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ARTICLE

Potential pathogenicity of *Aeromonas hydrophila* complex strains isolated from clinical, food, and environmental sources

Vicenta Albarral, Ariadna Sanglas, Montserrat Palau, David Miñana-Galbis, and M. Carmen Fusté

Abstract: *Aeromonas* are autochthonous inhabitants of aquatic environments, including chlorinated and polluted waters, although they can also be isolated from a wide variety of environmental and clinical sources. They cause infections in vertebrates and invertebrates and are considered to be an emerging pathogen in humans, producing intestinal and extra-intestinal diseases. Most of the clinical isolates correspond to *A. hydrophila*, *A. caviae*, and *A. veronii* bv. Sobria, which are described as the causative agents of wound infections, septicaemia, and meningitis in immunocompromised people, and diarrhoea and dysenteric infections in the elderly and children. The pathogenic factors associated with *Aeromonas* are multifactorial and involve structural components, siderophores, quorum-sensing mechanisms, secretion systems, extracellular enzymes, and exotoxins. In this study, we analysed a representative number of clinical and environmental strains belonging to the *A. hydrophila* species complex to evaluate their potential pathogenicity. We thereby detected their enzymatic activities and antibiotic susceptibility pattern and the presence of virulence genes (*aer*, *alt*, *ast*, and *ascV*). The notably high prevalence of these virulence factors, even in environmental strains, indicated a potential pathogenic capacity. Additionally, we determined the adhesion capacity and cytopathic effects of this group of strains in Caco-2 cells. Most of the strains exhibited adherence and caused complete lysis.

Key words: Aeromonas hydrophila complex, virulence factors, enterotoxins, Caco-2.

Résumé : Les membres d'*Aeromonas* sont des habitants autochtones d'environnements aquatiques, dont les eaux chlorées et polluées, bien qu'on puisse également les isoler d'une variété de sources environnementales et cliniques. Ils causent des infections chez les vertébrés et les invertébrés et on les considère comme des pathogènes émergents pour l'humain, chez lequel ils provoquent des maladies intestinales et extra-intestinales. La plupart des isolats cliniques correspondent à *A. hydrophila*, *A. caviae* et *A. veronii* bv. Sobria, qu'on met en cause dans des infections de lésions, la septicémie et la méningite chez les personnes immununocompromises, et la diarrhée et la dysenterie chez les personnes âgées et les enfants. Les facteurs pathogènes associés à *Aeromonas* touchent de multiples aspects, dont les éléments structuraux, les sidérophores, les mécanismes de détection du quorum, les systèmes de sécrétion, les enzymes extracellulaires et les exotoxines. Dans la présente étude, nous avons analysé un nombre représentatif de souches cliniques et environnementales appartenant au complexe de l'espèce *A. hydrophila* afin d'évaluer leur pathogénicité potentielle. À cet égard, nous avons mesuré leurs activités enzymatiques et leur profil de susceptibilité aux antibiotiques, et détecté la présence de gènes de virulence (*aer*, *alt*, *ast* et *ascV*). La prévalence remarquablement élevée de ces facteurs de virulence, même chez des souches environnementales, laisse croire à un potentiel pathogénique latent. En outre, nous avons mesuré la capacité d'adhérence et les effets cytopathiques de ce groupe de souches chez des cellules Caco-2. La plupart des souches ont su adhérer aux cellules et les lyser complètement. [Traduit par la Rédaction]

Mots-clés : complexe d'*Aeromonas hydrophila*, facteurs de virulence, Caco-2, enterotoxins

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Introduction

The genus *Aeromonas* Stanier 1943 belongs to the family *Aeromonadaceae* within the class *Gammaproteobacteria* (Martin-Carnahan and Joseph 2005). *Aeromonas* are autochthonous inhabitants of aquatic environments, including chlorinated and polluted waters, although they can also be isolated from a wide variety of environmental and clinical sources. They cause infections in vertebrates and invertebrates, such as frogs, birds, various fish species, and domestic animals. *Aeromonas* is considered to be an emerging pathogen in humans (Janda and Abbott 1998), producing intestinal and extra-intestinal diseases. Most of the clinical isolates correspond to *A. hydrophila*, *A. caviae*, and *A. veronii* bv. Sobria, which are described as the causative agents of wound infections, septicaemia, and meningitis in immunocompromised people, and diarrhoea and dysenteric infections in the elderly and children (Kirov et al. 2004).

The pathogenic factors associated with *Aeromonas* are multifactorial and involve structural components (flagella, fimbriae, capsule, S-layers, lipopolysaccharide, and outer membrane proteins), siderophores, quorum-sensing mechanisms, secretion systems (T2SS, T3SS, T4SS, and T6SS), extracellular enzymes (proteases with caseinase and elastase activities, lipases such as phospholipases and cholesterol acyltransferase), and exotoxins (Tomás 2012; Beaz-Hidalgo and Figueras 2013; Grim et al. 2013).

