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Putative virulence properties of *Aeromonas* strains isolated from food, environmental and clinical sources in Italy: A comparative study

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ABSTRACT

The distribution of virulence properties in 142 strains of *Aeromonas* isolated from diarrhoeic patients, food and surface water in Italy and identified by biochemical and molecular methods was investigated. The virulence properties studied were the presence of genes for the aerolysin (*aerA*), heat-stable cytotonic enterotoxin (*ast*), heat-labile cytotonic enterotoxin (*alt*), cytotoxic enterotoxin (*act*); and cytotoxicity for Vero cells and adhesion on Hep-2 cells. *A. hydrophila* and *A. caviae* were the species most commonly isolated from clinical and environmental samples (9/30; 30.0% and 5/27; 18.5%, respectively) while mesophilic *A. salmonicida* was most common in food samples (19/80; 23.7%). Out of 142 strains, 86 (60.6%) were positive for at least one of the virulence properties. All the toxin genes were present in 4/18 (22.3%) of clinical strains. Most of the food isolates (54/55; 98.2%) were cytotoxic and most of the environmental strains (12/13; 92.3%) were adhesive. The *aerA* gene was present in most toxigenic strains (72/86; 83.7%), irrespective of their origin. The growth temperature affected the expression of cytotoxicity and adhesivity. *Aeromonas* strains from food and surface water frequently had toxin gene patterns similar to those of clinical strains and expressed virulence properties at human body temperature. These findings indicate that aeromonads have the potential to cause human illness and confirm the role of food and water as vehicles for *Aeromonas* diseases.

1. Introduction

Members of the genus *Aeromonas* have received increasing attention as opportunistic as well as primary pathogens in humans and aquatic and terrestrial animals. Human infections include gastrointestinal tract syndromes, wound and soft tissue infections, blood-borne dyscrasias and various other infections (Janda and Abbott, 2010). However, gastroenteritis is the most common of these diseases, affecting mainly young, elderly and immunocompromised individuals (Janda and Abbott, 2010). Aeromonads are common aquatic microorganisms, frequently found in chlorinated and non chlorinated drinking water supplies, and bottled water (Biscardi et al., 2002; Massa et al., 2001). These microorganisms have also been isolated from food such as vegetables, meat, ham, offal, sausage, poultry, raw milk, fish and shellfish (Kingombe et al., 2004; Ottaviani et al., 2006). Thus, food and water are probable sources of human infection (Khajanchi et al., 2010).

The taxonomy of the genus is complex and has undergone numerous changes. To date, the genus includes 14 well-recognized species (Joseph and Carnahan, 2000) to which 7 new species have been added in the last 5 years (Janda and Abbott, 2010). Among the established species, *A. hydrophila*, *A. caviae*, and *A. veronii* biovar *sobria* are the most commonly known to cause intestinal and extraintestinal human infections, while *A. veronii* biovar *veronii*, *A. jandaei*, *A. trota*, *A. schubertii*, *A. popoffii*, *A. bestiarum*, *A. salmonicida*, *A. media*, and *A. eucrenophila* are infrequently or rarely involved in gastroenteritis (Janda and Abbott, 2010). The roles of the new species as human pathogens have still to be investigated although the involvement of *A. tecta* and *A. aquariorum* in intestinal and extraintestinal diseases has been documented (Demarta et al., 2008; Figueras et al., 2009). At the molecular level, a combination of *16S rDNA-RFLP* analysis (Figueras et al., 2000) and sequencing of the housekeeping genes *rpoD* and *gyrB* (Soler et al., 2004) is necessary for a correct identification of *Aeromonas* strains to the genospecies level. At the phenotypic level, to distinguish each species from its nearest neighbours, it is necessary to integrate the conventional biochemical schemes (Abbott et al., 2003) with additional key tests recently described (Beaz-Hidalgo et al., 2010).

The pathogenicity of aeromonads is attributed to a series of factors, including cell structural lipopolysaccharides (LPS), outer-membrane proteins (OMPs), pili and flagella, a type III secretion system (T3SS) acting as adhesion structures, and extracellular factors such as

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enzymes and toxins (Janda and Abbott, 2010; Kirov, 1997). Among the exotoxins, aerolysin (Heuzenroeder et al., 1999), heat-stable cytotonic enterotoxin, heat-labile cytotonic enterotoxin (Albert et al., 2000) and cytolitic enterotoxin (Xu et al., 1998) seem to play an important role in pathogenesis. Traditional methods for the detection of the virulence properties in *Aeromonas* are based on biological assays *in vitro* and *in vivo*, using cell lines and animal models, respectively (Heuzenroeder et al., 1999; Kirov, 1997). However, these only reveal the phenotypic characteristics of the strains, while the expression of the putative virulence-associated factors in *Aeromonas* appears to be affected by environmental conditions (Kirov, 1997; Merino et al., 1998; Tso and Dooley, 1995). For this reason, these methods could in some instances fail to indicate the potential pathogenicity of isolates.

In recent years several molecular methods, particularly polymerase chain-reaction-(PCR)-based methods, have been developed for routine identification of the species of *Aeromonas* most frequently involved in human disease (Sen, 2005) and to genetically detect putative virulence genes (Chang et al., 2008; Kannan et al., 2001).

