their labs (Figure 1; Table 1). Therefore, we contacted those authors again in order to obtain more detailed information about the exact origin of the isolates confirming that three pairs of isolates were duplicates deposited under different labels in the authors' collections. However, the four isolates with identical patterns again had a labelling problem because although 2 by 2 were proven to belong to the same strain, their origins were different (blood, ATCC 49569 and feces LMG 13064). In a recent study using numerical taxonomy, isolates ATCC 49569 and LMG 13064 presented a percentage of phenotypic similarity of 89.2% (Jaccard coefficient) and 94.3% (Simple Matching coefficient), which were considered within the cut-off values for a duplicate of a single strain (Valera & Esteve, 2002). This all together suggests that a mistake must have occurred on the labelling of those isolates. Our results highlight the confusion generated by re-labelling or re-numbering strains. We therefore recommend keeping the original nomenclature for the strains used in different studies in order to avoid this problem. The ERIC-PCR dendrogram showed two main clusters, the first containing *A. popoffii* isolates (outgroup), and the second, *A. jandaei* isolates (Figure 1). The latter was

split into two subclusters, the first containing strains from Spain, and the second those from other geographical areas, mainly from the USA. Isolates showed a tendency to cluster according to their geographical origin as described for other *Aeromonas* species using the same technique (Soler et al., 2003).

The percentage of distribution of the virulence genes was calculated by considering identical isolates as only one strain, therefore only 26 strains were considered.

The first step for bacterial infection is colonization. Swarming motility, due to the lateral flagella, has been thought to be an adaptation to life on surfaces, and its expression has been associated with better adherence and colonization (Gavín et al., 2002). Mutants for the genes *laf*A1, *laf*A2, *fli*U or *laf*T, which encode for lateral flagella, decreased the adherence level of *Aeromonas* to human epithelial cells and the capacity to form biofilms (Gavín et al., 2002). When the presence of genes involved in the synthesis of the lateral flagella were studied by dot blot in 20 strains belonging to the species *A. hydrophila*, *A. veronii* and *A. caviae*, all the strains were found to possess those genes using three different probes (*laf*B-C, *laf*X-S, and *laf*T-U) (Gavín et al., 2002); in contrast Kirov et al. (2002) found only 49% of 84 *Aeromonas* isolates belonging to the species *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, *A. caviae* and *A. veronii* were positive when using a probe for the *laf*A gene. Only the *A. jandaei* type strain was investigated by the latter author and found to possess the *laf*A gene. In the present study, twelve isolates were negative for the presence of the *laf*A gene by PCR but positive by Dot-blot hybridization, and were therefore considered positive (Table 3). Since no homology with other genes was detected by BLAST analysis with the *laf*A gene, we considered that the negative amplifications by PCR was due to a primer mismatch on the primer binding sites, as occurred with GCAT when it was investigated by PCR or colony blot (Chacón et al., 2002). Two PCR fragments of the *laf*A gene were sequenced (AY228331, strain CECT 4228^T and AY228330, strain A919). After a CLUSTAL W analysis, 73% of homology was obtained among them. Control strains were Dot-blot and PCR negative (Table 3), indicating the specificity of the primers designed for the genus

Aeromonas. The *laf*A gene was found widely distributed among all the isolates independent of their origin, since 16 (61.5%) presented the gene (Table 3). These findings are not surprising since the advantages of possessing lateral flagella are important in both environments, for colonization and adherence in clinical isolates, and for biofilm formation in environmental isolates.