Two main types of enterotoxins have been described in this genus: cytotonic and cytotoxic. Cytotoxic enterotoxins produce severe damage in the small intestine mucous membrane. Aer toxin (also named aerolysin or Act), the main enterotoxin described in *Aeromonas*, shows haemolytic, cytotoxic, and enterotoxic activities; is thermolabile; inhibits phagocytosis; and increases the TNF-α and interleukin IL-1β intracellular levels in macrophages. The *Aeromonas* cytotonic enterotoxins, such as Alt (thermolabile) and Ast (thermostable), do not destroy the intestinal mucous membrane. Instead, they increase the intracellular levels of prostaglandin in the enterocytes and activate the adenylate cyclase, thereby increasing cAMP synthesis and triggering a fluid secretion, which causes liquid diarrhoea (Aguilera-Arreola et al. 2007; Chang et al. 2008; Janda and Abbott 2010; Ottaviani et al. 2011). Some strains possess a type III secretion system (T3SS), which enables many pathogenic Gram-negative bacteria to secrete and inject pathogenicity proteins (effectors) into the cytosol of eukaryotic cells via needle-like structures called needle complexes or injectisomes. T3SS was first identified in pathogenic strains of *Yersinia* spp. (Michiels et al. 1990). In *Aeromonas*, T3SS has been identified in *A. salmonicida*, *A. caviae*, *A. veronii* bv. Sobria, and *A. hydrophila* (Vilches et al. 2004; Krzymińska et al. 2012). Since this mechanism correlates with bacterial pathogenicity, its presence is used as a general indicator of bacterial virulence.

Potential pathogenicity of *Aeromonas* strains has also been associated with adherence and cytopathogenic effects in Hep-2 (derived from a human laryngeal carcinoma), Vero (derived from normal kidneys of the African green monkey), and HT29 and Caco-2 (both derived from human colorectal adenocarcinoma) cell lines (Snowden et al. 2006; Couto et al. 2007; Janda and Abbott 2010; Ottaviani et al. 2011).

The *A. hydrophila* species complex (AHC) consists of a group of genetically closely related species that are phenotypically difficult to discriminate (Miñana-Galbis et al. 2002), which includes *A. hydrophila*, with 3 subspecies (*A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *dhakensis*, and *A. hydrophila* subsp. *ranae*), and *A. bestiarum*, *A. popoffii*, and *A. salmonicida*, divided into 5 subspecies (*A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *pectinolytica*, and *A. salmonicida* subsp. *smithia*) (Miñana-Galbis et al. 2002; Martin-Carnahan and Joseph 2005; Fusté et al. 2012). Two additional species have been recently described in this group, *A. aquariorum* and *A. piscicola* (Martínez-Murcia et al. 2008; Beaz-Hidalgo et al. 2009), while *A. hydrophila* subsp. *dhakensis* and *A. aquariorum* have been reclassified in *A. dhakensis* (~~Beaz-Hidalgo and Figueras 2013~~).

In this study, we analysed a representative number of clinical, food, and environmental strains belonging to the AHC to evaluate their potential pathogenicity. We thereby detected their enzymatic activities and antibiotic susceptibility pattern, the presence of virulence genes (*aer*, *alt*, *ast* and *ascV*), and their adhesion capacity and cytopathic effects in Caco-2 cells.

Materials and methods

Bacterial strains

A total of 127 strains belonging to the "*Aeromonas hydrophila* complex" (26 *A. bestiarum*, 15 *A. dhakensis*, 29 *A. hydrophila*, 11 *A. piscicola*, 6 *A. popoffii*, and 40 *A. salmonicida*), isolated from fresh waters, marine bivalve molluscs, and clinical samples, were analysed in this study (supplementary Table S1[1]). Strains were obtained from different culture collections, our own collection, or donated by different researchers. These strains were previously identified in a taxonomic study on the basis of multilocus sequence analysis of *cpn60*, *dnaJ*, *gyrB*, and *rpoD* genes (Fusté et al. 2012; Albarral 2013). Strains were grown on tryptic soy agar (TSA) plates with 1% NaCl, at 25–30 °C. Cultures were maintained in TSA (1% NaCl) tubes. For long-term preservation, strains were kept at –80 °C using glycerol 20% (*v*/*v*).

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjm-2015-0466.

Table 1. List of primers used for PCR amplification.

Gene	Primer	Sequence 5′→3′	Reference	Size product (bp)
aer	aer-f-d	CCTAYRGCCTGAGCGAGAAG	Modified from Soler et al. 2002*	402
	aer-r-d	CCAGTTCCARTCCSACCACT		
	F-Ah-a	ATCATATCCGGCCTGCTGA	This study	1161
alt	altF2	AAAGCGTCTGACAGCGAAGT	Aguilera-Arreola et al. ~~2007~~	320
	altR2	AGCGCATAGGCGTTCTCTT		
ast	astF1	TCTCCATGCTTCCCT TCCACT	Sen and Rodgers 2004	331
	astR1	GTGTAGGGATTGAAGAAGCCG		
	astF2	ATCGTCAGCGACAGCTTCTT	Aguilera-Arreola et al. ~~2007~~	504
	astR2	CTCATCCCTTGGCTTGTTGT		
ascV	ascV-F	CTCGAACTGGAAGAGCAGAATG	Martino et al. 2011	577
	ascV-R	GAACATCTGGCTCTCCTTCTCGATG		
	ascV-R+	GATGGTGATGATGAGGAAGATG	This study	397

*Degenerate primers were designed in this study.