During the past decades, the prevalence of *Aeromonas* in Italian water and food samples has been well documented in Italy (Ottaviani et al., 2006; Pianetti et al., 2004) but few studies have been performed to determine the potential pathogenicity of the isolates, and none to compare the genotypic and phenotypic virulence properties of *Aeromonas* from food and clinical sources. Therefore, an investigation of the distribution of genes encoding the most important enterotoxins in *Aeromonas* strains isolated from food, environmental and clinical sources; and comparison of their potential pathogenicities, by evaluation of their adhesive and cytotoxic properties were undertaken. The influence of growth temperature on the expression of some putative virulence properties was also investigated.

2. Materials and methods

2.1. Bacterial strains

A total of 142 previously isolated *Aeromonas* strains, were used in this study. Thirty-two strains were isolated from faeces of patients with diarrhoea linked to *Aeromonas* infections, 81 from various retail foods (meat, fish, shellfish, pork salted meat, cheese, bottled drinking water, and fresh pasta) of Italian origin and 29 from surface waters used for crops irrigation. Stock cultures were maintained at −80 °C in Tripticase Soy Broth (TSB; Oxoid, Milan, Italy) containing glycerol at 20% (v/v). All strains were grown on Tryptone Soya Agar (TSA, Oxoid) for 24–48 h. Environmental and food strains were grown at 28 °C, clinical strains at 37 °C.

2.2. Phenotypic identification

All strains were biochemically identified at the genus level by evaluating: oxidase, catalase and nitrate production, growth in nutrient broth containing 0 and 3% NaCl, resistance to O/129 (2,4-diamino 6,7diisopropylpteridine) 150 μg vibriostatic discs (Oxoid), production of acid from D-trehalose, failure to utilize malonate as the sole carbon source, fermentation of inositol, D-xylose and dulcitol, and growth in nutrient broth containing 6% NaCl (Ottaviani et al., 2006).

For species identification, each strain identified as *Aeromonas* was examined using a battery of biochemical tests (Abbott et al., 2003), including lysine decarboxylase, ornithine decarboxylase, and arginine dihydrolase; esculin hydrolysis, lipase and indole production (Kovac's method); methyl red, Voges-Proskauer; the utilization of citrate, DL-lactate, urocanic acid; acid and gas from D-glucose; acid from L-arabinose, cellobiose, lactose, glycerol, mannitol, rhamnose, salicin, sorbitol, pyrazinamidase, glucose 1-phosphate and sucrose; hydrolysis of urea; ONPG production; and susceptibility to ampicillin (10 μg). Further, selected supplementary biochemical tests were performed for better discrimination of the strains at the phenospecies level, introducing new key tests recently proposed (Beaz-Hidalgo et al., 2010): i.e. growth at 42 °C; acid from melibiose; hydrolysis of starch and gelatine; H_2S from cysteine; ß-haemolysis on sheep-blood agar; and motility in semisolid agar at 37 °C.

For preparing culture media, commercial reagents were used (Sigma-Aldrich, Milan, Italy). All these tests were performed using previously published methods (Abbott et al., 2003; Martin-Carnahan and Joseph, 2005). Using the Abbott schemes, each isolate was allocated to a "species-group" or to one of the following species: *A. encheleia*, *A. popoffii*, *A. veronii* biovar *veronii*, *A. allosaccharophila*, or *A. sobria*. Each "species-group" includes 3 different phenotypic species. In particular, the *A. hydrophila* group includes *A. hydrophila*, *A. bestiarum* and *A. salmonicida* (both motile and non-motile species), the *A. caviae* group includes *A. caviae*, *A. media*, and *A. eucrenophila*, and the *A. veronii*/*sobria* group includes *A. veronii* biovar *sobria*, *A. jandaei*, *A. schubertii* and *A. trota*. All the strains presumptively identified as *A. hydrophila* were further tested to discriminate this species from the new species *A. aquariorum* and *A. piscicola* by interpreting the results of the utilization of L-lactate according to Beaz-Hidalgo et al. (2009) and Figueras et al. (2009). All strains presumptively identified as *A. caviae* were further tested by interpreting the results of lysine decarboxylase, gas from D-glucose, and H_2S from cysteine (Figueras et al., 2009) to discriminate this species from the recently described new species *A. aquariorum*. Indole production and acid from glycerol were evaluated according to Demarta et al. (2008) for all strains presumptively identified as *A. eucrenophila* and *A. encheleia* (discrimination from the new recently described species *A. tecta*). Indole, starch hydrolysis and lysine decarboxylase were used to discriminate all strains presumptively allocated to the *A. caviae* group from the new species *A. molluscorum* and *A. bivalvium* (Miñana-Galbis et al., 2004, 2007). The group of strains presumptively allocated in the *A. veronii*/*sobria* group was examined for ß-haemolysis (Harf-Monteil et al., 2004), to exclude the new species *A. simiae*. If an isolate could not be definitely placed in one species group or in one of the phenospecies *A. encheleia*, *A. popoffii*, *A. veronii* biovar *veronii*, *A. allosaccharophila*, or *A. sobria*, it was considered to be an "atypical" *Aeromonas*. All typical aeromonads, presumptively identified to the species level or only allocated into a species group were molecularly confirmed by a multiplex-PCR assay.

2.3. Molecular characterization

For bacterial DNA extraction, each isolate was grown overnight in 5 ml of Nutrient Broth (Oxoid) at 28 °C for environmental and food strains or at 37 °C for clinical strains. Approximately 1 ml of the culture was centrifuged for 5 min at 15,700 × *g* and the bacterial pellet was suspended in 900 μl of L6 Buffer (Severn Biotech Ltd., Worcester, UK, distributed by Società Italiana Chimici Divisione Scientifica). The culture was denatured at 95 °C for 10 min and centrifuged for 15 s at 15,700 × *g*. Extraction matrix (100 μl; Severn Biotech Ltd.) was added to the supernatant and the mixture was shaken for 20 min on an orbital shaker. The pellet was washed twice using 200 μl of L2 Buffer (Severn Biotech Ltd.), twice with 200 μl of 80% ethanol and once with 200 μl of acetone. The pellet was then dried at 56 °C for 15 min and the DNA eluted by incubation with 150 μl of Milli-Q water at 56 °C for 5 min. The DNA was recovered by centrifugation at 15,700 × *g* for 2 min and stored at −20 °C.