The primers designed here were unable to amplify the *ast* and aerolysin/hemolysin genes on strains of other genera (Table 3), which demonstrates the specificity of the primers designed for the genus *Aeromonas*. In the case of *ast* and aerolysin/hemolysin genes, PCR and Dot-blot gave the same result (Table 3). When investigating the presence of the *alt* gene only the PCR method was applied, since this gene presents a high homology with other *Aeromonas* lipase gene sequences (i.e. 94% similarity with *pla*; 90% with *lip*H3; 85% with *plc*; 84% with *lip*). An important number of control strains from other genera gave a PCR fragment of identical size to that expected for the *alt* gene. However, after sequencing, those PCR products were found to be a hypothetical protein, with no known function, that did not resemble the *alt* gene (data not shown). Our results showed that only one clinical isolate of *A. jandaei* (strain 17E, from a child with diarrhoea) presented the *ast* gene. In addition, this strain possessed simultaneously the *alt* and *laf*A genes (Table 4). A statistical association between the presence of the *ast* and *alt* genes in *Aeromonas* strains isolated from patients with diarrhoea was described (Albert et al., 2000), and it was postulated that both genes would act synergistically to induce severe diarrhoea. Moreover, double deletion mutants for the *ast* and *alt* genes lead to a marked fluid secretion reduction in a ligated ileal loop assay on a mouse model (Sha et al., 2002). However, we found most human diarrhoeal isolates (11 isolates from a total of 12; 91.6%) without this association. Single deletion mutants for the *alt* gene lead to a fluid secretion reduction of 48% (Sha et al., 2002). However, the presence of the *alt* gene alone did not show any significant differences between diarrhoeal and control children (Albert et al., 2000). In our study only one diarrhoeal isolate presented this gene alone (Table 4).

Aerolysin presents a 79-93% homology with the *act* gene at the aminoacid level (Chopra et al., 1993), which results in differential neutralization of Act with Act- and aerolysin-specific monoclonal antibodies (Chopra et al., 1991), which could be due to a differential folding of the molecules. This homology is up to 96% at the DNA level, making impossible the design of PCR primers able to amplify each gene independently. For these reasons we talk here about aerolysin/hemolysin instead of the *act* gene. The role of both genes in *Aeromonas* pathogenicity has already been demonstrated (Chakraborty et al., 1987; Sha et al., 2002). In our study the aerolysin/hemolisin gene was present in 42.3% of the *A. jandaei* isolates. This results are clearly different from those obtained in previous studies on isolates of the same species, i.e. 0% by Kingombe et al. (1999) and 83.3% by Chacón et al. (2003), although those authors used a limited number of strains. In the present study, the *A. jandaei* type strain presented the aerolysin/hemolysin gene as stated by Chacón et al. (2003) and in contrast to results from Kingombe et al. (1999). Our results showed that this gene was more frequent in animal (66.6%) and human clinical (44.4%) isolates than in environmental ones (20%). The presence of the aerolysin/hemolysin gene was found associated with the *laf*A gene in 50% human extra-intestinal, 33.4% human intestinal and 33.3% of the animal isolates.

Triple deletion mutants for *alt*, *ast* and *act* genes lead to a 100% reduction in fluid secretion in a mouse model (Sha et al., 2002). In the present study none of the *A. jandaei* isolates presented the three mentioned genes simultaneously. Only 40% (2/5) of the environmental isolates presented any of the four studied virulence genes versus 72.2% (13/18) of the clinical ones (Table 4).

In conclusion, the *laf*A gene was found to be the most widely distributed among the strains (61.5%), followed by the aerolysin/hemolysin gene (42.3%), the *alt* (11.5%) and the *ast* (3.8%) genes. Simultaneous presence of the *laf*A and the aerolysin/hemolysin genes was observed in 34.7% of the isolates regardless of their origin, while association between the *ast* and *alt* genes was only found in one of the human clinical isolates. Finally, we do not

recommend re-labelling isolates received from different authors, and we encourage the use of ERIC-PCR as a typing technique for *Aeromonas* isolates.

Acknowledgements

This work has been supported by the grants: FIS 99/0944 from the Spanish Ministry of Health, Fundació Ciència i Salut and a fellowship from the Universitat Rovira i Virgili. We would like to thank the Colección Española de Cultivos Tipo (CECT), Dr. P.E. Granum, Dr. J.M. Janda and Dr. C. Esteve for kindly providing isolates.

References

Abrami, L., Fivaz, M. and van der Goot F.G. (2000) Adventures of a pore-forming toxin at the target cell surface. Trends Microbiol. 8, 168-72.