Enzymatic activities

Casein hydrolysis, DNase, hemolysis of sheep blood, and starch hydrolysis tests were performed as previously described (Reddy et al. 2007). Gelatinase activity was determined on TSA supplemented with 0.4% gelatin (Pickett et al. 1991; Miñana-Galbis et al. 2004). Elastase production was assessed according to Popoff and Lallier (1984). Lecithinase activity was tested following the recommendations of Esselmann and Liu (1961). All media used contained 1% (*w*/*v*) NaCl (Holt et al. 1994). Incubations were performed at 30 °C, with the exception of the psychrotrophic strains, which were grown at 25 °C.

Antibiotic susceptibility tests

Bacterial susceptibility to different antibiotics was determined using the disc diffusion method (Murray and Baron 2003). Antimicrobial agents tested included 8 β-lactams, 4 aminoglycosides, and 8 antibiotics of other groups. Antibiotics and the concentrations tested are shown in supplementary Table S2[1].

Detection of virulence genes

The presence of genes encoding exotoxins, namely aerolysin Aer, heat-labile enterotoxin Alt, heat-stable enterotoxin Ast, and the T3SS inner membrane component (*ascV*) was determined by polymerase chain reaction (PCR). Genomic DNA extraction was carried out using a commercial kit (Realpure genomic DNA extraction kit, Durviz). Table 1 shows the primers used for the PCR.

PCR amplification for *aer* was carried out in a total volume of 50 μL containing 400 ng of genomic DNA as a template, PCR buffer (500 mmol·L^{-1} KCl, 150 mmol·L^{-1} Tris–HCl (pH 8.0), 15 mmol·L^{-1} $MgCl_2$), 0.3 mmol·L^{-1} dNTPs, 2.5 U of AmpliTaq Gold DNA polymerase, and 1 μmol·L^{-1} (each) primer (aer-f-d and aer-r-d). The reaction mixtures were subjected to the following thermal cycling program: denaturation at 95 °C for 5 min; 35 cycles of extension at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 10 min. In the cases where the gene was not detected, a 2-stage nested PCR analysis was performed to increase its sensitivity and specificity. In the first round of PCR, a fragment containing 1161 bp was amplified using the F-Ah-a and aer-r-d primers, with the same conditions and temperature cycles as mentioned above. For the second round of PCR, primers aer-f-d and aer-r-d, which targeted an internal fragment of the first-round PCR product, were employed using 50 ng of PCR product as a template.

PCR for the *alt* gene was performed in a final volume of 50 μL containing 200 ng of genomic DNA as a template, PCR buffer (500 mmol·L^{-1} KCl, 150 mmol·L^{-1} Tris–HCl (pH 8.0), 15 mmol·L^{-1} $MgCl_2$), 0.8 mmol·L^{-1} dNTPs, 1.25 U of AmpliTaq Gold DNA polymerase, and 0.2 μmol·L^{-1} (each) primer (altF2 and altR2). The reaction mixtures were subjected to the following thermal cycling program: denaturation at 95 °C for 5 min; 35 cycles of extension at 94 °C for 45 s, 52 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 10 min. When the gene was not detected, amplification was performed using another pair of primers (altF1 and altR1) with the same thermal cycling program.

PCR amplification of the *ast* gene was carried out in a total volume of 50 μL containing 450 ng of genomic DNA as a template, PCR buffer (500 mmol·L^{-1} KCl, 150 mmol·L^{-1} Tris–HCl (pH 8.0), 15 mmol·L^{-1} $MgCl_2$), 0.4 mmol·L^{-1} dNTPs, 1.25 U of AmpliTaq Gold DNA polymerase, and 0.8 μmol·L^{-1} (each) primer. The reaction mixtures were subjected to the following thermal cycling program: denaturation at 95 °C for 5 min; 35 cycles of extension at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 10 min.

A PCR reaction for *ascV* was conducted as described by Martino et al. (2011) in the 59 strains selected for their adherence capacity and cytopathic effect properties. An alternative PCR was performed to detect *ascV* in negative strains with a new primer (ascV-R+). The reactions were carried out in a final volume of 50 μL containing 250 ng of genomic DNA as a template, PCR buffer (500 mmol·L^{-1} KCl, 150 mmol·L^{-1} Tris–HCl (pH 8.0), 15 mmol·L^{-1} $MgCl_2$), 1 mmol·L^{-1} dNTPs, 2.5 U of AmpliTaq Gold DNA polymerase,

and 1 μmol·L^{-1} (each) primer (ascV-F and ascV-R+). The reaction mixtures were subjected to the following thermal cycling program: denaturation at 95 °C for 5 min; 35 cycles of extension at 95 °C, 54 °C, and 72 °C for 1 min; and final extension at 72 °C for 5 min.

Adhesion capacity and cytopathic effect

According to the previous results obtained with the assayed virulence factors (enzyme activities, profiles of antibiotic sensitivity, and detection of *aer*, *alt*, and *ast* genes), we selected 59 strains from a total of 127 (supplementary Table S1[1]) and determined their adherence capacity and cytopathic effect on the cellular line Caco-2. In this selection we included strains with a variable range of virulence.

Strains were cultured on double concentrate tryptic soy broth without dextrose and incubated at 30 °C overnight. The strains were then washed twice with Dulbecco's phosphate-buffered saline (PBS-D) (Invitrogen 14190-094). Bacterial cell growth was estimated by viable counting and optical density, until reaching a biomass of 1 × 10^8 CFU·mL^{-1} (OD = 0.35 at 580 nm). Our aim was to achieve a Caco-2 cell to bacterial cell ratio of 1:10 (Couto et al. 2007).