For the identification of *Aeromonas* species, three multiplex PCRs were performed, according to Sen (2005). Six primer sets, which are common to two or more *Aeromonas* species, to detect lipase, elastase, DNAgyraseB (gyrB)-veronii, gyrB-bestiarum, hydrolipase, and Veronii-16S genes; and four primer sets corresponding to species-specific signature regions of the 16S rRNA genes of *A. popoffii*, *A. caviae*, *A. jandaei* and *A. schubertii* were developed (Table 1). Each *Aeromonas* strain was analysed using all three

Table 1
Identification key of *Aeromonas* spp. based on the combination of amplicons generated by the multiplex PCRs performed according to Sen (2005).

Species	Multiplex PCR-1			Multiplex PCR-2				Multiplex PCR-3		
	Hydrolipase (760 bp)	Popoffii-16S (323 bp)	Schubertii-16S (322 bp)	gyB-veronii (101 bp)	Elastase (540 bp)	Jandaeii-16S (322 bp)	Caviae-16S (322 bp)	Lipasi (383 bp)	gyrB-bestiarum (180 bp)	Veronii-16S (688 bp)
A. hydrophila	+	−	−	+	+	−	−	+	−	−
A. salmonicida[a]	−	−	−	−	+	−	−	+	+	−
A. eucrenophila[a]	−	−	−	−	+	−	−	+	+	−
A. popoffii	+	+	−	−	+	−	−	+	+	−
A. caviae	−	−	−	+	+	−	+	+	−	−
A. media	−	−	−	−	+	−	−	−	−	−
A. bestiarum	+	−	−	−	+	−	−	+	+	−
A. encheleia	−	−	−	−	+	−	−	+	+	+
A. sobria	−	−	−	−	+	−	−	−	−	+
A. veronii bv *sobria*[b]	−	−	−	+	−	−	−	−	−	+
A. veronii bv *veronii*[b]	−	−	−	+	−	−	−	−	−	+
A. allosaccharophila	−	−	−	−	−	−	−	−	−	+
A. schubertii	−	−	+	−	+	−	−	−	−	−
A. jandaei	−	−	−	−	+	+	−	−	−	−
A. trota	−	−	−	+	−	−	+	+	−	−

[a] A species-specific signature region of the *gyrB* gene from *A. eucrenophila* was used to discriminate between this species and *A. salmonicida*.
[b] Ornithine decarboxylase and arginine dihydrolase reactions were valued in *A. veronii* to discriminate between *sobria* and *veronii* biovars.

multiplex PCRs and definitively allocated to a species by combining the results of the presence/absence of PCR amplicons (Table 1).

PCR reactions were performed in a final volume of 25 µl containing 20 ng of DNA, 0.1–0.3 µM of each primer, and 1× of HotStarTaq Master mix containing $MgCl_2$, HotStarTaq DNA polymerase and deoxynucleotide triphosphate mix (dNTPs) (Qiagen, Milan, Italy). Positive controls (see reference strains in the Strain collection section) were processed in parallel in each test, as were the test samples. For negative controls, DNA preparation was replaced by PCR water. PCR conditions were as follows: 95 °C for 15 min, 30 cycles at 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, followed by a final elongation at 72 °C for 7 min. The PCR products (12 µl) were analysed by electrophoresis on 2.5% agarose gels stained with ethidium bromide (Promega, Milan, Italy) using 1× Tris-Acetate-EDTA buffer and were visualized by UV transillumination. For the detection of enterotoxin genes by PCR analysis the act-F/act-R, alt-F/alt-R, and ast-F/ast-R primers were used as previously described (Chang et al., 2008), for the amplification of a 250 bp fragment of the *Aeromonas* cytotoxic enterotoxin gene (*act*), a 153 bp fragment of the *Aeromonas* heat-labile cytotonic enterotoxin gene (*alt*), and a 99 bp fragment of the *Aeromonas* heat-stable cytotonic enterotoxin gene (*ast*), respectively. A 16S rDNA sequence (356 bp) was used as an internal positive control. PCR amplification was performed in a final volume of 25 µl containing 5 ng of DNA, 0.2 mM dNTPs deoxynucleoside triphosphate mix (Promega), 1× PCR buffer (Diatheva, Fano, Italy), 1.8 mM $MgCl_2$ (Diatheva), 0.5 µM (each) A16SF/A16SR primers; 1.5 µM (each) act-F/act-R, alt-F/alt-R primers; 2 µM (each) ast-F/ast-R primers; and 1.5 U of Hot-Rescue DNA polymerase (Diatheva). PCR conditions were as previously described (Chang et al., 2008). Positive and negative controls were included in each batch of tests. PCR products (12 µl) were analysed by electrophoresis in 2.0% agarose gel as before.