Albert, M.J., Ansaruzzaman, M., Talukder, K.A., Chopra, A.K., Kuhn, I., Rahman, M., Fauque, A.S.G., Islam, M.S., Sack, R.B. and Mollby, R. (2000) Prevalence of enterotoxin genes in *Aeromonas* ssp. isolated from children with diarrhea, healthy controls, and the environment. J. Clin. Microbiol. 38, 3785-3790.

Borrell, N., Acinas, S.G., Figueras, M.J. and Martínez-Murcia, A. (1997) Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. J. Clin. Microbiol. 35, 1671-1674.

Buckley, J.T. and Howard, S.P. (1999) The cytotoxic enterotoxin of *Aeromonas hydrophila* is aerolysin. Infect. Immun. 67,466-467.

Carnahan, A., Fanning, G.R. and Joseph, S.W. (1991) *Aeromonas jandaei* (formerly genospecies DNA group 9 A. sobria), a new sucrose-negative species isolated from clinical specimens. J. Clin. Microbiol. 29, 560-564.

Carson, J., Wagner, T., Wilson, T. and Donachie, L. (2001) Miniturized tests for computer-assisted identification of motile *Aeromonas* species with an improved probability matrix. J. Appl. Microbiol. 90, 190-200.

Chacón, M.R., Castro-Escarpulli, G., Soler, L., Guarro, J. and Figueras, M.J. (2002). A DNA probe specific for *Aeromonas* colonies. Diagn. Microbiol. Infect. Dis. 44, 221-225.

Chacón, M.R., Figueras, M.J., Castro-Escarpulli, G., Soler, L. and Guarro, J. (2003) Distribution of virulence genes in clinical and environmental isolates of *Aeromonas* spp. Antonie van Leeuwenhoek. (in press).

Chakraborty, T., Huhle, B., Hof, H., Bergbauer, H. and Woebel, W. (1987) Marker-exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demostrates the role of aerolysin in *A. hydrophila*-associated systemic infections. Infect. Immun. 55, 2274-2280.

Chopra, A.K. and Houston, C.W. (1999) Enterotoxins in *Aeromonas* associated gastroenteritis. Microb. Infect. 1, 1129-1137.

Chopra, A.K., Houston, C.W., Genaux, C.T., Dixon, J.D. and Kurosky, A. (1986) Evidence for production of an enterotoxin and cholera toxin cross-reactive factor by *Aeromonas hydrophila*. J. Clin. Microbiol. 24, 661-664.

Chopra, A.K., Houston, C.W. and Kurosky, A. (1991). Genetic variation in related cytolytic toxins produced by different species of *Aeromonas*. FEMS Microbiol. Lett. 62, 231-237.

Chopra, A.K., Houston, C.W., Peterson, J.W. and Jin, G.F. (1993) Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. Can. J. Microbiol. 39, 513-523.

Chopra, A.K., Pham, R. and Houston, C.W. (1994) Cloning and expression of putative cytotonic enterotoxin-encoding genes from *Aeromonas hydrophila*. Gene 139, 87-91.

Chopra, A.K., Peterson, J.W., Xu, X.J., Coppenhaver, D.H. and Houston, C.W. (1996) Molecular and biochemical characterization of a heat-labile cytotonic enterotoxin from *Aeromons hydrophila*. Microb. Pathog. 21, 357-377.

Chopra, A.K., Xu, X., Ribardo, D., Gonzalez, M., Kuhl, K., Peterson, J.W. and Houston, C.W. (2000) The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. Infect. Immun. 68, 2808-2818.

Ferguson, M.R., Xu, X.J., Houston, C.W., Peterson, J.W., Coppenhaver, D.H., Popov, V.L. and Chopra, A.K. (1997) Hyperpoduction, purification and mechanism of action of the cytotoxic enterotoxin produced by *Aeromonas hydrophila*. Infect. Immun. 65, 4299-4308.

Figueras, M.J., Guarro, J. and Martínez-Murcia, A.J. (2000a) Clinically relevant *Aeromonas* species. Clin. Infect. Dis. 30, 988-989.