A cell monolayer was grown in 75 cm^2 flasks (Cultek 430641) containing DMEM (Dulbecco's Modified Eagle Medium high glucose, glutaMax) (Invitrogen 31966-021) supplemented with 10% FBS (heat inactivated fetal bovine serum) (Invitrogen 10500-064) and 1% penicillin–streptomycin (Invitrogen 15140-122), and was then incubated at 37 °C and 5% CO_2 in a Nuaire incubator (NU-425-400E). The cell culture medium was changed every 48 h (Freshney 2005). The Caco-2 monolayer was used at a semiconfluence of 80%. Thus, 1 mL of a Caco-2 cell suspension at a concentration of 3 × 10^5 cells·mL^{-1} was grown on 13 mm diameter glass coverslips placed in 24-well tissue culture plates (Cultek 3524) in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin and was incubated in a 5% CO_2 atmosphere at 37 °C. For the adhesion assays, the monolayers were used 24 h after seeding. The monolayers were washed twice with PBS-D, inoculated in triplicate with 1 mL of bacterial suspension containing 1 × 10^7 CFU·mL^{-1}, and incubated for 90 min at 37 °C and 5% CO_2 (Guimarães et al. 2002; Couto et al. 2007). After 2 washes with PBS-D to remove nonadherent bacteria on the cell monolayers, cell cultures were fixed with methanol for 2 min and stained with Giemsa solution (Merck) (1:10 with tap water) and washed twice with water. The coverslips were removed from the wells, mounted on glass slides, and examined by optical microscopy.

Results and discussion

The majority of published studies on virulence factors in the genus *Aeromonas* are based on the detection of strains isolated from diverse backgrounds (Kingombe et al. 1999; Chacón et al. 2003; Kingombe et al. 2004; Ottaviani et al. 2011), specific sources such as treated and untreated water for human consumption (Scoaris et al. 2008; Carvalho et al. 2012), food (Chang et al. 2008), fish and seafood (Nam and Joh 2007; Martino et al. 2011; Sreedharan et al. 2013), diarrhoeal stools (Kannan et al. 2001), etc. In this work we determined the presence of virulence factors in a set of 127 AHC strains of different origins (environmental, food, and clinical) to verify if there were any differences among them and to determine their potential pathogenicity.

Enzymatic activities

The majority of strains included in this study were positive for the enzymatic activities tested (Table 2 and supplementary Table S1[1]). Most strains showed proteolytic (caseinase, gelatinase) and saccharolytic (amylase) activity: 96% hydrolyzed casein (92.5%–100%, depending on the species), while 2 *A. hydrophila* strains (AE215, AE216) and 3 *A. salmonicida* strains (CECT 5219, CECT 896^T, LMG 14900^T) were negative. In contrast, other authors detected casein hydrolysis in only 59%–74% of the strains (Soler et al. 2002; Castro-Escarpulli et al. 2003; Chacón et al. 2003; Zacaría et al. 2010), perhaps due to the use of different culture media, such as Luria broth or Mueller–Hinton supplemented with 1%–10% skim milk (Castro-Escarpulli et al. 2003; Zacaría et al. 2010), or other methods such as the azocasein method (Soler et al. 2002; Chacón et al. 2003). T2

Gelatin hydrolysis was detected in 96.9% of strains (93.1%–100%, depending on the species), with only 4 negative strains (*A. hydrophila* AE215, *A. hydrophila* AE216, *A. bestiarum* CECT 5217, *A. salmonicida* subsp. *achromogenes* LMG 14900^T), coinciding with other studies (Chacón et al. 2003; Beaz-Hidalgo et al. 2010).

Amylase activity, although considered a virulence factor (Campbell et al. 1990; Pemberton et al. 1997; Emele 2001), is usually described only in taxonomic studies (Miñana-Galbis et al. 2002; Valera and Esteve 2002). In our case, the percentage of strains showing this activity was 97% (93.1%–100%, depending on the species), with only 2 *A. hydrophila* (AE215, AE216) and the *A. salmonicida* subsp. *achromogenes* LMG 14900^T strains negative, results similar to those described in the literature (Miñana-Galbis et al. 2002; Valera and Esteve 2002).

The determination of lipolytic activity is controversial, because different lipases can be detected depending on the medium used. According to Merino et al. (1999), *Aeromonas* can produce 2 types of phospholipases: phospholipase A1, which is cytotoxic and hemolytic, and whose activity is determined in tributyrin agar, and phospholipase C or lecithinase, whose activity is detected using egg yolk agar. Phospholipase C displays cytotoxic and hemolytic activities in fish but not in sheep blood. We detected lecithinase activity in 89.06% of the AHC strains (84.6%–100%, depending on the species), all the *A. popoffii* strains being positive (Table 2 and supplementary Table S1[1]). In contrast, Scoaris et al. (2008) detected this activity in

Table 2. Enzymatic activities, detection of virulence genes, adhesion capacity, and cytopathic effect.