A 480 bp fragment of the aerolysin (*aerA*) gene was amplified with the primer pairs (Aer-1 and Aer-2) reported by Kannan et al. (2001). PCR amplification was performed in a final volume of 25 µl containing 12 ng of DNA, 1× PCR buffer (Diatheva), 1.0 mM of $MgCl_2$, 0.2 mM dNTPs, 0.8 µM (each) Aer-1/Aer-2 primers, and 1.5 U of Hot-Rescue DNA Polymerase (Diatheva). The amplification procedure consisted of an initial denaturation step at 95 °C for 10 min, followed by 30 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 8 min. A positive control and a negative control were included in each batch of tests. The PCR products (12 µl) were analysed by electrophoresis in 1.5% agarose gels (Promega) as before.

2.4. Adhesivity and cytotoxicity tests

For the adhesive properties assay, the cultures from TSA were inoculated into 5 ml of glucose-free double-strength Tryptic Soy Broth (TSB-2X Oxoid) and incubated at 4 °C, 24 °C and 37 °C until an absorbance of 0.07 at 600 nm (A_{600}), which corresponded to 5.0×10^6 CFU/ml, was reached.

The HEp-2 cell lines cultured in Minimum Essential Medium (MEM) with 10% foetal calf serum (Sigma) were used for studying the adhesive properties, as previously described (Barbieri et al., 1999). Briefly, cells were seeded at numbers of 5.0×10^4 cells/ml on glass coverslips in 24-well plates that were incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2. One millilitre of bacterial culture was added to each well and the plates were incubated at 37 °C for 90 min. To remove nonadherent bacteria, the coverslips were gently washed several times in Phosphate Buffer Saline (PBS, Oxoid). Finally cells were fixed in methanol and stained with 1% Giemsa. The numbers of adherent bacteria were determined microscopically. All samples and negative controls were assayed in duplicate. For each assay at least 50 cells containing adherent bacteria were observed and the mean number of bacteria per cell was calculated. The adhesion was considered as positive when more than 10 bacteria adhered on the surface of each cell.

For the cytotoxicity test, cultures from TSA were inoculated into 25 ml flasks of TSB-2X that were incubated at 4 °C, 24 °C and 37 °C until an A_{600} value of 0.125 which corresponded to 1.5×10^8 CFU/ml, was obtained. Cell-free filtrates were prepared by centrifugation at $9300\times g$ and at 4 °C for 30 min followed by filtration of the supernatant through a 0·45 mm filter. The Vero cell lines cultured in MEM with 10% foetal calf serum were used for cytotoxin production. Cell monolayers were seeded at numbers of 5.0×10^4 cells/ml on 96-well plates that were incubated at 37 °C for 24 h in an atmosphere containing 5% CO_2. Not diluted and serial twofold dilutions of filtrate from 1:2 to 1:256 were incubated for 24 to 48 h (Fiorentini et al., 1998). Supernatants heated at 65 °C for 15 min were used as negative controls. All samples and negative controls were assayed in duplicate. The cytotoxicity was measured by microscopic examination of the cells. The dead cells were rounded and detached from the monolayer. We defined the cytotoxic titre as the reciprocal of the highest dilution of the culture filtrate that caused destruction of 50% of the Vero cells.

2.5. Strain collection

The following reference strains were used in parallel with test strains as positive controls for the interpretation of doubtful biochemical reactions: *A. hydrophila* ATCC 7966, *A. salmonicida* ATCC 33658, *A. allosaccharophila* ATCC 35942, *A. eucrenophila* ATCC 23309, *A. veronii* biovar *sobria* ATCC 9071, *A. veronii* biovar *veronii* ATCC 35624, *A. bestiarum* ATCC 51108, *A. caviae* ATCC 15468, *A. media* ATCC 35950, *A. sobria* ATCC 43979, *A. popoffii* ATCC BAA-243, *A. trota* ATCC 49657 and ATCC 49659, *A. jandaei* ATCC 49568, *A. schubertii* ATCC 43700 and *A. encheleia* ATCC 51929.

A. hydrophila ATCC 7966 was used as positive control for PCR amplification of lipase, elastase, hydrolipase, gyrB-veronii, *alt*, *ast*, *act* and *aer*A genes and for adhesivity and cytotoxicity assays.

A. popoffii ATCC BAA-243 was used as positive control for PCR amplification of gyrB-bestiarum and Popoffii-16S.

A. caviae ATCC 15468, *A. jandaei* ATCC 49568, *A. schubertii* ATCC 43700, *A. veronii* biovar *veronii* ATCC 35624 and *A. eucrenophila* ATCC 23309 were used as positive controls for PCR amplification of Caviae-16S, Jandeii-16S, Schubertii-16S, Veronii-16S, and gyrB-eucrenophila, respectively.

2.6. Statistical analysis

The statistical significance of the data was determined by a chi-square test (χ^2) and a probability value (P) ≤ 0.05 was regarded as statistically significant.

3. Results

3.1. Species identification of the Aeromonas strains

The results of the species identification by biochemical and molecular tests are presented in Table 2.

Of 142 strains biochemically examined, 137 (96.5%) presented typical reactions, while five strains (3.5%), two clinical, two environmental and one food strains could not be allocated to one species group or phenospecies. Of the 137 typical strains, 55 (40.1%) were placed in one of the three species groups, 60 (43.8%) were phenotypically identified to the species level and 22 (16.1%) could be assigned to two species. The multiplex PCR analysis allowed definitive identification of all strains within the three species groups and discrimination between the two species for each of the strains for which phenotypical identification was ambiguous. For 6 (10%) of 60 strains biochemically identified to the species level, for which there was no correspondence between the biochemical and molecular identifications, the latter identification was assumed.

3.2. Prevalence of the Aeromonas strains

The results are presented in Table 3.