Figueras, M.J., Soler, L., Chacón, M.R., Guarro, J. and Martínez-Murcia, A.J. (2000b) Extended method for discrimination of *Aeromonas* spp. by 16S rDNA-RFLP. Int. J. Syst. Evol. Microbiol. 50, 2069-2073.

Gavin, R., Rabaan, A.A., Merino, S., Tomás, J.M., Gryllos, I. and Shaw, J.G. (2002) Lateral flagella of *Aeromonas* species are essential for epithelial cell adherence and biofilm formation. Mol. Microbiol. 43, 383-397.

González-Serrano, C.J., Santos, J.A., García-López, M.J. and Otero, A. (2002) Virulence markers in Aeromonas hydrophila and Aeromonas veronii biovar sobria isolates from freshwater fish and from a diarrhoea case. J. Appl. Microbiol. 93, 414-419.

Granum, P.E., O'Sullivan, K., Tomas, J.M. and Ormen, O. (1998) Possible virulence factors of *Aeromonas* spp. from food and water. FEMS Immunol. Med. Microbiol. 21, 131-137.

Janda, J.M. (2001) *Aeromonas* and *Plesiomonas* p. 1237-1270. *In* M. Sussman (ed.), Molecular Medical Microbiology. Academic Press, San Diego, USA.

Janda, J.M. and Abbott, S.L. (1998) Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. Clin. Infect. Dis. 27, 332-344.

Joseph, S.W., Carnahan, A.M., Brayton, P.R., Fanning, G.R., Almazan, R., Drabick, C., Trudo, E.W., JR. and Colwell, R.R. (1991) *Aeromonas jandaei* and *Aeromonas veronii* dual infection of a human wound following aquatic exposure. J. Clin. Microbiol. 19, 565-569.

Kingombe, C.I.B., Huys, G., Tonolla, M., Albert, M.J., Swings, J., Peduzzi, R. and Jemmi, T. (1999) PCR detection, characterization, and distribution of virulence genes in *Aeromonas* spp. Appl. Environm. Microbiol. 65, 5293-5302.

Kirov, S.M., Tassell, B.C., Semmler, A.B.T., O'Donovan, L.A., Rabaan, A.A. and Shaw, J.G. (2002) Lateral flagella and swarming motility in *Aeromonas* species. J. Bacteriol. 184, 547-555.

Overman T.L. and Janda, J.M. (1999) Antimicrobial susceptibility patterns of *Aeromonas jandaei*, *A. schubertii*, *A. trota*, and *A. veronii* biotype veronii. J. Clin. Microbiol. 37, 706-708.

Pidiyar,V., Kaznowski, A., Narayan, N.B., Patote, M. and Shouche, Y.S. (2002). *Aeromonas culicicola* sp. nov., from the midgut of *Culex quinquefasciatus*. Int. J. Syst. Evol. Microbiol. 52, 1723-1728.

Rose, J.M., Houston, C.W., Coppenhaver, D.H., Dixon, J.D. and Kurosky, A. (1989) Purification and chemical characterization of a cholera toxin-cross-reactive cytolytic enterotoxin produced by a human isolate of *Aeromonas hydrophila*, Infect. Immun. 57, 1165-1169.

Sarma, P.S. (2002) *Aeromonas jandaei* cellulitis and bacteremia in a man with diabetes. Am. J. Med. 112, 325.

Sha, J., Kozlova, E.V. and Chopra, A.K. (2002) Role of various enterotoxins in *Aeromonas hydrophila*-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxin activity. Infect. Immun. 70, 1924-1935.

Soler, L., Figueras, M.J., Chacón, M.R., Guarro, J. and Martinez-Murcia, A.J. (2003) Comparison of three molecular techniques for typing *Aeromonas popoffii* isolates. Antonie van Leweenhoek. (In press).

Soler, L., Figueras, M.J., Chacón, M.R., Vila, J., Marco, F., Martínez-Murcia, A.J. and Guarro, J. (2002). Potential virulence and antimicrobial susceptibility of *Aeromonas popoffii* recovered from freshwater and seawater. FEMS Immun. Med. Microbiol. 32, 243-247

Valera, L. and Esteve, C. (2002) Phenotypic study by numerical taxonomy of strains belonging to the genus *Aeromonas*. J. Appl. Microbiol. 93, 77-95.