	A. bestiarum (*n* = 26)*	*A. dhakensis* (*n* = 15)*	*A. hydrophila* (*n* = 29)*	*A. piscicola* (*n* = 11)*	*A. popoffii* (*n* = 6)*	*A. salmonicida* (*n* = 40)*
Enzymatic activity						
Casein hydrolysis	100	100	93.1	100	100	92.5
DNase	88.5	100	93.1	90.9	100	92.5
Elastase	61.5	93.3	86.2	54.5	0	80
Gelatinase	96.2	100	93.1	100	100	97.5
Hemolysis	88.4	100	72.4	90.9	100	85
Lecithinase	84.6	93.3	86.2	90.9	100	95
Starch hydrolysis	100	100	93.1	100	100	97.5
Virulence gene detection						
aer	100	100	86.2	100	100	100
alt	100	100	100	100	100	100
ast	100	100	100	100	100	100
ascV†	53 (40)	33 (17)	77 (54)	100 (100)	0 (0)	20 (15)
Adherence	80	100	84	100	33	65
Cytopathic effect	93	100	92	100	33	95

Note: Results are expressed as percentages; *n*, number of strains.

*For detection of virulence gene *ascV*, and for adhesion capacity and cytopathic effect, *n* = 15 for *A. bestiarum*, *n* = 5 for *A. dhakensis*, *n* = 13 for *A. hydrophila*, *n* = 2 for *A. piscicola*, *n* = 3 for *A. popoffii*, and *n* = 20 for *A. salmonicida*.

†Results obtained using primers ascV-F and ascV-R+. Values in parentheses are results obtained using primers ascV-F and ascV-R.

only 1 out of 5 *A. hydrophila* strains, also in egg yolk agar. However, studies that detect lipolytic activity using tributyrin agar (Soler et al. 2002; Castro-Escarpulli et al. 2003; Chacón et al. 2003) or TSA supplemented with Tween 80 (Beaz-Hidalgo et al. 2009; Carvalho et al. 2012) obtained results very similar to ours, reporting in all cases values higher than 90%.

Another virulence factor analysed in this work was DNase activity, which is usually determined for taxonomic purposes (Chacón et al. 2003). The high incidence of this activity detected in our study, 93.0% (88.5%–100%, depending on the species), agrees with the results published by other authors: for example, Chacón et al. (2003) reported that 87% of *A. hydrophila*, *A. bestiarum*, *A. salmonicida*, and *A. popoffii* strains tested positive, with the DNase-encoding gene detected in 91%.

Some tables of key phenotypic tests for the differentiation of *Aeromonas* species include elastase activity (Miñana-Galbis et al. 2010; Figueras et al. 2011), but this is the first time that this test has been used in a study of virulence factors in this genus. Elastase activity is essential for the pathogenicity of *A. hydrophila* and is associated with its virulence in fish (Pemberton et al. 1997; Cascón et al. 2000; Janda and Abbott 2010). In our strains, the results were variable: all the *A. popoffii* strains tested negative, while 54.5%–93.3% of the other AHC species were positive.

Hemolytic toxin production is considered to be evidence of the pathogenic potential of *Aeromonas*. β-Hemolytic activity is usually due to aerolysins but also to enzymes with lipase activity. Discrete β-hemolysis was found in 83.6% of the strains (72.4%–100%, depending on the species), while α-hemolysis was determined in only 2.34%. All the *A. dhakensis* and *A. popoffii* and most of the *A. piscicola*, *A. bestiarum*, and *A. salmonicida* strains were hemolytic, while *A. hydrophila* showed the lowest hemolytic activity (Table 2). In contrast with our results, Huys et al. (1997) and Soler et al. (2002) described this activity in only 15% of the *A. popoffii* strains tested. Such discrepancies may be due to the blood type, media, and the incubation temperature used for detection. The results obtained with the other AHC species coincide with published data (Huys et al. 2002; Chacón et al. 2003; Martínez-Murcia et al. 2005; Martin-Carnahan and Joseph 2005; Martínez-Murcia et al. 2008; Beaz-Hidalgo et al. 2009), since more than 85% of the strains were β-hemolytic.

Antibiotic susceptibilities

We also determined the susceptibility of our strains to different antibiotics (supplementary Table S2[1]): β-lactams (Fig. 1), aminoglycosides (Fig. 2), and a mixed antibiotic group with variable activities (Fig. 3). All strains were resistant to ampicillin except for 2: *A. salmonicida* CECT 894^T and *A. popoffii* LMG 17542. *Aeromonas bestiarum*, *A. piscicola*, *A. hydrophila*, and *A. salmonicida* strains were sensitive to cefoxitin, aztreonam, imipenem, and ceftriaxone (83%–100%). *Aeromonas popoffii* strains were sensitive to cephalothin, cefoxitin, ceftriaxone, and imipenem (83%–100%), and were also the only strains resistant to aztreonam. Sensitivity to amoxicillin–clavulanic acid, ampicillin, and ticarcillin varied among the strains. *Aeromonas dhakensis* showed the highest β-lactam resistance, although 100% were sensitive to aztreonam, 67% to ceftriaxone, 53% to imipenem, and 6.7% to ticarcillin (see supplementary Table S2[1]).

Figure 2 shows strains sensitivity to aminoglycoside antibiotics. All the *A. popoffii* strains were sensitive to these antibiotics. The majority of the remaining species were sensitive to amikacin and gentamicin (73%–100%)

Fig. 1. Percentage of sensitivity to β-lactams: AMC30, amoxicillin–clavulanic acid; AM10, ampicillin; ATM, aztreonam; CF30, cephalothin; FOX30, cefoxitin; CRO30, ceftriaxone; IMP10, imipenem; TIC75, ticarcillin.