Overall, *A. hydrophila* and *A. salmonicida* (motile strains) were the two most prevalent species, followed by *A. bestiarum*, *A. allosaccharophila* and *A. caviae*. Considering the different sources, *A. hydrophila* and *A. caviae* were most prevalent in human and environmental samples, while *A. salmonicida*, *A. bestiarum* and *A. allosaccharophila* were most prevalent in foods. *A. veronii* biovar *sobria* and *A. eucrenophila* were most prevalent in surface water. *A. media* was similarly prevalent in human and environmental samples. *A. veronii* biovar *veronii* was isolated only from foods.

3.3. Putative virulence properties of the Aeromonas strains

The results are presented in Tables 4, 5 and 6.

Of 142 strains analysed, 86 (60.6%) were positive for at least one of the putative virulence properties, 55/81 (67.9%), 18/32 (56.2%), and 13/29 (44.8%) from food, clinical, and environmental samples, respectively. Only the difference between the prevalence in food and environmental samples was significant ($p<0.05$).

The 14 clinical strains negative to all the virulence properties included three strains of *A. caviae*, two strains of each of the atypical strains, *A. salmonicida*, *A. media*, and *A. allosaccharophila*, and one strain of each of *A. hydrophila*, *A. veronii* biovar *sobria*, and *A. bestiarum*. Of the 26 negative food strains, five were *A. salmonicida*, eight were *A. allosaccharophila*, eight were *A. bestiarum*, two were *A. media*, and single strains were *A. eucrenophila*, *A. sobria* and an atypical strain. Of the 16 environmental *Aeromonas* negative strains, 2, 4, 3 and 2 were atypical strains, *A. eucrenophila*, *A. media* and *A. caviae*, respectively, and single strains were *A. salmonicida*, *A. bestiarum*, *A. trota*, *A. jandaei* and *A veronii* biovar *sobria*.

Between the 86 strains showing at least one of the virulence properties 79 (91.9%) had one or more toxin genes, 47 (54.6%) and 72 (83.7%), respectively, showed adhesivity and cytotoxicity at one temperature at least. At least one toxin gene was present in 17/18 (94.4%) clinical, 53/55 (96.4%) food, and 9/13 (69.2%) environmental strains. The prevalence of toxin genes was significantly less ($p<0.05$) in environmental than in food strains.

All the toxin genes were present in ten of 29 (34.5%) *A. hydrophila* strains. Of these, 4/18 (22.2%), 5/55 (9.1%), 1/13 (7.7%) were from clinical, food, and environmental samples, respectively.

Table 2
Biochemical and molecular identification of *Aeromonas* spp. isolated from different sources.

Biochemical identification by the Abbott schemes	Molecular identification by multiplex PCRs	No. of strains	Sources
A. hydrophila group	*A. hydrophila*	16	Food
	A. salmonicida	18	
	A. allosaccharophila	1	
	A. bestiarum	6	
	A. hydrophila	6	Clinical samples
	A. allosaccharophila	1	
	A. veronii bv *sobria*	2	
	A. bestiarum	1	
	A. salmonicida	1	Surface water
	A. bestiarum	1	
	A. caviae	1	
A. caviae group	*A. media*	1	Clinical samples
A. hydrophila	*A. hydrophila*	3	Clinical samples
	A. hydrophila	5	Surface water
A. bestiarum	*A. bestiarum*	9	Food
	A. popoffii	1	Food
A. salmonicida	*A. salmonicida*	2	Clinical samples
A. caviae/salmonicida	*A. salmonicida*	1	Food
A. caviae	*A. eucrenophila*	1	Food
	A. caviae	9	Clinical samples
	A. caviae	4	Surface water
A. media	*A. media*	2	Food
	A. media	2	Clinical samples
A. media/salmonicida	*A. salmonicida*	1	
A. media	*A. media*	2	Surface water
	A. caviae	1	Food
A. eucrenophila	*A. eucrenophila*	2	Food
	A. eucrenophila	1	Clinical samples
	A. eucrenophila	4	Surface water
	A. media	1	
A. sobria	*A. sobria*	1	Food
	A. sobria	2	Surface water
A. veronii bv *sobria*	*A. veronii* bv *sobria*	1	Food
	A. allosaccharophila	1	Clinical samples
	A. veronii bv *sobria*	4	Surface water
A. jandaei	*A. jandaei*	1	Surface water
A. trota	*A. trota*	1	Surface water
A. veronii/allosaccharophila	*A. allosaccharophila*	12	Food
	A. veronii bv *veronii*	8	
Atypical Aeromonads	–	2	Clinical samples
		1	Food
	–	2	Surface water
		142	Total

–, not assayed.

Table 3
Prevalence of the isolates belonging to each *Aeromonas* species from food, clinical and surface water samples.

	No. of typical strains identified (%)													
	A. hydrophila	*A. caviae*	*A veronii* bv *sobria*	*A. salmonicida*	*A. eucrenophila*	*A. trota*	*A. media*	*A. allosaccharophila*	*A. bestiarum*	*A. veronii* bv *veronii*	*A. popoffii*	*A. sobria*	*A. jandaei*	Total (%)
Stool	9 (30.0)	9 (30.0)	2 (6.6)	3 (10.0)	1 (3.3)	0	3 (10.0)	2 (6.6)	1 (3.3)	0	0	0	0	30 (100)
Food	16 (20.0)	1 (1.2)	1 (1.2)	19 (23.7)	3 (3.7)	0	2 (2.5)	13 (16.2)	15 (18.7)	8 (10.0)	1 (1.2)	1 (1.2)	0	80 (100)
Surface water	5 (18.5)	5 (18.5)	4 (14.8)	1 (3.7)	4 (14.8)	1 (3.7)	3 (11.1)	0	1 (3.7)	0	0	2 (7.4)	1 (3.7)	27 (100)
Total	30 (21.9)	15 (10.9)	7 (5.1)	23 (16.8)	8 (5.8)	1 (0.7)	8 (5.8)	15 (10.9)	17 (12.4)	8 (5.8)	1 (0.7)	3 (2.2)	1 (0.7)	137 (100)

Of the 86 strains, 29 (33.7%) had genes for three toxins, although in different combinations. Of these, 7/18 (38.8%), 19/55 (34.5%), and 3/13 (23.1%) were from clinical, food, and environmental samples, respectively.