Vila, J., Marcos, M.A. and Jimenez De Anta, M.T. (1996). A comparative study of different PCR-based DNA fingerprinting techniques for typing of the *Acinetobacter calcoaceticus-A. baumannii* complex. J. Med. Microbiol. 44, 482-489.

Table 1. Strains used in the present study.

***A. jandaei* strains**	**Other collection designations**	**Origin**
433c		Feces. 54 year-old man. SPAIN
400c		Feces. Child with simultaneous infections with *A. caviae* and *C. jejuni*. SPAIN
141c		Feces. Man with fever and diarrhoea. SPAIN
371c		Feces. 71 year-old man. SPAIN
578c		Feces. Traveller's diarrhoea. SPAIN
441c		Feces. Child with simultaneously infection with *C. jejuni*. SPAIN
574c		Bile. Clinical sample. SPAIN
764		Seawater. Badalona. SPAIN
799		River mouth. Seawater. La Platjola. SPAIN
392		River mouth. Muga river mouth. SPAIN
566c		Feces. > 65 year-old man. SPAIN
613		River water. Ter River. SPAIN
344		Freshwater. Banyoles Lake. SPAIN
343c		Feces. Clinical sample. SPAIN
MDL-90A-5119		Wound. Clinical sample. Provided by Dr. J.M Janda.
AER 206		Feces. < 1year-old female with gastroenteritis. New York. USA (Carnahan et al., 1991) Provided by Dr. J.M. Janda.
CECT4231		Prawn. Hawaii. Isolated from a prawn. Honolulu (Carnahan et al., 1991). Provided by CECT.
1E	AS167; CECT4231; ATCC49572	Prawn. Hawaii. Isolated from a prawn. Honolulu (Carnahan et al., 1991). Provided by Dr. C. Esteve
CECT 4228^{T}		Feces. Male with diarrhoea and dehydratation. Oregon. USA (Carnahan et al., 1991). Provided by CECT.
3E	CECT4228^{T}; ATCC49568^{T}	Feces. Male with diarrhoea and dehydratation. Oregon. USA (Carnahan et al., 1991). Provided by Dr. C. Esteve.
AER 14		Blood. 71-year-old male with adult-onset diabetes mellitis and multiple myeloma. New York. USA. (Carnahan et al., 1991) PProvided by Dr. J.M. Janda.
46E	AS14 (AER14); CECT4838; ATCC49569	Blood. 71-year-old male with adult-onset diabetes mellitis and multiple myeloma. New York. USA. (Carnahan et al., 1991) Provided by Dr. C. Esteve.
A179		Feces. Switzerland. Provided by Dr. CIB. Kingombe.
18E	A179; CECT4815; LMG13064	Feces. Switzerland. Provided by Dr. C. Esteve.
15E	A92; CECT4818; CDC2417-86	Rainbow trout. Kidney. Provided by Dr. C. Esteve
AER235		Blood. 96-year-old male with an acute myelogenous leukemia. New York. USA. (Carnahan et al., 1991). Provided by Dr. J.M. Janda.
CECT4229	WRII/4658	Wound below left eye. 10 year-old male with an injury under the lacrimal sac. Maryland. USA. (Carnahan et al., 1991, Joseph et al., 1991) Provided by CECT.
AH31b		Crab. Norway. (Granum et al., 1998)
A919		Feces. USA. Provided by Dr. C.I.B. Kingombe
NMRI-6		Leg wound. Soft tissue of the leg. USA navy diver with a simultaneous infection with *A. hydrophila*. Infection secondary to a puncture wound sustained during diver training in the Anacostia River (near Washington DC). (Carnahan et al., 1991) Provided by Dr. J.M. Janda.