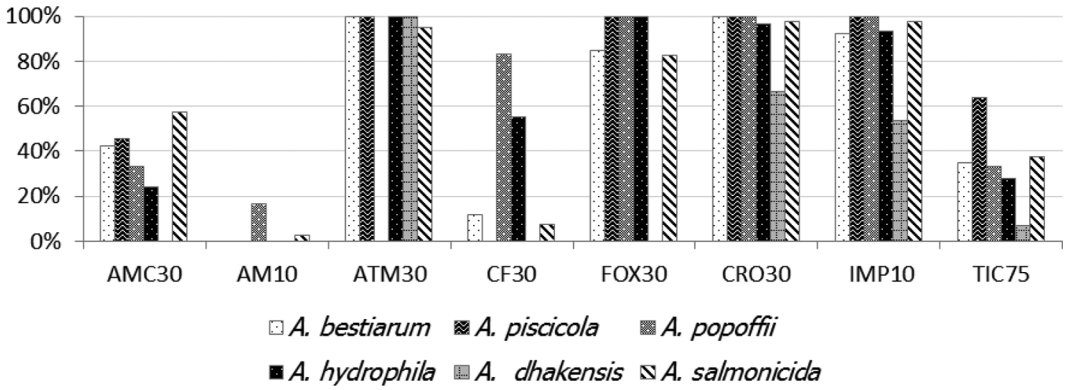
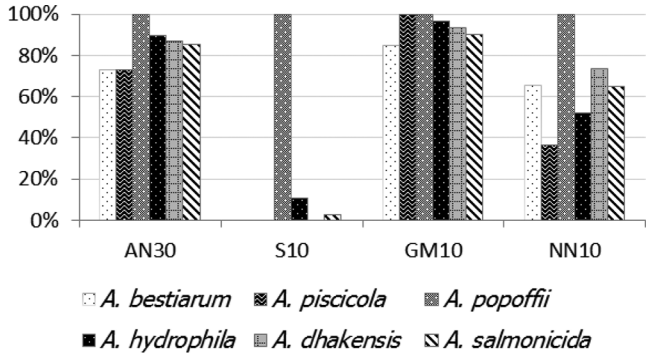


Fig. 2. Percentage of sensitivity to aminoglycosides: AN30, amikacin; S10, streptomycin; GM10, gentamicin; NN10, tobramycin.



and resistant to streptomycin (97.5%–100%), while sensitivity to tobramycin was variable.

Figure 3 shows the results obtained with the mixed antibiotic group. Most strains were sensitive to ciprofloxacin, chloramphenicol, fosfomycin, tetracycline, and trimethoprim–sulfamethoxazole (87%–100%), except *A. dhakensis*, which showed diverse sensitivity to chloramphenicol, tetracycline, and trimethoprim–sulfamethoxazole (53%–67%). Only *A. salmonicida* subsp. *achromogenes* LMG 14900^T and *A. dhakensis* LMG 19566 were sensitive to erythromycin. Variable results were obtained with colistin.

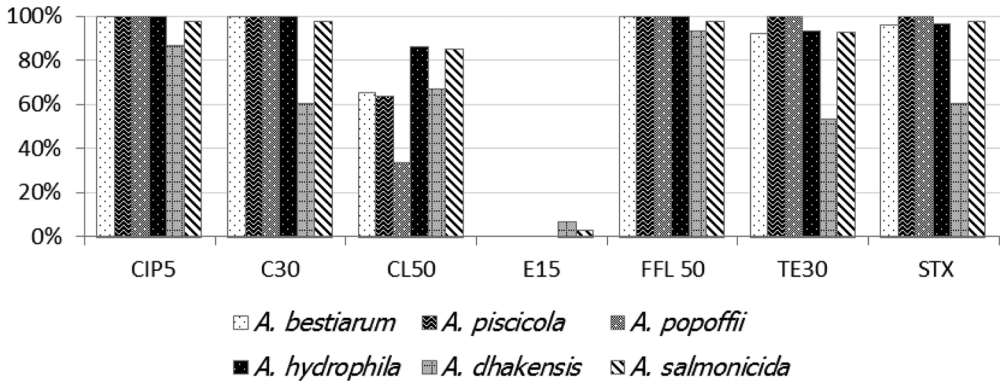
Besides helping to select the appropriate antibiotic for infection therapy, the pattern of sensitivity to antibiotics can also be used as a phenotypic trait to identify *Aeromonas* (Awan et al. 2009; Igbinosa et al. 2012). Of the 25 antibiotics used in this study, ciprofloxacin and fosfomycin were the most effective, with most of the strains analysed being sensitive (97.6% and 98.4%, respectively). On the contrary, the majority of the strains were resistant to ampicillin and erythromycin. The sensitivity pattern shown by *A. dhakensis* and *A. popoffii* was different compared with the other AHC species, independently of the origin of the strains (Figs. 1–3). These results agree with those published in other studies (Huys et al. 1997; Martínez-Murcia et al. 2008; Beaz-Hidalgo et al. 2009; Janda and Abbott 2010).