Overall, 30/86 strains (34.9%) had genes for two toxins. Of these, 23/55 (41.8%) were food, 3/13 (23.1%) were environmental and 4/18 (22.2%) were clinical strains.

Of the 86 strains, 46 (53.5%) had the *act* gene, 9/13 (69.2%), 10/18 (55.5%), and 27/55 (49.1%) from environmental, clinical and food samples, respectively.

In all these cases, differences between the prevalence were not significant.

Overall, the *aerA* gene was present in 72/86 (83.7%) strains. Of these, 51/55 (92.7%), 16/18 (88%), and 5/13 (38.5%) were food, clinical and environmental strains, respectively. The prevalence of *aer*A gene was significantly less ($p<0.01$) in environmental than in food and clinical strains.

The *alt* gene was overall present in 55/86 (63.9%) strains. Of these, 42/55 (76.4%) were food, 10/18 (55.5%) were clinical, and 3/13 (23.1%) were environmental strains. Only the difference between prevalence in food and environmental strains was significant ($p<0.01$).

Finally, 24 of 86 strains (27.9%) had the *ast* gene, 11/18 (61.1%), 4/13 (30.8%), and 9/55 (16.4%) from clinical, environmental, and food samples, respectively. Only the difference between prevalence in clinical and food strains was significant ($p<0.01$).

Fifty-four of 55 (98.2%) food, 10 of 13 (76.9%) environmental, and 8 of 18 (44.4%) clinical strains were cytotoxic. Only the difference between prevalence in food and clinical samples was significant ($p<0.01$). Of the most representative species, *A. caviae* had a prevalence of cytotoxic strains (2/10; 20%) significantly less ($p<0.01$) than *A. hydrophila* (27/29; 93.1%), *A. salmonicida* (14/15; 93.3%), *A. allosaccharophila* (4/5; 80%), and all the other species (100%).

Adhesivity was present in 12/13 (92.3%), 9/18 (50%), and 26/55 (47.3%) of environmental, clinical and food strains, respectively. The prevalence of adhesivity was significantly more ($p<0.05$) in environmental than in clinical and food strains. With regard to the most representative species, *A. caviae*, *A. veronii* biovar *veronii* and *A. hydrophila* had a prevalence of adhesive strains (8/10; 80%, 6/8; 75% and 19/29; 65.5%, respectively) significantly more ($p<0.05$) than *A. bestiarum*, *A. salmonicida*, and *A. allosaccharophila* (3/7; 42.8%, 4/15; 26.6% and 1/5; 20%, respectively).

Table 4
Virulence properties of *Aeromonas* spp. isolated from clinical samples.

No. of strains	Species	Adhesivity			Toxin genes				Toxin titre on VERO cells[a]		
		37 °C	24 °C	4 °C	*act*	*ast*	*alt*	*aerA*	37 °C	24 °C	4 °C
8	*A. hydrophila*	−	−	−	+	+	−	+	−	−	−
		−	−	−	+	+	+	+	−	−	−
		−	−	−	−	+	+	+	64	128	4
		−	−	−	+	−	−	+	64	128	2
		−	−	−	+	+	−	+	64	128	8
		+	−	−	+	+	+	+	128	256	16
		+	−	−	+	+	+	+	256	256	16
		+	+	−	+	+	+	+	128	256	8
6	*A. caviae*	+	+	+	−	−	−	−	−	−	−
		+	+	+	−	−	+	+	−	−	−
		+	−	−	−	−	−	+	−	−	−
		+	+	−	−	+	+	+	−	−	−
		+	+	+	−	+	+	+	−	−	−
		−	−	−	+	+	−	+	64	128	8
1	*A. eucrenophila*	−	−	−	+	−	−	+	−	−	−
1	*A. media*	−	−	−	−	−	−	+	−	−	−
1	*A. salmonicida*	−	−	−	−	−	+	+	−	−	−
1	*A. veronii* bv *sobria*	+	−	−	+	+	+	−	128	256	4

+, presence; −, absence.
[a] The results express absolute values because they are identical for both the replicates of each test sample.

3.4. Influence of the growth temperature on cytotoxic and adhesive properties of the Aeromonas strains

The results are presented in Tables 4, 5 and 6.

Of 72 cytotoxic strains, 8/8 (100%) clinical, 50/54 (92.6%) food, and 9/10 (90.0%) environmental strains, respectively, had this property at all the tested temperatures. However, irrespective of their origin, 69/72 (95.8%), 2/72 (2.7%), 1/72 (1.4%) strains had the highest toxin titres at 24 °C, 37 °C, and 4 °C, respectively.