Table 1

***A. jandaei* strains**	**Other collection designations**	**Origin**
110E	NMRI-6; CECT4901; ATCC49571	Leg wound. Soft tissue of the leg. US navy diver with a simultaneous infection by *A. hydrophila* and *A. jandaei*. Infection secondary to a puncture wound sustained during diver training in the Anacostia River (near Washington DC). (Carnahan et al., 1991) Provided by Dr C. Esteve.
17E	CDC1530-81; CECT4813	Feces. Children. Georgia. USA (Carnahan et al., 1991).Provided by Dr. C. Esteve.

Control strains	**Origin**
Pseudomonas aeruginosa ATCC25668	Human exhudate
Escherichia coli O:157H7 CECT 4076	Enterohemorragic
E. coli O:128K67 CECT742	Enteropathogenic
E. coli O:25 H42 CECT685	Entero toxygenic
E.coli O:158 K-:H34 CECT744	Feces
Vibrio parahaemolyticus CECT 612	Sea water
V. parahaemolyticus CECT 613	Marine plankton
V. parahaemolyticus CECT 611	Sea water
V. parahaemolyticus CECT 588	Mussels
Salmonella typhimurium ATCC 14028	Septicemic liver from bovine

ATCC, American Type Culture Collection Rockville, USA; CECT, Colección Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; CDC, Centers for Disease Control, United States Public Health Service, Atlanta, USA; LMG, Culture Collection of the Laboratorium voor Microbiologie Gent, Universiteit Gent, Ghent, Belgium; E, Dr. C. Esteve collection.

Table 3. Distribution of virulence factors among *A. jandaei* isolates

***A. jandaei* isolates**	**PCR (Dot-blot)**			**PCR**
	ast	***laf*A**	**aerolysin/hemolysin**	***alt***
Clinical isolates				
433c	- (-)	- (-)	- (-)	-
400c	- (-)	- (-)	- (-)	-
141c	- (-)	- (+)	+ (+)	-
371c	- (-)	- (-)	- (-)	-
578c	- (-)	- (-)	- (-)	-
441c	- (-)	- (-)	- (-)	+
566c	- (-)	- (-)	+ (+)	-
343c	- (-)	- (+)	+ (+)	-
CECT4228^T	- (-)	+ (+)	+ (+)	-
AER 206	- (-)	+ (+)	- (-)	-
17E	+ (+)	+ (+)	- (-)	+
A919	- (-)	+ (+)	+ (+)	-
574c	- (-)	- (+)	+ (+)	-
MDL-90A-5119	- (-)	+ (+)	- (-)	-
AS14 (AER14)	- (-)	- (+)	- (-)	-
AER235	- (-)	- (+)	+ (+)	-
CECT4229	- (-)	- (-)	- (-)	-
NMRI-6	- (-)	+ (+)	+ (+)	-
	1/18 (5.5%)	11/18 (61.1%)	8/18 (44.4%)	2/18 (11.1%)
Environmental isolates				
764	- (-)	- (-)	- (-)	-
799	- (-)	- (+)	+ (+)	-
392	- (-)	- (-)	- (-)	-
613	- (-)	- (-)	- (-)	-
344	- (-)	+ (+)	- (-)	-
	0/5 (0%)	2/5 (40%)	1/5 (20%)	0/5 (0%)
Animal isolates				
CECT4231 (AS167)	- (-)	- (+)	+ (+)	-
15E	- (-)	- (+)	+ (+)	+
AH31b	- (-)	+ (+)	- (-)	-
	0/3 (0%)	3/3 (100%)	2/3 (66.6%)	1/3 (33.3%)
TOTAL	**1/26 (3.8%)**	**16/26 (61.5%)**	**11/26 (42.3%)**	**3/26 (11.5%)**
Control strains				
Salmonella typhimurium ATCC 14028	- (-)	- (-)	- (-)	-
Escherichia coli O:157 H7 CECT4076	- (-)	- (-)	- (-)	+
E. coli O:25 H42 CECT 685	- (-)	- (-)	- (-)	+
E. coli O:128 K67 CECT 742	- (-)	- (-)	- (-)	+
E. coli O:158 K:H34 CECT 744	- (-)	- (-)	- (-)	+
Pseudomonas aeruginosa CECT 118	- (-)	- (-)	- (-)	-
Vibrio parahaemolyticus CECT 588	- (-)	- (-)	- (-)	+
V. parahaemolyticus CECT 611	- (-)	- (-)	- (-)	+
V. parahaemolyticus CECT 612	- (-)	- (-)	- (-)	+
V. parahaemolyticus CECT 613	- (-)	- (-)	- (-)	+