Virulence genes

Detection of virulence genes is important to determine the pathogenic potential of the *Aeromonas* strains, since enteropathogenicity in *Aeromonas* has been attributed to the production of exoenzymes, exotoxins, and adhesins, although the exact mechanism associated with virulence has not been well established (Pemberton et al. 1997). The virulence gene encoding aerolysin, the most studied in *Aeromonas*, has suffered some controversy in the bibliography, also being referred to as β-~~hemolisin~~, or cytolytic enterotoxin. Nowadays, some authors consider that both the *act* (corresponding to the cytolytic enterotoxin) and *aerA* gene (corresponding to aerolysin) encode the same protein owing to the high homology of their nucleotide sequences (Buckley and Howard 1999; Chopra and Houston 1999).

Our results show that genes encoding aerolysin (*aerA*), a cytotonic heat-labile toxin (*alt*), and cytotonic thermostable toxin (*ast*) were highly prevalent among the AHC strains, being detected in all of them except the *aerA* gene, which was not determined in 3 *A. hydrophila* (1074611, AE150, LMG 21080) and 1 *A. hydrophila* subsp. *ranae* (CIP 107985^T) strain (Table 2). Other authors (Chacón et al. 2003; Sen and Rodgers 2004; Aguilera-Arreola et al. 2007; Khajanchi et al. 2010; Ottaviani et al. 2011; Carvalho et al. 2012) also obtained high prevalence values for the *aerA* gene (>70%), although Wu et al. (2007) only detected it in 45% of the strains. The *ast* gene was detected by Wu et al. (2007), Ottaviani et al. (2011), and Carvalho et al. (2012) in only 27.5%, 25%, and 27.9% of strains, respectively. On the other hand, similar to our study, Aguilera-Arreola et al. (2007) and Khajanchi et al. (2010) detected the *ast* gene in 80%–90% of strains. The *alt* gene detection rates published by several authors (64%–97%) (Aguilera-Arreola et al. 2007; Wu et al. 2007; Kingombe et al. 2010; Khajanchi et al. 2010; Ottaviani et al. 2011) coincide with the high prevalence found among the strains analysed in the current study.

Fig. 3. Percentage of sensitivity to other antibiotics: CIP5, ciprofloxacin; C30, chloramphenicol; CL50, colistin; E15, erythromycin; FFL 50, fosfomycin; TE30, tetracycline; STX, trimethoprim–sulfamethoxazole.



Another virulence factor is the T3SS used by Gram-negative bacteria to export and deliver effector proteins into the cytosol of host cells (Sierra et al. 2010; Piqué et al. 2015). In this study, the T3SS-encoding gene (*ascV*) was detected in 44% of the strains assayed overall, with variability among the species (Table 2, supplementary Table S3[1]): 77% *A. hydrophila*, 53% *A. bestiarum*, 33% *A. dhakensis*, 20% *A. salmonicida*, 100% *A. piscicola* (2 strains), and 0% *A. popoffii* (3 strains). Similar results (45% positive) were obtained by Krzymińska et al. (2012), but a lower prevalence was reported by Martino et al. (2011) (24%) and Castelo-Branco et al. (2015) (17%). These discrepancies could be explained by the number of strains and the *Aeromonas* species analysed in the different studies. No differences were observed between clinical and environmental strains in the present study, which is in concordance with Castelo-Branco et al. (2015).

Using PCR to detect these 4 virulence genes has limitations, since this technique can give false negatives, as observed in this work. However, we obtained more positive results than other authors, probably due to applying more than one strategy, such as the use of different pairs of primers, the addition of additives like DMSO to the PCR, or carrying out nested PCRs in cases with negative or equivocal results.