Twenty of 47 (42.5%) adhesive strains had this property at all the tested temperatures. Twenty-five of 26 (96.1%) food, 11 of 12 (91.6%) environmental, and 5 of 9 (55.5%) clinical strains, respectively, were adhesive at 24 °C. Nine of 9 (100%) clinical, 23 of 26 (88.5%) food, and 8 of 12 (66.6%) environmental strains were adhesive at 37 °C. Finally, 14/26 (53.8%) food, 3/9 (33.3%) clinical, and 4/12 (33.3%) environmental strains were adhesive at 4 °C.

4. Discussion

In this study, *Aeromonas* strains were identified at the phenotypic level by integrating a conventional biochemical scheme with new biochemical key tests able to avoid a misidentification of each species with the nearest neighbours. The strains allocated into the well-recognized species were also genetically characterized by using a molecular approach based on multiplex PCR assays. Biochemical and molecular species identifications corresponded for the majority of the strains. Moreover, PCR analysis allowed a definitive discrimination of the strains for which the biochemical species identification was ambiguous. Therefore, our results confirm the suitability of this integrated approach in identifying aeromonads and its reliability for a routine use in microbiology laboratories.

In agreement with previous studies (Figueras, 2005; Janda and Abbott, 1998, 2010), *A. hydrophila* and *A. caviae* were the most prevalent species found in clinical samples. Moreover, in accordance with other researches (Chang et al., 2008; Ørmen and Østenvik, 2001; Pianetti et al., 2004; Snowden et al., 2006), *A. hydrophila* and *A. veronii* biovar *sobria* were the most frequently isolated species from food and environmental samples, respectively. Interesting in this study was the high number of

Table 5
Virulence properties of *Aeromonas* spp. isolated from food.

No. of strains	Species	Adhesivity			Toxin genes				Toxin titre on VERO cells[a]		
		37 °C	24 °C	4 °C	*act*	*ast*	*alt*	*aerA*	37 °C	24 °C	4 °C
16	*A. hydrophila*	−	−	−	+	−	+	+	16	16	4
		+	+	−	+	−	+	+	32	256	16
		−	−	−	−	−	+	−	32	64	4
		−	−	−	+	−	−	+	64	64	16
		−	−	−	+	+	+	+	256	16	2
		+	+	−	+	+	+	+	8	128	4
		+	+	+	−	−	+	+	8	16	nd[b]
		+	+	−	+	+	+	+	16	64	−
		+	+	+	−	−	+	+	16	16	nd
		+	+	+	−	−	+	+	16	256	4
		+	+	+	+	+	+	+	16	32	4
		+	+	+	+	−	+	+	64	128	16
		+	+	−	−	−	+	+	64	128	16
		+	+	−	+	+	+	+	64	256	4
		+	+	+	+	−	−	+	256	256	16
		+	−	−	+	−	+	+	256	256	2
14	*A. salmonicida*	−	−	−	−	−	+	+	16	128	4
		−	−	−	−	−	+	+	2	16	64
		−	−	−	−	−	+	+	16	128	4
		−	−	−	+	−	+	+	4	16	4
		+	+	−	+	−	+	+	16	128	4
		−	−	−	−	−	−	+	4	32	nd
		−	−	−	+	−	+	−	16	32	4
		−	−	−	−	−	−	+	128	256	nd
		−	+	−	+	−	+	+	4	32	2
		−	−	−	−	−	+	+	16	32	8
		−	+	−	+	−	+	+	16	64	4
		−	−	−	+	−	−	+	8	128	nd
		+	+	+	−	−	+	+	4	128	16
		−	−	−	−	−	+	+	2	64	nd
8	*A. veronii* bv *veronii*	+	+	+	+	−	+	+	128	256	4
		+	+	−	+	−	+	+	4	8	−
		−	−	−	+	−	+	+	4	128	16
		+	+	+	+	−	+	+	4	64	4
		+	+	+	+	−	+	+	64	−	−
		+	+	+	+	−	+	+	256	256	4
		+	+	+	+	−	+	+	256	256	−
		−	−	−	+	+	−	+	32	128	16
7	*A. bestiarum*	−	−	−	−	−	−	−	8	128	2
		+	+	−	−	−	+	+	4	64	2
		−	−	−	−	−	+	+	64	256	4
		−	−	−	−	−	+	+	4	16	4
		−	−	−	−	−	−	+	4	64	2
		−	+	+	−	−	−	+	16	128	8
		+	+	−	−	−	+	+	16	128	16
5	*A. allosaccharophila*	−	−	−	−	−	−	+	4	64	16
		−	−	−	−	+	+	+	32	32	4
		−	−	−	+	−	−	+	4	32	4
		+	+	+	−	−	−	−	64	256	8
		−	−	−	−	−	+	+	−	−	−
2	*A. eucrenophila*	−	−	−	−	−	+	+	64	256	16
		−	−	−	−	+	+	+	4	32	4
1	*A. veronii* bv *sobria*	−	−	−	+	+	−	+	32	128	4
1	*A. popoffii*	−	−	−	−	−	+	+	2	4	2
1	*A. caviae*	−	−	−	−	−	+	+	32	128	4

+, presence; −, absence.
[a] The results express absolute values because they are identical for both the replicates of each test sample.
[b] Not diluted.

isolates belonging to *A. salmonicida*, which was prevalent in foods. In disagreement with other researches (Hänninen and Siitonen, 1995; Janda et al., 1996; Martin-Carnahan and Joseph, 2005), strains of this species were also found in clinical samples. Irrespective of their origin, all the *A. salmonicida* strains investigated here were motile at 37 °C, suggesting that mesophilic strains were common in the studied area.