Table 2. Selected sequences and primers designed for the detection of *Aeromonas* spp. virulence genes

Gene	Selected primers	Primer site location		Amplified Fragment size in bp (gene)	Sequence accession number	Organism (strain)	Reference
ast	ast-F: 5'-ATCGTCAGCGACAGCTTCTT-3' ast-R: 5'-CTCATCCCTTGGCTTGTTGT-3'	2411-2430	2895-2914	504 (*ast*)	AF419157	*A. hydrophila* (SSU)	Sha et al., 2002
alt	alt-F : 5'-AAAGCGTCTGACAGCGAAGT-3' alt-R : 5'-AGCGCATAGGCGTTCTCTT-3'	163-172	464-482	320 (*alt*)	L77573	*A. hydrophila* (SSU)	Chopra et al., 1996
aerolysin/hemolysin *	aer-F: 5'-CCTATGGCCTGAGCGAGAAG-3' aer-R: 5'-CCAGTTCCAGTCCCACCACT-3'	1295-1314	1706-1725	431 (*aer*A)	M16495	*A. hydrophila* (Ah65)	Howard et al. 1987
		1188-1207	1598-1619		X65044	*A. hydrophila* (28SA)	Hirono et al. 1992
		905-924	1316-1335		X65045	*A. hydrophila* (AH1)	Hirono et al. 1992
		1189-1208	1621-1640		X65043	*A. hydrophila* (28SA)	Hirono et al. 1992
		1647-1666	2058-2077		M84709	*A. hydrophila* (SSU)	Chopra et al. 1993
		971-990	1382-1401		U40711	*A. caviae* (A1833)	Wang et al. 1996
		1160-1179	1571-1590		AF064068	*A. trota* (ATCC49659)	Khan et al. 1998
		1625-1644	2036-2055		Y00559	*A. sobria* (AB3)	Husslein et al. 1988
		1103-1123	1514-1533		X65046	*A. sobria* (33)	Hirono et al. 1992
		1105-1124	1516-1535		X65048	*A. salmonicida* (17-2)	Hirono and Aoki 1993
*laf*A	lafA-F: 5'-CCAACTT(T/C)GC(C/T)TC(T/C)(C/A)TGACC-3' lafA-R: 5'-TCTTGGTCAT(G/A)TTGGTGCT(C/T)-3'	826-845	1548-1569	743 (*laf*A1)	AF348135	*A. caviae* (Sch3)	Kirov et al., 2002
		1840-1859	2579-2598	736 (*laf*A2)			

221-240	939-958	737 (*lafA*)	AY028400	*A. hydrophila*	Gavin et al., 2002

*aerolysin/hemolysin primers have been published by Soler et al. (2002) and Chacon et al. (2003).

Table 4. Combinations of the virulence genes found on the *A. jandaei* isolates.

Origin	number of isolates	*lafA*	aerolysin/hemolysin	*alt*	*lafA* + aerolysin/hemolysin	*ast* + *lafA* + *alt*	*lafA* + aerolysin/hemolysin + *alt*	none
Human intestinal	12	1 (8.3%)	1 (8.3%)	1 (8.3%)	4 (33.4%)	1 (8.3%)		4 (33.4%)
Human extra-intestinal	6	2 (33.3%)			3 (50%)			1 (16.7%)
Environment	5	1 (20%)			1 (20%)			3 (60%)
Animal	3	1 (33.3%)			1 (33.3%)		1 (33.3%)	
TOTAL	26	5 (19.3%)	1 (3.8%)	1 (3.8%)	9 (34.7%)	1 (3.8%)	1 (3.8%)	8 (30.8%)