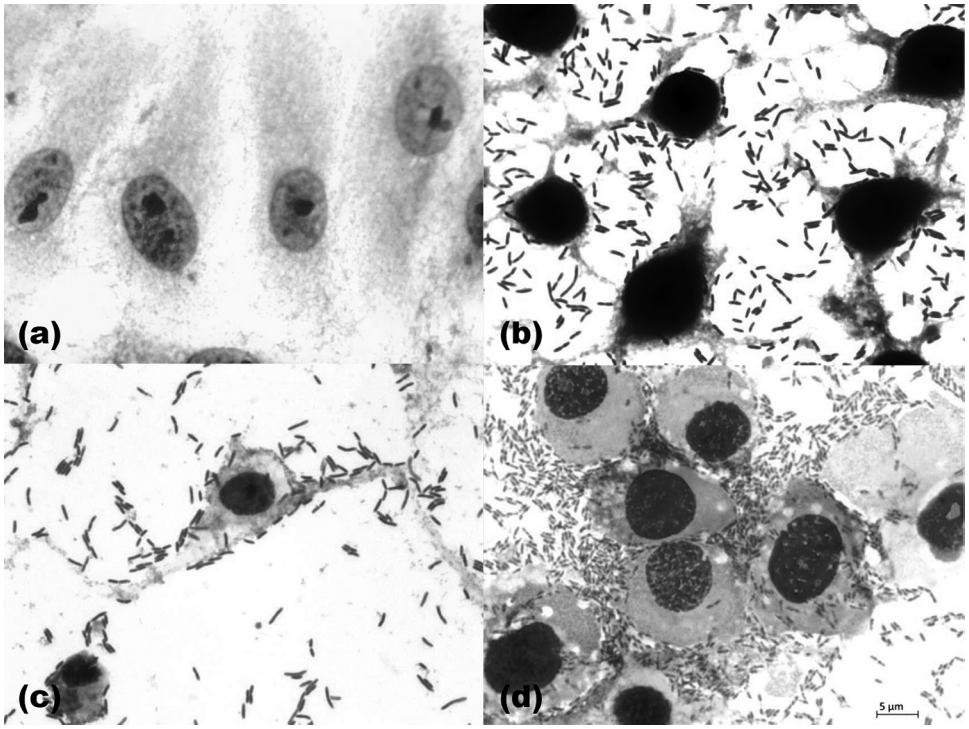
Adhesion capacity and cytopathic effect

Different authors have performed adherence, cytotoxic, and viability assays with Hep-2, Vero, HT29, and Caco-2 cell lines inoculated with *Aeromonas* strains (Kirov et al. 1995; Thornley et al. 1996; Guimarães et al. 2002; Castro-Escarpulli et al. 2003; Harf-Monteil et al. 2004; Snowden et al. 2006; Couto et al. 2007; Nam and Joh 2007; Ottaviani et al. 2011). In this study, the adhesion capacity and cytopathic effect were analysed with Caco-2 cells, using 59 strains representative of all AHC species (supplementary Table S1[1]), with different pathogenic potential. This cell line was chosen because, being derived from a colon carcinoma, it exhibits morphological and functional characteristics of the small intestinal cells. Caco-2 cells are recognized as a substitute for human intestinal epithelial cells and are used to study the adhesion of enteric pathogens (Nishikawa et al. 1994).

Adhesion capacity was considered as positive when more than 10 bacteria were found adhered to the surface of each Caco-2 cell (Ottaviani et al. 2011). Most strains (76.3%–81.4%) exhibited adherence, with variable prevalence among the AHC species (Table 2): 100% *A. dhakensis* and *A. piscicola*, 84% *A. hydrophila*, 80% *A. bestiarum*, 65% *A. salmonicida*, and 33.3% *A. popoffii* strains. It was difficult to compare our results with those of other authors (Thornley et al. 1996; Castro-Escarpulli et al. 2003; Couto et al. 2007), when the criteria establishing adherence capacity was not defined. However, we found a higher number of strains with positive results than other studies using the same criteria (>10 bacteria per host cell): around 50% of *Aeromonas* strains were reported to have adhesive ability by Kirov et al. (1995) and Snowden et al. (2006), and 33% by Ottaviani et al. (2011). Some authors differentiate 2 patterns of adhesion, diffuse and aggregative, depending on the distribution of the bacteria around the cell (Nishikawa et al. 1994; Thornley et al. 1996; Castro-Escarpulli et al. 2003; Couto et al. 2007). Cell culture analysis using optical microscopy showed that the same bacterial strain can adhere around Caco-2 cells in both a diffuse and aggregative way, depending on the bacterial cell density, thus questioning the differentiation between these 2 adherence patterns.

To evaluate the cytopathic effect, the following parameters were taken into consideration: destruction of the cell line monolayer, nuclear pyknosis, cell rounding, and presence of vacuoles in the cytoplasm (Martins et al. 2007). An example of these cytopathic activities is shown in Fig. 4. Although several authors observed complete F4
disruption of the cell monolayer after 120 min infection with several *Aeromonas* strains (Guimarães et al. 2002; Nam and Joh 2007), it is noteworthy that in our study most *Aeromonas* strains (66%) caused complete lysis of the host cells in 90 min (Table 2). For this reason, nuclear pyknosis, but not cell rounding or cytoplasm vacuolization, was observed (supplementary Table S3[1]). Although

Fig. 4. Light micrographs of Caco-2 monolayer cells infected with *Aeromonas* strains: (*a*) uninfected cells, (*b*) *A. bestiarum* CECT 4227^T showing nuclear pyknosis, (*c*) *A. bestiarum* H73 showing adhesion to and destruction of the monolayer cell line, (*d*) *A. hydrophila* CECT 5174 showing rounding and cytoplasmatic vacuoles. See supplementary Fig. S1[1] for colour version.



A. salmonicida strains showed a lower percentage of adherence to the cell line (65%) than the majority of the species studied, probably because the temperature used for infection (37 °C) was higher than the optimum growth temperature for some *A. salmonicida* strains, their cytopathic effect was higher (95% of strains). No differences were observed between strains of different origin, as observed by Couto et al. (2007), in contrast with Ottaviani et al. (2011), who found a higher adherence in environmental strains than in clinical and food strains, although these authors used a different cell line (Hep-2). Additionally, with the possible exception of *A. hydrophila*, in this study no correlation was observed between *ascV* detection and adherence and cytopathic effect, which is in contrast with Krzymińska et al. (2012), who showed a strong association between this gene and the cell-contact cytotoxicity.

Conclusions

In our study a very high prevalence of virulence-related enzymatic activities was detected in all the species belonging to the *Aeromonas* phenotypic group (AHC), except for that of elastase. Virulence genes *aerA*, *alt*, and *ast* were detected in all strains of the AHC species, with the exception of 4 *aerA* negative *A. hydrophila* strains. However, *ascV* was less prevalent (44%), although it was detected in all AHC species, except *A. popoffii*. Most strains exhibited an adhesion capacity and (or) cytopathic effect on Caco-2 cells, except *A. popoffii*. No differences in virulence factors were found among our strains with respect to the source of isolation (environmental, food, and clinical). In conclusion, the *Aeromonas* population studied showed a high potential pathogenic capacity, suggesting that the presence of *Aeromonas* strains could represent a risk to public health, given their ubiquitous nature.

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