Due to the complex pathogenesis, for aeromonads no single putative virulence-associated factor can be unequivocally pinpointed as responsible for particular symptoms or diseases (Albert et al., 2000). Moreover, previous studies suggest that putative virulence-associated or colonization factors of pathogenic strains can be lost upon *in vitro* passage (Morgan et al., 1985). All these findings could explain because a lot of the clinical strains investigated here, although identified as the cause of acute gastroenteritis, lacked all the putative virulence properties tested. Another explanation for this could be that the patients with gastroenteritis were healthy carriers of *Aeromonas*, and real aetiological agents of the disease were not identified.

In agreement with previous studies (Albert et al., 2000; Krzymińska et al., 2001; Sechi et al., 2002), we found a high heterogeneity in the distribution of toxin genes among the tested strains. Surprisingly, similar prevalence of toxin genes was frequently found in food, clinical and environmental strains.

Table 6
Virulence properties of *Aeromonas* spp. isolated from surface water.

No. of strains	Species	Adhesivity			Toxin genes				Toxin titre on VERO cells[a]		
		37 °C	24 °C	4 °C	*act*	*ast*	*alt*	*aerA*	37 °C	24 °C	4 °C
5	*A. hydrophila*	+	+	−	+	+	−	+	64	128	2
		−	−	−	+	−	−	+	64	128	8
		+	+	+	−	−	−	−	64	128	4
		+	+	+	+	−	−	+	128	256	2
		+	+	+	+	+	+	+	256	256	4
3	*A. veronii* bv *sobria*	−	+	−	+	+	+	−	32	64	−
		−	+	−	+	−	−	−	64	128	2
		+	+	+	+	+	−	+	64	128	2
3	*A. caviae*	+	+	−	−	−	−	−	−	−	−
		+	+	−	−	−	−	−	−	−	−
		+	−	−	−	−	−	−	−	−	−
2	*A. sobria*	−	+	−	+	−	+	−	64	128	2
		−	+	−	+	−	−	−	64	128	4

+, presence; −, absence.
[a] The results express absolute values because they identical for both the replicates of each test sample.

Some studies reported a correlation between the higher number of toxin genes harboured in an isolate of *Aeromonas* and its potential to determine diarrhoea (Albert et al., 2000; Chang et al., 2008; Sha et al., 2002. The evidence that the *Aeromonas* strains isolated in this study from food and environmental samples often had two or more toxin genes, although in different combinations, highlights their potential pathogenicity.

Irrespective of their origin, a lot of the *A. hydrophila* strains investigated here had all the toxin genes. This finding confirms that this species possesses a more extensive array of virulence genes than the other species of clinical relevance (Aguilera-Arreola et al., 2007).

With regard to *A. caviae*, a lot of clinical isolates lacked all the toxin genes or harboured *aerA* gene alone. This evidence suggests for this species the possible involvement of virulence factors not investigated in this study, and confirms the relevant role of the aerolysin in the pathogenesis (Aguilera-Arreola et al., 2007; Snowden et al., 2006).

In disagreement with some studies (Albert et al., 2000; Chang et al., 2008) but in accordance with others (Khajanchi et al., 2010), the *act* gene was frequently found in the tested strains, irrespective of their origin. Moreover, two environmental strains surprisingly harboured both *act* and *ast* genes, a combination that is rarely reported (Albert et al., 2000; Chang et al., 2008). Intriguing in this work was also the presence of the *ast* gene in strains of *A. caviae* and *A. veronii* biovar *sobria* of clinical and environmental origin, although previous evidence consolidated the opinion that these species lack the *ast* gene (Aguilera-Arreola et al., 2007). Albert et al. (2000) postulated that different prevalence of the toxin genes may be related to geographical distribution of the strains. Unfortunately, in order to clarify if the toxigenic patterns reported here are typical of the studied area, previous data on the distribution of toxin genes in Italian strains of *Aeromonas* were not available for a comparison.

Carrello et al. (1988) found that clinical strains of *A. hydrophila* were more adhesive on Hep-2 cells than environmental strains, while the opposite was found by Sechi et al. (2002) for isolates of *A. veronii* biovar *sobria*. Overall, in this study, adhesive and cytotoxic properties were less present in clinical than in environmental and food strains. In fact, surprisingly, a lot of the toxigenic strains of clinical origin were not adhesive and/or not cytotoxic. A reasonable explanation for this could be that the experimental conditions used in this study affected the expression of the genes involved in adhesivity and cytotoxicity. Therefore, this matter will be further investigated.

Previous research reported cytotoxic and adhesive properties at 37 °C for clinical isolates and at refrigeration temperature for food and environmental strains (Kirov, 1997). In disagreement with this, our results showed that most of the food and environmental strains were cytotoxic and adhesive at both 24 °C and 37 °C, but not at 4 °C, demonstrating their ability to express these putative virulence properties at the human body temperature. However, it is important to note that infections by potentially pathogenic aeromonads may not always lead to disease, due to host factors such as susceptibility or immune status and infectious dose of the microorganism. For these reasons, gastroenteritis caused by aeromonads in humans remains relatively rare.

In conclusion, *Aeromonas* strains isolated in this study from environmental and food samples showed toxin gene patterns similar to those of clinical strains. Moreover, these strains were frequently found to be cytotoxic and adhesive at 37 °C. These findings indicate that aeromonads have the potential to cause human illness and confirm the role of food and water as vehicles for *Aeromonas* diseases.

This study also demonstrated that characterisation of the potential pathogenicity of aeromonads at the genotypic and phenotypic level using an integrated molecular and biological approach is important, considering the multi-factorial nature of the diseases and the influence of environmental conditions in the expression of the putative virulence properties.

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